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References

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P2X₁ Ion Channels Promote Neutrophil Chemotaxis through Rho Kinase Activation¹

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ATP, released at the leading edge of migrating neutrophils, amplifies chemotactic signals. The aim of our study was to investigate whether neutrophils express ATP-gated P2X₁ ion channels and whether these channels could play a role in chemotaxis. Whole-cell patch clamp experiments showed rapidly desensitizing currents in both human and mouse neutrophils stimulated with P2X₁ agonists, $\alpha\beta$ -methylene ATP ($\alpha\beta$ MeATP) and $\beta\gamma$ MeATP. These currents were strongly impaired or absent in neutrophils from P2X₁^{-/-} mice. In Boyden chamber assays, $\alpha\beta$ MeATP provoked chemokinesis and enhanced formylated peptide- and IL-8-induced chemotaxis of human neutrophils. This agonist similarly increased W-peptide-induced chemotaxis of wild-type mouse neutrophils, whereas it had no effect on P2X₁^{-/-} neutrophils. In human as in mouse neutrophils, $\alpha\beta$ MeATP selectively activated the small RhoGTPase RhoA that caused reversible myosin L chain phosphorylation. Moreover, the $\alpha\beta$ MeATP-elicited neutrophil movements were prevented by the two Rho kinase inhibitors, Y27632 and H1152. In a gradient of W-peptide, P2X₁^{-/-} neutrophils migrated with reduced speed and displayed impaired trailing edge retraction. Finally, neutrophil recruitment in mouse peritoneum upon *Escherichia coli* injection was enhanced in wild-type mice treated with $\alpha\beta$ MeATP, whereas it was significantly impaired in the P2X₁^{-/-} mice. Thus, activation of P2X₁ ion channels by ATP promotes neutrophil chemotaxis, a process involving Rho kinase-dependent actomyosin-mediated contraction at the cell rear. These ion channels may therefore play a significant role in host defense and inflammation. *The Journal of Immunology*, 2009, 183: 2801–2809.

Neutrophils are key cells of the innate immune system that participate in a vast majority of inflammation-related diseases, such as coronary artery diseases, rheumatoid arthritis, and sepsis (1–3). They are programmed to exit the circulation and chemotax toward epicenters of inflammation, guided by gradients of a variety of inflammatory mediators (4). Neutrophils become polarized morphologically in response to chemotactic stimuli. These morphological changes are accompanied by a strongly polarized distribution of intracellular signal transduction components. The 3'-phosphoinositol lipids (PI3Ps)⁴ and PI3Ks;

Rho GTPases, such as RhoA, Rac, and Cdc42; and the actin and microtubule cytoskeletons play key roles in signaling polarity. Attractant receptors, acting on different G proteins, trigger two divergent pathways that contribute to polarity (5). Frontness depends upon G_i-mediated production of PI3Ps, the activated form of Rac, and F-actin. G₁₂ and G₁₃ trigger backness signals, including activation of Rho, a Rho-dependent kinase, and myosin II. Functional incompatibility causes the two resulting actin assemblies to aggregate into separate domains, making the leading edge more sensitive to attractant than the back (5).

In addition, mechanisms of signal amplification have been proposed that contribute to maintain signaling polarity even in a shallow gradient. These mechanisms involve the release of ATP from neutrophil leading edge and its rapid metabolism to ADP, AMP, and adenosine (6). As a neutrophil moves toward an attracting stimulus, a rapid and transient release of ATP further enforces its directional movement by autocrine signaling via P2Y₂ and A3 receptors. In support of this, neutrophils lacking P2Y₂ receptors showed a loss in gradient sensing, whereas cells without A3 receptors showed correct directionality, but diminished speed (6). Although the intracellular mechanisms remain undefined, these findings demonstrate the importance of extracellular ATP in the control of neutrophil chemotaxis.

Because ATP is released by activated platelets and leukocytes, as a result of multiple stress stimuli, and upon cell lysis, its concentration can become elevated at sites of inflammation (7). By acting on neutrophils in a paracrine manner, ATP could thus help these cells to locate bacteria and tissue damage. We therefore wondered whether neutrophils expressed other ATP receptors that could contribute to chemotaxis. ATP acts on specific cell surface P2 receptors belonging to two subclasses, the G protein-coupled P2Y receptors and the ATP-gated P2X-nonselective cation channels (8, 9). Eight human P2Y receptors, P2Y₁, P2Y₂, P2Y₄, P2Y₆,

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⁴ Abbreviations used in this paper: PI3P, 3'-phosphoinositol lipid; CaM, calmodulin; CI, chemotactic index; GEF, guanine nucleotide exchange factor; MeATP, methylene ATP; MLC, myosin L chain; WT, wild type.

and P2Y₁₁₋₁₄, and seven P2X subtypes, P2X₁₋₇, have been identified (9–11). Neutrophils express multiple P2X subtype mRNAs: P2X_{1,4,5,7} (6, 12–14). However, the presence of functional P2X ion channels in neutrophils has never been shown, and their contribution to neutrophil migration has never been investigated. P2X ion channels are widely distributed in various cell types and tissues (15). Only P2X₇ receptors are to date established as having physiological roles in inflammation, mostly via a rapid activation of caspase-1 with subsequent release of the proinflammatory cytokine IL-1 β from activated macrophages and microglia (15, 16). P2X₇ is also an important modulator of T cell functions (17–20).

In this study, we demonstrate that both human and mouse neutrophils express P2X₁ ion channels that play a significant role in the neutrophil response to chemotactic stimuli.

Materials and Methods

Reagents

$\alpha\beta$ -methylene ATP ($\alpha\beta$ MeATP), $\beta\gamma$ MeATP, fMLP, and BSA were from Sigma-Aldrich. W-peptide was from Innovagen. Mouse type IV collagen was from BD Biosciences. Homemade polyclonal rabbit anti-P2X₁ Ab was described previously (21). Polyclonal goat anti-myosin L chain (MLC)2 and anti-phospho MLC (Thr¹⁸/Ser¹⁹), mouse anti-RhoA, and mouse anti-Rac2 Abs were from Santa Cruz Biotechnology. Mouse anti-Rac1 (23A8) was from Upstate Biotechnology. Mouse anti-Cdc42 was from BD Biosciences. AlexaFluor488-coupled phalloidin was from Invitrogen.

Mice

Animals used in this study were 8- to 12-wk-old C57BL/6 mice (wild type (WT) and P2X₁^{-/-} described previously; Ref. 22) that were housed in specific pathogen-free animal facilities. All experiments were conducted following the guidelines of and in agreement with the local ethics committee.

Human neutrophil isolation

Fresh acid-citrate-dextrose (93 mM sodium citrate, 7 mM citric acid, and 0.14 mM dextrose (pH 6.5))-anticoagulated peripheral blood was drawn by venipuncture from healthy volunteers. Institutional Review Board approval was obtained from the Centre Hospitalier Universitaire de Liège, and informed consent from volunteers was obtained in accordance with the Declaration of Helsinki. Neutrophils were isolated by plasma/Percoll gradient centrifugation, mainly as previously described (23).

Mouse peritoneal neutrophil isolation

Mice were injected i.p. with 500 μ l of 4% thioglycolate in PBS. Three hours after injection, peritoneal lavage was performed with 5 ml of ice-cold PBS. Neutrophils were isolated by positive magnetic selection using an anti-Gr1 Ab (Miltenyi Biotec). Cells were counted and resuspended at a density of 1×10^6 cells/ml in HBSS containing 1 mM CaCl₂, 2 mM MgCl₂, and 0.2% BSA (referred as to HBSS buffer).

Western blotting

Western blotting detection of P2X₁, phospho-MLC, and MLC proteins in neutrophil extracts was performed, as previously described (24–26). For phospho-MLC and MLC detection, neutrophils were treated with 2.7 mM diisopropyl fluorophosphate for 15 min before stimulation with agonist for indicated times.

Rho GTPase pull-down assays

After pretreatment with 2.7 mM diisopropyl fluorophosphate for 15 min, neutrophils were stimulated with $\alpha\beta$ MeATP for the indicated times. Reaction was stopped by lysing the cells in the following ice-cold buffer: 0.5% Triton X-100, 25 mM HEPES (pH 7.3), 150 mM NaCl, 5 mM MgCl₂, 0.5 mM EGTA, 5 mM DTT, 4% glycerol, containing complete protease inhibitors, 10 mM sodium fluoride, 2 mM β -glycerophosphate, 2 mM sodium orthovanadate, and 1 mM PMSF. Lysates were centrifuged for 8 min at $13,000 \times g$. An aliquot of each supernatant was denatured in Laemmli buffer to measure the total RhoGTPase content by Western blotting. For pull-down assays, supernatants were incubated for 30 min with 30 μ g of GST-p21 binding domain of PAK1 protein containing the Cdc42 and Rac binding region of PAK-1B, or GST-Rho binding domain of Rhotekin protein containing the Rho binding region of rhotekin affinity linked to glutathione-Sepharose beads. The beads were washed four times in lysis

buffer and boiled in Laemmli buffer. Lysates were loaded on SDS-PAGE, and Western blotting was performed (25).

Electrophysiology

During isolation, both murine and human neutrophils were treated with 15 U/ml apyrase (grade I; Sigma-Aldrich) to remove extracellular ATP and prevent P2X₁ desensitization. Isolated neutrophils were adhered on a tissue culture dish and placed in a perfusion chamber on an inverted microscope that was continuously perfused with external solution containing 147 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 10 mM HEPES, and 13 mM glucose (pH 7.4 with NaOH). Whole-cell patch clamp recordings were made at room temperature ($23 \pm 1^\circ\text{C}$) using an Axopatch-1D amplifier and pCLAMP8 software (Axon Instruments). Pipette resistances ranged from 5 to 12 M Ω when filled with internal solution containing 147 mM NaCl, 10 mM EGTA, and 10 mM HEPES (pH 7.3 with NaOH). Gigaseals were obtained and currents were sampled at 10 KHz after low-pass filtering at 1 KHz. Series resistance was not routinely compensated. The membrane potential was clamped at -70 mV throughout the experiments. Fast solution changes were achieved with the complete VC-6 fast-step perfusion system (Harvard apparatus) together with a multibarrel glass pipette that was positioned ~ 50 μ m from the cell during drug application. Analysis of data was achieved with Clampfit 8.0 (Axon Instruments) and Prism 4.0 (GraphPad).

Boyden chamber assays

Forty-eight-well microchemotaxis plates (NeuroProbe) and 8- μ m (human neutrophils) or 5- μ m (mouse neutrophils) pore-size polyvinylpyrrolidone-free polycarbonate membranes (VWR) were used. Chemoattractant diluted in HBSS buffer was placed in the chamber bottom wells, and neutrophil suspension (1×10^6 cells/ml) was added to the top wells. HBSS buffer was used as a negative control. The plate was incubated for 1 h at 37°C . Membrane was removed, fixed, and stained using a Diff-Quick stain set (Medion Diagnostics). The number of neutrophils that migrated to the lower side of the membrane was counted in 10 random microscope fields using the $\times 40$ objective (Olympus). The chemotactic index (CI) is defined as follows: (mean neutrophils per field in chemoattractant – mean neutrophils per field in HBSS)/mean neutrophil per field in HBSS.

F-actin staining and confocal microscopy

Murine peritoneal neutrophils (1×10^6 /ml in HBSS buffer) were let to adhere on collagen type IV-coated microslides (Ibidi; Integrated Bio-Diagnostics) for 15 min at room temperature. Neutrophils were incubated with 100 nM W-peptide for the indicated times, before fixation in 4% paraformaldehyde. Cells were incubated in HBSS containing 0.25% Triton X-100, 2% BSA, and AlexaFluor488-coupled phalloidin. Microslides were analyzed with a Leica TCS SP2 confocal microscope equipped with a $\times 63$ objective (Leica Microsystems).

Time-lapse video microscopy: chemotaxis microslides

Chemotaxis microslides coated with type IV collagen or fibronectin (Ibidi) were used to study two-dimensional neutrophil migration. Murine neutrophils (3×10^6 cells/ml in HBSS buffer) were adhered to coated microslides for 15 min at room temperature. Microslide reservoirs were then filled with buffer, and chemoattractant was added. After 15 min, to allow diffusion of the chemoattractant and establishment of a linear gradient, neutrophil migration was observed using a Nikon Eclipse TS100 inverted microscope, equipped with a Nikon digital sight DS-2Mv camera. The Basic Research NIS-Elements Imaging software (Nikon) was used for time-lapse imaging. Phase-contrast images were captured every minute. Cell-tracking software (Chemotaxis and Migration Software tool based on ImageJ; Ibidi) was used to characterize chemotaxis from the captured images. The average circularity ratio of cells is a well known compactness measure of a shape computed by ImageJ and defined as $C = 4\pi(\text{area})/(\text{perimeter})^2$. For a perfect circle, C equals 1, whereas for a long, thin shape, C tends to 0. This ratio was computed for each cell at each capture by successively applying the Canny edge detector using FeatureJ plugin, thresholding using the Iso-data algorithm, and morphological operations (fill holes, erosion, and dilation). Parameter default values were used for all experiments.

Static adhesion assay

Ninety-six-well culture plates were coated with mouse type IV collagen (40 μ g/ml). Neutrophils from WT or P2X₁^{-/-} mice were allowed to adhere to collagen for 15 min at 37°C . Unattached cells were gently removed, and wells were rinsed once with HBSS buffer. Neutrophil adhesion was determined using the CellTiter-Glo luminescent cell viability assay (Promega).

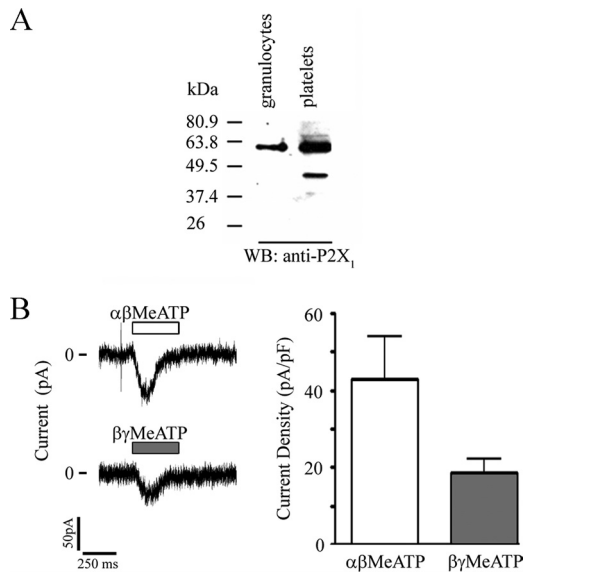


FIGURE 1. Human neutrophils express functional P2X₁ channels. *A*, Western blotting detection of P2X₁ proteins in neutrophil or platelet protein extracts. *B*, Whole-cell currents recorded at -70 mV holding potential. The average cell capacitance of human peripheral neutrophils was 2.1 ± 1.0 pF (mean \pm SD). Representative traces of inward currents induced by optimal concentrations of $\alpha\beta$ MeATP ($10 \mu\text{M}$) and $\beta\gamma$ MeATP ($10 \mu\text{M}$). The histograms depict current density. Averaged data are from at least three independent experiments with neutrophils of different individuals.

Luminescence was measured in a Victor2 multilabel microplate reader (PerkinElmer).

Mouse peritonitis model

WT mice ($n = 4-6$ mice per group; 3 independent experiments) were i.p. injected with $100 \mu\text{l}$ of $\alpha\beta$ MeATP ($0.16 \mu\text{mol/kg}$) or vehicle 15 min before i.p. injection of $150 \mu\text{l}$ of *Escherichia coli* (American Type Culture Collection 25922) (50×10^6 CFU/ml) or PBS. Mice were then injected with $100 \mu\text{l}$ of $\alpha\beta$ MeATP after 1 and 2 h. For experiments with P2X₁^{-/-} mice, animals were injected once with $150 \mu\text{l}$ of *E. coli* or PBS. After 3 h, the mice were killed, and peritoneal exudate cells were harvested by five successive washes with 1 ml of PBS containing 0.5% BSA and 2 mM EDTA. Total amounts of peritoneal cells were counted, and differential cell counts were determined by microscopic analysis of Giemsa-Wright-stained cytopins.

Statistics

Data are represented as mean \pm SD of at least three independent experiments. Statistical analyses were performed using either Student's *t* test or, for multiple comparisons, one-way ANOVA, followed by Bonferroni's test.

Results

Human neutrophils express functional P2X₁ channels

In agreement with previous studies (6, 13), real-time RT-PCR analyses indicated that human peripheral neutrophils mainly expressed P2X₁ subtype mRNAs (data not shown). Lower levels of P2X₄, P2X₅, and P2X₆ mRNA subtypes were also detected. We assessed whether P2X₁ proteins were expressed in neutrophil membranes and formed functional ion channels. Expression of P2X₁ proteins was analyzed by Western blotting (Fig. 1*A*). The apparent molecular mass of neutrophil P2X₁ proteins was ~ 60 kDa, similar to that of the glycosylated form of platelet P2X₁ (27). Functionality of the P2X₁ ion channels was studied by whole-cell patch clamp analyses. Application of the P2X₁ selective agonist $\alpha\beta$ MeATP ($10 \mu\text{M}$) induced rapidly desensitizing inward currents of ~ 40 pA/pF (Fig. 1*B*). Currents of lower density (20 pA/pF)

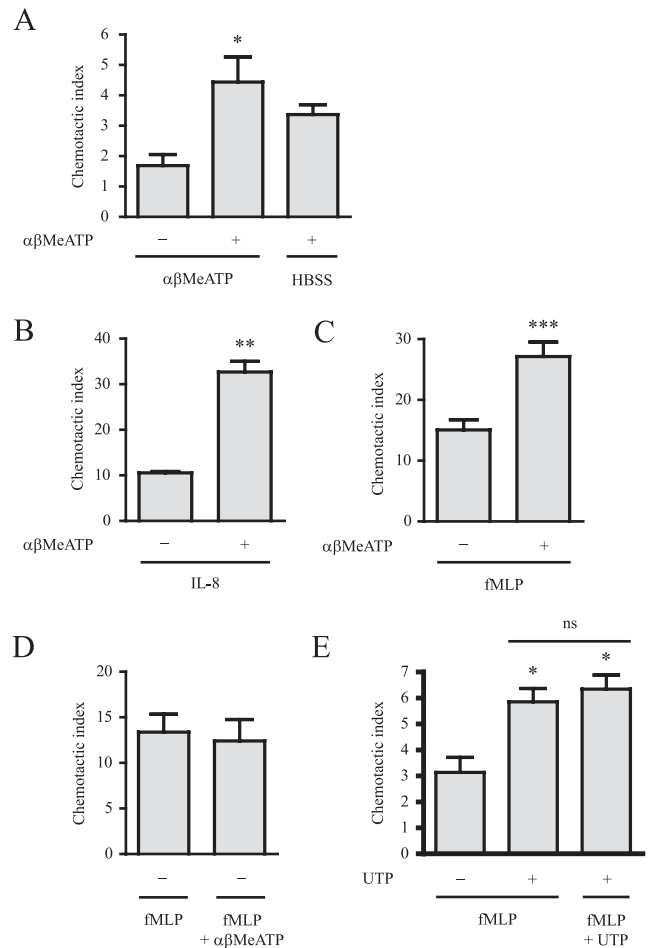


FIGURE 2. $\alpha\beta$ MeATP induces random migration of human peripheral neutrophils and enhances chemotaxis. Boyden chamber assays on human peripheral neutrophils. *A*, $\alpha\beta$ MeATP ($10 \mu\text{M}$) or HBSS (neutrophil resuspension buffer); *B*, IL-8 (100 ng/ml); or *C-E*, fMLP (100 nM). *D*, $\alpha\beta$ MeATP ($10 \mu\text{M}$) or *E*, UTP ($10 \mu\text{M}$) was combined or not with fMLP (100 nM) and placed in the bottom wells of the chamber, as indicated below solid lines. The “+” or “-” symbols above solid lines indicate that neutrophils were prestimulated or not with the indicated agonists ($\alpha\beta$ MeATP in *A-D*, or UTP in *E*) for 1 min before addition to the upper wells of the chamber. Averaged data are from at least three independent experiments with neutrophils of different individuals. *A-C*, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs absence of $\alpha\beta$ MeATP in the upper wells. *E*, *, $p < 0.05$ vs absence of UTP.

were observed upon application of the same concentration of the other P2X₁ agonist, $\beta\gamma$ MeATP, which is consistent with P2X₁ pharmacological properties described in other cell types (28, 29). Moreover, applying ATP ($100 \mu\text{M}$) gave rise to similar rapidly desensitizing currents (data not shown), suggesting that human neutrophils do not express functional P2X ion channels characterized by slow desensitization kinetics (composed of P2X₄, P2X₅, and/or P2X₆ subunits).

$\alpha\beta$ MeATP induces random migration and enhances chemotaxis of human peripheral neutrophils

The effect of $\alpha\beta$ MeATP on neutrophil chemotaxis was investigated using Boyden chamber assays (30) (Fig. 2*A*). We placed neutrophils into the chamber top wells and allowed them to migrate in the bottom wells. $\alpha\beta$ MeATP ($10 \mu\text{M}$), placed in the bottom wells, did not induce significant neutrophil chemotaxis. However, pretreating neutrophils with $\alpha\beta$ MeATP significantly increased CI. In the absence of $\alpha\beta$ MeATP in the bottom wells, the

migratory activity of $\alpha\beta$ MeATP-treated neutrophils also tended to be augmented. Similar results were obtained when $\beta\gamma$ MeATP was used as P2X₁ agonist (data not shown).

Neutrophils chemotaxed efficiently in response to IL-8 (CI = 10.5; Fig. 2B). We found that pretreating the cells with $\alpha\beta$ MeATP markedly enhanced chemotaxis induced by IL-8 (CI = 32.6; Fig. 2B), at any tested IL-8 concentration (from 10 to 500 ng/ml) (data not shown). A similar phenomenon was observed when formylated peptide (fMLP, 100 nM) was used as chemoattractant (Fig. 2C). Only short treatment with the agonist (~1 min) led to the increased chemotaxis (supplemental Fig. S1C).⁵ When longer preincubation periods were used (from 5 min), $\alpha\beta$ MeATP failed to enhance neutrophil migration, pointing to the importance of the kinetics of P2X₁-mediated signaling. Thus, stimulation of neutrophils with P2X₁ agonists promoted neutrophil chemotaxis in response to both endogenous (IL-8) and exogenous bacterial (fMLP) chemoattractant. The $\alpha\beta$ MeATP-induced increase of chemotaxis toward fMLP was fully inhibited by the selective P2X₁ antagonist, NF449, and was similar to the effect of the nonhydrolyzable ATP analog adenosine 5'-O-(3-thiotriphosphate) (supplemental Fig. S1, A and B). ATP was more potent to enhance migration than the two analogues, possibly because of ATP conversion to adenosine during cell migration (supplemental Fig. S1B). Strikingly, the addition of $\alpha\beta$ MeATP in the chemoattractant medium failed to increase chemotaxis (Fig. 2D). Taken together, these results indicated that $\alpha\beta$ MeATP was not chemotactic by itself, but could induce random neutrophil migration (chemokinesis). This is in sharp contrast with the effect of the P2Y₂ receptor agonist, UTP, that was able to increase fMLP-induced chemotaxis when added together with fMLP in the bottom wells of the Boyden chamber, as well as when it was placed in direct contact with the cells (Fig. 2E).

$\alpha\beta$ MeATP-induced migration involves the Rho pathway

We then investigated the molecular mechanisms underlying the $\alpha\beta$ MeATP-induced migration. To analyze the activity of Rho GTPases, central players in signaling polarity during cell migration (4), we performed GST pull-down assays using lysates of $\alpha\beta$ MeATP-stimulated human neutrophils.

We found that $\alpha\beta$ MeATP (10 μ M) rapidly and selectively activated RhoA (Fig. 3). The amount of GTP-bound RhoA increased by 4.2 \pm 0.4-, 4.1 \pm 1.4-, and 3.7 \pm 1.7-fold after 10 s, 30 s, and 1 min of stimulation, respectively. In contrast, $\alpha\beta$ MeATP failed to activate Rac1, Rac2, or Cdc42 (Fig. 3B). Consistently with the activation of the Rho pathway, we also found that $\alpha\beta$ MeATP increased MLC phosphorylation, being maximal after 1 min and disappearing after 5 min (Fig. 3C).

To determine whether the Rho pathway was involved in $\alpha\beta$ MeATP-induced chemokinesis, neutrophils were incubated with two different inhibitors of the Rho kinase, Y27632 and H1152, and Boyden chamber assays were performed. Both inhibitors fully blocked neutrophil migration induced by $\alpha\beta$ MeATP (Fig. 3D). Under our experimental conditions, Y27632 (5 μ M) alone failed to inhibit chemotaxis toward fMLP, whereas it abolished the effect of $\alpha\beta$ MeATP (Fig. 3E). On the contrary, inhibiting PI3K γ with AS252424 (31, 32) failed to modify $\alpha\beta$ MeATP-triggered chemokinesis (data not shown), whereas it totally blocked the fMLP-induced chemotaxis (data not shown). Taken together, these results indicate the involvement of the RhoA/Rho kinase pathway in P2X₁-mediated neutrophil chemokinesis.

Characterization of P2X₁^{-/-} neutrophils

To further characterize P2X₁ function in neutrophil chemotaxis and to assess agonist specificity, we used cells isolated from P2X₁^{-/-} mice (22). The hematological profile of these mice has been analyzed in a previous study, revealing normal erythrocyte and leukocyte counts, hemoglobin, and hematocrit (33). The distribution of leukocyte cell types was also similar between WT and knockout mice. In transmission electron microscopy, we showed that P2X₁^{-/-} peripheral neutrophils had no apparent ultrastructural abnormalities (data not shown). We then performed whole-cell patch clamp experiments on mouse peritoneal neutrophils. As observed in human cells, neutrophils from WT mice displayed rapidly desensitizing inward currents upon stimulation with the two P2X₁ agonists, $\alpha\beta$ MeATP (10 μ M) and $\beta\gamma$ MeATP (10 μ M). Current density was 136.9 \pm 44.9 pA/pF and 51.6 \pm 22.1 pA/pF (means \pm SEM), respectively (Fig. 4). $\beta\gamma$ MeATP-induced currents were absent in P2X₁^{-/-} neutrophils. A remaining rapidly desensitizing current could still be induced by $\alpha\beta$ MeATP in cells lacking P2X₁ (mean current density = 70.7 \pm 20.2 pA/pF). P2X₁ characteristics (sensitivity to activation by $\alpha\beta$ MeATP and rapid desensitization kinetics) are shared by P2X₃ homomeric channels (28). Although $\beta\gamma$ MeATP is reported to be equipotent to $\alpha\beta$ MeATP at P2X₁, it is ~30- to 50-fold less potent at P2X₃. Real-time RT-PCR analyses indicated that, in contrast to human neutrophils, mouse neutrophils expressed P2X₃ subtype mRNAs (data not shown). P2X₃ channels might thus mediate the residual $\alpha\beta$ MeATP-induced current in P2X₁^{-/-} neutrophils.

We then investigated the chemotactic properties of P2X₁^{-/-} neutrophils in vitro. We first used Boyden chamber assays to assess direction sensing (30). Migration toward the chemotactic peptide W-peptide was similar for both P2X₁^{-/-} and WT neutrophils (Fig. 5A), and ~10-fold higher than toward the HBSS buffer control (data not shown). This indicated that P2X₁ was not required for direction sensing. Nevertheless, pretreating WT neutrophils with $\alpha\beta$ MeATP (10 μ M) significantly enhanced W-peptide-induced chemotaxis (Fig. 5A), as observed for human neutrophils. The migratory activity of P2X₁^{-/-} cells was not affected by $\alpha\beta$ MeATP (Fig. 5A), demonstrating that the $\alpha\beta$ MeATP effects on migration require P2X₁ ion channels.

As observed for human cells, P2X₁-mediated neutrophil migration also involved the Rho pathway in murine neutrophils. In Boyden chamber assays, the enhancing effect of $\alpha\beta$ MeATP on W-peptide-induced migration of WT neutrophils was fully blocked when cells were pretreated with inhibitors of the Rho kinase (Fig. 5B). Using GST pull-down assays, we found that P2X₁ activation with $\alpha\beta$ MeATP was able to rapidly increase RhoA activity in WT neutrophils, whereas RhoA activity in P2X₁^{-/-} neutrophils was not affected by this agonist (Fig. 5C).

Reduced speed and static adhesion for P2X₁^{-/-} neutrophils

We next investigated two-dimensional chemotaxis of P2X₁^{-/-} neutrophils using Ibidi chemotaxis microslides and time-lapse video microscopy. Mouse peritoneal neutrophils were adhered on collagen type IV and placed in a gradient of W-peptide. Both WT and P2X₁^{-/-} neutrophils migrated toward the W-peptide gradient (supplemental videos 1 and 2). Cell tracking showed that the forward migration index determined for P2X₁^{-/-} neutrophils was comparable to the one of WT cells, indicating that neutrophils can orient normally in the absence of P2X₁. Nevertheless, further analyses revealed diminished migration speed for P2X₁^{-/-} neutrophils (Fig. 6A). The addition of the ATP/ADP scavenger, apyrase, reduced the migration speed of WT neutrophils to a similar extent as for P2X₁^{-/-} cells, pointing to a contribution for ATP released by

⁵ The online version of this article contains supplemental material.

FIGURE 3. Role of RhoA-Rho kinase pathway in $\alpha\beta$ MeATP-triggered migration of human neutrophils. *A* and *B*, RhoGTPase activities assessed by GST pull-down assays. Human neutrophils were stimulated with $\alpha\beta$ MeATP (10 μ M) for the indicated times. Immunoblots depicting GST-bound and total GTPase levels are shown. *A*, A representative immunoblot of GST-bound and total RhoA and levels of GST-bound RhoA normalized to the signal detected in the absence of $\alpha\beta$ MeATP are shown. *B*, Representative immunoblots of GST-bound and total Rac1, Rac2, and Cdc42. *C*, Representative immunoblot of phosphorylated (P-MLC) and total MLC in neutrophils stimulated with $\alpha\beta$ MeATP (10 μ M) for the indicated times. *Bottom panel*, Results of band quantification. Levels of P-MLC were normalized to the signal detected in the absence of $\alpha\beta$ MeATP. *D*, Boyden chamber assays. $\alpha\beta$ MeATP-treated neutrophils were preincubated or not with the Rho kinase inhibitors, Y27632 (5 μ M) or H1152 (1 μ M), for 15 min. $\alpha\beta$ MeATP (10 μ M) was added in the bottom wells. Cell migration in the presence of HBSS is also shown (control). *E*, fMLP (100 nM) was placed in the bottom wells of the Boyden chamber. Neutrophils preincubated or not with the Rho kinase inhibitor Y27632 (5 μ M) were placed in the upper wells in the presence or absence of $\alpha\beta$ MeATP (10 μ M), as indicated. Data represent the means \pm SD of at least three independent experiments with neutrophils of different individuals. *A*, *, $p < 0.05$ vs control. *C*, **, $p < 0.01$ vs control. *D*, **, $p < 0.01$ vs HBSS control; #, $p < 0.05$ vs $\alpha\beta$ MeATP. *E*, ***, $p < 0.001$ vs fMLP alone (control); ###, $p < 0.001$ vs $\alpha\beta$ MeATP.

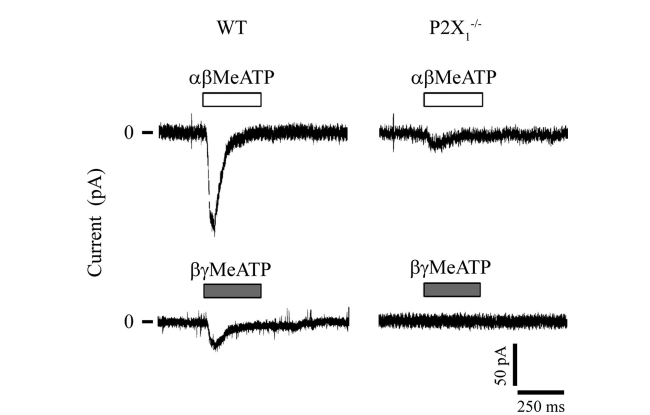
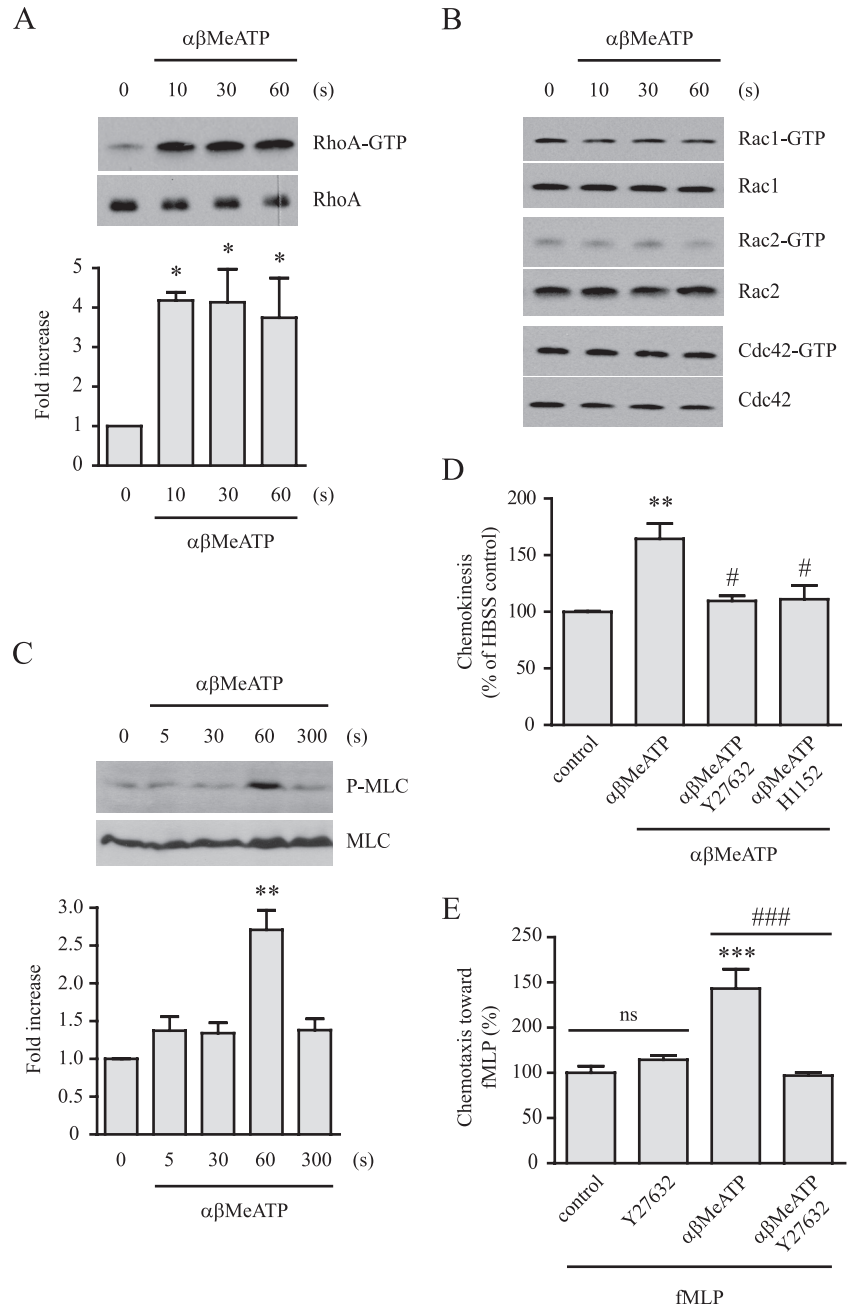


FIGURE 4. Characterization of $P2X_1^{-/-}$ neutrophils. Whole-cell currents recorded at -70 mV holding potential. The average cell capacitance of mouse peritoneal neutrophils was 1.7 ± 0.3 pF (mean \pm SD). Representative traces of $\alpha\beta$ MeATP (10 μ M) and $\beta\gamma$ MeATP (10 μ M) induced inward currents in WT and $P2X_1^{-/-}$ peritoneal neutrophils.

migrating cells during the assays (supplemental Fig. S2B). A reduced migration speed was also observed when $P2X_1^{-/-}$ neutrophils were adhered to fibronectin (supplemental Fig. S2A), indicating that this defect was not substrate specific. Moreover, the detachment of fine trailing appendages at the rear of $P2X_1^{-/-}$ neutrophils appeared to be delayed as compared with WT cells (Fig. 6B). Among 80 cells analyzed, 75% of $P2X_1^{-/-}$ neutrophils showed a tail persisting for 45 min, whereas this percentage was 16.7% for WT cells (46.7% of the WT cells displayed a tail persisting for 31–45 min, and 23.3% for 16–30 min). To support these observations, we quantified cell deformation during the time course of the experiments. For this purpose, we calculated circularity ratios for all cells (see *Materials and Methods*). During migration of WT neutrophils, quite constant and repetitive oscillations of the averaged ratios occurred (representing cell deformation), but the cells always recovered their original shape (Fig. 6C). Notably, although such oscillations were also observed

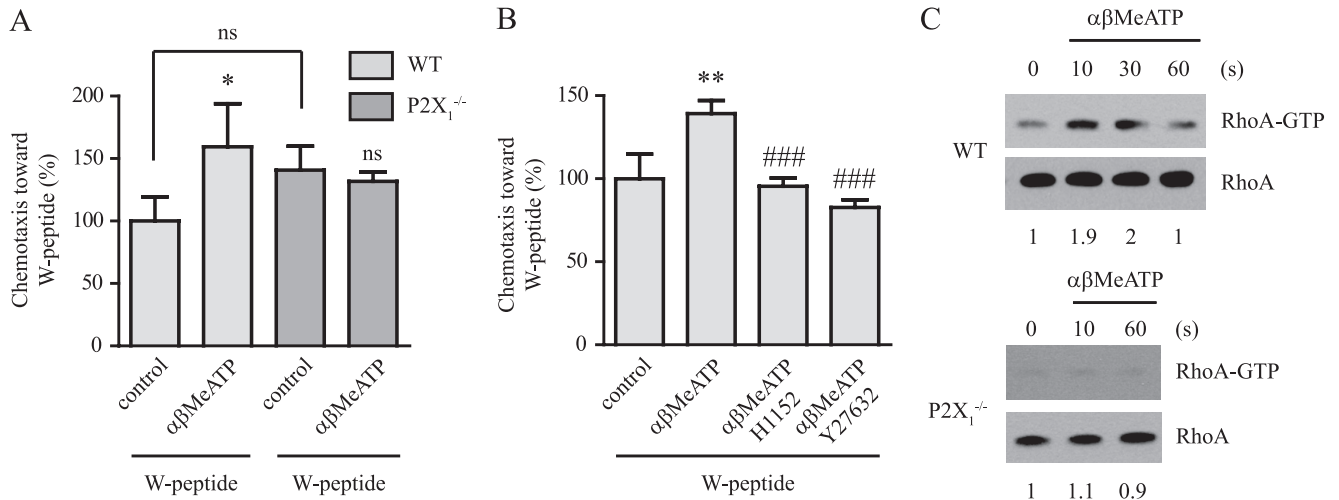


FIGURE 5. Chemotaxis and RhoA signaling in WT and P2X₁^{-/-} neutrophils. *A* and *B*, Boyden chamber assays. *A*, W-peptide (100 nM) was placed in the bottom wells of the chamber. WT and P2X₁^{-/-} peritoneal neutrophils were pretreated or not (control) with $\alpha\beta$ MeATP (10 μ M) for 1 min prior to being placed in the upper wells. *B*, WT neutrophils were preincubated or not with the Rho kinase inhibitor H1152 (1 μ M) or Y27632 (5 μ M) before pretreatment with $\alpha\beta$ MeATP, as indicated. Migrating neutrophils were counted after 1 h. Averaged data are from at least three independent experiments with neutrophil pools from three mice. *A*, *, $p < 0.05$ vs absence of $\alpha\beta$ MeATP (control). *B*, **, $p < 0.01$ vs absence of $\alpha\beta$ MeATP (control); ###, $p < 0.001$ vs absence of inhibitors. *C*, RhoA GTPase activity was assessed in WT and P2X₁^{-/-} neutrophils stimulated with $\alpha\beta$ MeATP (10 μ M) for the indicated times. Numbers below the immunoblots represent the fold increase of RhoA activity normalized to the signal detected in the absence of $\alpha\beta$ MeATP. Data are representative of two independent experiments with neutrophil pools from eight animals.

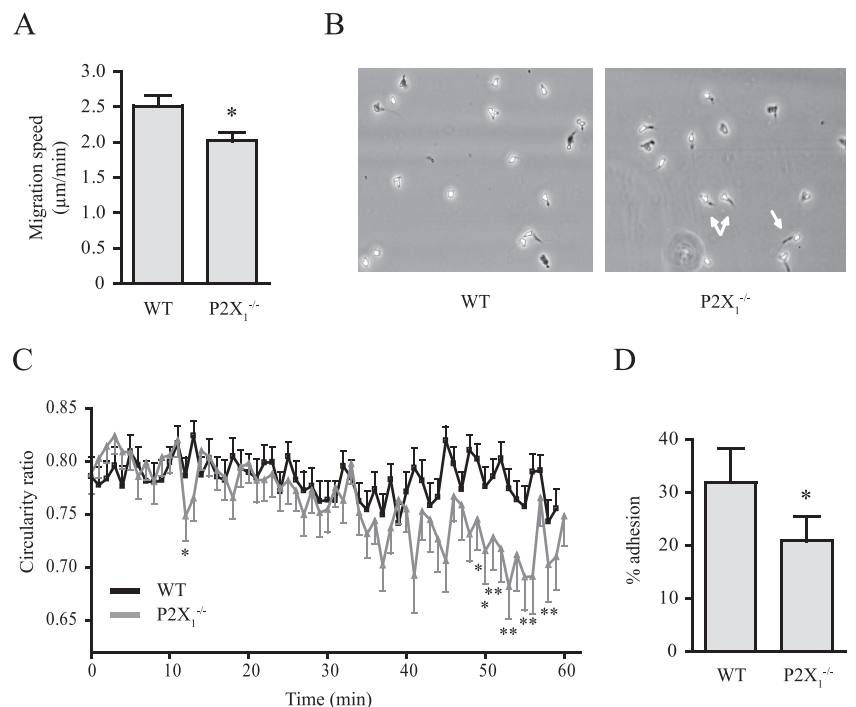
for P2X₁^{-/-} neutrophils, from ~35 min of migration up to 60 min, the shape of these cells remained thinner and longer than at earlier time steps, possibly reflecting an impaired trailing edge retraction. Furthermore, static adhesion of P2X₁^{-/-} neutrophils to collagen IV was found to be reduced by ~30% (Fig. 6D). Thus, activation of P2X₁ by released ATP could promote cell movement during chemotaxis, facilitating cell deformation and adhesion dynamics. In agreement with the Boyden chamber assays, P2X₁ would not contribute to cell orientation (gradient sensing). To determine whether P2X₁ would be involved in actin cytoskeleton reorganization in migrating neutrophils, we performed F-actin staining in

WT and P2X₁^{-/-} neutrophils uniformly stimulated with W-peptide. Polarized actin polymerization appeared to occur normally in cells lacking P2X₁ (Fig. 7).

P2X₁ activation promotes neutrophil recruitment in vivo in a peritonitis model

The role of P2X₁ in neutrophil chemotaxis was further investigated in vivo by assessing cell recruitment in the peritoneal cavity of mice i.p. injected with living *E. coli* bacteria. $\alpha\beta$ MeATP-treated mice showed significantly increased numbers of neutrophils in the

FIGURE 6. Impaired static adhesion, diminished speed, and increased time to trailing edge retraction for P2X₁^{-/-} neutrophils. *A–C*, Study of WT and P2X₁^{-/-} neutrophil two-dimensional migration using collagen IV-coated Ibidi chemotaxis microslides. *A*, Migration speed. *B*, Representative image captured at 60 min showing several P2X₁^{-/-} neutrophils with a persistent trailing edge (white arrows). *C*, Averaged circularity ratio of cells during the time course of three independent experiments. Between 40 and 100 cells were assigned scores. *, $p < 0.05$; **, $p < 0.01$. *D*, Static cell adhesion assay. Peritoneal neutrophils were placed onto collagen IV-coated wells for 15 min, and the percentage of adherent cells was determined. Averaged data are from three independent experiments with neutrophil pools from two animals. *, $p < 0.05$ vs WT.



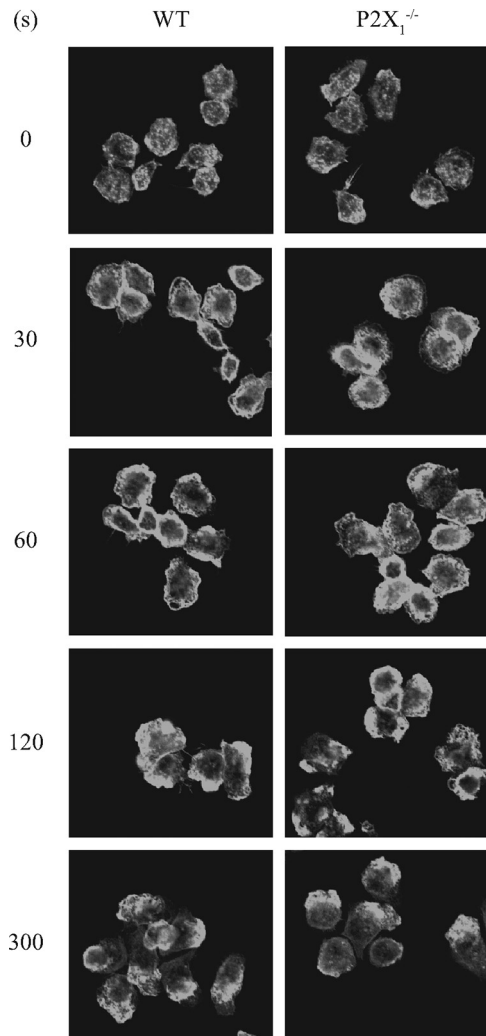


FIGURE 7. Normal F-actin polymerization in $P2X_1^{-/-}$ neutrophils uniformly stimulated with W-peptide. Confocal images of murine peritoneal neutrophils adhered to collagen IV and incubated with 100 nM W-peptide for the indicated times before labeling with AlexaFluor488-labeled phalloidin (magnification $\times 210$).

peritoneum as compared with untreated animals (Fig. 8A), indicating that $P2X_1$ activation promotes bacteria-induced neutrophil recruitment in vivo. Accordingly, we found that *E. coli*-induced neutrophil recruitment was impaired in the $P2X_1^{-/-}$ mice as compared with WT animals (Fig. 8B).

Discussion

Our data demonstrate for the first time that human and mouse neutrophils express functional $P2X_1$ ion channels. Whole-cell patch clamp revealed that two $P2X_1$ agonists evoked rapidly desensitizing inward currents that were absent ($\beta\gamma\text{MeATP}$) or impaired ($\alpha\beta\text{MeATP}$) in neutrophils isolated from $P2X_1^{-/-}$ mouse peritoneum.

Our work significantly extends the knowledge on $P2X_1$ expression in various blood cell types. In platelets, $P2X_1$, strongly expressed as a heavily glycosylated protein (27), contributes to thrombus formation under high shear stress conditions and has been proposed to be an interesting new target for antithrombotic drugs (24, 33, 34). Previous studies of $P2X_1$ receptor expression in other human hematopoietic cell types have reported $P2X_1$ receptor mRNA in total human blood leukocytes (35). Expression of $P2X_1$ receptor protein has also been found in a variety of leukemic hu-

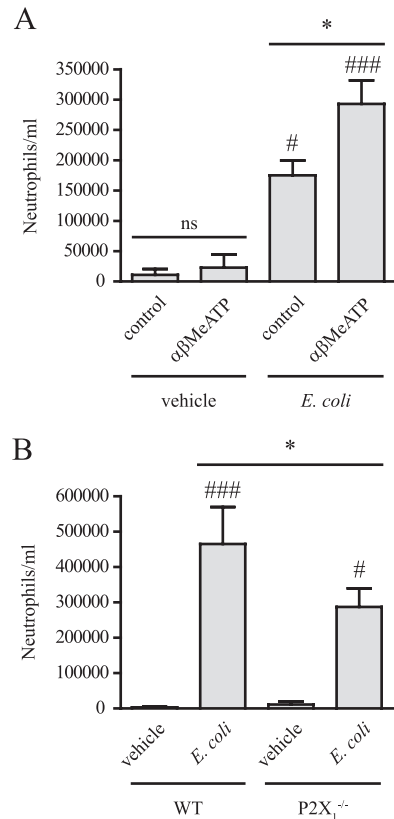


FIGURE 8. $P2X_1$ promotes neutrophil recruitment in vivo in a model of *E. coli*-induced peritonitis. **A**, WT mice injected i.p. with either PBS (vehicle) or *E. coli*. These mice were injected i.p. with $\alpha\beta\text{MeATP}$ or vehicle (control) before and after *E. coli* injection. First treatment was administered 15 min before *E. coli*/vehicle; second and third treatments were given after 1 and 2 h, respectively. Mice were sacrificed after 3 h. Neutrophils were counted in the peritoneal lavages. **B**, WT and $P2X_1^{-/-}$ mice injected i.p. with PBS (vehicle) or *E. coli*. **A** and **B**, Averaged data are from three independent experiments performed with groups of four to six mice. **A**, *, $p < 0.05$ vs control; #, $p < 0.05$ vs vehicle; ###, $p < 0.001$ vs vehicle. **B**, *, $p < 0.05$ WT *E. coli* vs $P2X_1^{-/-}$ *E. coli*; #, $p < 0.05$ vs vehicle; ###, $p < 0.001$ vs vehicle.

man cell lines, including THP1 monocytes and dibutyryl cAMP-differentiated HL-60 cells (27), and $P2X_1$ receptor function has been described in PMA-differentiated HL-60 cells (36). However, until now, no $P2X_1$ protein could be detected in circulating neutrophils and monocytes, so that $P2X_1$ expression was thought to be repressed at the most distal stages of phagocyte differentiation (27). Our data and a recent study describing the expression of functional $P2X_1$ ion channels in mouse peritoneal macrophages (37) question this hypothesis.

We present evidence that $P2X_1$ ion channels contribute to the control of neutrophil chemotaxis. First, the selective $P2X_1$ agonists, $\alpha\beta\text{MeATP}$ and $\beta\gamma\text{MeATP}$, are not chemoattractants by themselves, but they cause random migration (chemokinesis) and amplify chemotaxis induced by endogenous (IL-8) and bacterial exogenous chemoattractants (Boyden chamber assays), as well as by living bacteria (*E. coli*) (mouse peritonitis model). Second, $P2X_1^{-/-}$ mice displayed impaired neutrophil recruitment in their peritoneum upon *E. coli* injection. Third, two-dimensional chemotaxis assays using collagen IV- or fibronectin-coated microslides indicated that $P2X_1^{-/-}$ neutrophils moved with diminished speed when placed in a gradient of a bacterial mimetic peptide. We also observed that static adhesion of $P2X_1^{-/-}$ neutrophils to collagen IV was impaired. The reduced migration rates of these cells could

therefore be partially explained by defective adhesion dynamics required for efficient cell movement. P2X₁^{-/-} neutrophils showed normal orientation in the gradient, migrated normally in Boyden chamber assays, and well polarized F-actin assembly still occurred when these cells were uniformly stimulated with W-peptide. These observations suggest that P2X₁ channels do not contribute to actin polymerization at the cell front and are not involved in gradient sensing. Accordingly, in Boyden chamber assays, pretreating human neutrophils with the P2X₁ selective antagonist, NF449, did not affect the fMLP-induced chemotaxis (supplemental Fig. S1A). This is in sharp contrast with the role of P2Y₂ receptors that, upon activation by ATP released from cell leading edge, are required for proper cell orientation in the gradient of W-peptide (6) and contribute to actin polymerization at the front (38). In agreement with these data, we noted that UTP, acting at P2Y₂ receptors, was able to increase fMLP-induced chemotaxis when added together with fMLP in the bottom wells of the Boyden chamber, whereas $\alpha\beta$ MeATP could only promote chemotaxis when placed at the contact of the cells. Our findings thus emphasize differential roles for P2Y₂ and P2X₁ receptors in neutrophil migration. Because these two receptors are both activated by extracellular ATP (9), their differential effects could be related to different receptor localization (or relocalization) in membranes of migrating neutrophils and/or to the activation of distinct signaling pathways downstream from these receptors. The concentrations of ATP required to activate the two receptors in native cells may also differ. Furthermore, because P2X₁ receptor-mediated responses can be potentiated by G α_q -coupled receptors (39), P2X₁ and P2Y₂ receptors could act cooperatively to promote chemotaxis.

The fact that P2X₁^{-/-} neutrophils move more slowly in a gradient of chemoattractant, although polarizing normally, indicates that structural front-rear polarization is not sufficient to allow efficient and rapid cell movement. Conversely, it has been shown that cells with very weak morphological polarization move more slowly, but, similarly to P2X₁^{-/-} neutrophils, can chemotax with normal efficiency (40).

Signaling polarity plays an important role in determining cell movement. Cells can indeed detect differences in the concentration of chemoattractant between points on their surface (41) and translate the extracellular gradient into an intracellular derivative, encoded by the signaling networks engaged by the chemoattractant. Localized accumulation of PI3Ps is a critical signal coordinating chemotaxis or movement and depends on PI3K γ and SHIP-1 (31, 40, 42). The study of Chen et al. (6) proposed that ATP released from developing leading edges can act to control chemotaxis. However, the intracellular signals involved in this process remain undefined.

In our study, we found that signals elicited upon P2X₁ activation involved RhoA, Rho kinase, and MLC phosphorylation. Several recent studies convincingly described a gradient of Rho activity along the axis of polarized neutrophils (low at the front) (43), driving myosin-based contraction at the rear (5, 44, 45). This backness pathway is mutually exclusive (in spatial terms), but partially dependent upon a frontness network, driven by the same chemoattractant and mediated via G α_q , PI3K γ , Rac, and F-actin (44). In agreement with normal morphological polarization and F-actin assembly at the front of P2X₁^{-/-} neutrophils, we could not find any evidence for PI3K γ , Rac, or Cdc42 activation downstream from P2X₁. Nevertheless, such frontness-backness interactions could, at least partially, explain how P2X₁-mediated Rho activation at the rear would promote migration elicited by a chemoattractant. Moreover, in neutrophils, actomyosin-mediated contraction at the rear of the cell seems to be a major factor in both initiating movement and forcing the cell forward (45, 46). We thus propose that P2X₁

activation by released ATP could trigger Rho-dependent amplification signals, facilitating membrane retraction at the receding end and possibly favoring migration-linked adhesion dynamics (47, 48). In agreement with this proposition, our time-lapse video microscopy analyses revealed more pronounced and persistent deformation of P2X₁^{-/-} neutrophils and delayed detachment of trailing edge appendages as compared with WT cells. This observation and the fact that the selective P2X₁ agonist, $\alpha\beta$ MeATP, increases RhoA activity and promotes migration of WT neutrophils in a Rho kinase-dependent manner, whereas it had no effect on P2X₁^{-/-} cells, strongly suggest that the P2X₁-RhoA axis is required for efficient neutrophil movement, by favoring tail retraction.

The molecular link between P2X₁ activation and RhoA remains unknown. RhoA is currently thought to be exclusively activated via G $\alpha_{12/13}$ and G α_q (49) acting at guanine nucleotide exchange factors (GEFs) and GTPase-activating protein (50). There are at least four RhoA GEFs that link G α subunits to RhoA. Among them, Lsc has been shown to be required for normal RhoA activity, polarization, migration, and adhesion of fMLP-stimulated neutrophils (51). Lsc^{-/-} neutrophils showed inappropriate formation and retraction of supernumerary pseudopodia. They also exhibit enhanced chemokinesis, reduced directionality, and adhesion, but normal chemotaxis. Except for the fact that they both show normal gradient sensing, Lsc^{-/-} and P2X₁^{-/-} neutrophils thus display very distinct phenotypes. Surprisingly, Lsc^{-/-} neutrophils did not show any defect of tail retraction. It is thus unlikely that P2X₁ would signal via Lsc.

Interestingly, a recent work describes that a Ca²⁺/calmodulin (CaM)-dependent protein kinase increases Rac activity through its interaction with the GEF β Pak-interacting exchange factor and GIT1 in hippocampal neurons (52). Noteworthy, in platelets, P2X₁-triggered Ca²⁺ influx causes CaM- and MLC kinase-dependent increase of MLC phosphorylation, leading to cell shape change (26). Although we cannot rule out the possibility that P2X₁ could also signal through Ca²⁺-dependent pathways independently of RhoA, it would be interesting to determine whether Ca²⁺- and CaM-dependent protein kinase-dependent mechanisms of RhoA activation exist in neutrophils.

In conclusion, our study indicates that P2X₁ ion channels contribute to the ATP-dependent control of neutrophil chemotaxis by activating signals that, in the presence of a chemoattractant, facilitate cell movement, thereby enhancing chemotaxis. Further investigations are awaited to evaluate the importance of P2X₁-dependent signals *in vivo* and to determine whether these receptors could constitute therapeutic targets for the treatment of inflammatory diseases.

To our knowledge, this is the first evidence that P2X₁ ion channels contribute to cell migration. Besides the presently described role in neutrophils, it is possible that similar mechanisms apply to other P2X₁-expressing cell types. It is striking to note that P2X₁ ion channels are abundantly expressed on vascular smooth muscle cells (35, 53, 54) and on microglia (55), and might therefore contribute to various disorders related to migration of these cells that include development of restenosis, diabetic microvascular disease, chronic allograft rejection, pulmonary hypertension, as well as neurological disorders.

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Disclosures

The authors have no financial conflict of interest.

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