P2X$_1$-mediated ERK2 Activation Amplifies the Collagen-induced Platelet Secretion by Enhancing Myosin Light Chain Kinase Activation*

Emese Toth-Zsamboki‡§¶, Cécile Oury‡§‖, Heidi Cornelissen‡, Rita De Vos**, Jos Vermylen‡, and Marc F Hoylaerts‡ ‡‡

*From the ‡Center for Molecular and Vascular Biology and **Laboratory of Morphology and Molecular Pathology, University of Leuven, B-3000 Leuven, Belgium*

The ATP-gated P2X$_1$ ion channel is the only P2X subtype expressed in human platelets. Via transmission electron microscopy, we found that P2X$_1$ mediates fast, reversible platelet shape change, secretory granule centralization, and pseudopodia formation. In washed human platelets, the stable P2X$_1$ agonist α,β-methylene ATP (α,β-meATP) causes rapid, transient (2–5 s), and dose-dependent myosin light chain (MLC) phosphorylation, requiring extracellular Ca^{2+}. Phosphorylation was inhibited by the calmodulin (CaM) inhibitor W-7, but not by the Rho kinase inhibitor HA-1077, *i.e.* it is exclusively regulated by Ca^{2+}/CaM-dependent MLC kinase. Correspondingly, the P2X$_1$-induced platelet shape change was inhibited by W-7 and by the MLC kinase inhibitor ML-7 but not by HA-1077. W-7, ML-7, the protein kinase C inhibitor GF109203-X, and the Src family kinase inhibitor PP1 inhibited the collagen and convulxin-induced early platelet degranulation, shape change, and subsequent aggregation, indicating a role for Ca^{2+}/CaM and MLC kinase in these glycoprotein VI-related platelet responses. The secreted ATP-mediated P2X$_1$-dependent ERK2 activation induced by low collagen concentrations contributes to MLC kinase activation since P2X$_1$ desensitization or blockade of ERK2 phosphorylation by U0126 strongly attenuated MLC phosphorylation, degranulation, and aggregation. We therefore conclude that at low doses of collagen, glycoprotein VI activation leads to early protein kinase C- and MLC kinase-dependent degranulation. Rapidly released ATP triggers P2X$_1$-mediated Ca^{2+} influx, activating ERK2, in turn amplifying platelet secretion by reinforcing the early MLC kinase phosphorylation. Hence, the P2X$_1$-ERK2-MLC axis contributes to collagen-induced platelet activation by enhancing platelet degranulation.

P2X receptors are oligomeric non-selective ATP-gated cation channels expressed in many excitable and non-excitable cells, where they mediate a variety of physiological processes including central and peripheral neurotransmission, smooth muscle contraction, and hormone secretion (1, 2). Seven P2X receptor subtypes have been identified so far, but relatively little is known about the intracellular signaling pathways subserving their biological actions.

The ATP-gated P2X$_1$ receptor is a rapidly desensitizing, non-selective cation channel, mediating fast non-selective influx of Na$^+$ and Ca^{2+} ions across the cell membrane, resulting in cell depolarization (3). The direct influx of extracellular Ca^{2+} through this channel rapidly increases intracellular levels and regulates cellular processes as diverse as muscle contraction, fertilization, cell proliferation, vesicular function, and apoptosis (4). As Ca^{2+} enters the cytosol, it interacts with calmodulin (CaM).[1] Upon Ca^{2+} binding, CaM changes its conformation, induces dimerization and active site remodeling, and displaces the auto-inhibitory domains of many different target proteins (5).

In response to specific agonists, platelets undergo morphological alterations known as shape change, secrete the contents of their granules, and aggregate (6). These processes require reorganization of the platelet cytoskeletal structure and therefore involve Ca^{2+}/CaM-regulated pathways. Hence, phosphorylation of the myosin light chain (MLC) by the Ca^{2+}/CaM-dependent MLC kinase is considered a key step in platelet activation (7, 8), indicating that platelet actomyosin is regulated similarly to that found in smooth muscle. Phosphorylation of the regulatory light chain subunit of myosin at serine 19 results in a marked increase of actin-activated myosin ATPase activity, leading to attachment of myosin to actin via cross-bridging (9). These events result in the assembly of actin microfilaments, folding of the cell membrane, and a contractile wave centralizing the platelet secretory granules.

Rho kinase is also implicated in the regulation of MLC phosphorylation (10, 11). Activated by the small GTP-binding protein RhoA, Rho kinase phosphorylates the myosin-binding subunit of myosin phosphatase and inhibits its activity. Rho kinase also directly phosphorylates the myosin light chain, activating myosin *in vitro*, and induces smooth muscle contraction in the absence of Ca^{2+}. Both the Rho/Rho kinase and Ca^{2+}/CaM-regulated MLC kinase pathways mediate MLC phosphorylation independently of each other in human platelets (11, 12) as a function of the agonist applied.

[1] The abbreviations used are: CaM, calmodulin; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; PKC, protein kinase C; PRP, platelet-rich plasma; PLC, phospholipase C; α,β-meATP, α,β-methylene ATP; GPVI, glycoprotein VI.

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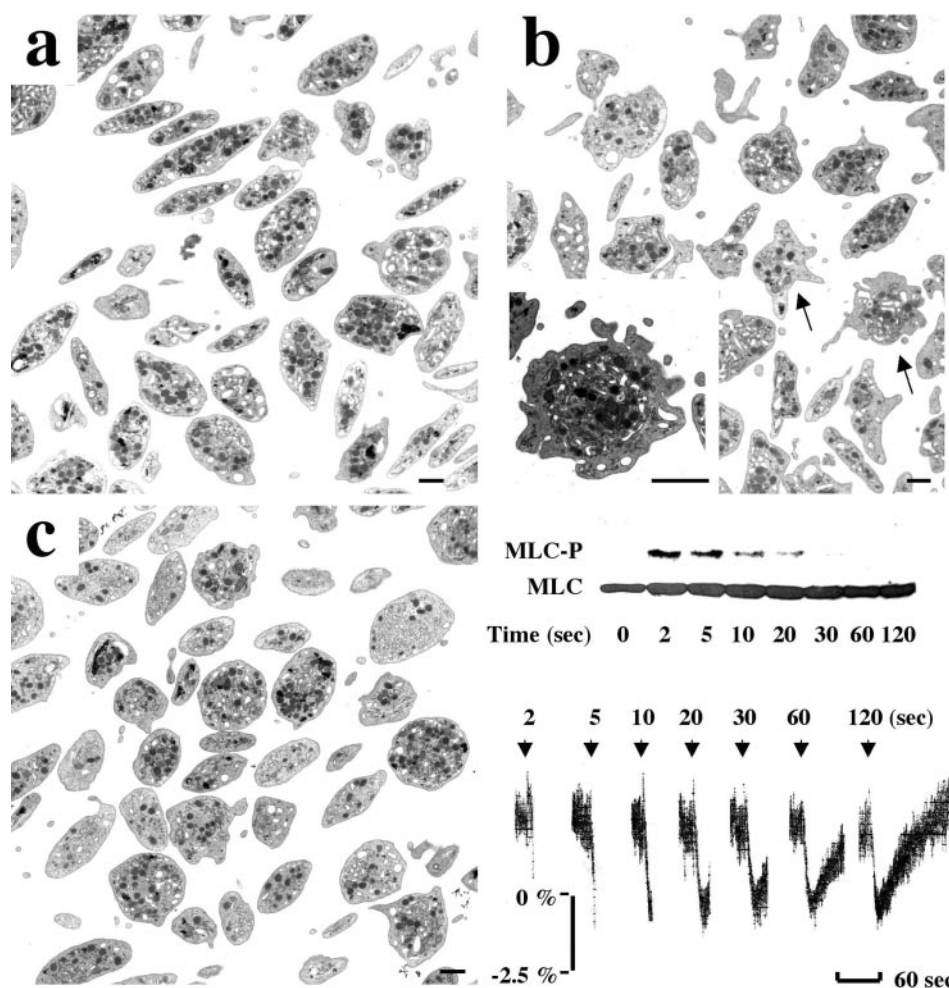FIG. 1. **P2X₁-mediated platelet shape change.** Transmission electron microscopy of resting platelets (*a*) and platelets activated with 2.5 μM α,β-meATP, fixed 25 s after exposure (*b*) or 90 s after exposure (*c*) is shown. The *inset* shows centralization of platelet granules; other examples are indicated by *arrows*. *Scale bar* is 1 μm. Also shown is the time course of MLC phosphorylation (*MLC-P*) following activation of washed platelets with 2.5 μM α,β-meATP, as a function of time, when compared with the total MLC levels indicated in the *lower lanes*; phospho-MLC and control MLC (*lower panels*) antibodies were used. For the shape change studies, *arrows* represent the moment of agonist application; moments of sampling for phosphorylation studies and corresponding light transmission tracings and scales are indicated.

P2X₁ is highly expressed in human platelets and megakaryocytes (13, 14); because of the role of Ca^{2+}/CaM in platelet MLC kinase activation, we have presently investigated the P2X₁-mediated Ca^{2+} influx in relation to MLC phosphorylation and platelet shape change. We have previously shown that the P2X₁-mediated Ca^{2+} influx triggers phosphorylation of the extracellular signal-regulated kinase (ERK2), a reaction found to contribute to platelet activation and secretion induced by low concentrations of collagen (15, 16). We therefore have also investigated the relation between MLC kinase and ERK2 activation during collagen-induced platelet activation, in relation to P2X₁-mediated Ca^{2+} influx.

Activation of platelets by collagen triggers immediate phospholipase Cγ2-dependent MLC phosphorylation in a Ca^{2+}/CaM-regulated manner, an event instrumental in the early dense granule release. This degranulation is necessary to complete platelet aggregation via amplification pathways, activated by released ADP and ATP. We presently found that released ATP-triggered P2X₁ activation results in Ca^{2+}/CaM-dependent phosphorylation of ERK2, which in turn intensifies the ongoing activation of MLC kinase. Hence, our work uncovers a central link between ERK2 activation and MLC phosphorylation during collagen-induced platelet activation, secretion, and aggregation.

EXPERIMENTAL PROCEDURES

Materials—The stable, specific P2X₁ receptor agonist α,β-meATP, the inositol 1,4,5-trisphosphate receptor antagonist 2-aminoethoxydiphenyl borate (2-APB), and the ectonucleotidase apyrase (grade I) were purchased from Sigma. The α,β-meATP was purified by high pressure liquid chromatography on a Adsorbosphere HS C18, 7-μm, 250 × 4.6-mm column (Alltech, Bad Segeberg, Germany) (15, 16). Purified samples, lyophilized and adjusted to pH 7.0 with potassium phosphate buffer, were kept at −80 °C and were stable over the time interval studied. The Rho kinase inhibitor HA-1077, the calmodulin inhibitor W-7, the MLC kinase inhibitor ML-7, and the Src family kinase inhibitor PP1 were purchased from Calbiochem. The protein kinase C (PKC) inhibitor GF109203-X and the MEK1/2 inhibitor U0126 were from BioMol Research Laboratories (Plymouth Meeting, MA). Collagen (collagen reagent Horm) was from Nycomed (Munich, Germany); the purified snake venom toxin, convulxin, was obtained through Dr. K. J. Clementson (Theodor Kocher Institute, University of Berne, Berne, Switzerland). Hirudin (lepirudin) was from Hoechst, Frankfurt, Germany.

Preparation of Platelet-rich Plasma, Washed Platelets, and Shape Change Analyses—Platelet-rich plasma (PRP) was prepared from fresh blood, taken on acid citrate dextrose (93 mM sodium citrate, 7 mM citric acid, 0.14 mM dextrose, pH 6.5) as an anticoagulant, supplemented with 1 unit/ml apyrase (grade I), by double centrifugation for 15 min at 150 × *g*. PRP was diluted 3-fold with acid citrate dextrose containing 1 unit/ml apyrase, and platelets were pelleted at 800 × *g* for 10 min. Washed platelets were resuspended in Tyrode's buffer (137 mM NaCl, 12 mM NaHCO₃, 2 mM KCl, 0.34 mM Na₂HPO₄, 1 mM MgCl₂, 5.5 mM glucose,
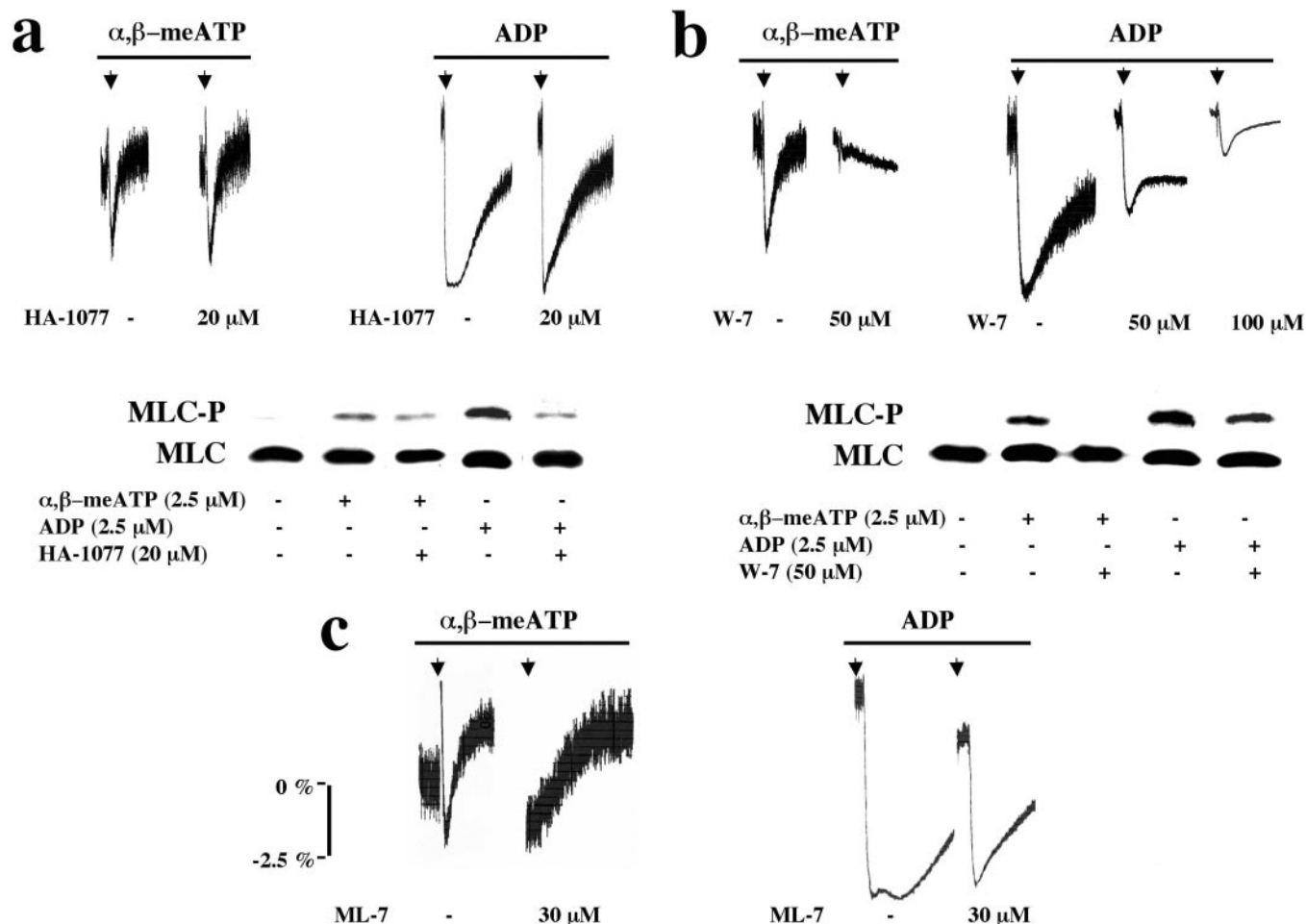
FIG. 2. **Mechanism of P2X$_1$-mediated platelet shape change.** Inhibition of the α,β-meATP- and ADP-induced shape change and MLC phosphorylation (*MLC-P*) by the specific Rho kinase inhibitor HA-1077 (*a*), the specific calmodulin antagonist W-7 (*b*), or the MLC kinase inhibitor ML-7 (*c*) is shown. The MLC phosphorylation in stimulated washed platelets is shown for the indicated concentrations of α,β-meATP and ADP after preincubation with the indicated concentrations of inhibitor. *Arrows* represent the moment of agonist application.

and 5 mM HEPES, pH 7.3) containing 2 units/ml apyrase at a density of $2.5–3.5 \times 10^5$ platelets/μl. High concentrations of apyrase were needed to fully recover P2X$_1$ functionality in the final platelet suspension. Platelet shape change was detected as a decrease in light transmission in an ELVI 840 aggregometer with 5-fold amplification of the signal. CaCl$_2$ (2 mM) was added prior to the recordings; aliquots of washed platelets were preincubated with the inhibitors as indicated for 1 min prior to the addition of the agonist. The ADP-induced platelet shape change was analyzed in the presence of 1.25 μg/ml of the $\alpha_{IIb}\beta_3$ antagonist tirofiban (Merck) to avoid platelet aggregation. In each case, at least three independent experiments were performed on different individuals.

Platelet Aggregation and ATP Secretion Analyses—Light transmission during collagen- or convulxin-induced platelet aggregation was recorded in apyrase-treated washed platelets with a Chrono-Log Aggregometer. ATP secretion was monitored in washed platelets in parallel with platelet aggregation by adding firefly luciferase and luciferin and comparing the luminescence generated by platelet ATP release with an ATP standard (Chrono-Lume, Kordia, The Netherlands). At least three independent experiments were performed on platelets from different individuals.

Electron Microscopy—To preserve physiological Ca^{2+} levels, blood was taken on hirudin (20 μg/ml) and centrifuged at $150 \times g$ for 15 min to yield PRP. Platelet numbers in PRP were diluted to 250,000/μl with autologous platelet-poor plasma. PRP (300 μl) was then stirred at 1000 rpm and stimulated with 2.5 μM α,β-meATP for 25 and 60 s and immediately fixed at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, at pH 7.2 overnight, after which centrifugation at $800 \times g$ for 10 min yielded a condensed platelet pellet. After postfixation in 1% OsO$_4$, 0.1 M phosphate buffer (pH 7.2), and dehydration in graded series of ethanol, these pellets were embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined in

a Zeiss FM 10 electron microscope (Oberkochen, Germany).

Western Blot Detection of Myosin Light Chain and ERK2 Phosphorylation—Aliquots of washed platelets (300 μl) were incubated in an aggregometer cuvette with the agonists under stirring at 37 °C. Platelet activation was stopped by adding 100 μl of 4× concentrated SDS sample buffer at different time points, as indicated. Samples were boiled for 5 min and loaded on a 12% SDS-PAGE gel. After electrophoretic transferal of the proteins to nitrocellulose, membranes were blocked in Tris-buffered saline-Tween-milk buffer and incubated overnight with the appropriate primary antibodies: anti-phospho-MLC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or the monoclonal anti-phospho-p44/42 MAP antibody (New England Biolabs). After adding horseradish peroxidase-conjugated secondary antibodies, the immunoreactive bands were visualized by ECL (Amersham Biosciences). For detection of total proteins, membranes were stripped and reprobed with anti-MLC and p44/42 antibodies. At least three independent platelet extracts from different individuals were analyzed.

RESULTS

P2X$_1$ Stimulation Leads to Myosin Light Chain Phosphorylation—The stable P2X$_1$-specific agonist α,β-meATP causes a fast and dose-dependent, but reversible, platelet shape change (17, 18). Analysis by transmission electron microscopy of this transient platelet shape change (Fig. 1, *a–c*) shows extremely rapid pseudopodia formation but also illustrates in some platelets a fast and reversible centralization of secretory granules, in addition to some platelet sphering. These findings indicate that the α,β-meATP-mediated Ca^{2+} influx suffices to cause functionally relevant morphological changes in platelets, without inducing aggregation or secretion itself. When measured in
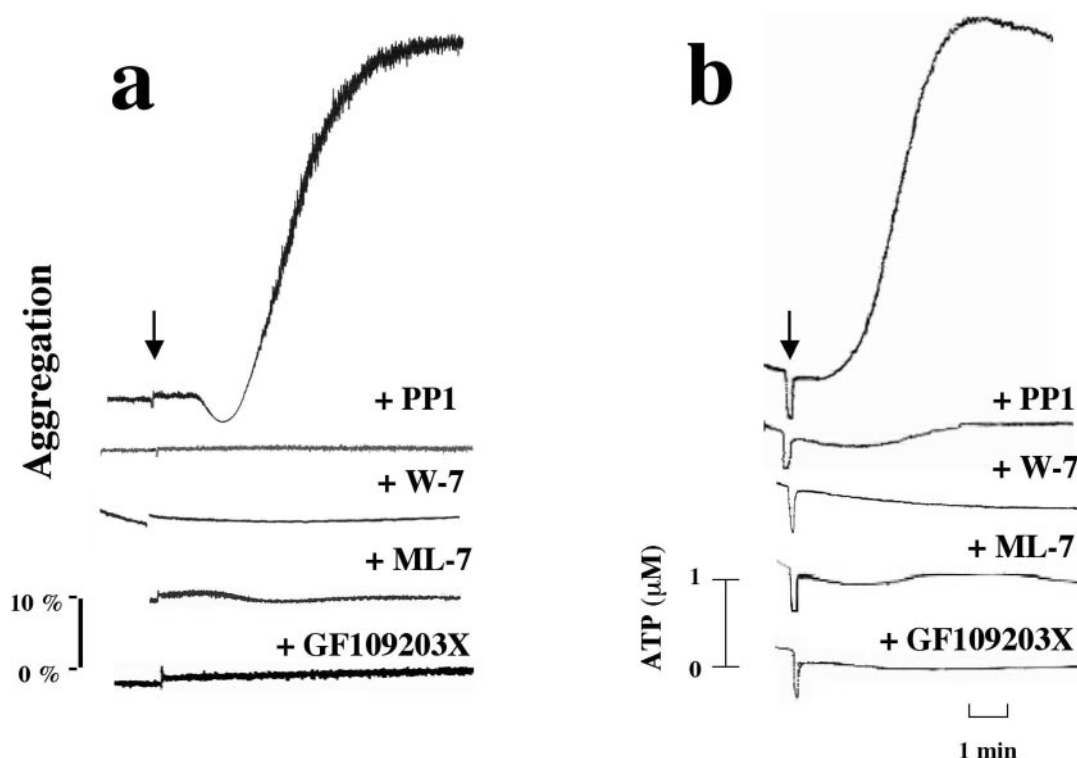
FIG. 3. **Role of Ca²⁺/CaM and MLC kinase in platelet aggregation and degranulation.** Aggregation tracings (*a*) and ATP secretion profiles (*b*) following activation of washed platelets, with collagen (1 μg/ml), in the absence (*upper tracings*) or in the presence of the Src family kinase inhibitor PP1 (20 μM), the Ca²⁺/CaM antagonist W-7 (50 μM), the MLC kinase inhibitor ML-7 (30 μM), or the PKC inhibitor GF109203X (10 μM) are shown.

apyrase-treated washed platelets, α,β-meATP also caused a rapid, transient, and dose-dependent MLC phosphorylation (Fig. 1, *lower right panel*). MLC phosphorylation was observed as early as 2 s and paralleled the actual platelet shape change response; it was over 30 s after agonist application, *i.e.* coinciding with the reversibility of the platelet shape change. With increasing concentrations of α,β-meATP, the degree of MLC phosphorylation paralleled the intensity of the platelet shape change (not shown).

As was the case for the P2X₁-dependent α,β-meATP-induced shape change, no MLC phosphorylation occurred in the absence of added extracellular CaCl₂, and it dropped to very low levels when apyrase was omitted from the platelet suspension (not shown), leading to P2X₁ desensitization. The inositol 1,4,5-trisphosphate receptor inhibitor, 2-APB (100 μM), did not affect the P2X₁-induced platelet shape change and MLC phosphorylation (not shown), compatible with an event that relies on the influx of Ca²⁺ exclusively, without mobilizing calcium from intracellular stores. In contrast, the ADP-induced MLC phosphorylation and shape change, dependent on P2Y₁-mediated Ca²⁺-mobilization, were not inhibited when extracellular Ca²⁺ was omitted, although manifestation of full shape change and MLC phosphorylation also required apyrase to protect P2Y₁ from desensitization (not shown). In agreement with its behavior as a (non-selective) P2 receptor antagonist, suramin abrogated the α,β-meATP-induced shape change and MLC phosphorylation (not shown).

Mechanism of P2X₁-mediated MLC Phosphorylation and Platelet Shape Change—Phosphorylation of the MLC can be dichotomously regulated by the Ca²⁺ calmodulin-regulated MLC kinase and by Rho kinase (11); these two pathways can be activated independently of each other, depending on the type and concentration of the platelet agonist. We have therefore investigated the contribution of both kinases to the P2X₁-mediated MLC phosphorylation by studying α,β-meATP-induced

phosphorylation and platelet shape change in the presence of the specific calmodulin inhibitor W-7 or the Rho kinase inhibitor HA-1077. Fig. 2*a* shows that the α,β-meATP-induced platelet shape change was not affected by the presence of HA-1077 (20 μM); in contrast, the reversible second phase of the ADP-induced platelet shape change was detectably and repeatedly reduced at this concentration of HA-1077. Correspondingly, HA-1077 hardly affected the α,β-meATP-induced MLC phosphorylation, but strongly reduced the ADP-induced MLC phosphorylation, without abolishing it (Fig. 2*a*). On the other hand, the α,β-meATP-induced shape change was dose-dependently and completely inhibited by W-7 at 50 μM, whereas the ADP-induced platelet shape change could not be neutralized entirely at 100 μM (Fig. 2*b*). Correspondingly, the α,β-meATP-induced MLC phosphorylation was eliminated by 50 μM W-7, whereas this concentration only partially inhibited the ADP-induced MLC phosphorylation (Fig. 2*b*). The α,β-meATP-induced shape change was also inhibited by the MLC kinase inhibitor ML-7 at 30 μM, whereas ML-7 had an effect on the ADP-induced shape change, comparable with that of W-7. These experiments confirm that platelet shape change brought about by P2X₁-mediated Ca²⁺ influx does not involve Rho kinase but relies on rapid and Ca²⁺/CaM-dependent MLC kinase-triggered MLC phosphorylation, responsible for rearranging myosin and linked morphological changes during the transient platelet shape change reaction.

Ca²⁺/CaM Plays a Central Role in Collagen-induced Platelet Activation—In view of the role that the P2X₁-mediated ERK2 phosphorylation plays in platelet activation by low doses of collagen (16), we have analyzed whether the P2X₁-mediated Ca²⁺/CaM activation would be involved in this process. We therefore have reanalyzed the collagen-induced platelet aggregation in the presence of W-7. Fig. 3*a* shows that at 1 (and 2, not shown) μg/ml collagen, platelet shape change and aggregation were completely inhibited by W-7, in agreement with older
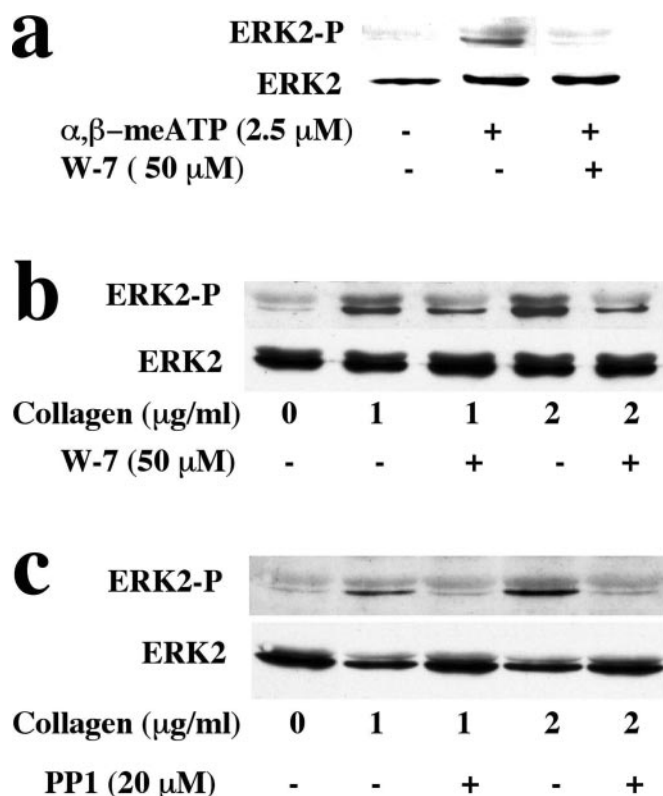
FIG. 4. **Role of Ca^{2+}/CaM in ERK2 activation.** The figure shows P2X$_1$-mediated ERK2 phosphorylation in washed platelets activated with α,β-meATP in the absence or presence of W-7, as indicated (*a*); collagen-induced ERK2 phosphorylation in the absence or presence of W-7, as indicated (*b*); and collagen-induced ERK2 phosphorylation in the absence or presence of PP1, as indicated (*c*). Phospho-ERK1/2 MAP kinase (*ERK2-P*) and control MAP kinase (ERK2, *lower panels*) antibodies were used.

findings (19, 20). Parallel recordings of ATP secretion indicated complete inhibition of degranulation by W-7 (Fig. 3*b*). Likewise, strong inhibition of aggregation, shape change, and ATP secretion was achieved with ML-7. In agreement with our previous findings (16), the PKC inhibitor GF109203X also abrogated these events (Fig. 3). The fact that the Src family kinase inhibitor PP1 equally prevented platelet aggregation and ATP secretion almost entirely, confirmed that the collagen-induced platelet activation was the result of GPVI-mediated platelet activation. Indeed, PP1 is a potent inhibitor of Fyn, a Src family kinase involved in early steps of GPVI signaling (21, 22). Accordingly, parallel experiments using the GPVI-selective agonist convulxin, yielded similar results (not shown). These findings therefore imply that both PKC and Ca^{2+}/CaM-dependent MLC kinase activation are instrumental in the early platelet dense granule release induced by collagen.

During platelet activation by 1 μg/ml collagen, the phosphorylation of ERK2 depends on ATP-triggered Ca^{2+} influx via P2X$_1$ (16). We presently found that the α,β-meATP-induced ERK2 phosphorylation was fully inhibited by W-7 (Fig. 4*a*), compatible with Ca^{2+}/CaM-dependent activation of ERK2. We therefore expected that, during the collagen-induced platelet aggregation, W-7, by abrogating both platelet degranulation and the ATP-mediated P2X$_1$-dependent activation, would prevent ERK2 phosphorylation. Fig. 4*b* confirms that W-7 largely prevents the low dose (1 μg/ml) collagen-induced phosphorylation of ERK2. The inhibition by W-7 of ERK2 phosphorylation induced by higher doses of collagen ($\geq$2 μg/ml), which is independent of P2X$_1$, however, suggested the existence of addi-

tional links between Ca^{2+}/CaM and ERK2 (see below). Similar results were found for the convulxin-induced ERK2 phosphorylation (not shown).

However, the collagen-induced ERK2 phosphorylation was entirely eliminated in the presence of PP1, compatible with an event requiring GPVI-mediated Fyn and Lyn phosphorylation and subsequent phospholipase Cγ2 activation. These experiments, at a low dose of collagen, confirmed that when the early secretion of ATP was abrogated, ERK2 phosphorylation was absent.

P2X$_1$-mediated ERK2 Activation Reinforces MLC Kinase Activation—We have previously shown that desensitization of P2X$_1$ by pretreating the platelets with α,β-meATP inhibits low dose collagen-induced secretion, aggregation, and related ERK2 phosphorylation (15). Fig. 5*a* shows that the collagen-induced MLC phosphorylation is a slow process at low concentrations of this agonist but speeds up at higher concentrations. ML-7 and W-7 abolish MLC phosphorylation elicited by collagen, supportive of a role for Ca^{2+}/CaM and MLC kinase in this event (Fig. 5*b*). Interestingly, at a low dose of collagen (1 μg/ml), MLC phosphorylation can also partially be inhibited by U0126, at a concentration that prevents ERK2 activation. This finding uncovers a role for ERK2 in the activation of MLC kinase. In agreement with a role for P2X$_1$ in this process, MLC phosphorylation was also strongly reduced by prior P2X$_1$ desensitization using α,β-meATP (Fig. 5*c*). Similarly to our previous findings on collagen-induced aggregation, at higher collagen concentrations (2 μg/ml), neither α,β-meATP nor U0126 had any effect on the level of MLC phophorylation. These data point to a role for P2X$_1$-ERK2-mediated MLC phosphorylation in platelet secretion and aggregation, elicited by low concentrations of collagen.

DISCUSSION

The use of transgenic (23) and knock-out (24) mouse models recently established a contribution for P2X$_1$ to platelet activation induced by low concentrations of collagen. These models have further demonstrated a role for platelet P2X$_1$ in thrombosis. Generally, platelet shape change is considered to be the first measurable response to platelet activation, involving Rho kinase and MLC kinase (11). It is characterized by spheration, contraction of the cell, cytoskeletal rearrangement, folding and ruffling of the surface membrane, and finally, formation of pseudopodia (25). We therefore have first investigated how P2X$_1$-mediated Ca^{2+} influx would activate these enzymes, and secondly, how these enzymes contribute to P2X$_1$-dependent pathways of platelet activation.

Collagen leads to rapid platelet activation, accompanied by protein kinase C-dependent release of nucleotides and subsequent P2 receptor activation. Released ATP-mediated P2X$_1$ activation leads to phosphorylation of the mitogen-activated protein kinase ERK2, thus enhancing platelet activation and granule release, in a poorly understood manner. We have now shown that P2X$_1$-activated ERK2 is functionally coupled to myosin light chain kinase activation and MLC phosphorylation, important in platelet shape change and degranulation, reinforcing downstream platelet activation.

The fast reversibility of the P2X$_1$-mediated platelet shape change has been reported before (16, 17), but transmission electron microscopy presently showed that platelet shape change is accompanied by secretory granule centralization. The specific, non-hydrolysable P2X$_1$ agonist, α,β-meATP, caused rapid, transient, and dose-dependent MLC phosphorylation in washed human platelets. These results are consistent with previous reports using saponin-permeabilized platelets and correlating phosphorylation of myosin and its interaction with microfilaments responsible for contracting
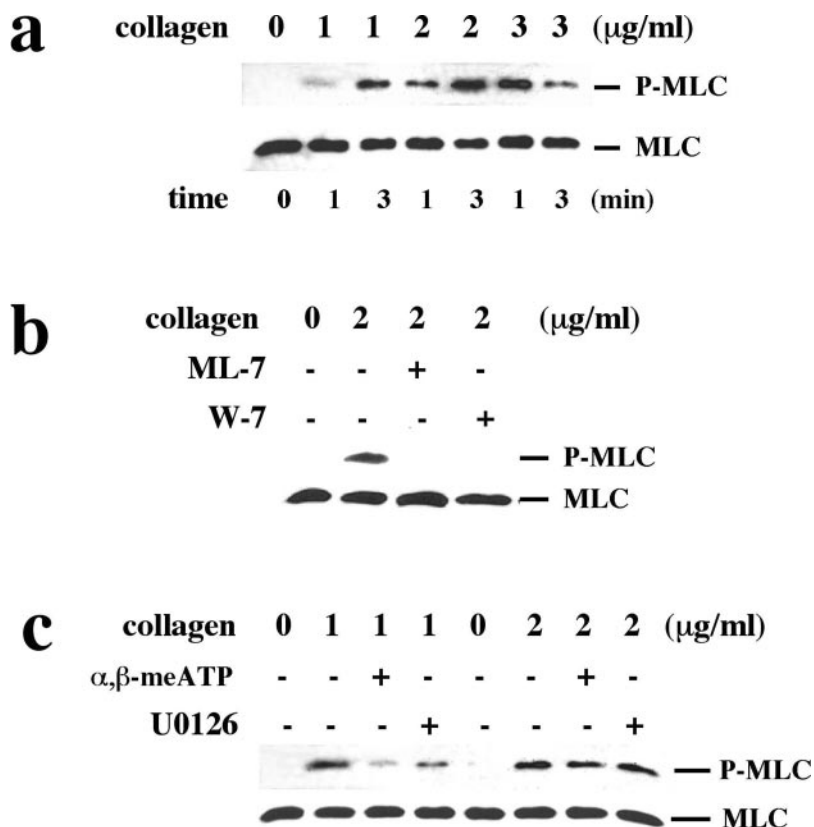
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FIG. 5. **P2X$_1$-mediated ERK2 phosphorylation enhances MLC kinase activation.** This figure shows the kinetics of MLC phosphorylation at the indicated concentrations of collagen (*a*); inhibition of MLC phosphorylation by the Ca^{2+}/CaM inhibitor W-7 and the MLC kinase inhibitor ML-7, at the indicated concentration of collagen (*b*); and inhibition of MLC phosphorylation after P2X$_1$ desensitization (with α,β-meATP added simultaneously to collagen, see Ref. 16) and by the MAPK kinase inhibitor U0126, at the indicated concentrations of collagen (*c*). Phospho-MLC (*P-MLC*) and control MLC (*lower panels*) antibodies were used.

the shell and moving the granules to the center of the platelet (26, 27). Rho kinase was not involved in the P2X$_1$-induced shape change and MLC phosphorylation, but the Ca^{2+}/CaM inhibitor W-7 and the MLC kinase inhibitor ML-7 dose-dependently inhibited these phenomena, in agreement with a direct link between Ca^{2+} influx and MLC phosphorylation.

In general, Ca^{2+}/CaM affects cellular growth and stress responses through ERKs. A number of proximal signaling pathways, including G-protein βγ subunits, PKC, as well as an increase in intracellular calcium levels, may converge on the ERK signaling cascade. In vascular smooth muscle, ERK1/2 activation in response to thrombin, angiotensin, or ATP involves both Ca^{2+}/CaM and PKC-dependent mechanisms (28–30). In platelets, we presently found that the P2X$_1$-mediated ERK2 phosphorylation depends on Ca^{2+}/CaM. However, ERK2 is not involved in the rapid P2X$_1$-mediated platelet shape change since inhibition of ERK2 phosphorylation by U0126 has no impact on α,β-meATP-induced shape change (not shown).

Early studies have indicated that Ca^{2+}/CaM-dependent MLC phosphorylation plays an important role in the platelet release reaction (19). In view of our earlier finding that P2X$_1$ contributes to the collagen-induced platelet secretion via the ERK2 pathway, we have studied in more detail the existence of cross-talk between MLC phosphorylation and ERK2 activation. The direct analysis of a role for P2X$_1$-dependent Ca^{2+}/CaM regulation appeared to be complicated because not only was the collagen-induced platelet aggregation fully inhibited by W-7 and by ML-7, in agreement with earlier findings, but also the early degranulation (and shape change) was abrogated by these inhibitors. Yet those findings pointed to the central role of myosin light chain phosphorylation, even in the early minor degranulation, in addition to a role for PKC.

Both integrin α$_2$β$_1$ and GPVI are recognized as major platelet receptors underlying interactions with collagen (31, 32). In its high affinity state, α$_2$β$_1$ has been proposed to be the major player in adhesion to collagen. GPVI, non-covalently associated

with the FcRγ chain, serves as the signal-transducing receptor in human platelets. Tyrosine phosphorylation of the FcRγ chain immunoreceptor tyrosine-based activation motif region by the Src family kinases Lyn and Fyn activates Syk and LAT, initiating enzyme cascades involving phosphatidylinositol 3-kinase and phospholipase Cγ2. Upon its phosphorylation, PLCγ2 migrates to the plasma membrane, where it generates inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. Finally, these events result in intracellular calcium mobilization, shape change, activation of PKC, and inside-out signaling, resulting in expression of α$_{IIb}$β$_3$-binding sites. We have presently confirmed that the P2X$_1$-mediated amplification reactions during the collagen-induced activation of platelets are the result of GPVI-mediated signaling, leading to early degranulation, in turn responsible for further nucleotide-dependent platelet activation. Indeed, the early degranulation and subsequent platelet aggregation were completely abrogated by the Src family kinase inhibitor PP1, as well as by W-7 and ML-7, illustrating that they depend on PLCγ2 activation, subsequent Ca^{2+}-mobilization, PKC activation, and Ca^{2+}/CaM-dependent MLC phosphorylation. Released ADP then triggers further Ca^{2+}-mobilization after activation of PLCβ.

We presently found that the weak MLC phosphorylation accompanying early collagen-induced platelet degranulation is up-regulated by ERK2. The finding that desensitization of P2X$_1$ either by prior α,β-meATP or by U0126 both strongly reduce the amplification of MLC phosphorylation provides a mechanistic basis for the role of P2X$_1$ in activating both enzymes, as illustrated in Fig. 6. Thus, released ATP contributes via P2X$_1$ to the collagen-mediated activation of platelets because it amplifies MLC phosphorylation. ERK2, activated by the P2X$_1$-mediated Ca^{2+} influx, by phosphorylating MLC kinase would accelerate MLC phosphorylation, and ultimately, platelet activation. Accordingly, in other cell types, MLC kinase activity was shown to be regulated by MAP kinase-dependent phosphorylation in functional assays; for example, the
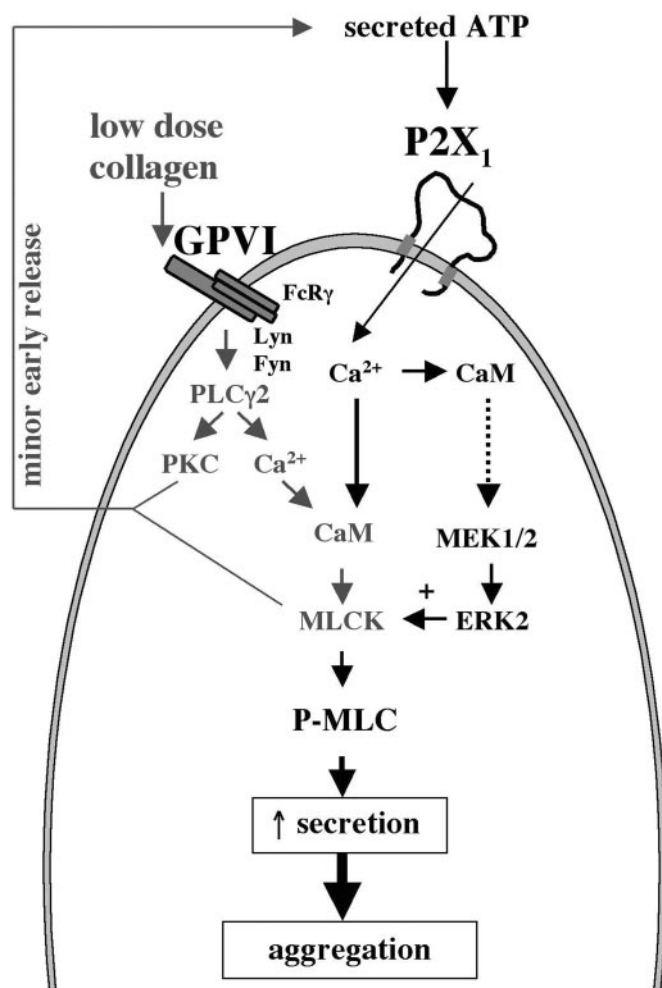
FIG. 6. **Diagram of the P2X$_1$-triggered signaling reactions during collagen-induced platelet activation.** At low concentrations of collagen, GPVI-collagen interactions trigger PLCγ2 activation, leading to PKC activation, Ca^{2+}-mobilization, and Ca^{2+}-calmodulin-dependent activation of MLC kinase (*MLCK*), both enzymes contributing to minor early platelet degranulation. Released ATP causes P2X$_1$-mediated Ca^{2+} influx, leading to progressive Ca^{2+}/CaM-dependent ERK2 phosphorylation. The collagen-induced Ca^{2+}/CaM-mediated phosphorylation of MLC is strongly enhanced by the activated ERK2 through phosphorylation dependent amplification of MLC kinase activation. The resulting up-regulated MLC phosphorylation (*P-MLC*) then contributes to further platelet degranulation, completing platelet aggregation.

phosphorylation, following Ca^{2+}-mobilization, in agreement with the role of MLC kinase in the ADP-induced platelet shape change (Fig. 2). In conclusion, the present work shows that P2X$_1$ activation by ATP leads to both activation of MLC kinase and of ERK2 and that the highly regulated interaction between both enzymes contributes to collagen-mediated platelet aggregation.

functional interaction between MAP kinase and MLC kinase has been implicated in carcinoma cell migration (33). Several molecular interactions depend on calmodulin; therefore, the pharmacological action of W-7 on ERK2 phosphorylation by α,β-meATP does not necessarily rely on a single mechanism, explaining the *dashed arrow* in Fig. 6. Indeed, we have shown before that ERK2 phosphorylation is also a PKC-dependent process (16). Fig. 6 highlights the role in platelets of secreted ATP. It is evident that released ADP will also stimulate MLC

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