Knock-out Mice Reveal the Contributions of P2Y and P2X Receptors to Nucleotide-induced Ca\(^{2+}\) Signaling in Macrophages*\(^{\dagger}\)

Received for publication, August 11, 2006 Published, JBC Papers in Press, September 15, 2006, DOI 10.1074/jbc.M607713200

Adriana del Rey\(^{1}\), Vijay Renigunta\(^{3}\), Alexander H. Dalpke\(^{1}\), Jens Leipziger\(^{4}\), Joana E. Matos\(^{5}\), Bernard Robaye\(^{6}\), Marylou Zuzarte\(^{1}\), Annemieke Kavelaars\(^{7,8}\), and Peter J. Hanley\(^{1,11}\)

From the \(^{1}\)Institute of Physiology, Marburg University, Deutschhausstrasse 2, 35037 Marburg, Germany, the \(^{2}\)Department of Hygiene and Medical Microbiology, University of Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany, the \(^{3}\)Institute of Physiology and Biophysics, University of Aarhus, 8000 Aarhus, Denmark, the \(^{4}\)Institute of Interdisciplinary Research, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, 6041 Charleroi (Gosselies), Belgium, and the \(^{5}\)Laboratory for Psychoneuroimmunology, University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

Immune cell function is modulated by changes in extracellular nucleotide levels. Here we used reverse transcription-PCR analyses, single cell Ca\(^{2+}\) imaging, and knock-out mice to define the receptors mediating nucleotide-induced Ca\(^{2+}\) signaling in resident peritoneal macrophages. In Ca\(^{2+}\)-free buffer, the potent (\(K_{i,5} < 1 \mu M\)) stimulatory effect of UTP (or ATP) on endoplasmic reticulum (ER) Ca\(^{2+}\) release was abolished in cells isolated from P2Y\(_2\)/P2Y\(_4\) double knock-out mice. Moreover, P2Y\(_{2-}\)/, but not P2Y\(_{2/-}\), macrophages responded to UTP. In P2Y\(_{2/-}\) macrophages, we could elicit Ca\(^{2+}\) responses to “pure” P2X receptor activation by applying ATP in buffer containing Ca\(^{2+}\). Purified UDP and ADP were ineffective agonists, although modest UDP-induced Ca\(^{2+}\) responses could be elicited in macrophages after “activation” with lipopolysaccharide and interferon-\(\gamma\). Notably, in Ca\(^{2+}\)-free buffer, UTP-induced Ca\(^{2+}\) transients decayed within 1 min, and there was no response to repeated agonist challenge. Measurements of ER \([\text{Ca}^{2+}]\) with mag-fluo-4 showed that ER Ca\(^{2+}\) stores were depleted under these conditions. When extracellular Ca\(^{2+}\) was available, ER Ca\(^{2+}\) stores refilled, but Ca\(^{2+}\) increased to only \(\sim 40\%\) of the initial value upon repeated UTP challenge. This apparent receptor desensitization persisted in GRK2\(^{-/-}\) and GRK6\(^{-/-}\) macrophages and after inhibition of candidate kinases protein kinase C and calmodulin-dependent kinase II. Initial challenge with UTP also reduced Ca\(^{2+}\) mobilization by complement component C5a (and vice versa). In conclusion, homologous receptor desensitization is not the major mechanism that rapidly dampens Ca\(^{2+}\) signaling mediated by P2Y\(_2\), the sole G\(_{\alpha}\)-coupled receptor for UTP or ATP in macrophages. UDP responsiveness (P2Y\(_{\alpha}\) receptor expression) increases following macrophage activation.

---

\(^{*}\) This work was supported by a grant from the Kempkes-Stiftung (to P. J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{\dagger}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2 and supplemental Table 1.

\(^{1}\) To whom correspondence should be addressed. Tel.: 49-6421-286-6546; Fax: 49-6421-286-8960; E-mail: hanley@mail.uni-marburg.de.

---

Antigen-presenting cells such as monocytes, macrophages, and dendritic cells express two families of nucleotide receptors as follows: ATP-gated cation channels (P2X receptors) and P2Y receptors, a subset of the \(G\) protein-coupled receptor superfamily (1). In mouse, ATP and UTP are equipotent agonists at the \(G_{\alpha}\)-coupled receptors P2Y\(_2\) and P2Y\(_{12}\), whereas human P2Y\(_4\) is selectively activated by UTP and competitively antagonized by ATP (2). Moreover, in human, ATP additionally activates the dual \(G_{\alpha,\alpha}\)- and \(G_{\alpha,\gamma}\)-coupled P2Y\(_{11}\) receptor, absent in the mouse genome (3). The concentration of ATP or UTP in the local extracellular milieu is estimated to be around 10 \(nM\) (4, 5), which may be just sufficient to evoke local inositol 1,4,5-trisphosphate (IP\(_3\))-\(\alpha\)-trisphosphate (IP\(_3\))\(^2\)-induced Ca\(^{2+}\) puffs (6). However, mechanical stress, cellular injury, inflammation, degradation of mast cells, and other factors may increase ATP and UTP to levels sufficient to evoke larger and longer lasting global Ca\(^{2+}\) signals (5, 7, 8). ATP is also released as a cotransmitter from the sympathetic nervous system, a potentially important neuroimmune interaction (9, 10).

Local increases in ATP or UTP levels are transient because of both diffusion and the activity of ecto-nucleotidases such as CD39 (NTPDase 1), which catalyzes the sequential hydrolysis of ATP and UTP to their respective monophosphates (8, 11). A subset of P2Y receptors is selectively activated by nucleotide diphosphates. In particular, P2Y\(_1\) and P2Y\(_{12}\) receptors are preferentially activated by ADP and play important roles in platelet function (12). UTP degradation by CD39 yields transiently the intermediate UDP, which is a specific agonist for P2Y\(_{6}\) receptors (13). During prolonged agonist stimulation, the response of P2Y and P2X receptors is typically switched off within minutes (14–16). In the case of G protein-coupled receptors, the activity of the activated G protein is terminated by GTPase-activating proteins, which catalyze the hydrolysis of GTP bound to the \(\alpha\)-subunit (17). Agonist-induced “desensitization” is thought to involve phosphorylation of the C terminus (or intracellular loops) by G protein-coupled receptor kinases (GRKs) or second messenger-induced protein kinase C (PKC). Phosphorylation...
by GRK promotes the rapid binding of β-arrestin, which blocks further G protein activation (18, 19).

Because of the lack of specific agonists and antagonists, it is difficult to assign unequivocally P2Y and P2X receptor subtypes to a particular cell type. In this study, by using P2Y2−/− and/or P2Y4−/− mice, as well as RT-PCR analyses, we show that P2Y4 is the dominant receptor in resident peritoneal macrophages. Furthermore, we dissected the interplay of P2Y2 receptors, P2X receptors, Ca2+ release-activated Ca2+ channels, and Ca2+-ATPases (Ca2+ pumps) in nucleotide-induced Ca2+ signaling. Finally, we assessed the role of PKC, calmodulin-dependent kinase II (CaMKII), GRK2, and GRK6 in the rapid desensitization of P2Y2 receptors using inhibitors and GRK-deficient mice.

**EXPERIMENTAL PROCEDURES**

**Knock-out (KO) Mice—**P2Y2−/−, P2Y4−/−, and P2Y2/P2Y4 double KO mice were generated as described recently (20). Heterozygous P2Y2 mice originally were kindly donated by Dr. Beverly H. Koller (University of North Carolina, Chapel Hill), and the inactivation of the P2Y4 gene, which is located on the X chromosome, has been described previously (21). GRK2−/− mice were kindly provided by Dr. Marc G. Caron (Duke University Medical Center, Durham, NC), and GRK6-deficient mice were generously provided by Dr. Richard T. Premont and Dr. Robert J. Lefkowitz (Duke University Medical Center). The genetic backgrounds of the male littermate wild-type (WT) and KO mice used in this study were as follows: P2Y2−/− (mixed B6D2 × SV129); P2Y4−/− (mixed CD-1 × SV129); P2Y2/P2Y4 double KO (mixed B6D2 × CD-1 × SV129); GRK2−/− (C57BL/6); and GRK6−/− (C57BL/6). Heterozygous GRK2 mice were used because homozygous mutations are embryonically lethal.

**Isolation of Peritoneal Macrophages—**Mice were killed by cervical dislocation, and resident peritoneal cells were harvested by lavage with 10 ml of ice-cold Hanks’ physiological salt solution. After centrifugation, cells were resuspended in RPMI medium containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded onto glass coverslips and incubated at 37 °C in air with 5% CO2. After 2 h, nonadherent cells were removed by washing the coverslip. In selected experiments, macromolecules were activated by incubation for >24 h in RPMI medium (with 10% calf serum) containing 100 ng/ml lipopolysaccharide and 100 units/ml interferon-γ.

**Flow Cytometry—**B220−CD11b+ cells (macrophages) were purified by depletion of B220+ cells (B lymphocytes), followed by positive selection for CD11b+ cells (supplemental Fig. 1) using a magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). Note that B1 cells are the major subpopulation of B lymphocytes in the peritoneal cavity and weakly express CD11b (22). Aliquots before and after purification were stained with phycocerythrin-conjugated anti-B220 (clone RA3–6B2; Pharmingen), allophycocyanin-conjugated anti-CD11b (clone M1/70; Pharmingen), and a fluorescein isothiocyanate-conjugated antibody (clone Cl:A3-1; Serotec, Düsseldorf, Germany) against the macrophage marker F4/80. Phenotypical analysis was performed by flow cytometry using a FACS Canto flow cytometer (BD Biosciences).
RESULTS

P2Y Receptor Signaling—To determine the effects of extracellular nucleotides on ER Ca\(^{2+}\) release in macrophages, Ca\(^{2+}\)-free buffer was used to obviate the potentially confounding effects of store-operated Ca\(^{2+}\) entry and Ca\(^{2+}\) influx via P2X receptors. When single WT macrophages were superfused with UTP in Ca\(^{2+}\)-free buffer, a concentration-dependent increase in peak cytosolic [Ca\(^{2+}\)] ([Ca\(^{2+}\)\(_i\)]\(_{peak}\)) was evoked (Fig. 1A); UTP increased peak [Ca\(^{2+}\)] with a \(K_0.5\) value of 0.7 \(\mu\)M. Activation of either P2Y\(_2\) or P2Y\(_4\) receptors could be responsible for the transient Ca\(^{2+}\) response to UTP in Ca\(^{2+}\)-free buffer. Both of these receptor subtypes have recently been reported to be expressed in mouse macrophages (23). To explore the relative roles of P2Y\(_2\) and P2Y\(_4\) receptors, we first challenged macrophages isolated from P2Y\(_2\) and P2Y\(_4\) knock-out mice with UTP. Concentrations of UTP between 0.1 and 100 \(\mu\)M, or as high as 250 \(\mu\)M (\(n = 7\); not shown), had no effect on [Ca\(^{2+}\)]\(_i\) in macrophages isolated from P2Y\(_2\)/P2Y\(_4\) double knock-out mice (Fig. 1B), indicating that P2Y\(_2\) or P2Y\(_4\) are the only possible G\(_q\)-coupled receptors mediating UTP-induced Ca\(^{2+}\) signaling.

P2Y\(_2\)/P2Y\(_4\) double knock-out mice exhibited no obvious phenotype (20), and macrophages isolated from these animals were indistinguishable from WT cells (Fig. 2, A and B). Moreover, similar to WT cells, macrophages isolated from double knock-out mice expressed mRNA specific for P2Y\(_1\) and P2Y\(_6\) (Fig. 2C), and P2Y\(_2\), but not P2Y\(_4\), could be detected in WT cells. Mouse whole brain RNA was used as positive control for the P2Y\(_4\)-specific primer (not shown). Both WT and double knock-out macrophages expressed several ATP-gated, nonselective cation channels as follows: P2X\(_1\), P2X\(_4\), and P2X\(_7\) receptors (Fig. 2D). The upper band in the P2X\(_4\) lanes was not identified.

To assess the relative contributions of P2Y\(_2\) and P2Y\(_4\) receptors, we compared the effects of UTP on Ca\(^{2+}\) signaling in macrophages isolated from P2Y\(_2\)/P2Y\(_4\) double knock-out mice. Similar to WT cells, application of 10 \(\mu\)M UTP (in Ca\(^{2+}\)-free buffer) elicited a transient and oscillatory increase in cytosolic Ca\(^{2+}\) in macrophages isolated from P2Y\(_2\)/P2Y\(_4\) double knock-out mice, as shown in Fig. 3A. However, as illustrated by the example in Fig. 3B, macrophages isolated from P2Y\(_2\)-deficient mice did not respond to UTP, but these cells responded to ATP, provided that Ca\(^{2+}\) was present in the buffer (Fig. 3B). The concentration-response relations for macrophages isolated from P2Y\(_4\)- and P2Y\(_2\)/P2Y\(_4\) mice are presented in Fig. 3, C and D. Thus, consistent with the RT-PCR data, the concentration-response data reveal that P2Y\(_2\) is the sole G\(_q\)-coupled receptor for UTP in resident macrophages. UTP is sequentially degraded to UDP and UMP extracellularly, a reaction catalyzed by the surface membrane enzyme...
P2Y and P2X Receptors in Macrophages

FIGURE 3. P2Y<sup>−/−</sup> but not P2X<sup>−/−</sup> macrophages respond to UDP. A, typical UDP-induced Ca<sup>2+</sup> response of a macrophage isolated from a P2Y<sup>−/−</sup> mouse. B, UDP fails to increase Ca<sup>2+</sup> in a macrophage isolated from a P2Y<sup>−/−</sup> mouse. However, in the presence of extracellular Ca<sup>2+</sup>, 100 µM ATP induces a small, transient Ca<sup>2+</sup> signal. C and D, plots of [UDP] versus peak [Ca<sup>2+</sup>], for macrophages isolated from P2Y<sup>−/−</sup> (C) and P2Y<sup>−/−</sup> (D) mice. Each data point is the mean ± S.E. of 4–6 cells.

CD39. Because we detected mRNA for P2Y<sub>6</sub> in both WT and double knock-out macrophages, we expected to observe ER Ca<sup>2+</sup> release induced by UDP. In preliminary experiments, 100 µM UDP (97% pure by high performance liquid chromatography) consistently induced ER Ca<sup>2+</sup> release. However, after contaminating UTP (or ATP) was scavenged with hexokinase (24), 10 of 11 WT macrophages did not respond at all to 100 µM UDP, whereas all cells responded to ATP, used as positive control (Fig. 4A). Note that when extracellular Ca<sup>2+</sup> was reintroduced after stimulating cells with high ATP (or UTP; not shown) concentrations in Ca<sup>2+</sup>-free buffer, an increase in intracellular Ca<sup>2+</sup>, consistent with store-operated Ca<sup>2+</sup> entry (25), was always observed (Fig. 4A). Moreover, similar to WT, 12 of 17 P2Y<sub>2/P2Y<sub>4</sub></sub>-deficient macrophages did not respond to 100 µM UDP, and the response in the remaining five cells was characterized by a small, single Ca<sup>2+</sup> transient (Fig. 4B). Taken together, the [UDP]-response relations for macrophages from both WT and P2Y<sub>2/P2Y<sub>4</sub></sub>-deficient double knock-out mice were essentially flat in the 1–100 µM range (Fig. 4C). These data reveal that, functionally, P2Y<sub>6</sub> is weakly expressed in resident macrophages. However, when P2Y<sub>2/P2Y<sub>4</sub></sub>-deficient macrophages were activated for 48–72 h with lipopolysaccharide and interferon-γ, responsiveness increased, such that most cells (9 of 11) produced a small oscillatory Ca<sup>2+</sup> response to 100 µM UDP (peak F/F<sub>0</sub> 3.0 ± 0.2; not shown).

To explore the potential role of P2Y<sub>1</sub> receptors, we challenged macrophages with ADP under Ca<sup>2+</sup>-free conditions. Application of 100 µM ADP (purified with hexokinase) had no effect on [Ca<sup>2+</sup>], in 9 of 11 WT cells, whereas ATP produced a strong positive control response (Fig. 5A). Concentration-response relations for ADP and ATP in WT macrophages are overlaid in Fig. 5B. Similar to WT, ADP also had little or no effect in macrophages isolated from P2Y<sub>2/P2Y<sub>4</sub></sub> double knock-out mice, and no response to ATP was observed (Fig. 5C).

P2X Receptor Signaling—Although we could not elicit a Ca<sup>2+</sup> response to UTP in macrophages isolated from P2Y<sub>2</sub>-deficient (P2Y<sub>2</sub><sup>−/−</sup> or double knock-out) mice, we could observe a small transient response to ATP provided that Ca<sup>2+</sup> was present in the buffer (Fig. 6). Thus, targeted disruption of the P2Y<sub>2</sub> gene reveals the Ca<sup>2+</sup> response to pure P2X receptor activation. The Ca<sup>2+</sup> response elicited by ATP was small and diminished upon repeated application of agonist (Fig. 6, A and B). The concentration-response relation in the 0.1–250 µM ATP range is shown in Fig. 6C (data from P2Y<sub>2</sub><sup>−/−</sup> and double knock-out mice were combined). Note that in RT-PCR analyses we could detect P2X<sub>1</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub>, in macrophages isolated from both WT and P2Y<sub>2/P2Y<sub>4</sub></sub> double knock-out mice (see Fig. 2D). For comparison, the pure P2Y receptor response to ATP is overlaid in Fig. 6. It can be seen that the P2Y receptor-induced Ca<sup>2+</sup> response greatly dominates over the P2X receptor response in the 0.1–10 µM ATP range.
GRK6 Is Not Essential for Rapid Desensitization of P2Y₂ Receptors—When extracellular Ca²⁺ was available, application of 100 μM UTP typically produced a rapid increase in [Ca²⁺], followed by fast and slow components of decline (Fig. 7A), the latter of which was absent in Ca²⁺-free conditions, and can be attributed to store-operated Ca²⁺ entry. Furthermore, the Ca²⁺ response to repeated UTP challenge was greatly diminished (Fig. 7A), suggesting that the initial challenge may have desensitized the receptor. GRKs have been implicated in the desensitization mechanism of various G protein-coupled receptors, and to test whether GRK6 is involved in the rapid desensitization of P2Y₂ receptors, we isolated macrophages from GRK6⁻/⁻ mice. Compared with WT cells, there was no enhancement of the second UTP-induced Ca²⁺ response in GRK6-deficient macrophages, even when cells were pretreated with inhibitors of the candidate regulatory kinases PKC (staurosporine) and CaMKII (KN93) (Fig. 7B). RT-PCR analyses of RNA extracted from purified macrophages indicated that GRK2 and GRK5 were also expressed, but only weak signals for GRK3 and GRK4 were detected (Fig. 7C). We also found that apparent desensitization, assayed by repeated challenges with 100 μM UTP, was not reduced in macrophages isolated from GRK2⁻/⁻ mice, which express 50% protein compared with WT (26). Thus, the experiments with WT, GRK2⁻/⁻, and GRK6⁻/⁻ macrophages summarized in Fig. 7D (see also supplemental Fig. 2) suggest that the kinases GRK2, GRK6, PKC, and CaMKII are not necessary for the rapid dampening of P2Y₂ receptor signaling in macrophages.

Stimulation of P2Y₂ Receptors Decreases the Subsequent Response to a Non-P2Y Gq-coupled Receptor—If the decreased Ca²⁺ response to repeated UTP application is because of homologous P2Y₂ receptor desensitization, then the response to a non-P2Y Gq-coupled receptor should be unaffected following initial UTP challenge. To test this possibility, we used complement factor C5a as a second agonist, the receptor of which has been shown using WT versus Gq/15 mice to be coupled to Gq/15 (27), whereas P2Y₂ receptors are coupled to Gq/11. When a macrophage was first challenged with 100 μM UTP followed by 100 nM C5a, the second Ca²⁺ response was decreased (Fig. 8A) and vice versa (Fig. 8B), indicating that heterologous mechanisms, such as phosphatidylinositol 4,5-bisphosphate depletion, are contributing to the decayed Ca²⁺ responses to repeated UTP challenges. On average, the peak of the second Ca²⁺ response was ~40% of the initial response when UTP was applied twice, whereas the second response was ~60% (relative to the first response) when different agonists were used (Fig. 8C). Another potentially important factor determining the magnitude of the second agonist-induced Ca²⁺ response is the availability of ER Ca²⁺. Indeed, there was no response at all to repeated UTP challenge when experiments were performed in Ca²⁺-free buffer (Fig. 8C), an unphysiological condition that promotes Ca²⁺ extrusion via the plasma membrane Ca²⁺-ATPase (Ca²⁺ pump).

Contribution of ER Ca²⁺ Depletion to Apparent P2Y₂ Receptor Desensitization

**FIGURE 5.** Lack of P2Y₂ receptor responsiveness in macrophages from WT and P2Y₂/P2Y₄ double knock-out mice. A, application of 100 μM ADP (purified with hexokinase) under Ca²⁺-free conditions had no effect on [Ca²⁺]. ATP was used as positive control. B and C, overlay of concentration-response relations for ATP (open symbols) and ADP (solid symbols) in macrophages isolated from WT (B) and P2Y₂/P2Y₄ double knock-out (DKO) (C) mice. Each data point is the mean ± S.E. of 6–10 cells.

**FIGURE 6.** P2Y₂-deficient cells unmask the Ca²⁺ response to P2X receptor activation. A and B, in the presence of extracellular Ca²⁺, application of 100 μM ATP (A) or 250 μM ATP (B) to P2Y₂-deficient macrophages elicits a small and transient Ca²⁺ response, which is decreased upon repeated agonist challenge. C, plot of [ATP] versus peak [Ca²⁺] in P2Y₂-deficient macrophages. This plot essentially shows the peak Ca²⁺ response to P2X receptor activation. For comparison, the P2Y₂ receptor response, obtained using WT macrophages under Ca²⁺-free conditions, is overlaid (dashed line). DKO, double knock-out mice.

**FIGURE 7.** Staging of P2Y₂ and P2X receptors in macrophages.
Desensitization—Mag-fluo-4 was selectively loaded into the ER lumen to measure \([\text{Ca}^{2+}]_\text{ER}\). When 100 \(\mu\)M UTP was applied to macrophages, a decrease in \([\text{Ca}^{2+}]_\text{ER}\) was observed (Fig. 9A), which on average recovered 77.3 \(\pm\) 4.5\% (\(n = 6\)). Compared with fluo-3, mag-fluo-4 was much more susceptible to photobleaching, and thus the extent of recovery was probably underestimated in these experiments. In \(\text{Ca}^{2+}\)-free buffer, the recovery of \([\text{Ca}^{2+}]_\text{ER}\) after transient application of UTP was less than 15\% (\(n = 5\)), as shown in Fig. 9B. However, the ER lumen was rapidly refilled with \(\text{Ca}^{2+}\) after switching to buffer containing 1.3 mM \(\text{Ca}^{2+}\) (Fig. 9B).

**DISCUSSION**

Calcium is an important regulatory ion inside cells, and in immune cells it has been implicated in diverse functions,
P2Y and P2X Receptors in Macrophages

In P2Y₂-deficient macrophages, ATP-induced Ca²⁺ mobilization was abolished in strictly Ca²⁺-free conditions. This lack of responsiveness to ATP underscores that there is no equivalent to the human P2Y₁₁ receptor in mouse macrophages. In the presence of extracellular Ca²⁺, a small Ca²⁺ transient induced by ATP is observed and most likely represents the pure P2X receptor response to this agonist. We found that mouse macrophages express P2X₁, P2X₄, and P2X₇ mRNA, which seems to be a common pattern of expression for macrophages from various species (23, 29, 33). P2X₄ receptors do not form heteromeric receptors with other P2X subunits, but heteromeric P2X₁/P2X₄ receptor assembly has been described recently in an oocyte expression system (34). Whether heteromeric P2X₁/P2X₄ receptors, or even P2Y receptor heteromerization (35), play a functional role in native cells remains to be established. At this stage, we assume that homomeric P2X₄ receptors are the major contributors to Ca²⁺ influx in the concentration range we tested (0.1–250 μM) because the P2X₁ receptor response has been reported to terminate within 1 s, and the P2X₄ receptor is much less sensitive to ATP (15). Under physiological conditions, P2X receptor signaling is not only switched off by receptor desensitization, which we could observe in superfused cells, but also by rapid degradation of ATP by ecto-nucleotidases.

ADP is the preferential agonist for the Gₛ-coupled P2Y₁ receptor, and RT-PCR analyses showed that this P2Y₁ receptor subtype is expressed in mouse macrophages along with P2Y₄. In peritoneal macrophages isolated from BALB/c mice, this agonist has been reported to have no effect on [Ca²⁺]ᵢ at 10 μM but to have a modest effect (possibly overestimated because of contaminating ATP) at 100 μM (23). We also observed that ADP (purified with hexokinase) had negligible effect in macrophages isolated from WT or P2Y₂-deficient mice. We additionally detected P2Y₆ receptors in RT-PCR analyses; however, UDP-induced Ca²⁺ release was scant in resident macrophages isolated from either P2Y₂/P2X₄ double knock-out or WT mice. A modest response to UDP, though, was observed after macrophages had been activated with lipopolysaccharide and interferon-γ, suggesting that, functionally, P2Y₆ receptors are weakly expressed in resting macrophages but, following activation by stimuli such as Toll-like receptor ligands, P2Y₆ gene expression is switched on, increasing the scope of uridine nucleotide-induced signaling.

Signal transduction by G protein-coupled receptors, including P2Y receptors, is tightly controlled by mechanisms that dampen signal transmission. The rapid termination of P2Y₂ receptor-induced Ca²⁺ signaling and refractoriness to repeated agonist challenge we observed could, in principle, be due to several mechanisms. The activated Gₛ₁₁ subunit may be inhibited by GTPase-activating proteins, such as members of the regulators of G protein signaling family (17, 36), and direct receptor phosphorylation by PKC or GRKs (promoting the recruitment of β-arrestin) may uncouple the receptor. Our data obtained using GRK₂⁻/⁻ and GRK₆⁻/⁻ mice, as well as pharmacological inhibition of PKC, suggest that GRK6, PKC, or normal levels of GRK2 are not essential for switching off Ca²⁺ signaling induced by P2Y₂ receptor activation. Consistent with our observations, González and co-workers (37, 38) deduced...
that PKC was not responsible for agonist-induced P2Y2 receptor desensitization, but they provided evidence that receptor phosphorylation by an unidentified kinase was involved. We also found that inhibition of CaMKII did not prevent apparent P2Y2 receptor desensitization. Similarly, Tulapurkar et al. (39) reported that inhibition of CaMKII did not affect desensitization of P2Y1 receptors, but it blocked internalization of activated receptors.

Local depletion of phosphatidylinositol 4,5-bisphosphate (the source of IP$_3$), which has been nicely shown in myocytes to have low mobility in the surface membrane (40), could contribute to switching off signal transmission by activated G$_q$-coupled receptors. This could explain why the Ca$^{2+}$ response is greater when the second agonist is C5a instead of UTP after initial challenge with UTP, i.e., the C5a receptor has access to a spatially distinct lipid pool. Downstream of IP$_3$ generation, ER Ca$^{2+}$ stores may become depleted, intraluminal Ca$^{2+}$ cycling may be rate-limiting, or the IP$_3$ receptor may be rendered refractory. ER [Ca$^{2+}$] measurements suggest that Ca$^{2+}$ stores are rapidly replenished following P2Y$_2$ receptor stimulation, but under certain conditions favoring plasma membrane Ca$^{2+}$ transport systems (Ca$^{2+}$-free buffer) ER stores are depleted, and there is no response to repeated agonist challenge. Thus, the interplay of Ca$^{2+}$ transport systems is an important determinant of G$_q$-coupled receptor-mediated Ca$^{2+}$ signaling.

In conclusion, we can summarize the main findings of this study in the schematic diagram shown in Fig. 10. Various stimuli such as mechanical stress, cell injury, or inflammation release UTP and ADP into the extracellular space. Macrophages sense the increased nucleotide levels through the dominant G$_q$-coupled P2Y$_2$ receptor that is activated by UTP = ATP but insensitive to UDP and ADP. ATP additionally induces Ca$^{2+}$ influx via P2X receptors, and UDP activates the P2Y$_6$ receptor, functionally expressed in activated macrophages. Downstream of the activated G$_q$-coupled receptors, IP$_3$ is generated and releases Ca$^{2+}$ from the endoplasmic reticulum. At the same time, the recently identified Ca$^{2+}$-release sensor STIM1 probably translocates to the surface membrane (Fig. 10, dashed arrow) and activates Ca$^{2+}$-release-activated Ca$^{2+}$ channels to promote Ca$^{2+}$ entry and refilling of stores (25). Two Ca$^{2+}$-ATPases compete to clear Ca$^{2+}$ from the cytosol as follows: the sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase and the plasma membrane Ca$^{2+}$-ATPase. Conditions favoring plasma membrane Ca$^{2+}$-ATPase activity will lead to diminished ER refilling.

**REFERENCES**

1. Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J. M., Morelli, A., Torboli, M., Bolognesi, G., and Baricordi, O. R. (2001) Blood 97, 587–600
2. Herold, C. L., Qi, A. D., Harden, T. K., and Nicholas, R. A. (2004) J. Biol. Chem. 279, 11456–11464
3. Communi, D., Robaye, B., and Boeynaems, J. M. (1999) Br. J. Pharmacol. 128, 1199–1206
4. Lazarowski, E. R., and Harden, T. K. (1999) Br. J. Pharmacol. 127, 1272–1278
5. Lazarowski, E. R., Boucher, R. C., and Harden, T. K. (2003) Mol. Pharmacol. 64, 785–795
6. Koizumi, S., Saito, Y., Nakazawa, K., Nakajima, K., Sawada, J. I., Kohsaka, S., Illes, P., and Inoue, K. (2002) Life Sci. 72, 431–442
7. Osipchuk, Y., and Cahalan, M. (1992) Annu. Rev. Biochem. 61, 1272–1278
8. Lazarowski, E. R., Homolya, L., Boucher, R. C., and Harden, T. K. (1997) J. Biol. Chem. 272, 24348–24354
9. Haskó, G., and Szabó, C. (1998) Biochem. Pharmacol. 56, 1079–1087
10. Westfall, D. P., Todorov, L. D., and Mihaylova-Todorova, S. T. (2002) J. Pharmacol. Exp. Ther. 303, 439–444
11. Lazarowski, E. R., Boucher, R. C., and Harden, T. K. (2000) J. Biol. Chem. 275, 31061–31068
12. Gachet, C. (2005) Annu. Rev. Pharmacol. Toxicol. 46, 277–300
13. Lazarowski, E. R., Rochelle, L. G., O’Neal, W. K., Ribeiro, C. M. P., Grubb, B. R., Zhang, V., Harden, T. K., and Boucher, R. C. (2001) J. Pharmacol. Exp. Ther. 297, 43–49
14. Koshimizu, T., Koshimizu, M., and Stojilkovic, S. S. (1999) J. Biol. Chem. 274, 37651–37657
15. North, R. A. (2002) Physiol. Rev. 82, 1013–1067
16. Hardy, A. R., Conley, P. B., Luo, I., Benovic, J. L., Poole, A. W., and Mundell, S. J. (2005) Blood 105, 3552–3560
17. Ross, E. M., and Wilkie, T. M. (2000) Annu. Rev. Biochem. 69, 795–827
18. Krasel, C., Bünnemann, M., Lorenz, K., and Lohse, M. J. (2005) J. Biol. Chem. 280, 9528–9535
19. Lefkowitz, R. J., and Shenoy, S. (2005) Science 308, 512–517
20. Matos, J. E., Robaye, B., Boeynaems, J. M., and Leipziger, J. (2005) J. Physiol. (Lond.) 564, 269–279
21. Robaye, B., Ghaheri, E., Wilkin, F., Fokan, D., van Driessen, W., Schurmans, S., Boeynaems, J. M., and Beauwens, R. (2003) Mol. Pharmacol. 63, 777–783
22. Montecino-Rodriguez, E., Leathers, H., and Dorshkind, K. (2006) Nat. Immunol. 7, 293–301
23. Coutinho-Silva, R., Ojcius, D. M., Góręcki, D. C., Persechini, P. M., Bisaglia, A. N., Mendes, A. N., Marks, J., Burnstock, G., and Duno, P. M. (2005) Biochem. Pharmacol. 69, 641–655
24. Harden, T. K., Lazarowski, E. R., and Boucher, R. C. (1997) Trends Pharmacol. Sci. 18, 43–46
25. Zhang, S. L., Yu, Y., Roos, J., Kozak, J. A., Deerinck, T. J., Elismas, M. H., Stauderman, K. A., and Cahalan, M. D. (2005) Nature 437, 902–905
26. Vroom, A., Kavelaars, A., Limmoorth, V., Lombardi, M. S., Goebel, M. U., van Dam, A. M., Caron, M. G., Scheldowsky, M., and Heijnen, C. J. (2005) J. Immunol. 174, 4400–4406
27. Davignon, I., Catalina, M. D., Smith, D., Montgomery, J., Swantek, J., Croy, J., Siegelman, M., and Wilkie, T. M. (2000) Mol. Cell. Biol. 20, 797–804
28. Marteau, F., Communi, D., Boeynaems, J. M., and Suarez-Gonzalez, N. (2004) J. Leukocyte Biol. 76, 796–803

**FIGURE 10. Schematic diagram of nucleotide-induced Ca$^{2+}$ signaling in a resident macrophage.** Abbreviations: PLC$\beta$, phospholipase C$\beta$; PMCA, plasma membrane Ca$^{2+}$-ATPase; CRAC, Ca$^{2+}$-release-activated Ca$^{2+}$ channel; SERCA, sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase.
35155

P2Y and P2X Receptors in Macrophages

29. Hanley, P. J., Musset, B., Renigunta, V., Limberg, S. H., Dalpke, A. H., Sus, R., Heeg, K. M., Preisig-Müller, R., and Daut, J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9479–9484
30. Kaufmann, A., Musset, B., Limberg, S. H., Renigunta, V., Sus, R., Dalpke, A. H., Heeg, K. M., Robaye, B., and Hanley, P. J. (2005) J. Biol. Chem. 280, 32459–32467
31. Gallo, E. M., Cante-Barrett, K., and Crabtree, G. R. (2006) Nat. Immun. 7, 25–32
32. Mak, D.-O. D., McBride, S., and Foskett, J. K. (2001) J. Gen. Physiol. 177, 435–446
33. Bowler, J. W., Bailey, R. J., North, R. A., and Surprenant, A. (2003) Br. J. Pharmacol. 140, 567–575
34. Nicke, A., Kerschensteiner, D., and Soto, F. (2005) J. Neurochem. 92, 925–933
35. Yoshioka, K., Saitoh, O., and Nakata, H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7617–7622
36. Hollinger, S., and Hepler, J. R. (2002) Pharmacol. Rev. 54, 527–559
37. Otero, M., Garrad, R. C., Velázquez, B., Hernández-Pérez, M. G., Camden, J. M., Erb, L., Clarke, L. L., Turner, J. T., Weisman, G. A., and González, F. A. (2000) Mol. Cell. Biochem. 205, 115–123
38. Flores, R. V., Hernández-Pérez, M. G., Aquino, E., Garrad, R. C., Weisman, G. A., and González, F. A. (2005) Mol. Cell. Biochem. 280, 35–45
39. Tulapurkar, M. E., Zündorf, G., and Reiser, G. (2006) J. Neurochem. 96, 624–634
40. Cho, H., Kim, Y. A., Yoon, J.-Y., Lee, D., Kim, J. H., Lee, S. H., and Ho, W.-K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 15241–15246