Glutamate mediates platelet activation through the AMPA receptor

Craig N. Morrell,[1] Henry Sun,[2] Masahiro Ikeda,[8] Jean-Claude Beique,[3,4] Anne Marie Swaim,[1] Emily Mason,[1] Tanika V. Martin,[1] Laura E. Thompson,[1] Oguz Gozen,[5,9] David Ampagoomian,[1] Rolf Sprengel,[10] Jeffrey Rothstein,[3,5] Nauder Faraday,[6] Richard Huganir,[3,4] and Charles J. Lowenstein[2,7]

[1]Department of Molecular and Comparative Pathobiology, [2]Department of Medicine, [3]Department of Neuroscience, [4]Howard Hughes Medical Institute, [5]Department of Neurology, [6]Department of Anesthesia and Critical Care, and [7]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205
[8]Department of Veterinary Pharmacology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan
[9]Department of Physiology, Ege University School of Medicine, 35100 Izmir, Turkey
[10]Max Planck Institute for Medical Research, Department of Molecular Neurobiology, D-69120, Heidelberg, Germany

Glutamate is an excitatory neurotransmitter that binds to the kainate receptor, the *N*-methyl-D-aspartate (NMDA) receptor, and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (AMPAR). Each receptor was first characterized and cloned in the central nervous system (CNS). Glutamate is also present in the periphery, and glutamate receptors have been identified in nonneuronal tissues, including bone, heart, kidney, pancreas, and platelets. Platelets play a central role in normal thrombosis and hemostasis, as well as contributing greatly to diseases such as stroke and myocardial infarction. Despite the presence of glutamate in platelet granules, the role of glutamate during hemostasis is unknown. We now show that activated platelets release glutamate, that platelets express AMPAR subunits, and that glutamate increases agonist-induced platelet activation. Furthermore, we demonstrate that glutamate binding to the AMPAR increases intracellular sodium concentration and depolarizes platelets, which are important steps in platelet activation. In contrast, platelets treated with the AMPAR antagonist CNQX or platelets derived from GluR1 knockout mice are resistant to AMPA effects. Importantly, mice lacking GluR1 have a prolonged time to thrombosis in vivo. Our data identify glutamate as a regulator of platelet activation, and suggest that the AMPA receptor is a novel antithrombotic target.

CORRESPONDENCE
Craig Morrell:
cmorrell@jhmi.edu

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; CNS, central nervous system; CPP, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; GPCR, G protein–coupled receptor; JST, Joro spider toxin; NHE, Na^+/H^+ exchanger; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; PRP, platelet-rich plasma; SBFI, sodium-binding benzofuran isophthalate; TRAP, thrombin receptor–activating peptide.

Cardiovascular disease and stroke are major causes of morbidity and mortality. Although many pathophysiologic processes play a role in the chronic development of cardiovascular disease, thrombosis is often the event that precipitates stroke and acute coronary syndromes. Thrombosis is initiated by receptors on resting platelets responding to activation initiators such as vWf, thrombin, ADP, and collagen. Despite the acknowledged principal role of platelet activation in initiating or exacerbating cardiovascular disease, there are currently few platelet-specific drugs available, making investigation into mechanisms of platelet activation very important.

After the initiation of platelet activation, subsequent pathways are activated, including conformational changes in receptors (e.g., GPIIb/IIIa), granule exocytosis, and the secretion of vasoactive mediators. Activation of resting platelets triggers a variety of intraplatelet signaling pathways, including changes in intracellular ion concentrations such as a burst of intracellular Na^+ (1–3). The primary identified means of increasing intracellular Na^+ is via the Na^+/H^+ exchanger (NHE), which is relatively slow and does not account for the large rapid increase in Na^+ influx (4, 5). It has also been demonstrated that membrane depolarization in megakaryocytes and other cells mediates an increase in ligand affinity and downstream signaling of G protein–coupled receptors (GPCR), such as the platelet purinic receptors (P2Y) (6–8). Therefore, efficient generation of inward currents by ion flux may be an important modulator of

The online version of this article contains supplemental material.

platelet response to agonists such as ADP, thrombin, and thromboxane.

Many molecules and receptors involved in platelet function also have important roles in neuronal function, including exocytosis regulatory molecules, serotonin/serotonin receptor, ADP/purinergic receptors, PAR receptors, ApoE/ApoER, α-synuclein, acetylcholine, epinephrine, and substance P (9–11). Platelet-dense granules also contain and release glutamate, which is an important neurotransmitter (12–15). A major class of glutamate receptors includes the ionotropic glutamate receptors (*N*-methyl-D-aspartate [NMDA], kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA] receptors [AMPARs]). Each receptor has been known to respond to glutamate released from a presynaptic neuron during excitatory neurotransmission, but glutamate-mediated signaling may also be important in cells that are not commonly considered excitatory (16). For example, AMPA can stimulate insulin release from β cells in the pancreas, and NMDA receptors have been noted in osteoblasts, osteoclasts, and platelets (17–19). NMDA receptor signaling is reported to have a role in regulating platelet production from megakaryocytes, in addition to potentially inhibiting platelet activation (20–23). Other sites where glutamate receptors have been identified outside the central nervous system (CNS) include the heart, spleen, testis, and kidney (16, 24). Several of these peripheral glutamate receptors have been cloned and sequenced and are identical to those in the CNS (16).

Blood glutamate concentration is relatively high compared with the CNS, and it is tightly controlled by peripheral glutamate transporters (16). Platelets express glutamate uptake transporters (EAATs) to clear glutamate from the extracellular environment and vesicular glutamate (vGlut) transporters to load glutamate into granules (12, 13, 15). Studies primarily aimed at using platelets as peripheral markers of CNS diseases have demonstrated that glutamate is released upon platelet activation (12, 14, 25, 26). After stroke, plasma glutamate concentrations rise and remain elevated for up to 2 wk (14, 27). Concentrations >200 μM have been reported in patients upon admission for stroke, perhaps contributing to an increased thrombotic risk (25, 27). However, functional studies of peripheral glutamate signaling and its role in vascular physiology are limited.

Although described in many tissues, AMPAR is best characterized in the brain as a glutamate-sensitive ion channel of neuronal activation (16, 20). AMPARs are comprised of heteromeric tetramer complexes of subunits designated GluR1–GluR4 (28, 29). The AMPAR subunits combine to form a transmembrane receptor with a central ion-permeable channel. Glutamate binding to AMPAR triggers Na^+ influx and facilitates transduction of an electrical current (30). Calcium permeability of the AMPAR is dictated by the GluR2 subunit isoform that is present. GluR2 isoform expression varies between regions of the brain, and with it, AMPAR permeability to calcium also varies (31, 32).

We now demonstrate the importance of glutamate as a modulator of platelet function. AMPAR is expressed on platelets and has a functional role in regulating platelet agonist response. Furthermore, mice lacking a complete AMPAR have prolonged time to vessel occlusion and platelet function in vivo.

RESULTS

Glutamate release during thrombus generation increases platelet activation

Platelets store glutamate, express glutamate transporters, and release glutamate during activation, but vascular targets of glutamate have not been characterized (13, 14, 16, 24, 25, 33). To define the role of platelet-derived glutamate in the vasculature, we first quantified platelet glutamate release locally within a developing thrombus using a glutamate-sensitive enzymatic probe. The probe provides continuous real-time glutamate concentration measurements by glutamate oxidase–catalyzed conversion of glutamate to α-ketoglutaric acid and hydrogen peroxide. Hydrogen peroxide is then ampomerically detected (34). We diluted whole blood in Tyrode's buffer (1:1), and glutamate concentration was measured during thrombin-initiated (0.5 U/ml) platelet aggregation. Glutamate increases rapidly during platelet aggregation to concentrations exceeding 400 μM (Fig. 1, A and B).

We next determined whether glutamate is a mediator of platelet activation. Platelet-rich plasma (PRP) was diluted 1:20 in Tyrode's buffer and incubated with glutamate (0–250 μM) for 5 min before activating platelets with a moderate concentration of thrombin receptor–activating peptide (TRAP-6; 5 μM) or a high concentration of TRAP (20 μM). Platelet activation was measured by FACS analysis for active conformation of surface GP IIb/IIIa using PAC-1 antibody (Fig. 2 A; or by surface P-selectin expression, Fig. S1 B, available at

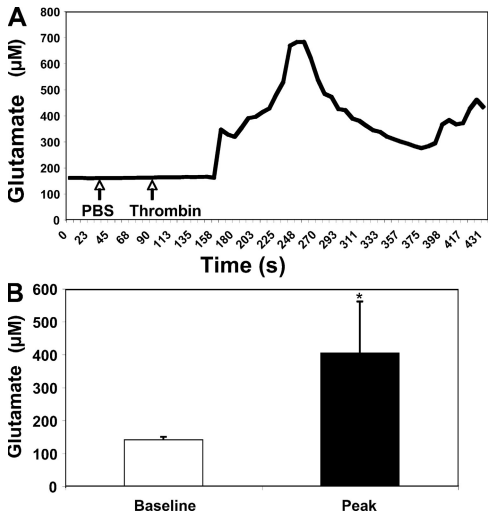

Figure 1. Glutamate is released in thrombus formation. (A) Whole blood was diluted in Tyrode's buffer (1:1), and glutamate concentration was measured with a voltage-sensitive enzymatic probe at rest after addition of PBS (no change) or 0.5 U/ml thrombin (representative tracing). (B) Quantification. Baseline and peak glutamate concentrations during thrombus formation. $n = 3–4$. *, $P < 0.05$.

http://www.jem.org/cgi/content/full/jem.20071474/DC1). Glutamate does not increase resting platelet activation (Fig. 2 A), but in the presence of glutamate, 5 μM of TRAP significantly increases platelet activation (Fig. 2 A). Glutamate has no effect at high TRAP concentrations (Fig. 2 A). A dose–response curve with glutamate and moderate TRAP concentration (5 μM) was also performed, and PAC-1 binding was expressed as the percentage of increase in platelet mean fluorescence versus resting control platelets. Glutamate dose dependently increases platelet activation in response to TRAP (Fig. 2 B). As little as 150 μM of glutamate increases TRAP-induced platelet activation by >50% compared with control-treated platelets (Fig. 2 B). These are physiologically relevant plasma glutamate concentrations; control patient plasma glutamate is ∼100 μM at baseline, and in stroke patients it can rise to >200 μM on admission. Our data in Fig. 1 (A and B) demonstrate that local glutamate concentrations in platelet aggregation can exceed 400 μM (27, 35). Glutamate also dose dependently increases platelet activation in response to the thromboxane mimetic U46619 (Fig. 2 C).

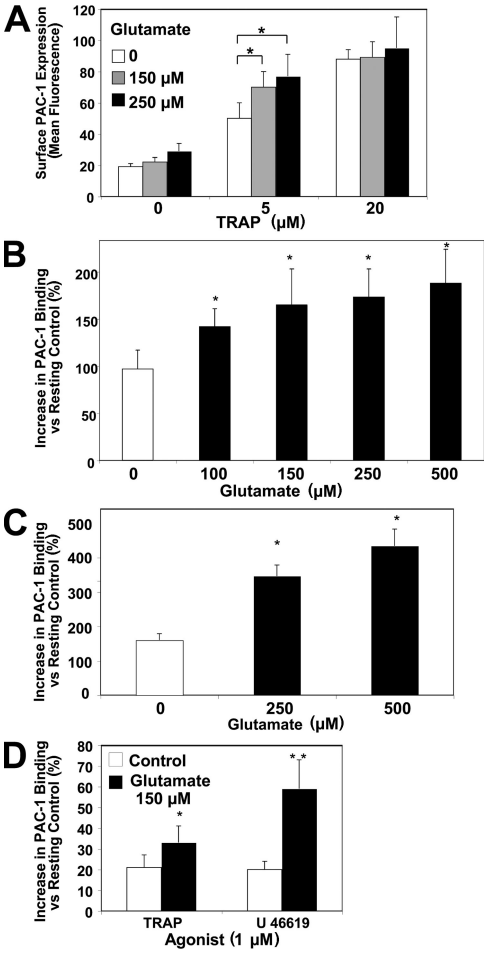

Figure 2. Glutamate mediates platelet activation. (A) Glutamate increases platelet activation. Platelets were incubated with glutamate and activated with a moderate concentration of TRAP (5 μM) or high concentration of TRAP (20 μM). Activation was measured by FACS analysis for surface PAC-1 antibody binding. $n = 4 \pm$ the SD. *, $P < 0.02$ versus 0.5 μM TRAP. (B) Dose response. Platelets were incubated with glutamate and activated with a moderate concentration of TRAP (5 μM). Activation was measured by FACS analysis for surface PAC-1 antibody binding and expressed as the percentage of increase in fluorescence versus resting platelets. $n = 5$–$7 \pm$ the SD. *, $P < 0.01$ versus 0. (C) AMPAR regulates platelet activation. Glutamate increases thromboxane mimetic-induced (U46619; 10 μM) platelet activation measured by PAC-1 antibody binding. $n = 3 \pm$ the SD. *, $P < 0.01$ versus 0 μM. (D) Glutamate is highly effective at low-dose agonist stimulation. Platelets were incubated with 150 μM glutamate and activated with 1 μM TRAP or U46619. $n = 5 \pm$ the SD. *, $P < 0.05$; **, $P < 0.01$ versus control.

Because glutamate appears to have a modulatory role in platelet activation, we determined its affects at concentrations that are most relevant, low concentrations of glutamate (150 μM), and low agonist concentrations (1 μM TRAP and U46619). Glutamate-mediated increase in platelet activation after low concentration thromboxane mimetic is approximately three times greater than control, and approximately two times greater in response to TRAP (Fig. 2 D).

Glutamate mediates platelet activation and aggregation through the AMPAR

We next sought to identify the glutamate receptor that augments platelet activation. Others have demonstrated the presence of the NMDA type glutamate receptor (NMDAR) on platelets, but this appears to have a different role in platelet function (20, 22, 23). To determine whether NMDAR mediates glutamate activity, we blocked the NMDAR with (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and treated platelets with glutamate before stimulating with TRAP. NMDAR inhibition does not inhibit glutamate-mediated increase in platelet activation (Fig. 3 A). We therefore explored whether platelets express another type of ionotropic glutamate receptor. We demonstrated the presence of AMPAR subunit proteins GluR1-4 by immunoblot (Fig. 3 B). We also localized expression of the AMPAR subunit GluR1 to the platelet surface using flow cytometry (FACS) with an antibody specific for an external region of GluR1 (Fig. 3 C). These data demonstrate that platelets express the AMPAR.

To define AMPAR's potential role as a mediator of platelet activation, we incubated PRPs with control or AMPA (0.1 – 1 mM) and activated the platelets with TRAP. Similar to glutamate, pretreatment of platelets with AMPA has no affect on resting platelets, but dose dependently increases agonist-induced platelet activation (Fig. 4 A). AMPA also increases platelet activation in response to U46619 (Fig. S1 C).

We next used additional pharmacological approaches to demonstrate signaling through the AMPAR and its potential as a target for antithrombotic therapy. First, we incubated human platelets with control, the AMPAR inhibitor CNQX (100 μM), AMPA, or both, and then we measured platelet activation in response to TRAP. CNQX is an AMPAR inhibitor with some kainate receptor blocking, but has a 20–150-fold selectivity for AMPAR subunits versus kainate receptor subunits. As before, AMPA increases TRAP-induced PAC-1 antibody binding (Fig. 4 B). CNQX inhibits TRAP-induced

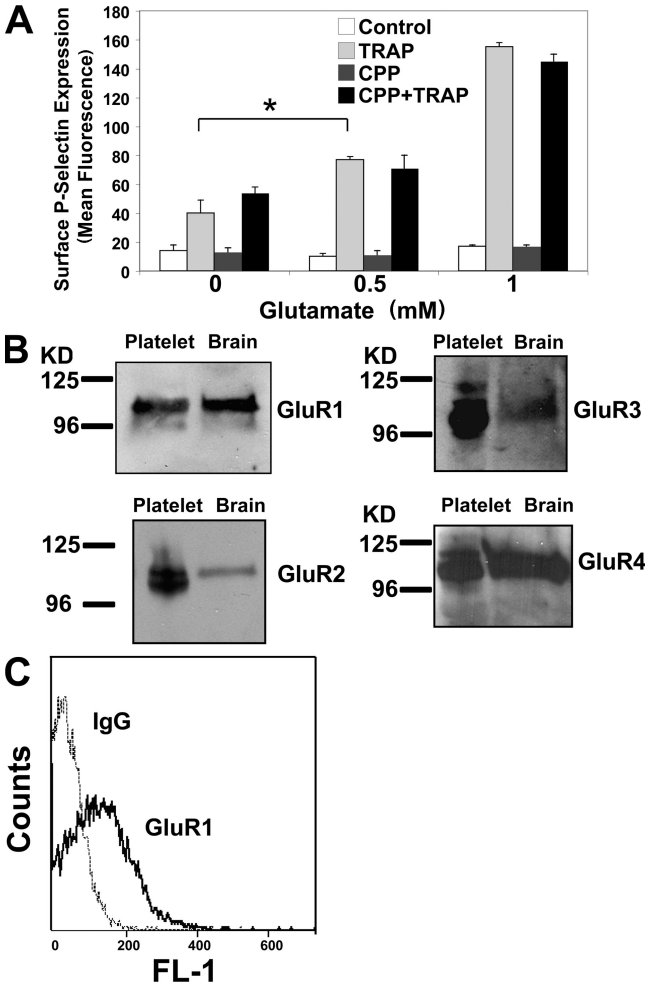

Figure 3. Platelets express the AMPAR. (A) Glutamate does not act through the NMDA receptor. Platelets were incubated with control, glutamate, or glutamate after the NMDA receptor inhibitor CPP, and then activated or not with TRAP. $n = 3 \pm$ the SD. *, $P < 0.05$ versus 0 mM. (B) Platelets express AMPAR subunits. Human platelet and mouse brain lysates were immunoblotted for AMPAR subunit proteins GluR1–4. (C) GluR1 is localized to the surface of platelets. Platelets were incubated with control IgG or GluR1 antibody and surface expression assayed by FACS (representative of three experiments with similar results).

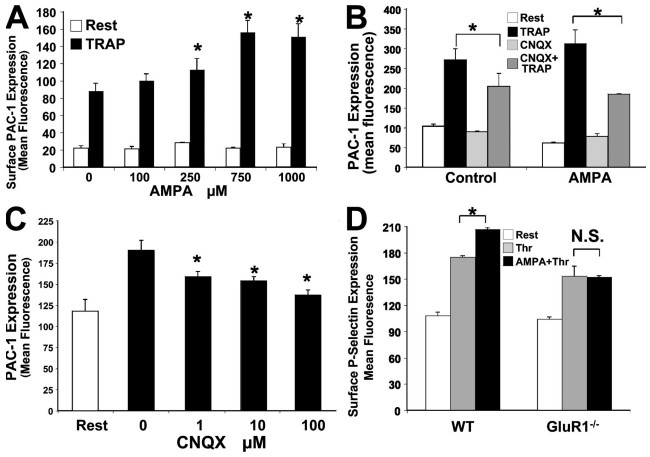

Figure 4. AMPAR signaling mediates platelet activation. (A) AMPA dose dependently increases agonist-induced platelet activation. Platelets were incubated with AMPA and activated with TRAP. Platelet activation was measured by FACS analysis for surface PAC-1 antibody binding. $n = 5 \pm$ the SD. *, $P < 0.05$ versus 0 mM. (B) AMPAR antagonist inhibits AMPA-mediated increase in platelet activation. Platelets were incubated with control, CNQX, AMPA, or AMPA after CNQX, and then activated with TRAP. $n = 3 \pm$ the SD. *, $P < 0.05$. (C) AMPAR antagonist decreases platelet activation. Platelets were incubated with control or CNQX, and then activated or not with TRAP. $n = 5 \pm$ the SD. *, $P < 0.05$. (D) GluR1$^{-/-}$ platelets do not respond to AMPA. Platelets from WT and GluR1$^{-/-}$ mice were incubated with control or AMPA, and activated with 0.25 U/ml thrombin (Thr). $n = 3 \pm$ SD. *, $P < 0.05$.

platelet activation and inhibits the effects of AMPA upon platelet stimulation (Fig. 4 B). Furthermore, as little as 10 μM of CNQX significantly decreases platelet activation, likely by inhibition of released endogenous glutamate activities, indicating an autocrine role of glutamate in platelet activation (Fig. 4 C and Fig. S1 D). Other AMPAR inhibitors, such as NBQX, have similar effects (Fig. S1 E).

The functional relevance of platelet AMPAR was explored by isolating platelets from WT and GluR1 subunit knockout mice (GluR1$^{-/-}$). Platelets from each type of mouse were incubated with vehicle or AMPA (1 mM) and activated or not activated with thrombin (0.25 U/ml). Unstimulated platelets from WT and GluR1$^{-/-}$ mice have similar levels of activation, as measured by P-selectin localized to the platelet surface membrane (Fig. 4 D). However, WT platelets stimulated with thrombin have a greater increase in P-selectin externalization than GluR1$^{-/-}$ platelets. Furthermore, AMPA increases activation of WT platelets, but not GluR1$^{-/-}$ platelets (Fig. 4 D).

Collectively, these data demonstrate that AMPAR signaling increases platelet activation.

Platelet AMPAR signaling induces sodium influx and platelet depolarization

In the open state, AMPARs are permeable to Na^+, and depending on its subunit isoform composition, can also be selectively permeable to Ca^{2+}. To determine if platelet AMPAR increases Na^+ influx into platelets, we preincubated platelets with the Na^+-sensitive dye sodium-binding benzofuran isophthalate (SBFI) and added control, AMPA (1 mM), CNQX (100 μM), or both. Platelets were then activated or not activated with TRAP. AMPA increases intracellular Na^+ concentration by approximately twofold upon activation, as compared with control (Fig. 5 A). CNQX not only decreases Na^+ concentrations in platelets treated with both AMPA and TRAP, but CNQX also decreases Na^+ levels in platelets treated with TRAP alone (Fig. 5 A). These data demonstrate that the AMPAR mediates an increase in intracellular Na^+.

To confirm that the AMPAR mediates Na^+ influx independent of the NHE, we inhibited the NHE with 100 μM amiloride (Fig. S1 F), and activated platelets or not with TRAP.

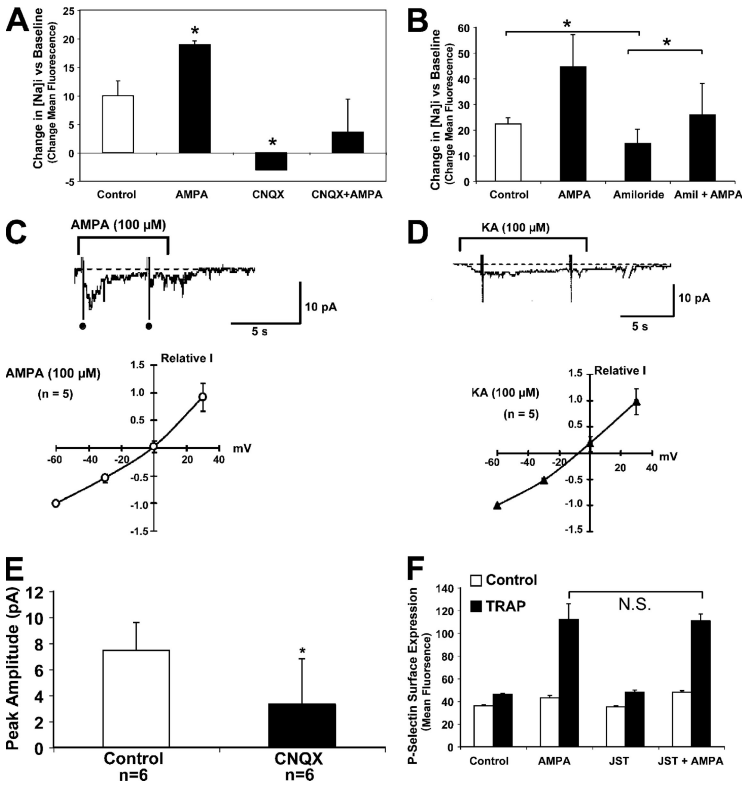

Figure 5. Platelet AMPAR mediates sodium transport. (A) AMPA increases intracellular sodium. Platelets were loaded with SBFI; treated with control, AMPA, CNQX, or AMPA after CNQX; and activated with TRAP. Change in intracellular sodium was measured by FACS. $n = 3 \pm$ the SD. *, $P < 0.05$ versus control. (B) AMPAR increases Na^+ influx independent of NHE. Platelets were treated with control, amiloride, AMPA, or amiloride before AMPA. Intracellular Na^+ was determined by FACS. $n = 3 \pm$ the SD. *, $P < 0.05$. (C) Fast application of AMPA to megakaryocytes induces a rapid inward current that reverses close to 0 mV. (top) A current trace showing an inward current induced by fast application of AMPA (time of application shown by the solid line). Rapid current steps for determining I–V relationships were applied before (not depicted) and during application of AMPA (rapid current deflection, black dots). (bottom) The I–V relationship obtained for the AMPA-induced current. The plot represents the averaged I–V curves obtained from 5 different cells, normalized to the current obtained at −60 mV. (D) Fast application of kainate to megakaryocytes induced a rapid inward current that reversed close to 0 mV. (top) A current trace and the averaged I–V curves for kainate application are shown on the bottom. (E) The inward current induced by 100 μM AMPA was blocked by administration of the AMPAR antagonist CNQX (30 μM; ± the SD) *, $P < 0.05$. (F) JST had no effect on the AMPA- triggered increase in platelet activation. Platelets were incubated with 1 μM JST and activated or not with TRAP. $n = 3 \pm$ the SD.

Inhibition of the NHE reduces Na^+ influx and platelet activation in response to TRAP (Fig. 5 B and Fig. S1 G). However, adding AMPA after amiloride restores Na^+ influx and platelet activation, again suggesting that the AMPAR can act as a Na^+ channel in platelet activation (Fig. 5 B).

To further characterize the signaling induced by platelet AMPAR activation, we performed electrophysiological recordings from bone marrow–isolated megakaryocytes. Whole-cell recordings showed that rapid application of AMPA (0.1 or 1 mM; $n = 7$; not depicted) or kainate (kainate; 0.1 or 1 mM; $n = 3$) did not induce a measurable current. In contrast, when the desensitization blocker cyclothiazide was included in the recording solution, a small but reproducible inward current was readily observed after rapid perfusion of AMPA (100 μM; 12.9 ± the SEM 7.1 pA; $n = 9$; Fig. 5 C) and kainate (100 μM; 4.8 ± SEM 1.4 pA; $n = 7$; Fig. 5 D). Whereas the inward current induced by kainate was of sustained amplitude throughout the duration of the application, that induced by AMPA was typically of slightly greater amplitude at the beginning of the application and subsided to a steady-state value (Fig. 5 C). To further demonstrate that the current induced by AMPA was mediated by activation of AMPARs, bone marrow megakaryocytes were isolated and electrophysiologic recordings performed in parallel using cells from a single megakaryocyte isolation. Administration of the AMPAR antagonist CNQX (30 μM) blocks the AMPA-induced current ($n = 6$; Fig. 5 E and Fig. S1 J).

We next sought to determine the reversal potential of the current induced by AMPA and kainate by determining the current–voltage relationship of the agonist-induced inward current. In the case of AMPA application, it was somewhat difficult to reliably and reproducibly match the voltage steps with the short-lived peak current obtained at the very beginning of the application (Fig. 5 C). As a consequence, I–V curves were constructed from the delayed, steady-state part of the AMPA-induced current. In these conditions, the current induced by both AMPA ($n = 5$) and kainate ($n = 5$) reversed at a potential close to 0 mV and did not show any obvious

rectification (Fig. 5, C and D). Together, the results from these electrophysiological recordings support our previous finding in showing that megakaryocytes express functional AMPARs on their surface. In addition, the linear current–voltage relationship suggests that these receptors are GluR2 subunit–containing AMPARs.

In addition to conferring a linear I–V relationship to the AMPAR complex, the incorporation of GluR2 subunit renders the AMPAR impermeable to calcium. Joro spider toxin (JST) allows pharmacological discrimination between these subtypes of AMPARs by selectively inhibiting GluR2 Ca^{2+}-permeable AMPARs (36). We next incubated platelets with 1 μM JST before AMPA. JST preincubation had no effect on AMPA-mediated increase in platelet activation (Fig. 5 F). Thus, these results suggest that platelet AMPARs are of the GluR2-containing, Ca^{2+}-impermeable AMPAR subtype.

Collectively, these data suggest that the AMPAR modulates platelet activation in part by regulating Na^+ influx.

AMPAR increases platelet activation in response to GPCR agonists

Others have demonstrated an increase in GPCR ligand affinity and activity subsequent to membrane depolarization (6–8). Glutamate increases both thrombin and thromboxane receptor (both GPCR) agonist-induced platelet activation. To determine if it similarly increases platelet activation in response to other GPCR agonists, platelets were incubated with glutamate (250 and 500 μM) before activation with epinephrine, which is a GPCR ligand, or collagen, which is a non-GPCR ligand. Glutamate significantly increases epinephrine-induced platelet activation (Fig. 6 A), but has no effect on collagen-induced platelet activation (Fig. 6 B). As further proof, we activated platelets with ATP in the presence of glutamate. ATP stimulates platelet $P2X_1$, which is a purine-dependent, ligand-gated ion channel for Ca^{2+} (37). ATP-mediated platelet activation is not increased by glutamate (Fig. 6 C). Platelet P2Y receptors are GPCR purine-dependent receptors, and activation of platelets by the P2Y-specific agonist 2-Me-ADP is increased by glutamate (Fig. 6 D). These data support the idea that platelet AMPAR activation increases GPCR ligand signaling activity.

AMPAR inhibition blocks aggregation and delays in vivo thrombosis

The functional relevance of targeting platelet AMPAR signaling was assayed by first measuring aggregation in response to TRAP (10 μM) after incubating platelets with control or CNQX (1–100 μM). CNQX dose dependently reduces platelet aggregation (Fig. 7, A and B). Exogenous AMPA (1 mM) was also added to platelets before aggregation. AMPA increases the rate of early aggregation. At 1 min after TRAP, control platelets are ~55% aggregated and AMPA-pretreated platelets are ~75% aggregated (Fig. 7 C). At later time points, the effect is less apparent (Fig. 7 C), which is likely a reflection of the large concentration of endogenous glutamate released into the growing platelet aggregate obscuring exogenous AMPA effects with time.

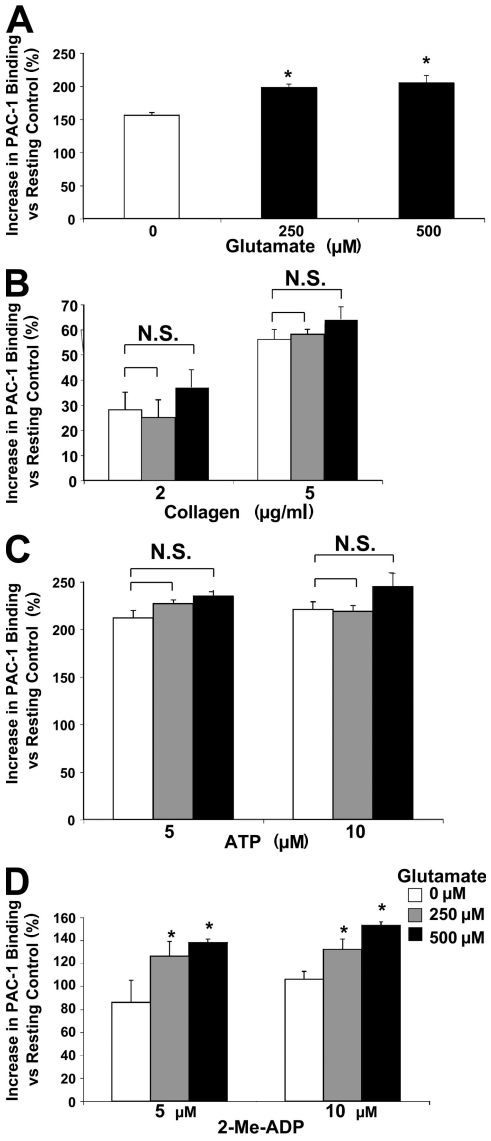

Figure 6. Glutamate mediates an increase in GPCR signaling. (A) Glutamate increases epinephrine-mediated platelet activation. Epinephrine-induced (10 μM) platelet activation was measured by PAC-1 antibody binding. $n = 5 \pm$ the SD. *, $P < 0.01$ versus 0 μM. (B) Glutamate does not effect collagen-mediated platelet activation. Platelets were incubated with glutamate and activated with collagen. Activation was measured by FACS analysis for surface PAC-1 antibody binding. $n = 5 \pm$ the SD. (C) Glutamate does not increase ATP-induced platelet activation. ATP induced platelet activation was measured by PAC-1 antibody binding. $n = 5 \pm$ the SD. (D) Glutamate increases P2Y-mediated platelet activation. The P2Y agonist 2-Me-ADP-induced platelet activation was measured by PAC-1 antibody binding. $n = 5 \pm$ the SD. *, $P < 0.01$ versus 0 μM.

These data demonstrate that platelet AMPAR presents a potential novel in vivo therapeutic target. We therefore performed in vivo studies to support this idea. We treated mice i.v. with control PBS or CNQX (0.1 mg/kg), and 20 min later, tail bleeding times were determined. Control mice have a mean bleeding time of ~200 s, with a cluster of mice that have very similar bleeding times (Fig. 8 A). AMPAR inhibitor–treated

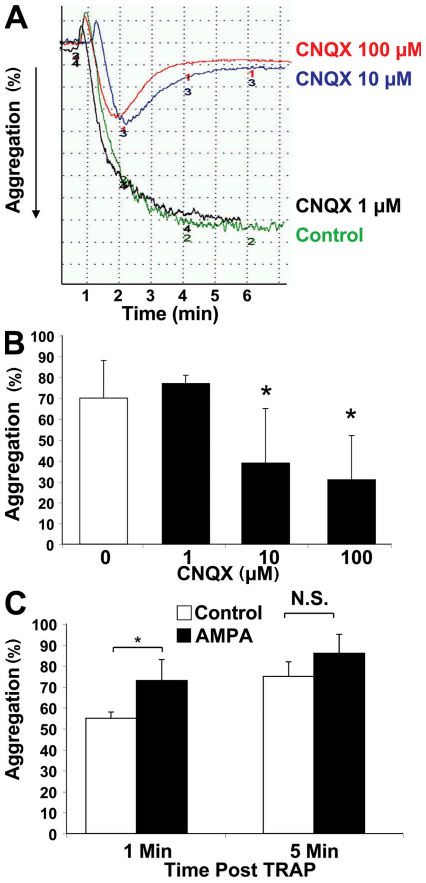

Figure 7. AMPAR signaling mediates platelet aggregation. (A) AMPAR inhibition decreases platelet aggregation. Platelets were incubated with control or CNQX and activated with 10 µM TRAP. Platelet aggregation was measured by an aggregometer. (B) Quantification. AMPAR inhibition decreases platelet aggregation. $n = 5–12 \pm$ the SD. *, $P < 0.01$. (C) Exogenous AMPA increases early platelet aggregation. $n = 5 \pm$ the SD. *, $P = 0.03$; NS, $P = 0.09$.

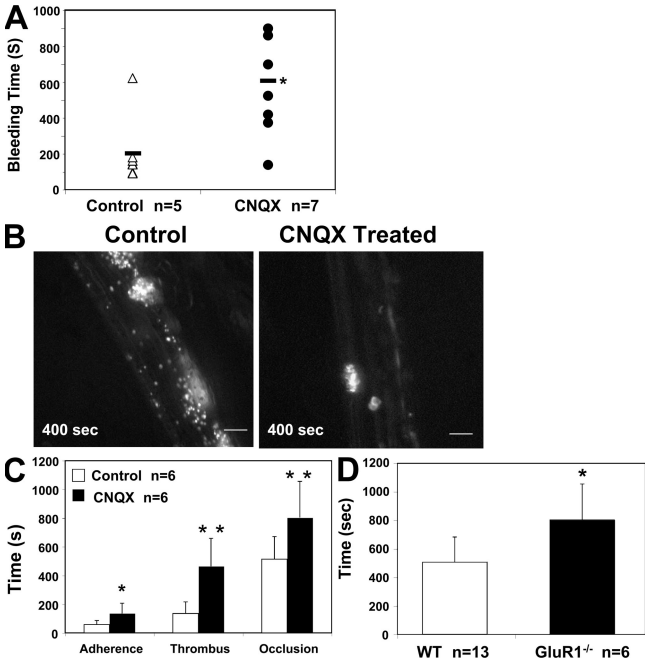

Figure 8. AMPAR mediates platelet function in vivo. (A) AMPAR inhibition with 0.1 mg/kg CNQX prolongs bleeding time in vivo. *, $P < 0.01$ versus control. (B) $FeCl_3$ was placed on externalized mesenteric arterioles, and platelets were imaged using intravital microscopy (representative images 400 s after $FeCl_3$). Bars, 25 µm. (C) AMPAR inhibition with 0.1 mg/kg CNQX prolongs platelet adherence, thrombus formation, and vessel occlusion in vivo. *, $P < 0.05$; **, $P < 0.01$ versus control. (D) GluR1$^{-/-}$ mice have prolonged time to vessel occlusion in vivo. *, $P < 0.02$ versus WT. Error bars represent the mean ± the SD.

mice have a prolonged mean bleeding time (~600 s) and a greater range of bleeding times (Fig. 8 A).

To confirm these data, we used intravital microscopy to examine the role of platelet AMPAR signaling in thrombus formation. Platelets were isolated from control mice, fluorescently labeled, and injected i.v. into recipient mice. Mice were then treated with CNQX or control PBS intravenously 20 min before mesentery externalization and $FeCl_3$ application. Images were collected to quantify in vivo platelet function (Fig. 8 B). Compared with PBS-treated mice, mice treated with CNQX have prolonged time to platelet adhesion (>10 platelets adherent for >10 s), thrombus formation (first stable thrombus >20 µm), and total vessel occlusion (Fig. 8 C).

To determine if mice lacking the AMPAR subunit GluR1 have in vivo changes in platelet function, isolated platelets from WT control or GluR1$^{-/-}$ mice were fluorescently labeled and injected into mice of the same genotype. Mesenteric arterioles were then damaged with $FeCl_3$, and thrombus formation was again imaged. Similar to mice treated with the AMPAR inhibitor, mice lacking GluR1 have prolonged time to vessel occlusion compared with WT control mice (Fig. 8 D).

DISCUSSION

The major findings of our study are that AMPARs modulate platelet activation and thrombosis. These results have important implications for thrombotic diseases such as stroke, where plasma glutamate levels can rise significantly (25, 27). After compromise of the blood–brain barrier, glutamate released by neurons or platelets may contribute to the pathophysiology of stroke, not only as an excitatory amino acid but also by acting as a prothrombotic messenger. The importance of these results is not limited to cerebral vascular disease. We show that large concentrations of glutamate are released locally in platelet aggregation, making glutamate signaling important in disease processes such as myocardial infarction and ischemia-reperfusion injury. AMPAR antagonists may therefore be useful in the treatment and prevention of stroke, myocardial infarction, and other thrombotic diseases.

We propose that glutamate contained within platelet-dense granules is released in high concentrations locally into "the platelet synapse" as platelets come into close apposition during thrombus formation. This glutamate released into the formed platelet synapse early in platelet activation acts in an

autocrine manner to promote complete platelet activation and in a paracrine fashion to rapidly increase the agonist sensitivity of recruited platelets. This helps drive and accelerate further thrombus formation. This model is supported by our data demonstrating that glutamate increases platelet sensitivity to low-dose agonist stimulation, that AMPAR antagonists inhibit platelet function, and that mice lacking the AMPAR subunit GluR1 have prolonged time to vessel occlusion. Our data does not rule out potential contributions of AMPARs expressed by other vascular cells, or the potential contribution of other platelet-expressed glutamate receptors, such as kainate receptors or metabotropic glutamate receptors. Taken as a whole, however, our ex vivo and in vivo data demonstrate an important role for platelet glutamate and AMPAR in platelet activation and thrombus formation.

Glutamate function in thrombosis is very similar to that of another neurotransmitter, serotonin (5-HT). At basal conditions, plasma 5-HT is relatively low and tightly regulated, but is rapidly released from dense granules upon platelet activation. Serotonin promotes continued platelet aggregation, as indicated by mice lacking the enzyme necessary for peripheral 5-HT production having prolonged time to vessel occlusion (38, 39). Similar to glutamate, 5-HT is a weak platelet agonist on its own and its effect is enhanced by the addition of other agonists (40). Serotonin reuptake inhibitors may provide myocardial infarction protection, but have not been extensively studied or used clinically as a preventative therapy (39).

We demonstrate that glutamate mediates an increase in platelet activation in response to the GPCR agonists thrombin, thromboxane, epinephrine, and ADP. However, glutamate has no effect on non-GPCR signaling pathways, such as through the collagen or P2X receptors. We also show that AMPAR signaling increases platelet Na^+ influx and induces an inward current that depolarizes megakaryocyte membranes. Membrane depolarization can lead to an increase in GPCR ligand affinity by creating a charge movement that increases G protein coupling with the GPCR (8, 37). Platelet G protein receptors contain positively charged internal amino acid regions that are possible voltage-sensing domains, providing further support to our hypothesis. Alternatively, independent of a potential depolarization function, Na^+ is an important ion in platelet activation. A rapid increase in intracellular Na^+ is necessary to drive the Na^+/Ca^{2+} exchanger and promote calcium-mediated events in platelet activation. The primary function of platelet AMPAR may be simply as a rapid sodium channel. Future studies are needed to determine the key regions of platelet GPCRs that AMPAR signaling and membrane depolarization may act upon to increase platelet activation.

Our electrophysiological studies support our data by showing that megakaryocytes express functional, most likely GluR2-containing, Ca^{2+}-impermeable AMPARs. Notably, the inward current induced by AMPA application in megakaryocytes was of modest amplitude and only observed in the presence of a desensitization blocker, cyclothiazide. This contrasts with neurons where robust AMPAR-mediated inward currents are readily observed in the absence of cyclothiazide. Microglial cells, however, are similar to megakaryocytes in that AMPAR-mediated inward currents are only observed in the presence of cyclothiazide (41). Several mechanisms may account for these cell type–specific biophysical properties of AMPARs. For instance, transmembrane AMPAR-regulating proteins are important modulators of AMPAR functions, and their differential expression among cell types is expected to translate into different functional properties of AMPARs (35, 42). Future studies will be required to directly address this possibility.

There have been few reports of the NMDA receptor on platelets and megakaryocytes (20–23). Signaling through the NMDA receptor has been demonstrated to increase platelet production from megakaryocytes (20, 23), and interestingly, NMDA receptor signaling in platelets has been shown to inhibit platelet aggregation (21, 22). We now show that glutamate-mediated increase in platelet activation is independent of the NMDA receptor (Fig. 3 A). Prior NMDA receptor studies primarily used aggregation as the measure of platelet function, but endogenous platelet glutamate must be taken into account. We demonstrate that a substantial concentration of glutamate is released in the developing thrombus that reaches peak levels very rapidly (Fig. 1), and in our studies, exogenous glutamate or AMPA addition has no effect on final aggregation, likely because the rapid release of endogenous glutamate obscures any effect. However, the NMDAR studies do raise important questions for future research. NMDAR signaling in neurons is a major means to regulate AMPAR trafficking by inducing an influx of Ca^{2+} that initiates the delivery of AMPARs from the recycling endosome to the surface membrane. Prior NMDAR studies in platelets demonstrate that NMDA induces an increase in Ca^{2+} influx, and as a result may mediate an increase in AMPAR surface localization (21, 22).

Other glutamate regulatory elements in platelets, such as the glutamate transporters (EAATs), have primarily been studied as peripheral markers of CNS disease. However, EAATs and other glutamate receptors may also have an important role in normal platelet function by controlling the plasma glutamate concentration. Individuals with polymorphisms in EAAT-2 have increased plasma glutamate concentrations with a higher frequency of early neurological worsening in stroke (27). It is interesting to consider that these individuals may also be at increased risk of stroke, or that increased neurological decline may be secondary to continued platelet activation and increased infarct size. Further study is needed to fully appreciate glutamate in the periphery and its relevance to thrombosis.

MATERIALS AND METHODS

Reagents

L-glutamate, AMPA, CPP, ADP, CNQX:HBC, mouse thrombin, choline chloride, amiloride, U46619, and JST were purchased from Sigma-Aldrich. Antibody to GluR1 was purchased from Calbiochem; antibodies to GluR2, GluR3, and GluR4 were purchased from Santa Cruz Biotechnology. PAC-1 and P-Selectin antibodies were purchased from BD Biosciences. FITC-IgG secondary antibody for FACS, SBFI, and BCEF were purchased from Invitrogen. RT-PCR reagents were purchased from Invitrogen. TRAP-6 was purchased from Bachem.

Platelet isolation and ex vivo experiments

Human platelets were isolated from healthy volunteers who had not taken aspirin or nonsteroidal antiinflammatory drugs for 10 d, under a protocol approved by the Johns Hopkins University School of Medicine Institutional Review Board. Blood was collected into citrate anticoagulant, and platelets were isolated as PRPs by centrifugation at 180 g for 15 min and diluted in Tyrode's buffer at a dilution of 1:20 for activation flow cytometry studies. To determine surface GluR1, a Fc blocking antibody was added to reduce nonspecific background antibody binding. Mouse platelets were isolated by collection into heparinized Tyrode's buffer and isolated by centrifugation. Washed platelets were resuspended in Tyrode's.

Intracellular ion concentrations were performed after incubating platelets diluted 1:20 in Tyrode's buffer with SBFI for 45 min before experiments.

Glutamate concentration

Whole blood was isolated as described in the previous section and diluted 1:1 in Tyrode's buffer. A 1-mM glutamate-sensitive probe (Pinnacle Technology, Inc.) was used to determine real-time glutamate concentrations. The glutamate biosensor relies on the glutamate oxidase–catalyzed conversion of glutamate to α-ketoglutaric acid and hydrogen peroxide. The enzymatically produced hydrogen peroxide is then ampomerically detected by its oxidation at the probe's platinum-iridium electrode with an applied potential of 600 mV, with high sensitivity to glutamate at low (micromolar) concentrations, producing a linear response over a wide concentration range. The rapid response (1–4 s) is recorded using probe-specific software to monitor the time course of a physiological glutamate release. Probe calibration was performed by incubating the probe in a gradient of glutamate concentrations. Voltage output and glutamate concentration were then correlated. While stirring, the biosensor probe was placed in stirring blood and voltage recordings were initiated. Thrombin was added to induce platelet activation and aggregation.

Immunoblotting

Platelets purchased from HemaCare and rat brain cerebellar lysate were lysed with NP-40 lysis buffer, and supernatants were fractionated on a 4–15% gel. Membrane-transferred proteins were immunoblotted with antibodies against GluR1-4.

In vivo studies

Mouse experiments were performed as approved by the Johns Hopkins University School of Medicine Animal Care and Use Committee.

Tail bleeding

6-wk-old male mice were anesthetized with ketamine and xylazine (80/13 mg/kg) and injected i.v. with either PBS or CNQX:HBC, with 0.1 mg/kg of active CNQX. 20 min later, the distal 3 mm of the tail was amputated and immersed in 37°C saline, and the time to visual cessation of bleeding was recorded.

Intravital microscopy

Platelets were isolated from mice as above and resuspended in Tyrode's buffer at a concentration of 10^8/100 μl, fluorescently labeled with 10 μM calcein-AM, and 100 μl was injected intravenously into a mouse anesthetized with ketamine and xylazine. The mesentery was externalized, thrombosis was initiated by the addition of a 5-mm^2 piece of Whatmann's paper soaked in 10% FeCl3, and thrombosis was recorded using a digital imaging camera and software (QCapture Pro; Retiga). GluR1$^{-/-}$ mice, on a C57Bl6/J background, were a gift from R. Sprengel (University of Heidelberg, Germany).

Electrophysiological experiments

Cell preparation and solutions. All procedures using animals were approved by the University of Miyazaki Institutional Animal Care and Use Committee. Megakaryocyte preparation was performed as previously reported (43). In brief, mouse bone marrow was flushed with Na^+-rich external solution (Na^+-ES, 150 mM NaCl, 1 mM $CaCl_2$, and 10 mM Hepes, pH adjusted to 7.2 using NMDG-OH). Cells were dispersed by repetitive pipetting, washed three times by gentle centrifugation, resuspended in Na^+-ES, and used within 90 min of isolation.

Electrophysiological studies. Whole-cell recordings were obtained from freshly isolated megakaryocytes using an intracellular solution of the following composition: 150 mM CsCl, 1 mM EGTA, and 10 mM Hepes. The pH was adjusted to 7.3 using NMDG-OH. The patch-clamp procedures were similar to those previously described (43). In brief, the experiments were performed in the tight-seal whole-cell configuration at room temperature, and electrical signals were acquired with an EPC-7 (HEKA). The pipette resistances ranged between 2 and 3 MΩ.

Current–voltage relationships (I–V curves) were obtained by clamping the cell to different potentials in 30-mV increments (−30, 0, and + 30 mV; each for 35 ms) from a holding potential of −60 mV. I–V curves obtained before the application of the agonists were subtracted from those (typically two) obtained in the presence of the agonists. The current responses were monitored on a chart recorder (WR7700; Graphtec) through a low-pass filter (200 Hz). Chemicals and reagents used were purchased from either Sigma-Aldrich or Wako Pure Chemicals.

Rapid perfusion of AMPA and Kainate was achieved by puff application from a nearby pipette of 30–35 μm diam to an isolated single megakaryocyte. The perfusion pipette was located within 30 μm of the cell, and the puff pressure was adjusted to obtain rapid and effective agonist application (< 10 ms).

Fig. 5 E was performed using two bathing chambers (test and control) placed on the stage of an inverted microscope. One chamber contained a megakaryocytes immersed in Na^+-ES–containing CNQX (test chamber), and the other contained the same batch of megakaryocytes immersed in Na^+-ES alone (control chamber). Traces used in the I–V curve were those which had good signal-to-noise ratio during the voltage steps.

Data analysis

Data are expressed as the mean ± the SD, unless otherwise stated. Statistical comparisons between two groups were performed using the Student's *t* test.

Online supplemental material

Fig. S1 shows glutamate probe calibration (A), data related to glutamate stimulation of platelet activation (B and C), AMPAR antagonist data (D and E), amiloride blocking of NHE (F and G), and inward current tracings (H–J). The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20071474/DC1.

This work was supported by a National Institutes of Health (NIH) grant to C.N. Morrell (5K08HL074945) and grants to C.J. Lowenstein from NIH (P01 HL56091, R01 HL074061, R01 HL78635, and P01 HL65608), the American Heart Association (EIG 0140210N), the Ciccarone Center, the John and Cora H. Davis Foundation, and the Clarence P. Doodeman Professorship in Cardiology.

All authors have no conflicting financial interests.

Submitted: 17 July 2007
Accepted: 24 January 2008

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