Extracellular ATP Induces Cytokine Expression and Apoptosis through P2X7 Receptor in Murine Mast Cells

Elena Bulanova, Vadim Budagian, Zane Orinska, Martina Hein, Frank Petersen, Lutz Thon, Dieter Adam and Silvia Bulfone-Paus

*J Immunol* 2005; 174:3880-3890; ;
doi: 10.4049/jimmunol.174.7.3880
http://www.jimmunol.org/content/174/7/3880

---

**References**

This article cites 58 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/174/7/3880.full#ref-list-1

---

**Why *The JI***? Submit online.

- **Rapid Reviews! 30 days**\(^*\) from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

\(^*\)average

---

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Errata  An erratum has been published regarding this article. Please see
next page or:
/content/186/4/2683.full.pdf
Extracellular ATP Induces Cytokine Expression and Apoptosis through P2X\(_7\) Receptor in Murine Mast Cells

Elena Bulanova,\(^1,2,\ast\) Vadim Budagian,\(^1\ast\) Zane Orinska,\(*\) Martina Hein,* Frank Petersen,* Lutz Thon,† Dieter Adam,† and Silvia Bulfone-Paus*

Extracellular ATP and other nucleotides act through specific cell surface receptors and regulate a wide variety of cellular responses in many cell types and tissues. In this study, we demonstrate that murine mast cells express several P2Y and P2X receptor subtypes including P2X\(_7\), and describe functional responses of these cells to extracellular ATP. Stimulation of bone marrow-derived mast cells (BMMC), as well as MC/9 and P815 mast cell lines with millimolar concentrations of ATP, resulted in Ca\(^{2+}\) influx across the cellular membrane and cell permeabilization. Moreover, brief exposures to ATP were sufficient to induce apoptosis in BMMCs, MC/9, and P815 cells which involved activation of caspase-3 and -8. However, in the time period between commitment to apoptosis and actual cell death, ATP triggered rapid but transient phosphorylation of multiple signaling molecules in BMMCs and MC/9 cells, including ERK, Jak2, and STAT6. In addition, ATP stimulation enhanced the expression of several proinflammatory cytokines, such as IL-4, IL-6, IL-13, and TNF-\(\alpha\). The effects of ATP were mimicked by submillimolar concentrations of 3-O-(4’-benzoyl)-benzoyl-benzoyl-ATP, and were inhibited by pretreatment of mast cells with a selective blocker of human and mouse P2X\(_7\) receptor, I[N,O-bis[5-isoxazolinesulphonyl]-N-methyl-L-tyrosyl]-4-phenylpiperazine, as well as oxidized ATP. The nucleotide selectivity and pharmacological profile data support the role for P2X\(_7\) receptor as the mediator of the ATP-induced responses. Given the importance of mast cells in diverse pathological conditions, the ability of extracellular ATP to induce the P2X\(_7\)-mediated apoptosis in these cells may facilitate the development of new strategies to modulate mast cell activities. The Journal of Immunology, 2005, 174: 3880–3890.

Mast cells are derived from CD34\(^+\) hemopoietic progenitor cells and circulate in blood and the lymphatic system before homing to tissues and acquiring their final effector characteristics under the influence of local tissue-microenvironmental factors (1–3). The expansion, homing, and maturation of mast cell progenitors are influenced by several cytokines, most important of which are stem cell factor and IL-3 (1). Mast cells are recognized as the key cells of allergic inflammatory reactions, but they are also implicated in the pathogenesis of a number of chronic inflammatory diseases, in wound healing, fibrosis, and in native immunity (2). They are described as long-living cells that keep relatively constant numbers in tissues under physiologic conditions, which depends both on the rate of production of mast cell precursors from the bone marrow and the length of survival of mature mast cells within tissues (2). Given, however, their pivotal role in the acute allergic reaction, mast cell numbers need to be tightly controlled by a balance between cell proliferation, development, and death, that link between mast cell activation during early stages of the allergic process and triggering of anti-apoptotic signaling pathways has been suggested as an important factor that contributes to the extended life of mast cells (2, 3). Antiapoptotic mechanisms limit the initiation of programmed cell death, thereby contributing to the multiple pathological conditions that involve mast cell activities.

Mast cells contain cytoplasmic granules that store several preformed mediators, such as TNF-\(\alpha\), IL-4, histamine, heparin, serotonin, \(\gamma\)-aminobutyric acid, and proteases, which are released immediately upon activation of mast cell after cross-linkage of FcERI (1, 2). Newly synthesized mediators include IL-1 through IL-8, TNF-\(\alpha\), IL-12, IL-13, IL-15, and IL-16, chemokines, prostaglandins and leukotrienes, and growth and angiogenesis factors, such as vascular endothelial factor and platelet-derived growth factor (1–3). Despite the well-characterized role of FcERI in mast cell activation, a variety of other agonists can activate mast cells. These include complement, lipid mediators, neuropeptides, cytokines, chemokines, microbial products, and extracellular nucleotides (2–6). In particular, rat mast cells migrate toward adenine nucleotides (7), whereas extracellular ATP has been demonstrated to induce pore formation in the plasma membrane of rat peritoneal mast cells (8). Furthermore, ATP induced release of hexosaminidase, an elevation in Ca\(^{2+}\) level and protein tyrosine phosphorylation in MC/9 mast cell line (9), and stimulated release of histamine and leukotriene from murine bone marrow-derived mast cells (BMMCs)\(^3\) (10–12). In contrast, no histamine release in response to ATP or adenosine was observed in human lung mast cells, although ATP was able to modulate anti-IgE-induced release of this compound (13), thus emphasizing functional heterogeneity of mast cells from different sources.

*Department of Immunology and Cell Biology, Research Center Borstel, Borstel, Germany; and Institute of Immunology, Christian-Albrechts-University, Kiel, Germany

Received for publication June 25, 2004. Accepted for publication January 15, 2005.

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.

1 E.B. and V.B. contributed equally to this work.

2 Address correspondence and reprint requests to Dr. Elena Bulanova, Department of Immunology and Cell Biology, Research Center Borstel, Parkallee 22, D-23845 Borstel, Germany. E-mail address: ebulanova@fz-borstel.de

3 Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; oATP, oxidized ATP; KN-62, 1\(\{\)N,N-bis[5-isoxazolinesulphonyl]-N-methyl-L-tyrosyl\(\}4\)-phenylpiperazine; BzATP, 3-O-(4’-benzoyl)-benzoyl-benzoyl-ATP; pTyr, phosphotyrosine; PARP, poly(ADP-ribose) polymerase; PL, phosphatidylinositol; AFC, 7-amido-4(trifluoromethyl)coumarin; CaMKII, Ca\(^2+\)/calmodulin-dependent protein kinase II.

Copyright © 2005 by The American Association of Immunologists, Inc.

0022-1767/05/S02.00
Extracellular ATP and other nucleotides act through specific cell surface receptors and can regulate a broad range of cellular responses, such as platelet aggregation, smooth muscle contractility, neurotransmission, vascular tone, mucociliary clearance, mitogenic stimulation, or induction of cell death (reviewed in Refs. 14 and 15). ATP is known to be released in a Ca2+-dependent manner from storage compartments in nerve terminals, chromaffin cells, circulating platelets, and mast cells (6). Relatively large amounts of ATP and UTP are liberated from epithelial and endothelial cells, smooth muscle, glial cells, fibroblasts, and hepatocytes upon mechanical stimulation, including shear stress, hypotonic swelling, stretch, and vascular injury (14). In addition, ATP can be released from exercising muscle, ischemic cells, inflammatory cells, and necrotic/apoptotic cells (6, 14). The presence of ATP in the extracellular space leads to a robust activation of purinergic receptors (6, 14, 15). Two primary classes of purinoreceptors, P1 and P2, mediate biological effects of extracellular nucleotides. The P2 receptors are subdivided in two mechanistically distinct subclasses, the metabotropic G protein-coupled P2Y receptors and the ionotropic ligand-gated channel P2X receptors (6, 14). Each purinoreceptor is defined by its relative response to different purinergic ligands. Adenosine has been demonstrated to be a selective agonist of P1 receptors (13). UTP serves as a high potency agonist for human P2Y3 and P2Y4 receptors, whereas at P2Y1 and P2Y11 it is inactive (14, 15). ADP activates P2Y12 and P2Y13, and was reported to be equipotent or even more potent as ATP for P2Y1, while for P2Y11 ATP is more potent than ADP (14). UTP selectively activates P2Y4 (14). The unique naturally occurring agonist of P2X receptors is ATP (14, 15–17).

P2X receptor shares similar to other P2X receptors overall membrane topology of two membrane-spanning domains, a large extracellular loop, and intracellular N- and C-terminal domains, but its COOH-terminal intracellular domain is ~200 amino acids longer (17, 18). P2X7 has a pharmacological profile similar to that receptor previously designated as P2X2, with prominent expression in many immune cells, particularly lymphocytes, monocytes, macrophages, bone marrow, dendritic, mast, mesangial, and microglial cells (6, 14, 17), as well as on a limited number of other cell types including parotid salivary cells, testis, and fibroblasts (14). P2X7 receptor requires millimolar levels of ATP in the presence of divalent cations to achieve activation (19, 20), which leads to the formation of a nonselective cationic channel with low affinity for monovalent cations to achieve activation (19, 20), which leads to the formation of a nonselective cationic channel with low affinity for monovalent cations (21, 22), equilibration of sodium and potassium gradients (21, 22). A large transmembrane pore can cause perturbations in ion homeostasis and finally result in cell death (14).

P2X7 receptor shares similar to other P2X receptors overall membrane topology of two membrane-spanning domains, a large extracellular loop, and intracellular N- and C-terminal domains, but its COOH-terminal intracellular domain is ~200 amino acids longer (17, 18). P2X7 has a pharmacological profile similar to that receptor previously designated as P2X2, with prominent expression in many immune cells, particularly lymphocytes, monocytes, macrophages, bone marrow, dendritic, mast, mesangial, and microglial cells (6, 14, 17), as well as on a limited number of other cell types including parotid salivary cells, testis, and fibroblasts (14). P2X7 receptor requires millimolar levels of ATP in the presence of divalent cations to achieve activation (19, 20), which leads to the formation of a nonselective cationic channel with low affinity for monovalent cations (21, 22), equilibration of sodium and potassium gradients (21, 22). A large transmembrane pore can cause perturbations in ion homeostasis and finally result in cell death (14).

A number of studies have implicated P2X7 in mediating ATP-induced apoptosis in macrophages, mesangial, dendritic, and microglial cells (14, 22–25). Zanovello et al. (26) demonstrated that extracellular ATP caused apoptosis in P815 mastocyte cell line. However, the ability of extracellular ATP to induce apoptosis in BMMCs as well as in MC9 and P815 mast cell lines through P2X7 receptor has not been investigated yet. In this report, we provide experimental evidence that extracellular ATP in millimolar range induces the P2X7-mediated apoptosis in BMMCs and MC9 mast cells. In agreement with earlier findings (26), ATP triggers apoptosis in P815 mastocyte cell line, and this effect is also P2X7-dependent. Finally, in the time lag between the commitment to apoptosis and actual cell death, extracellular ATP stimulates the phosphorylation of ERK1/2, Jak2, and STAT6 in mast cells, and transitorily up-regulates expression of several proinflammatory cytokines, such as IL-4, IL-6, IL-13, and TNF-α.

Materials and Methods

Reagents and Abs

ATP: N-[O-bis(5-isoquinolinylsulphonyl)-N-methyl-1-tyrosyl]-4-phenyl-piperazine (KN-62), and oxidized ATP (oATP), both antagonists of P2X7 receptor (27, 28); 3-O-(4′-benzoyl)-benzoyl-benzoyl-ATP (BzATP), agonist of P2X7 receptor (14); and EHTA were purchased from Sigma-Aldrich. Anti-phosphoryrosine (anti-pTyr) Abs (RC20H2) were obtained from BD Transduction Laboratories. Abs against STAT6 (M-20), phospho-STAT6 (pSTAT6 (Tyr641)), and P2X7 receptor were purchased from Santa Cruz Biotechnology. Abs against Jak2 phosphorylated at Tyr1007/1008 were from BioSource International, and Abs against intact poly(ADP-ribose) polymerase (PARP) and cleaved PARP (7c9) from Cell Signaling Technology. Concentration of IL-4, IL-6, IL-13, and TNF-α in cell supernatants was detected by standard ELISA procedure using DuoSet kits from R&D Systems.

Cell culture and stimulation

BMMCs were obtained from femoral bone marrow of 6-wk-old C57BL/6 mice as previously described (29), and cultured in IMDM supplemented with 10% FCS (PA Laboratories), 30 μM 2-ME, 2 mM l-glutamine (Sigma-Aldrich), 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate (all from Invitrogen Life Technologies). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2. To evaluate mRNA expression, cells were transfected with first and second Abs and washing with PBS, and finally detected by an ECL Western blotting detection reagents (Amersham Pharmacia). The Journal of Immunology

RT-PCR

RNA was extracted from cells using TRizol reagent (Invitrogen Life Technologies). cDNA was synthesized from 5 μg of total RNA using random oligonucleotides and SuperScriptII kit (Invitrogen Life Technologies). Sequences of the primers used are shown in Table I. All primers were purchased from Metabion. cDNA was amplified by standard PCR procedure as described previously (30). To evaluate mRNA expression semiquantitatively, in addition to the PCR product from 35 cycles, 15 μl of the PCR product from the 25 cycles and the 30 cycles were run simultaneously. β-Actin message was used to normalize the cDNA amount to be used. A mock PCR (without cDNA) was included to exclude contamination in all experiments.

Western blotting

Cell pellets were lysed for 15 min on ice in 1% Nonidet P-40 cell extraction buffer: 20 mM Tris-HCl (pH 8.0), 15 mM NaCl, 2 mM EDTA, 10 mM sodium fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 mM PMSF, and 100 mM sodium vanadate (all reagents from Sigma-Aldrich). The de-tergent-insoluble material was removed by centrifugation at 13,000 rpm for 15 min at 4°C. Samples were resuspended in SDS-PAGE loading buffer, boiled for 5 min, and analyzed by 10% SDS-PAGE. The resolved proteins were transferred onto nitrocellulose (Bio-Rad). Blots were blocked for 1 h in PBS with 0.05% Tween 20 and 3% BSA (Sigma-Aldrich). After incubations with first and second Abs and washing with PBS, blots were incubated with horseradish peroxidase-labeled Abs. Western blotting detection reagents (Amersham Pharmacia) according to the manufacturer’s instructions.

Downloaded from www.jimmunol.org on December 22, 2010

by guest on May 1, 2019
Apoptosis assay and flow cytometric analysis

For apoptosis induction cells were cultured at 1 × 10⁶ cells/ml in the presence or absence of ATP or BzATP. The percentage of apoptotic cells was evaluated by ApoTarget Annexin-V-FITC Apoptosis kit (BioSource International) according to the manufacturer’s protocol. Cell viability was determined by propidium iodide (PI) exclusion. Cells were analyzed by flow cytometry using an inverted fluorescence microscope (Nikon Diaphot 300) equipped with a thermostat plate reader.

Changes in plasma membrane permeability

ATP-dependent increase in the plasma membrane permeability was measured using the extracellular fluorescent tracer Lucifer yellow (Molecular Probes) as described earlier (31). For Lucifer yellow uptake, cells were incubated for 15 min at 37°C in PBS containing 250 μM sulfinpyrazone and 1 mg/ml Lucifer yellow, and stimulated with 3 mM ATP. After several washings to remove the extracellular dye, cells were analyzed with an inverted fluorescence microscope (Nikon Diaphot 300) using a ×40 objective and a fluorescein filter.

Intracellular Ca²⁺ measurement

Cells were preincubated with 2 μM membrane-permeable fura 2-AM (Molecular Probes) for 30 min at 37°C and Ca²⁺ influx was measured in PTI-RF-M2001 spectrophotometer (Photon Technology International) using 340/380 excitation filters and emission at 510 nm. After recording of background for 20 s, cells were stimulated with 3 mM ATP. Maximal and minimal fluorescence ratios were determined by lysing the cells with reduced Triton X-100 and subsequent addition of EGTA (final concentration 10 mM) and concentration of Ca²⁺ was calculated using equation of Grynkiewicz et al. (32).

Fluorogenic substrate assay for caspase activity

The enzymatic activity of caspase-3 and -8 was measured essentially as described (33). Cells were collected and lysed in a buffer containing 10 mM HEPES (pH 7.4), 142 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2% v/v Nonidet P-40, 1 mM DTT, and 2 mM Pefabloc. To measure caspase activity, 100 μl of caspase buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% w/v CHAPS, 10% w/v sucrose (pH 7.2)) containing 100 μM zDEVD-7-amido-4(trifluoromethyl)coumarin (APC) or zLETD-AFC were added to cytosolic extracts (25 μl) and incubated at 37°C. The release of AFC was measured as emission at 505 nm upon excitation at 405 nm using a Labsystems Fluoroscan II fluorometer equipped with a thermostat plate reader.

Data analysis

All experiments were performed in at least three independent assays, which yielded highly comparable results. Data are summarized as mean ± SD. Statistical analysis of the results was performed by Student’s t test for unpaired samples. A p-value of <0.05 was considered statistically significant.

Results

Mast cells express purinoreceptors of the P2X and P2Y subtypes

Extracellular nucleotides can reportedly induce a number of biologically relevant responses in mast cells, including the release of histamine, morphological changes, and chemotaxis (6, 7). To analyze the expression pattern of P2 receptors in BMMCs, MC/9, and P815 cells, a panel of mouse purinoreceptors was tested by RT-PCR using specific primers. The amplified bands were purified and analyzed with an Agilent 2100 Bioanalyzer.
from gel and sequenced to prove the identity of the products (data not shown). This approach revealed that primary mast cells and mast cell lines express varying amounts of mRNA coding for several members of the P2X family (P2X₁, P2X₂, P2X₅, P2X₆, and P2X₇). Notably, the expression of P2X₇ was absent in MC/9 cells, whereas message for P2X₁ was detected only in P815 cells (Fig. 1A). Interestingly, all mast cells exhibited a prominent expression of P2X₁ receptor. This fact was further corroborated by Western blotting analysis using Abs directed against P2X₁ receptor, which confirmed the presence of P2X₁ protein in the cell lysates (Fig. 1B). Furthermore, mast cells expressed varying amounts of mRNA for P2Y₁, P2Y₄, P2Y₆, P2Y₁₂, P2Y₁₃, and P2Y₁₄. However, P2Y₄ and P2Y₁₂ were absent in MC/9 cells, whereas P2Y₂ was found only in P815 (Fig. 1A). Thus, primary mast cells and mast cell lines express several P2 receptors subtypes, including P2X₇.

**Extracellular ATP induces cell membrane permeability in mast cells**

It has been repeatedly reported that signal transduction through P2X₇ receptor is associated with Ca²⁺ influx across the cellular membrane (14, 31), and may also open a nonselective pore capable of allowing uptake of low molecular mass hydrophilic solutes (up to 900 Da), such as Lucifer yellow and ethidium bromide (14). In addition, the ability of ATP to induce pore formation in the plasma membrane of rat peritoneal mast cells or mobilization of calcium in IL-dependent cultured mast cells has already been documented (8, 12). Thus, we tested whether these effects could be observed in BMMCs, MC/9, and P815 cells treated with various concentrations of ATP (0.1–3 mM). Indeed, concentrations of ATP in the range of 1–3 mM rendered the cells permeable for Lucifer yellow (Fig. 2A) or ethidium bromide (data not shown). The fluorescent dyes exhibited diffuse distribution in the cytoplasm of ATP-treated mast cells, indicating membrane permeabilization. Phase-contrast microscopy analysis demonstrated that mast cells did not become permeable to trypan blue (molecular mass 961 Da) after ATP stimulation, showing a molecular mass limitation for the phenomenon of permeabilization (data not shown). Furthermore, we also observed an increase in intracellular Ca²⁺, although this effect was rather weak in P815 cells (Fig. 2B). The stimulation of the cells in Ca²⁺-free medium in the presence of Ca²⁺ chelator EGTA (2 mM) abrogated ATP- or BzATP-induced Ca²⁺ uptake (data not shown), indicating that changes in the amount of intracellular Ca²⁺ are due to Ca²⁺ influx across the cellular membrane rather than release from the intracellular stores. The fact that only rather high concentrations of ATP (1–3 mM) were able to induce the cell membrane permeability and influx of calcium ions supports the involvement of P2X₇ receptor (20, 22).

To assess the role of P2X₇ receptor more rigorously, BMMCs and mast cell lines were treated with BzATP, KN-62, and oATP. BzATP is a more potent agonist for P2X₇ receptor than ATP and can induce the P2X₇-mediated responses at lower concentrations (14, 22). In contrast, an isoquinoline derivative KN-62 is widely used as a most potent and selective antagonist of both human and mouse P2X receptor due to its species-specific action, because it is inactive at rat receptor (27, 34). In addition, oATP is considered as an effective antagonist of P2X₇ because it covalently and irreversibly binds to the receptor and inhibits its effects (28), although data are available indicating that this agent also blocks currents at P2X₁ and P2X₂ receptors (22). In fact, treatment of mast cells with BzATP (100 μM) mimicked ATP action (Fig. 2A). In contrast, the pretreatment of cells with KN-62 (1 μM) for 5 min before ATP stimulation was sufficient to prevent the permeabilization of the cell membrane in BMMCs and P815 cells, and significantly attenuated this response in MC/9 cells (Fig. 2A). Furthermore, the ATP-induced Ca²⁺ influx was significantly inhibited in the presence of 1 mM KN-62 (Fig. 2B), which is in agreement with earlier findings (34). In addition, the pretreatment of mast cells with oATP at concentrations >300 μM for 30 min at 37°C before ATP stimulation also prevented the permeabilization of the cell membrane and Ca²⁺ uptake, further corroborating the involvement of P2X₇ (data not shown). The fact that KN-62 was not able to fully abrogate membrane permeability in MC/9 cells may reflect differences in the amount and/or kinetics of activation of functional P2X₇ receptors upon their surface. Taken together, these results show that ATP in millimolar range induces Ca²⁺ influx and permeabilization.
of the cell membrane in murine mast cells, whereas these effects are inhibited by KN-62 or oATP.

**Extracellular ATP induces the P2X<sub>7</sub>-mediated apoptosis of mast cells**

The ability of extracellular ATP to trigger apoptosis of mouse cell lines was documented in P815 mastocytoma and YAC lymphoid cells, and subsequently extended to macrophages and dendritic cells (22, 25, 26). However, the molecular mechanism of the ATP-induced apoptosis in P815 mastocytoma cell line is still unclear (26). Thus, we investigated the effects of ATP upon the viability of mast cells by incubating BMMCs, MC/9, and P815 cells with various amounts of extracellular ATP for different time intervals at 37°C. Then, the ATP-containing supernatant was replaced by fresh medium. After 18 h, the cells were collected and analyzed by Annexin V/PI staining and flow cytometry. In fact, ATP at millimolar concentrations (1–3 mM) induced apoptosis in BMMCs, and P815 cells at concentrations below 10 μM (data not shown). Importantly, KN-62 at concentration 1–3 μM was able to rescue BMMCs from the BzATP-induced cell death only partially, resulting in survival of ∼30% of the cells, whereas lower doses of this isquinoline had little effect (Fig. 3D). The partial antagonism of KN-62 at BzATP-activated human P2X<sub>7</sub> was already reported (34). Interestingly, the kinetic experiments demonstrated that stimulation with high doses of ATP induced apoptosis of mast cells already within the first minutes, and the removal of ATP did not result in cell recovery (data not shown), thereby indicating that some internal irreversible apoptotic mechanisms are activated by such treatment. The requirement of millimolar concentrations of ATP to induce cell death and the ability of KN-62 or oATP to inhibit this process strongly support the idea that apoptosis of mast cells is mediated through P2X<sub>7</sub> receptor.

**ATP-induced apoptosis of mast cells involves activation of caspases**

Caspases are ubiquitously expressed cysteine proteases involved in the activation and execution of apoptosis (35). A number of studies reported that P2X<sub>7</sub> mediates apoptosis in various cells through caspase-dependent mechanism (36–39). Stimulation of P2X<sub>7</sub> has been associated with activation of caspase-1, -3, and -8, and subsequent cleavage of the caspase substrates (39). Because caspases have been implicated as the principal mediators of apoptosis, we wanted to test whether these proteases are involved in the ATP-induced apoptosis in mast cells. As a first step, the cleavage of known caspase-3 substrate PARP (37), a DNA repair enzyme, was assessed. In fact, millimolar concentrations of extracellular ATP induced a characteristic cleavage of 117-kDa PARP protein into...
the 89-kDa fragment both in BMMCs and MC/9 cells, while pretreatment with 1 µM KN-62 for 5 min before ATP treatment, washed twice with medium, and incubated for 18 h at 37°C. Percentage of apoptotic cells was analyzed by Annexin V-FITC/PI staining and FACS. The results (mean ± SD) are based on three independent experiments. **, Data from mast cells treated with ATP were significantly different from control (p < 0.01). *, Data from mast cells treated with KN-62 + ATP were significantly different from ATP-treated mast cells (p < 0.05). B, BMMCs were incubated with different ATP concentrations (0.1–5 mM) for 30 min at 37°C, washed twice with medium, and incubated for 18 h at 37°C. Percentage of apoptotic cells was analyzed by Annexin V-FITC/PI staining and FACS. The ability of extracellular ATP to induce mast cell apoptosis is dose-dependent because ATP at concentrations below 1 mM (0.1–0.5 mM) did not affect the viability of BMMCs. C, BMMCs were incubated with ATP (0–3 mM) or pretreated with different concentrations of KN-62 (0.1–3 µM) for 5 min before ATP treatment to prevent apoptosis. After 18 h, the percentage of apoptotic cells was analyzed by Annexin V-FITC/PI staining followed by FACS analysis. D, BMMCs were incubated with BzATP (100 µM) or pretreated with different concentrations of KN-62 (0.1–3 µM) for 5 min before BzATP treatment to prevent apoptosis, and numbers of apoptotic cells were counted after Annexin V-FITC/PI staining by flow cytometry. Stimulation with BzATP induced apoptosis in BMMCs more potently than ATP, whereas KN-62 had only partial protective effect. E and F, BMMCs and MC/9 were treated with 3 mM ATP for 1 h or pretreated with KN-62 for 5 min before ATP treatment. Cell lysates were analyzed by 10% SDS-PAGE using Abs recognizing intact (117 kDa) and cleaved (89 kDa) PARP (E). Activity of caspase-3 and -8 in BMMCs was analyzed after 1 h of ATP treatment as described in Materials and Methods (F). Basal activity of caspases was considered as 100%. The data shown are representative of three independent experiments with similar results.

Extracellular ATP induces the phosphorylation of signaling molecules in mast cells
One of the early effects of P2X7 receptor activation is Ca2+ influx across the plasma membrane and the equilibration of membrane K+ and Na+ gradients (14). Such ionic perturbations can trigger activation of a number of intracellular signaling molecules, including MAPKs (40). It has been shown that ATP can induce phosphorylation of ERK in PC12 cells and fetal astrocytes (40, 41), and of JNK in BAC1 murine macrophages (22). In addition, we have recently demonstrated that stimulation with extracellular ATP mediates activation of p56lck, ERK, and JNK in Jurkat cells (42).
Based on these findings, our next goal was to determine whether ATP is able to induce activation of intracellular signaling molecules in mast cells. For this purpose, BMMCs, MC/9, and P815 cells were pulse-stimulated with extracellular ATP for relatively brief periods ranging from 5 to 15–30 min. We considered such pulses to represent a more “physiologic” stimulus, as mast cells are likely exposed to high ATP concentrations only for short time intervals due to the ubiquitous expression of ecto-ATPases/ectonucleotidases which enzymatically degrade extracellular ATP. In the experiments illustrated in Fig. 4, mast cells were stimulated with 3 mM ATP. At the indicated time points, the cells were collected, lysed, and assessed for patterns of tyrosine phosphorylation by Western blotting. Immunoprecipitation and probing with anti-pTyr Abs showed that a number of proteins were phosphorylated upon the exposure of cells to extracellular ATP. These included 10–18, 20–22, 27, 30, 33, 38, 45, 55–60, and 80–85 kDa proteins (Fig. 4, upper panel). Such phosphorylation pattern was already detectable within the first 5 min of ATP administration, reaching its maximum after 15 min, and returning to near basal level within 30 min (Fig. 4, and data not shown).

Notwithstanding, ATP stimulation induced rather weak changes in the pattern of tyrosine phosphorylation in P815 cells, as compared with untreated control cells (Fig. 4). The probing of the membranes with phosphospecific Abs allowed us to identify among the phosphorylated proteins STAT6, ERK1 (p44), ERK2 (p42), as well as weak phosphorylation of Jak2 (Fig. 4A). Kinetic experiments confirmed that ATP induced a rapid and transient phosphorylation of these signaling molecules. In contrast, the phosphorylation state of other members of Jak (Jak1, Jak3, Tyk2) and STAT family (STAT1, STAT3, STAT5), as well as p38 kinase, JNK1 (p54), and JNK2 (p52) was not affected, remaining at the same level throughout the time course of the experiment (data not shown). For loading control, membranes were stripped and reprobed with anti-STAT5, anti-ERK, or anti-Jak2 Abs, respectively. In contrast, these effects were abolished when cells were pretreated with KN-62 (Fig. 4A) or oATP (Fig. 4B) before ATP stimulation, suggesting that the observed phosphorylation is mediated through P2X<sub>7</sub> receptor. Taken together, these results show that extracellular ATP induces activation of multiple signaling molecules via P2X<sub>7</sub> receptor in murine mast cells.

**Extracellular ATP induces cytokine expression in mast cells**

Mast cells are efficient producers of many key inflammatory cytokines in response to stimulation with a variety of stimuli (1–3). To understand the biological significance of the observed activation of signaling molecules after ATP stimulation, a number of cytokines (e.g., IL-4, IL-2, IL-6, IL-7, IL-13, IL-15, IL-18, and TNF-α) were tested by semiquantitative RT-PCR using specific primers to detect changes in the level of transcription. To this end, BMMCs or MC/9 cells were stimulated with ATP at concentrations ranging from 100 μM to 3 mM, harvested for RNA preparation, and assessed for the level of cytokine transcription. Fig. 5, A and B, show that ATP in millimolar range was a potent stimulus for the transcription of IL-4, IL-6, IL-13, and TNF-α in BMMCs. However, only messages for IL-4 and IL-13 were up-regulated in MC/9 cells (Fig. 5C). Notably, the expression of IL-6 and TNF-α was already rather high in MC/9 cells, which might account for the inability of ATP to further enhance mRNA level of these cytokines. The increase in the transcription was most prominent after 3 h of ATP stimulation and undetectable after 12 h (data not shown), presumably due to the cell apoptosis. Furthermore, the stimulation of BMMCs with BzATP (100 μM) mimicked ATP action (3 mM), whereas the increase in the cytokine transcription was significantly inhibited by the pretreatment of cells with 1 μM KN-62 (Fig. 5D) or oATP (data not shown), further supporting the involvement of P2X<sub>7</sub>. Notably, KN-62 at concentrations below 0.5 μM had little, if any, effect on the transcription of these cytokines (data not shown).

Next, we wanted to test whether mast cells may release these cytokines to the culture medium in response to the stimulation with ATP or BzATP. To this end, BMMCs and MC/9 cells were incubated with these agents essentially as described above, and the cell supernatants were harvested for ELISA after 24 h. These experiments showed the ability of ATP or BzATP to up-regulate production of IL-6 and IL-13 in BMMCs and MC/9 cells, whereas KN-62 inhibited most of these effects (Fig. 5D). Interestingly, KN-62 had almost no effect upon the BzATP-induced release of IL-13 in MC/9 cells. In contrast, no changes in the release of TNF-α and IL-4 from BMMCs and MC/9 cells were observed (data not shown). Taken together, these experiments demonstrate the ability of ATP or BzATP to up-regulate transcription and production of several proinflammatory cytokines in murine mast cells.

**Discussion**

The present work demonstrates that murine mast cells express a functional P2X<sub>7</sub> receptor capable of initiating apoptosis after stimulation with millimolar concentrations of extracellular ATP. Concomitantly, we observed a rapid but transient phosphorylation of multiple intracellular proteins, such as ERK1/2, Jak2, and STAT6. Notwithstanding the development of apoptosis, an up-regulation in
the expression level of several proinflammatory cytokines was detected by RT-PCR and ELISA in BMMCs and MC/9 cells, including IL-4, IL-6, IL-13, and TNF-α. The identification of P2X7 as a receptor subtype responsible for the observed effects of ATP was supported by several lines of experimental evidence: 1) only millimolar concentrations of ATP were effective in inducing apoptosis, whereas BzATP mimicked ATP action at submillimolar doses; 2) the treatment with ATP or BzATP triggered a rapid transmembrane Ca2+ influx through channels permeable to small cations and also opened a nonselective pore capable of allowing an uptake of low molecular mass hydrophilic solutes; 3) the effects of ATP or BzATP on mast cells were abrogated by pretreatment with KN-62 or oATP; and 4) RT-PCR and Western blotting analysis confirmed the presence of P2X7 mRNA and protein in mast cells.

Thus, the requirement of ATP in millimolar range and the ability of submillimolar amounts of BzATP to mimic its effects, taken together with inhibitory properties of KN-62 and oATP, are typical for the pharmacological profile of P2X7, strongly suggesting a role for this receptor as the mediator of the ATP-induced effects in murine mast cells.

Although the best characterized pathophysiologic stimulus for the activation of mast cells is provided by cross-linking of FcεR on the cell surface by allergens complexed to IgE molecules, a variety of other stimuli are capable of inducing mast cell activation, including complement, lipid mediators, neuropeptides, cytokines, chemokines, and extracellular nucleotides (2, 3). Because ATP can be released to the extracellular environment in many physiologic or pathologic conditions, it represents a good candidate to the role of nonimmune activation stimulus for mast cells. However, such stimuli may induce mast cells to respond in a restricted way, which could result in a release of a limited subset of mediators or cytokines or affect the amount and/or kinetics of the production of mast cell-specific bioactive substances (44). In our hands, short exposures of murine mast cells to millimolar concentrations of extracellular ATP were sufficient to initiate a number of the P2X7-mediated biological responses, which finally culminated in the apoptotic cell death. The P2X7-mediated apoptosis of mast cells involved the activation of caspases-3 and -8, and the cleavage of caspase-3 substrate PARP. The ability of P2X7 to mediate activation of caspases is in agreement with recent findings in other cell systems (37–39). It remains to be elucidated whether the caspase-dependent apoptosis represents the main mechanism of the ATP-induced mast cell death, or other caspase-independent mechanisms may also be operative. Currently, we perform experiments to assess how broad-spectrum or specific protease inhibitors may affect the death-inducing properties of ATP in BMMCs and MC/9 cells. In addition, the irreversibility of the ATP-induced apoptosis in mast cells is intriguing and deserves a more profound investigation to define its biological significance.

The P2X7-initiated cascade of signaling events reportedly involves a defined sequence of phenotypic changes that result in apoptosis only at least several hours after the transient exposure to

**FIGURE 5.** ATP and BzATP induce cytokine expression in mast cells while KN-62 inhibits this effect. A, BMMCs were incubated with indicated concentrations of ATP for 1 h. BMMCs (B) and MC/9 (C) cells were treated with 3 mM ATP or 100 μM BzATP for 1 h or pretreated with 1 μM KN-62 for 5 min before stimulation with ATP or BzATP. Total RNA was extracted from cells, reverse-transcribed, and subjected to PCR amplification using specific primers for IL-4, IL-6, IL-13, and TNF-α as described in the Materials and Methods. For semiquantitative analysis, in addition to 35 cycles, 15-μl aliquots of the PCR product from 25 and 30 cycles were also evaluated. Picture shows the amplified bands after 35 cycles. The amount of cDNA was equalized by PCR amplification of β-actin. A mock PCR (no cDNA) was included as a negative control. The data represent three separate experiments with comparable results. D, BMMCs and MC/9 cells were treated as described above; the supernatants were collected after 24 h and analyzed for IL-6 and IL-13 by ELISA. Results represent mean ± SD of three independent experiments (*, p < 0.05 vs control, and **, p < 0.05 vs ATP and BzATP, respectively).
ATP induces apoptosis of mast cells (22). It has been suggested that in the time period between commitment to apoptosis and actual cell death, agonistic stimulation of P2X_{7} receptor also activates additional signaling pathways, which may lead to cytokine production in case of proteolytic processing and release of IL-1β from LPS-primed macrophages that precedes cell death (45), and activation of various transcription factors like NFAT or NF-κB (46), thus modulating the overall response to ATP. Accordingly, a rapid ATP-induced phosphorylation of ERK1/2, Jak2, and STAT6 was observed in murine BM-MCs and MC/9 cells, which returned to nearly basal levels after 30 min, whereas the phosphorylation state of other members of Jak and STAT families, p38 kinase, and JNK was not altered (data not shown). Additional experiments are underway to determine the identity of other tyrosine-phosphorylated proteins in BM-MCs and MC/9 cells upon ATP stimulation. Furthermore, we observed that ATP induced in a dose-dependent manner a significant increase in the transcription level of IL-4 and IL-13, and to a lesser extent, of IL-6 and TNF-α. Furthermore, ATP stimulation enhanced the release of IL-6 and IL-13 to the culture medium from BM-MCs and MC/9 cells. Moreover, BzATP, which is a more potent agonist for P2X_{7} receptor than ATP, mimicked the effects of ATP at lower concentrations. Notwithstanding, only ~40–45% of BM-MCs or MC/9 cells died via apoptosis after 18 h of ATP treatment. Thus, not all mast cells are equally susceptible to ATP, and the observed phosphorylation pattern as well as the increase in the cytokine expression may predominantly take place in the apoptosis-resistant mast cells, although additional experiments are required to confirm or reject this suggestion.

Gargett and Wiley (27) first reported the fact that an ionotropic receptor, Ka2-ATP, can act as a potent antagonist of P2X_{7} receptor. Although KN-62 is also a potent inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), an inactive analog of KN-62, KN-04, also blocked the P2X_{7} responses, indicating that the inhibition of P2X_{7} receptor by KN-62 is not mediated by CaMKII (27). Importantly, the action of KN-62 upon P2X_{7} does not involve its inhibitory properties on CaMKII in short-term studies, whereas prolonged exposures to KN-62 require caution in interpretation because of concomitant inhibition of this kinase (14, 27). Humphreys and colleagues (22, 34) have demonstrated the high selectivity of KN-62, which inhibits both human and mouse P2X_{7}, but is inactive at rat receptor, thus providing a useful tool for identifying the P2X_{7}-mediated functional responses. However, the molecular mechanism(s) that underlie the ability of KN-62 to inhibit both human and mouse P2X_{7} receptor remain unclear (22, 34). It has been suggested that KN-62 elicits its inhibitory action on human P2X_{7} receptor through direct binding to the amino-terminal half, which contains the large extracellular loop. Correspondingly, the introduction of the first 335 amino acids of the human receptor sequence conferred KN-62 sensitivity to rat P2X_{7} receptor (34). The pretreatment of mast cells with KN-62 or another irreversible P2X_{7} antagonist, αATP, inhibited the effects of extracellular ATP such as membrane permeabilization, protein phosphorylation, and cytokine expression. Moreover, these chemical agents significantly reduced the numbers of apoptotic BM-MCs, MC/9, and P815 cells as compared with untreated controls.

Notably, the antagonists were more effective in preventing the ATP-induced apoptosis in P815 cells, whereas KN-62 was able to antagonize the BzATP-induced effects only partially, which is in accord with previous findings (34). It is noteworthy that the amount and/or kinetics of activation of functional P2X_{7} receptors upon the cell surface of BM-MCs and these two mast cell lines may presumably vary, contributing to slightly different functional responses to ATP and the ability of the antagonists to inhibit them. Importantly, ATP induced transmembrane Ca^{2+} influx, which was most prominent in BM-MCs and MC/9 but rather weak in P815 cells, whereas KN-62 or αATP abrogated this effect. The ability of P2X_{7} receptor to trigger a long-lasting transmembrane Ca^{2+} influx is in agreement with earlier studies (14, 31).

The fact that brief exposures to millimolar concentrations of extracellular ATP result in the irreversible P2X_{7} receptor-mediated apoptosis raises the issue of the relevance of this process to mast cell physiology. Mast cells play critical roles in a variety of allergic, autoimmune, and inflammatory diseases (1, 3, 6, 47). The expression of an active P2X_{7} receptor capable of mediating apoptosis offers a possibility to quickly eliminate unwanted mast cells under circumstances which favor accumulation of extracellular ATP in rather high concentrations. Although it is still not clear whether such high levels of ATP could be achieved in the extracellular space, amounts of ATP in the protected compartments at the level of the cell membrane could easily reach concentrations sufficient to activate the low-affinity P2X_{7} receptor (14). Recent findings implicate mast cells in a variety of neuroinflammatory diseases, especially those worsened by stress (6), thereby questioning the role of neuronal mast cell activation in the development of migraines and multiple sclerosis (6). It remains to be elucidated whether extracellular ATP can contribute through the P2X_{7}-mediated apoptosis to the elimination of unwanted mast cells in such pathological conditions, and how an activation of antiapoptotic signaling pathways may oppose its action.

Given that many cell types including mast cells can release ATP, this chemical agent might be able to alter function of the bone or nearby cells by autocrine/paracrine mechanism. In fact, the amount of ATP released from one mast cell was shown to be sufficient to diffuse several tens of micrometers and elicit rises in intracellular Ca^{2+} in surrounding cells (6, 48). It has been suggested that the operation of an ATP-based autocrine/paracrine loop can support the P2X_{7}-mediated lymphoid cell growth in the absence of serum-derived growth factors (49). Many studies have documented that P2X_{7} receptor/pore participates in diverse monocyte, macrophage, microglia, lymphocyte, and dendritic cell responses such as cell membrane permeabilization, cytokine release, multinucleated giant cell formation, cell proliferation or apoptosis (6, 14, 15). For example, stimulation of P2X_{7} receptor by ATP induces posttranslational processing of IL-1β and IL-18 precursors in human monocytes (50, 51), altered cytokine production in mice lacking this receptor (52), stimulation of JNK activity and induction of apoptosis in murine macrophages (22), inhibition of osteoclastic resorption (53), and activation of transcription factor NF-κB (54) and NFAT (46) in microglial cell lines. Data from our laboratory suggest that ATP can trigger proliferation of Jurkat T lymphoblastoid cell line and mediate p56^lck-dependent phosphorylation of ERK and JNK, and activation of AP-1 transcription factor, simultaneously down-regulating p50/p65 NF-κB heterodimers (42). It is believed that in T and B lymphocytes, ATP acting over P2X_{7} generates a pore smaller than that seen in other cell types, such as macrophages (14). In regard to mast cell function, extracellular ATP has been demonstrated to stimulate degranulation and release of histamine from these cells (10, 11), and to induce cell membrane permeabilization (9, 12). Notably, ATP was shown to trigger apoptosis in P815 mastocytoma cell line, although the mechanism of the ATP-mediated cell death remained obscure (26). Further, ATP stimulated the release of hexosaminidase from MC/9 mast cell line through a mechanism that was distinct from the activation induced by the cross-linking of Fc receptors (9), and induced the release of histamine and leukotriene from BM-MCs (10–12). Several studies have reported a direct Ca^{2+}-dependent
histamine release and a potentiation of Ag- or ionophore A23187-induced histamine release from rat peritoneal mast cells in response to ATP stimulation (55, 56). The ability of extracellular ATP to induce degranulation of murine mast cells is in accordance with our findings (data not shown). Conversely, ATP only modulated anti-IgE-induced histamine release from human lung mast cells without having the ability to stimulate the release of this compound (13). Thus, mast cells derived from different body districts or distinct species exhibit a functional heterogeneity, which invites a cautious interpretation of mast cell responses in vitro with respect to their relevance in vivo.

ATP is found at a concentration 5–10 mM in the cytosol of most eukaryotic cell types, whereas its amount in platelets is significantly higher and can reach a concentration of ~1 M (14). Release of ATP to extracellular space by both lytic and nonlytic mechanisms has been observed from virtually all cell types and tissues under conditions of hypoxia, ischemia, inflammation, injury, and cell necrosis or apoptosis (14, 15). However, extracellular ATP concentrations are generally maintained at extremely low levels by a sequential action of ubiquitous cell surface enzymes such as ecto-ATPases/ectonucleotidases, which rapidly degrade it to ADP, AMP, and adenosine (6, 14). Conversely, ATP can be copackaged with serotonin in platelet granules and released locally in significant amounts during platelet activation (14, 57). Thus, platelets themselves are a major source of ATP, whereas cytosolic ATP stores can also be liberated by the sudden breakage of intact cells, as might occur during the rupture of blood vessels and other tissue injury (57). These two sources suggest that significant amounts of extracellular ATP may be generated at vascular sites of the injury formation and infection/inflammation. The key role of extracellular ATP and P2 receptors in hemostasis has already been emphasized (14). On the other hand, an emerging concept implicates mast cells as repair cells, which provide antimicrobial and/or profibroinflammatory mediators to prevent thrombus formation or to help dissolve thrombotic material in the course of vascular repair processes (58, 59). The profibrolytic potential of mast cells is determined by the relative abundance of either generated phospholipase A2 or plasminogen activator inhibitors. Mast cells act profibrolytically through expression of a key fibroinflammatory enzyme, tissue-type plasminogen activator, without coexpression of plasminogen activator inhibitors, and supernatants as well as cell lysates from cultured mast cells are capable of lysing a fibrin clot in a manner similar to recombiant tissue-type plasminogen activator (58). The recognition of mast cells as a profibrolytic cell raises a question of its potential pathophysiologic role in the prevention of repair of vascular thrombosis and tissue repair following fibrinogen extravasation. Mast cells can accumulate around thrombosed vessels and provide a number of important repair molecules including antithrombotic heparin, and other profibrolytic substances. Thus, mast cell recruitment and activation at the sites of vascular injury may result in local thrombolyis and prevention of coagulation, which may interfere with the healing of a vascular rupture at the initial stages of injury. Therefore, the apoptosis of mast cells in response to rather high concentrations of extracellular ATP which may be achieved at the sites of acute vascular injury due to its release from damaged blood cells, endothelial cells, and activated platelets appears to be physiologically relevant, because it presumably prevents their function as antithrombotic and/or profibroinflammatory cells at initial stages of vascular rupture.

In summary, our results demonstrate that ATP induces a number of the P2X,-mediated cell responses in murine mast cells, including the phosphorylation of signaling molecules, transcription of cytokines, membrane permeabilization, and induction of apoptosis. The high sensitivity of mast cells to the cytotoxic effects of extracellular ATP suggests that this process might have a biological relevance in certain physiologic or pathologic conditions. The fact that extracellular ATP can play an important role in maintaining mast cell numbers through the ATP,-mediated apoptosis may provide helpful insights to the development of new therapeutic approaches to intervene and limit excessive and/or undesired mast cell activities.

Acknowledgments

We are grateful to Manuel Fohlmeister and Katrin Strecek for excellent technical assistance and Renate Bergmann for the help with ELISA.

Disclosures

The authors have no financial conflict of interest.

References

1. Benoist, C., and M. Mathis. 2002. Mast cells in autoimmune disease. Nature 420:875.
2. Piliponsky, A. M., and F. Levi-Schaffer. 2000. Regulation of apoptosis in mast cells. Apoptosis 5:435.
3. Robb, M. Y., and M. A. Brown. 2002. The role of mast cells in allergy and autoimmunity. Curr. Opin. Immunol. 14:298.
4. Wedemeyer, M. L., and S. J. Galli. 2000. Roles of mast cells and basophils in innate and acquired immunity. Curr. Opin. Immunol. 12:624.
5. Theophilou, N., J. E. Glinavska, R. D. Wolburg, S. E. Gassmann, M. S. Grant, and C. F. Howlett. 2001. The pharmacology of mast cells in health and disease. Acta Physiol. Scand. 170:369.
6. Sato, H., N. Sakaguchi, M. Ebisawa, K. Kurihara, N. Sakaguchi, M. Ebisawa, K. Matsumoto, A. Akasawa, and Y. Ikura. 1991. The stimuli releasing histamine from murine bone marrow-derived mast cells. 1. The presence of P2X,-purinoceptors. J. Biol. Chem. 266:17559.
7. Sato, H., N. Sakaguchi, M. Ebisawa, K. Kurihara, N. Sakaguchi, M. Ebisawa, K. Matsumoto, A. Akasawa, and Y. Ikura. 1991. The stimuli releasing histamine from murine bone marrow-derived mast cells. 2. Activation induced by the cross-linking of Fc receptors. J. Immunol. 146:1370.
8. Tatham, P. E., and M. Lindau. 1990. ATP-induced pore formation in the plasma membrane of rat peritoneal mast cells. J. Gen. Physiol. 95:459.
9. Sudo, N., Y. Fuchita, I. Koga, M. Okumura, C. Kubo, and K. Nomoto. 1996. Expression of ATP,-receptor in mast cells via a mechanism that is different from the activation mediated by the cross-linking of Fc receptors. J. Immunol. 156:3970.
10. Nakamura, H., H. Sato, and Y. Ikura. 1989. The stimuli releasing histamine from monocytes and monocyte-derived mast cells. 1. The presence of P2X,-purinoceptors. J. Immunol. 143:9559.
11. Sato, H., M. Ebisawa, D. C. Reason, K. Ohno, K. Kurihara, N. Sakaguchi, A. Ohgimi, E. Saito, A. Akasawa, K. Akimoto, et al. 1991. Extracellular ATP stimulates interleukin-dependent cultured mast cells and eosinophils through calcium mobilization. Int. Arch. Allergy Appl. Immunol. 94:68.
12. Schulman, E. S., M. C. Glaum, T. Post, Y. Wang, D. C. Rabille, J. Mohanty, J. H. Butterfield, and A. Pelleg. 1999. ATP modulates anti-IgE-induced release of histamine from human lung mast cells. Am. J. Respir. Cell Mol. Biol. 20:530.
13. Di Virgilio, F., P. Chiozzi, D. Ferrari, S. Falzoni, J. M. Sano, A. Morelli, M. Torboli, G. Bolognesi, and O. R. Baricordi. 2001. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood 97:587.
14. North, R. A., and A. Suprenant. 2000. Pharmacology of cloned P2X receptors. Annu. Rev. Pharmacol. Toxicol. 40:563.
15. Fredholm, B. B., M. P. Abbracchio, G. Burnstock, J. W. Daly, T. K. Harden, K. A. Jakobson, P. Left, and M. Williams. 1994. Nomenclature and classification of purinoceptors. Pharmacol. Rev. 46:143.
16. Surprenant, A., F. Rassendren, E. Kawashima, R. A. North, and G. Buell. 1996. The cytolytic P2,-receptor for extracellular ATP identified as a P2X,-receptor. Science 272:735.
17. Riley, J. S., and G. R. Dubay. 1989. Extracellular adenosine triphosphate increases cation permeability of chronic lymphocytic leukaemic lymphocytes. Blood 73:116.
18. Greenberg, S., F. Di Virgilio, H. Steinberg, and S. C. Silverstein. 1988. Extracellular nucleotides mediate Ca2+ fluxes in 3T3/L1 macrophages by two distinct mechanisms. J. Biol. Chem. 263:10337.
19. Steinberg, T. H., A. S. Newman, J. A. Swanson, and S. C. Silverstein. 1987. ATP4- permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. J. Biol. Chem. 262:8884.
20. Rassendren, F., G. N. Buell, C. Virginio, G. Collo, R. A. North, and A. Suprenant. 1997. The permeabilizing ATP receptor, P2X,- Cloning and expression of a human cDNA. J. Biol. Chem. 272:2542.
21. Humphreys, B. D., J. Rice, S. B. Kertesy, and G. R. Dubyak. 2000. Stress-activated protein kinase/NFkappaB activation and apoptotic induction by the macrophage P2X,-nucleotide receptor. J. Biol. Chem. 275:26792.
22. Ferrari, D., M. Villalba, P. Chiozzi, S. Falzoni, P. Riccardi-Castagnoli, and F. Di Virgilio. 1996. Mouse microglial cells express a plasma membrane pore gated by extracellular ATP. J. Immunol. 156:1531.
23. Nihei, O. K., C. A. campos de Carvalho, W. Salvino, and L. A. Alves. 2000. Pharmacologic properties of P2Z/P2X,- receptor characterized in murine dendritic cells: role in the induction of apoptosis. Blood 96:996.
25. Coutinho-Silva, R., P. M. Persechini, R. Da Cunha Bisaggio, J.-L. Perfettini, A. C. Torres de Saneto, J. M. Kanellopoulos, I. Motta-Ly, A. Dautry-Varsat, and D. M. Ojcius. 1999. P2Z/P2X receptor-dependent apoptosis of dendritic cells. Am. J. Physiol. 276:C1139.

26. Gattetti, C. M., C. A. M. Zanfrilli, A. Rosato, P. Pizzo, and F. Di Virgilio. 1990. Responses of mouse lymphocytes to extracellular ATP. J. Immunol. 145:1545.

27. Gargani, C. E., J. S. Wiley. 1997. The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes. Br. J. Pharmacol. 120:169.

28. Murga, M., S. Hanau, P. Pizzo, M. Rippa, and F. Di Virgilio. 1993. Oxidized ATP: an irreversible inhibitor of the macrophage purinergic P2Z receptor. J. Biol. Chem. 268:8199.

29. Horta, A., L. S. Andrade, and C. S. Lantz. 1997. Culture of mast cells. In Immunology Methods Manual. I. Letkovits, ed. Academic Press, London, p. 1393.

30. Bulanova, E., V. Budagian, Z. Orinska, H. Krause, R. Paus, and S. Bulpin. 2003. Mast cells express novel functional interleukin-15 receptor α isoforms. J. Immunol. 170:5045.

31. Mutini, C., S. Falzoni, D. Ferrari, P. Chozio, A. Morelli, O. R. Baricordi, G. Cello, R. Ricciardi-Castanoli, and F. Di Virgilio. 1999. Mouse dendritic cells express the P2X4 purinergic receptor: characterization and possible participation in antigen presentation. J. Immunol. 163:1958.

32. Grzybowski, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3440.

33. Steinitz, H. R., and G. S. Salvesen. 1997. Biochemical characteristics of caspases-3, -6, -7, and -8. J. Biol. Chem. 272:25719.

34. Humphreys, B. D., C. Virginio, A. Suprenant, J. Rice, and G. R. Dubyak. 1998. Isoquinolines as antagonists of the P2X2 nucleotide receptor: high selectivity for the human versus rat receptor homologues. Mol. Pharmacol. 54:22.

35. Emmrich, W., V. L., M. Martins, and S. H. Kaufman. 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu. Rev. Biochem. 68:383.

36. Donnelly-Roberts, D. L., M. T. Namovic, C. R. Faltynek, and M. F. Jarvis. 2004. Mitogen-activated protein kinase and caspase signaling pathways are required for P2X2 receptor (P2X2-R)-induced pore formation in human THP-1 cells. J. Pharmacol. Exp. Ther. 208:1053.

37. Wen, L., T. C. Caldwell, and A. F. Knowles. 2002. Poly(ADP-ribose) polymerase and changes in Bax protein expression associated with extracellular ATP-mediated apoptosis in human embryonic kidney 293-P2X1 cells. Mol. Pharmacol. 63:706.

38. Hillman, K. A., H. Harada, C. M. Chan, A. Townsend-Nicholson, S. E. Morehouse, K. Miyamoto, Y. Suketa, G. Burnstock, R. J. Unwin, and P. D. Reed. 1998. Chicken DT40 cells stably transfected with the rat P2X2 receptor channel: a system suitable for the study of purine receptor-mediated cell death. Biochem. Pharmacol. 66:415.

39. Ferrari, D., M. Los, M. K. A. Bauer, P. Vandenabeele, S. Weidberg, and K. Schulze-Osthoff. 1997. P2Z purinergic receptor in human induces activation of caspases with distinct role in apoptotic and necrotic liberation of cell death. FEBS Lett. 447:71.

40. Swanson, K. D., C. Reigh, and G. E. Landreth. 1998. ATP-stimulated activations of the mitogen-activated protein kinase and rat brain nonglycolytic P2X2 purinergic receptors in PC12 cells. Differences in purine receptor sensitivity in two PC12 derivatives. J. Biol. Chem. 273:19686.

41. Neary, J. T., M. McCarthy, Y. Kang, and B. Zariga. 1998. Mitogen-regulated signals from P1 and P2 purinergic receptors to mitogen-activated protein kinase in human fetal astrocytic cultures. Neurosci. Lett. 242:159.

42. Badagni, V., E. Bulanova, L. Brovkov, Z. Orinska, R. Fayad, R. Paus and S. Bulpine-Paus. 2003. Signaling through P2X4 receptor in human T cells involves p56lck, MAP kinases, and transcription factors AP-1 and NFκB. J. Biol. Chem. 278:1549.

43. Vassort, G. 2001. Adenosine 5′-triphosphate: a P2-purinergic agonist in the myocardium. Physiol. Rev. 81:767.

44. Galli, S. J., M. Maurer, and C. S. Lantz. 1999. Mast cells as sentinels of innate immunity. Curr. Opin. Immunol. 11:53.

45. Ferrari, D., P. Chozio, S. Falzoni, M. Dal Susino, L. Melchiorri, O. R. Baricordi, and F. Di Virgilio. 1997. Extracellular ATP triggers IL-1β release by activating the purinergic P2Z receptor of human macrophages. J. Immunol. 159:1451.

46. Ferrari, D., C. Stroh, and K. Schulze-Osthoff. 1999. P2X2/P2Z purinoreceptor-mediated activation of transcription factor NFAT in microglial cells. J. Biol. Chem. 274:13205.

47. Seman, M., S. Adriouch, F. Scheppelein, C. Krebs, D. Freese, G. Glowacki, P. Deterre, F. Haag, and F. Koch-Nolte. 2003. NAD-induced T cells death: ADP-ribosylation of cell surface proteins by ART2 activates the cytolytic P2X2 purinoceptor. Immunity 19:571.

48. Osipchuk, Y., and M. Cahalan. 1992. Cell-to-cell spread of calcium signals mediated by ATP receptor in mast cells. Nature 359:241.

49. Baricordi, O. R., L. Melchiorri, E. Adinolfi, S. Falzoni, P. Chozio, G. Buel, and F. Di Virgilio. 1989. Increased proliferation rate of lymphoid cells transfected with P2X4 ATP receptor. J. Biol. Chem. 274:33260.

50. Perregaux, D. G., P. McNiff, R. Laliberte, M. Conklyn, and C. A. Gabel. 2001. ATP acts as an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood. J. Immunol. 165:4615.

51. Mattia, V. H., J. Han, and M. D. Wexler. 2001. ATP stimulated release of interleukin (IL)-1β and IL-18 requires purinergic but not dopaminergic receptors. J. Immunol. 166:5820.

52. Sotol, M., S. Tenem, D. G. Perregaux, F. Stani, N. Bubravsha, B. H. Koller, and C. A. Gabel. 2000. Altered cytokine production in mice lacking P2X2 receptors. J. Biol. Chem. 275:2625.

53. Grivas, C. E., S. J. Dixarit, and C. M. Sims. 2001. Activity-dependent development of P2X7 current and 5′-AZT entry into rabbit osteoclasts. J. Biol. Chem. 276:19107.

54. Ferrari, D., S. Weidberg, M. K. Bauer, and K. Schulze-Osthoff. 1997. Extracellular ATP induces transcription factor NF-κB through the P2Z purinoreceptor by selectively targeting NF-κB1a. J. Cell Biol. 137:1635.

55. Chatterton, N. 1990. The role of plasma membrane Ca2+/Mg2+-activated adenylate triphosphate or rat mast cells on histamine release. Acta Pharmacol. Toxicol. 67:229.

56. Gauer, S. K., and J. D. Gomperts. 1979. Activation and inhibition of calcium-dependent histamine secretion by ATP ions applied to rat mast cells. J. Physiol. 289:262.

57. Berti, R., R. Kobatake, M. Aisawa, and G. R. Duhyak. 1999. Detection of local ATP release from activated platelets using cell surface-attached fibre luciferase. Am. J. Physiol. 276:C267.

58. Bankl, H. C., and P. Valent. 2002. Mast cells, thrombosis, and fibrinolysis: the emerging concept. Thrombosis Res. 105:359.

59. Valent, P., M. Baghestanian, H. C. Bankl, C. Sillaber, W. R. Sper, J. Wojta, B. R. Binder, and K. Lechner. 2002. New aspects in thrombosis research: possible role of mast cells as pro fibrinolytic and antithrombotic cells. Thromb. Haemostasis 85:786.
Letter of Retraction

We wish to retract the article titled “Extracellular ATP Induces Cytokine Expression and Apoptosis through P2X7 Receptor in Murine Mast Cells” by Elena Bulanova, Vadim Budagian, Zane Orinska, Martina Hein, Frank Petersen, Lutz Thon, Dieter Adam, and Silvia Bulfone-Paus, *The Journal of Immunology*, 2005, 174: 3880–3890.

This retraction follows a formal investigation by the Research Center Borstel into scientific misconduct. The main findings of the investigation were the following:

1) Fig. 3: Although in principle the reported results are correct, the error bars could not be verified on the basis of the raw data.
2) Fig. 4: The figure seems to contain several severe manipulations. Various raw data are not available. Independent repetitions of the experiments shown in Fig. 4B regarding BMMC and MC9 (left and middle panels) confirmed the original data in principle.

The first two authors declined to sign this Letter of Retraction. All the other authors wish to retract the article. We deeply regret these irregularities and apologize to the scientific community for any inconvenience this might cause.

Zane Orinska
Martina Hein
Frank Petersen
Silvia Bulfone-Paus
Research Center Borstel
Borstel, Germany

Lutz Thon
Ferring GmBH
Kiel, Germany

Dieter Adam
University of Kiel
Kiel, Germany