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Impaired P2X1 Receptor–Mediated Adhesion in Eosinophils from Asthmatic Patients

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Eosinophils play an important role in the pathogenesis of asthma and can be activated by extracellular nucleotides released following cell damage or inflammation. For example, increased ATP concentrations were reported in bronchoalveolar lavage fluids of asthmatic patients. Although eosinophils are known to express several subtypes of P2 receptors for extracellular nucleotides, their function and contribution to asthma remain unclear. In this article, we show that transcripts for P2X1, P2X4, and P2X5 receptors were expressed in healthy and asthmatic eosinophils. The P2X receptor agonist α,β-methylene ATP (α,β-meATP; 10 μM) evoked rapidly activating and desensitizing inward currents (peak 18 ± 3 pA/pF at −60 mV) in healthy eosinophils, typical of P2X1 homomeric receptors, which were abolished by the selective P2X1 antagonist NF449 (1 μM) (3 ± 2 pA/pF). α,β-meATP–evoked currents were smaller in eosinophils from asthmatic patients (8 ± 2 versus 27 ± 5 pA/pF for healthy) but were enhanced following treatment with a high concentration of the nucleotidase apyrase (17 ± 5 pA/pF for 10 IU/ml and 11 ± 3 pA/pF for 0.32 IU/ml), indicating that the channels are partially desensitized by extracellular nucleotides. α,β-meATP (10 μM) increased the expression of CD11b activated form in eosinophils from healthy, but not asthmatic, donors (143 ± 21% and 108 ± 11% of control response, respectively). Furthermore, α,β-meATP increased healthy (18 ± 2% compared with control 10 ± 1%) but not asthmatic (13 ± 1% versus 10 ± 0% for control) eosinophil adhesion. Healthy human eosinophils express functional P2X1 receptors whose activation leads to eosinophil $\alpha_M\beta_2$ integrin–dependent adhesion. P2X1 responses are constitutively reduced in asthmatic compared with healthy eosinophils, probably as the result of an increase in extracellular nucleotide concentration. *The Journal of Immunology*, **2016, 196: 4877–4884.**

Asthma is a common chronic inflammatory airway disease that causes considerable morbidity. It is defined by variable airflow obstruction associated with airway hyperresponsiveness and an inflammatory process that is often, although not invariably, eosinophilic (1). The close association between eosinophils and asthma has been noted for many years, and it is emerging that eosinophils play a causal role in severe exacerbations of the disease, although their importance in the development of airway hyperresponsiveness and variable airflow obstruction is less certain (2). Eosinophils may also contribute to airway tissue repair and remodeling (3). The physiological regulation of eosinophil functions (e.g., priming, activation, and migration) involves a broad range of cell surface receptors, including chemokine, cytokine and inhibitory receptors, innate and Fc receptors and adhesion molecules (3), as well as P2 receptors for nucleotides (4).

Extracellular nucleotides (e.g., ATP and ADP) act via cell surface P2 receptors, a family composed of P2X ligand–gated ion channels (P2X1–7) (5) and metabotropic P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14) (6). Functional P2X receptors are trimers that can be homomeric or heteromeric, thereby increasing the number of potential P2X receptor subtypes that can contribute to cell activity (5, 7, 8). An increase in extracellular nucleotide concentration occurs following cell degranulation, tissue injury, apoptosis, osmotic shock, mechanical stress, hypoxia, and inflammation (9–11). In human airways, nucleotides can be derived from damaged airway epithelium, airway smooth muscle, the accumulation and activation of diverse immune cells (e.g., mast cells, T lymphocytes, eosinophils, and neutrophils) (12), and activated platelets (13). Increased ATP concentrations in bronchoalveolar lavage fluids were reported from chronic smokers (14) and patients with asthma (15), chronic obstructive pulmonary disease (14), cystic fibrosis (16), and idiopathic pulmonary fibrosis (17).

Transcripts for several P2 receptors were reported in human eosinophils, including P2X1, P2X4, P2X5, and P2X7 receptors; however, the presence of P2X7 mRNA in resting eosinophils remains controversial (18, 19). Extracellular nucleotides elevate

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Abbreviations used in this article: $[Ca^{2+}]_i$, intracellular calcium concentration; GINA, Global Initiative for Asthma; GMFI, geometric mean fluorescence intensity; α,β-meATP, α,β-methylene ATP; PAF, platelet-activating factor; qPCR, real-time PCR; ROS, reactive oxygen species.

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Table I. Clinical characteristics

	Asthmatic Patients (n = 62)	Healthy Volunteers (n = 57)
Female (**n** [%])	29 (53)	41 (72)
Age (y; mean [range])	55 (23–79)	47 (18–68)
Age at diagnosis (y; mean [range])	24 (0–65)	n.a.
Atopy (**n** [%])	52 (84)	n.a.
FEV_1 **(l)**[a]	2.3 (0.1)	n.d.
$FEV_{1\%}$ predicted	83 (3.6)	n.d.
FEV_1/FVC (%)	51 (4.6)	n.d.
Inhaled corticosteroid dose (μg)[b]	1208 (84)	n.a.
Percentage with GINA 2/3	44	n.d.
Percentage with GINA 4/5	56	n.d.

Asthmatic patients and healthy control volunteers were ≥18 y.
Data are mean (SEM), unless stated otherwise.
[a]Postbronchodilator.
[b]Beclomethasone dipropionate equivalent.
FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GINA 2, mild persistent; GINA 3, moderate persistent; GINA 4 and 5, severe persistent; n.a., not applicable; n.d., not determined.

eosinophil cytosolic calcium (18–21) and aggravate inflammatory states via the generation and release of cytotoxic eosinophil cationic protein, IL-8, and reactive oxygen species (ROS), in addition to upregulation of the integrin CD11b, enhanced chemotaxis, and eosinophil actin reorganization (18, 21–23). Allergen stimulation can upregulate P2Y2 receptor expression in eosinophils from patients with allergic asthma (24). Eosinophils isolated from P2Y2 receptor–deficient mice no longer migrate toward ATP, suggesting the importance of this receptor for eosinophil chemotaxis. P2X receptor(s) also were proposed to modulate eosinophil function; however, because of the indirect methods of characterization, the subtype(s) of receptor(s) involved are unclear (18, 19, 21–23).

The aims of this study were to characterize the P2X receptor(s) present on eosinophils from healthy and asthmatic donors and determine their contribution to eosinophil function.

Materials and Methods

Reagents

All reagents were from Sigma-Aldrich (Poole, U.K.), unless otherwise stated. 4,4′,4′′,4′′′-[Carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))] tetrakis-1,3-benzenedisulfonic acid (NF449) and platelet-activating factor (PAF) were from Tocris Biosciences (Bristol, U.K.). Fura-2, AM, RPMI 1640 medium+GlutaMAX I, and FCS were obtained from Life Technologies (Paisley, U.K.), and IL-5 was purchased from R&D Systems Europe (Abingdon, U.K.).

Eosinophil isolation

Asthmatic patients, with a physician diagnosis of persistent asthma, as per Global Initiative for Asthma (GINA) guidelines (25), were recruited from the specialist airways clinics at Glenfield Hospital (Leicester, U.K.). Asthmatic patients were stable with no recent exacerbation. Table I summarizes the clinical characteristics of the blood volunteers recruited for the present study. This work was approved by the Leicestershire, Rutland, and Northamptonshire Health Ethics committee. Human eosinophils were isolated, as previously described (26), and resuspended in buffer W (RPMI 1640 medium + GlutaMAX I + 2% FCS). Purity was ≥99.4 ± 0.0% (99 donors).

RT-PCR and real-time PCR

Total RNA from eosinophils was isolated using the QIAshredder and RNeasy kit with on-column DNase I digestion (QIAGEN, Crawley, U.K.). Total RNA (5.6 ng) was reverse transcribed using the Sensiscript Reverse Transcription kit (QIAGEN) in 20 μl. For negative control, the Sensiscript enzyme was replaced with H_2O. PCR reactions using BIOTAQ DNA Polymerase (1.25 U/reaction; Bioline, London, U.K.) were performed (27) with 2 μl cDNA or negative control. Real-time PCR (qPCR) was conducted using 2.5 μl iQ SYBR Green qPCR Kit (Bio-Rad, Hemel Hempstead, U.K.), 1 μl forward/reverse primer mix (0.75 μM each), and 1.5 μl cDNA in a LightCycler 480 real-time PCR System (Roche Diagnostics, Burgess Hill, U.K.). qPCR expression data were normalized to the geometric mean of β-actin, PPIB, and RPL13A1 mRNAs as a normalization factor using the $2^{-\Delta Ct}$ method. The primer list is shown in Supplemental Table I.

Patch-clamp recording

Resuspended eosinophils were plated onto glass coverslips and incubated at 37°C for ≥30 min with apyrase grade VII prior to use, to reduce P2X1 receptor desensitization by endogenous ATP release. The apyrase concentration was 0.32 IU/ml (concentration previously shown to prevent complete desensitization of platelet P2X1 responses) (28) or 10 IU/ml. Conventional whole-cell patch-clamp recordings were made at a holding potential of −60 mV at 21°C (29). The agonist was applied rapidly via a U-tube. To examine the effect of NF449 on agonist-evoked P2X1 receptor currents, cells were incubated for 5 min with the antagonist before concomitant application of the agonist.

Western blot

Eosinophil proteins were analyzed by Western blot (30). Then the membrane was stripped of bound Abs and reprobed with an anti-actin Ab (1:1000; Abcam, Cambridge, U.K.), followed by an HRP-conjugated goat anti-mouse IgG (1:5000 dilution; Jackson ImmunoResearch Europe, Suffolk, U.K.) using the same procedure as above. A total of 9 μg eosinophil protein sample was analyzed per donor for P2X1 protein expression. Human embryonic kidney cells (HEK293) stably transfected with P2X1 receptor expressed a very high level of P2X1 protein. Therefore, to avoid the obliteration of P2X1 protein signal in the neighboring samples on the

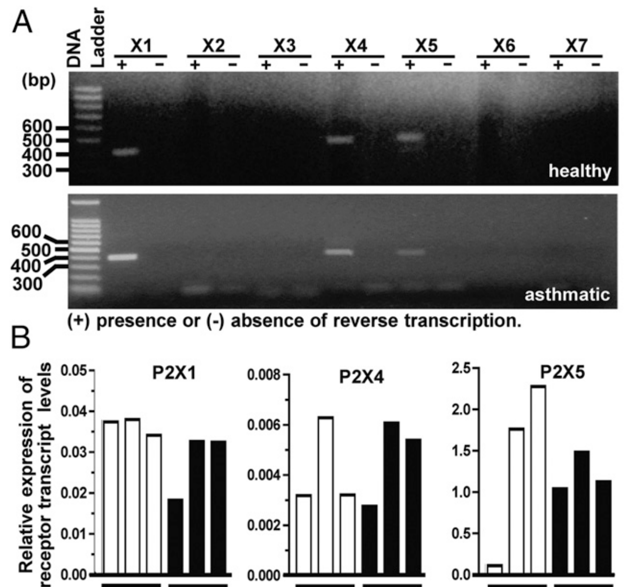

FIGURE 1. P2X receptor transcript expression in human eosinophils. (**A**) RT-PCR performed on eosinophils from one healthy and one asthmatic donor (representative of three healthy and three asthmatic donors). (**B**) qPCR performed on three healthy and three asthmatic additional donors.

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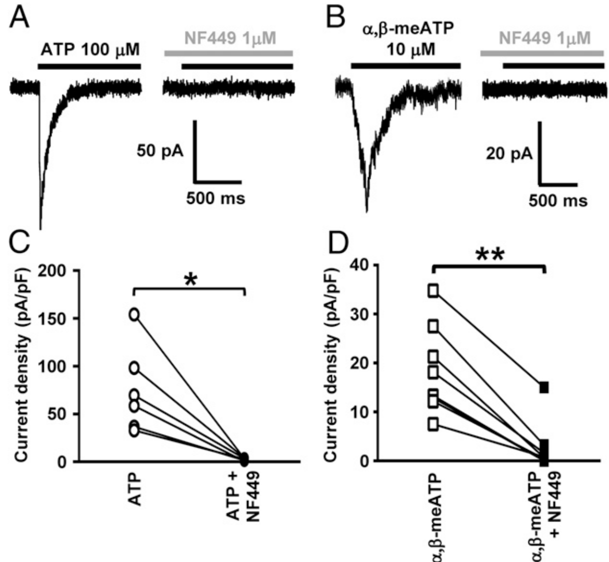

FIGURE 2. Presence of P2X1 receptor current in healthy human eosinophils. A rapidly activating and fast desensitizing current is elicited by ATP (100 μM) (**A**) and α,β-meATP (10 μM) (**B**) in healthy eosinophils and is abolished in the presence of NF449 (1 μM). Agonist and NF449 application durations are indicated by black and gray bars, respectively. Summary of NF449 antagonism on ATP-induced (**C**) and α,β-meATP–induced currents (**D**). $*p < 0.05$, $**p < 0.01$.

autoradiography film, we used 0.2 μg HEK cell protein/sample. Once P2X1 protein was analyzed, the hybridization membrane was stripped and reprobed for actin. Although 9 μg eosinophil protein sample/donor was adequate to detect a signal for actin protein, 0.2 μg protein for HEK cells was not sufficient to observe the actin signal on the autoradiography film.

Flow cytometric detection of eosinophil P2X1 total protein expression

Whole blood (100 μl) was stained with PE–Siglec-8 (1.5 μg/ml, 347104; BioLegend UK, London, U.K.) and PE–Cy7-CD39 (25 μg/ml, 25-0399-42; eBioscience, Hatfield, U.K.) for 15 min on ice. The cells were then treated with 1-Step Fix/Lyse Solution (00-5333-57; eBioscience), washed in 1× Permeabilization Buffer (00-8333-56; eBioscience), and incubated with 8 μg/ml rabbit anti-human P2X1 polyclonal Ab (APR001; Alomone Labs, Jerusalem, Israel) or a rabbit IgG Ab (X0936; Dako UK, Ely, U.K.) at room temperature for 30 min. Rabbit Abs were detected using Alexa Fluor 647–goat anti-rabbit secondary F(ab′)$_2$ (1 μg/ml, A21246; Life Technologies) for 15 min on ice. Cells were washed prior to fixation in FluoroFix buffer (BioLegend) and were acquired on a BD FACSCanto A flow cytometer (Becton Dickinson, Oxford, U.K.) and analyzed using FlowJo (V10) software (TreeStar). Compensation was applied to remove cell (FITC) autofluorescence from each detection channel using fluorescence minus one controls. Singlets were gated (data not shown), and eosinophils were identified as Siglec-8$^+$, high autofluorescent cells (boxed region, Supplemental Fig. 1C). From this gate, the geometric mean fluorescence intensity (GMFI) of P2X1 receptor, following subtraction of fluorescence minus one control treated cells, was calculated (ΔGMFI).

Intracellular calcium concentration measurements in platelet suspensions

Preparation of platelet-rich plasma and washed platelet suspensions, as well as ratiometric fluorescence measurements of intracellular Ca^{2+} from stirred suspensions of Fura-2–loaded human platelets, was conducted as previously described (31). Experiments were conducted at 37°C in a Cairn spectrofluorometer system (Cairn Research, Faversham, U.K.), and intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated using a dissociation constant for Ca^{2+} of 224 nM, according to Rolf et al. (28). Platelet count was measured using a z2 Coulter Counter (Beckman Coulter, High Wycombe, U.K.) and adjusted to yield an equal platelet density in paired healthy and asthmatic samples. Two-way ANOVA followed by a Bonferroni posttest were used for same-day paired healthy/asthmatic platelet $[Ca^{2+}]_i$ measurements (no significant difference was observed between the groups).

CD11b/CD11b activated form cell surface expression measurements

Eosinophils (10^5 cells/100 μl buffer W) were subjected to various agonist stimulations for 2 h at 37°C in a humidified atmosphere of 5% CO_2. The cells were washed and incubated with PE anti-human CD11b (clone ICRF44, 15 μg/ml), PE anti-human CD11b activated form (activation-sensitive anti-α_M mAb clone CBRM1/5, 20 μg/ml), or their respective PE isotype-control Ab (clone MOPC-21) at matching concentrations (BioLegend) in 100 μl buffer W for 15 min at room temperature. Finally, the cells were washed and resuspended in 400 μl FluoroFix Buffer, and ≥10,000 eosinophil events were acquired immediately on a BD FACSCanto A flow cytometer. Using FACSDiva software, eosinophils were identified using light scatter (side scatter versus forward scatter plot) and gated onto a PE histogram to measure the expression of CD11b and CD11b activated form. The data are expressed as the cellular-specific ΔGMFI following subtraction of isotype control–treated cells.

Eosinophil adhesion assay

Cell adhesion was assessed as residual eosinophil peroxidase activity of adherent eosinophils, as previously described (32). Variations to the original protocol were introduced as follows. The 96-well microplates were coated overnight at 4°C with 100 μl BSA (50 μg/ml) dissolved in HBSS (Life Technologies). Eosinophils (3×10^4 cells/100 μl) were treated with 3.2 IU apyrase and incubated or not with NF449 (1 μM) for 5 min at room temperature prior to cell stimulation with IL-5 (10 ng/ml), α,β-methylene ATP (α,β-meATP; 10 μM), or vehicle. Absorbance was measured at 490 nM in a microplate reader (TECAN Infinite M200; Tecan, Reading, U.K.).

Statistical analysis

Data were analyzed with GraphPad Prism 6 software and are presented as mean ± SEM. Unless stated in the figure legend, the Student t test was performed when Gaussian distribution was observed, otherwise the Wilcoxon matched-pairs and Mann–Whitney tests were used for paired and unpaired values, respectively. For electrophysiology experiments, currents from at least three cells were averaged per donor. CD11b flow cytometry experiment significance was assessed by the ratio paired t test. Significance was accepted at $p < 0.05$.

Results

Healthy human eosinophils express P2X1, P2X4, and P2X5 receptor transcripts

The expression of P2X receptor mRNAs in human eosinophils was determined by RT-PCR. Transcripts for P2X1, P2X4, and P2X5

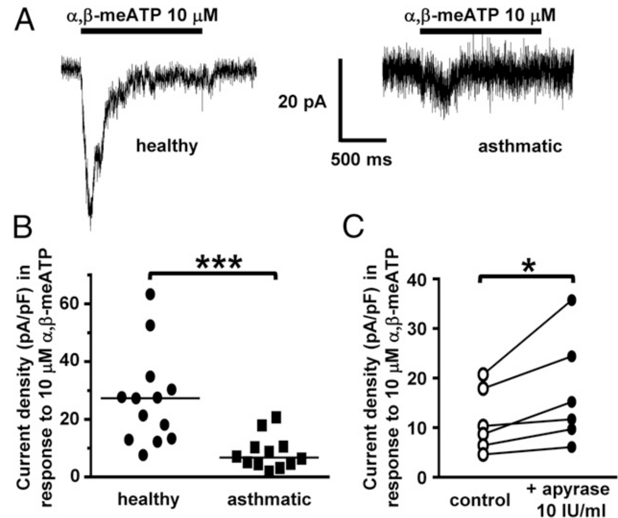

FIGURE 3. Eosinophils from asthmatic donors exhibit dramatically reduced P2X1 receptor currents. (**A**) α,β-meATP induced a rapidly activating and fast desensitizing current in healthy eosinophils that was significantly reduced in eosinophils from asthmatic donors. (**B**) Summary of α,β-meATP–induced currents in healthy and asthmatic eosinophils (horizontal line = median). (**C**) Apyrase (10 IU/ml) enhanced α,β-meATP–induced currents in asthmatic eosinophils compared with control conditions (0.32 IU/ml). $*p < 0.05$, $***p < 0.005$.

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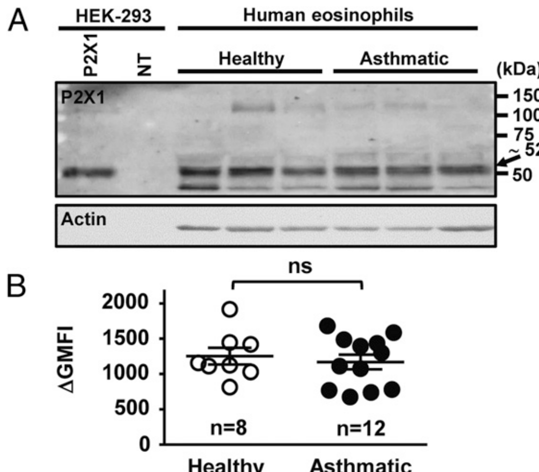


FIGURE 4. P2X1 receptor protein expression in human eosinophils. (**A**) Western blot analysis showing P2X1 protein expression in eosinophils from three healthy and three asthmatic donors and HEK293 cells—native (HEK293-NT) or stably expressing human P2X1 receptor (HEK293-P2X1). (**B**) Flow cytometric detection of eosinophil P2X1 total protein expression level in healthy and asthmatic donors. ns, not significant.

receptors were expressed in eosinophils from healthy and asthmatic donors (n = 3, Fig. 1A). P2X2, P2X3, P2X6, and P2X7 receptors were below the limit of detection. Real-time PCR, which was performed to more accurately assess the level of expression of the different P2X subtypes, confirmed the presence of P2X1, P2X4, and P2X5 mRNAs in healthy eosinophils (P2X5 >> P2X1 > P2X4) (n = 3; Fig. 1B), whereas the remaining P2X subtypes were not confidently detected.

Presence of P2X1 receptor currents in human eosinophils

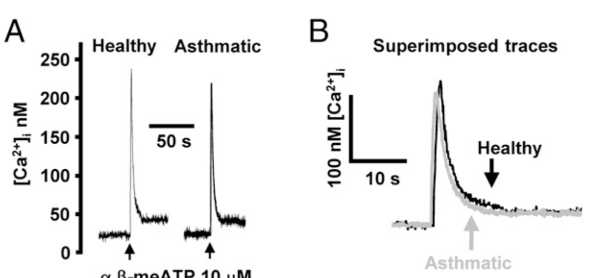
We used an electrophysiological approach to characterize eosinophil P2X receptors. The different receptor subtypes can be discriminated based on their time course and sensitivity to agonists and antagonists. ATP (100 μM) evoked rapidly activating and desensitizing inward currents (peak current 75 ± 19 pA/pF, time constants of rise of 7 ± 1 ms and monophasic decay 57 ± 4 ms, n = 3) in healthy human eosinophils (Fig. 2A). These characteristics are typical of recombinant P2X1 and P2X3 homomeric receptors (5). Because homomeric P2X4 (5) and P2X5 (33), as well as heteromeric P2X1/P2X4 (8) and P2X1/P2X5 (7), receptors exhibit slow desensitization, we can exclude their functional presence in eosinophils. The potent and selective P2X1 receptor antagonist NF449 (1 μM) (34) abolished the ATP-evoked current (3 ± 1 pA/pF, n = 6, p = 0.0313) (Fig. 2A, 2C). Therefore, this finding, together with the absence of detectable P2X3 transcripts (Fig. 1A and qPCR observations), rules out the expression of functional P2X3 receptor in eosinophils. The nonhydrolyzable P2X receptor agonist α,β-meATP (10 μM) also induced a fast transient current in eosinophils (18 ± 3 pA/pF; time constants of rise of 99 ± 17 ms and decay 58 ± 10 ms, n = 3), which was essentially abolished by 1 μM NF449 (3 ± 2 pA/pF, n = 8, p = 0.0078) (Fig. 2B, 2D). Together with the RT-PCR/qPCR data, these results indicate that human eosinophils from healthy subjects express functional P2X1 receptors.

P2X1 receptor currents are reduced in eosinophils from asthmatic donors

P2X1 receptor currents, induced by 10 μM α,β-meATP, were 3.2-fold smaller in eosinophils from asthmatic donors (8 ± 2 pA/pF, n = 12) compared with healthy donors (27 ± 5 pA/pF, n = 13, p < 0.0001) (Fig. 3A, 3B), without a change in whole-cell capacitance (1.8 ± 0.1 and 2.0 ± 0.2 pF, respectively). To determine whether the reduction in α,β-meATP–evoked current amplitude resulted from a decrease in P2X1 expression level in asthmatic eosinophils, we measured P2X1 transcript and protein levels. Similar levels of P2X1 (P2X4 and P2X5) mRNA were detected for eosinophils from healthy and asthmatic donors using RT-PCR and qPCR (Fig. 1). In addition, no change in eosinophil P2X1 total protein level was observed by Western blot or flow cytometry (Fig. 4) performed either directly on eosinophils stained in whole blood (ΔGMFI 1253 ± 119 for healthy, n = 8; 1170 ± 104 for asthmatic, n = 12) or on purified eosinophils (n = 4 for each, Supplemental Fig. 1A, 1B), therefore ruling out the possibility of a defective translational event. This suggests that other factors, such as post-translational events, regulatory proteins, or desensitization could be modulating P2X1 activity in asthmatic eosinophils. Interestingly, P2X receptor agonist α,β-meATP (10 μM) induced similar rapid transient increases in intracellular Ca^{2+} in platelets from asthmatic and healthy donors, suggesting that the intrinsic properties of P2X1 receptor in platelets are unaffected in asthma and that the loss of eosinophil P2X1 responses in asthmatic patients is not widespread among all blood cells (Fig. 5).

High concentrations of apyrase increase asthmatic eosinophil P2X1 receptor currents

The reduced P2X1 receptor currents observed in asthmatic eosinophils could result from increased receptor desensitization due to the presence of extracellular nucleotides. To test this hypothesis, eosinophils from asthmatic donors were treated with a substantial (10 IU/ml) or a standard (0.32 IU/ml) dose of the soluble nucleotidase apyrase before the recordings took place. The high apyrase concentration rescued P2X1 receptor activity in asthmatic eosinophils (α,β-meATP [10 μM]-induced currents of 17 ± 5 pA/pF for 10 IU/ml and 11 ± 3 pA/pF for 0.32 IU/ml of apyrase, n = 6, p = 0.0313) (Fig. 3C), but it had no effect on healthy eosinophils (12 ± 3 pA/pF for 10 IU/ml and 17 ± 6 pA/pF for 0.32 IU/ml, n = 3). These data suggest the desensitization of P2X1 receptor by extracellular nucleotides.



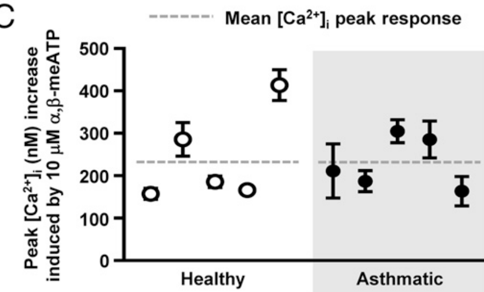


FIGURE 5. Platelets from asthmatic donors express functional P2X1 receptor. (**A**) α,β-meATP triggered comparable changes in platelet $[Ca^{2+}]_i$ from healthy and asthmatic donors. (**B**) Kinetics of platelet $[Ca^{2+}]_i$ response to α,β-meATP. (**C**) Comparison of the peak $[Ca^{2+}]_i$ response induced by α,β-meATP among five control and five asthmatic donors.

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P2X1 receptor activation upregulates the expression of CD11b activated form in healthy eosinophils

P2X1 receptor regulates CD11b (from the integrin complex $\alpha_M\beta_2$) expression in many hematic cell types (23, 35, 36). α,β-meATP (10 μM) caused an increase in CD11b activated form (37) on eosinophils from healthy donors (143 ± 21% of control response, donors $n = 13$, $p = 0.0409$) but not from asthmatic donors (108 ± 11% of control response, $n = 10$) (Fig. 6A, 6E, 6F). ATP (100 μM) induced similar responses on eosinophils from healthy (142 ± 34%, $n = 11$) and asthmatic (109 ± 7%, $n = 10$) donors (significance was not reached because of interdonor variability) (Fig. 6B, 6E, 6F). PAF (1 μM) and eotaxin (100 ng/ml) increased eosinophil CD11b activated form in healthy (184 ± 45%, $n = 10$, $p = 0.0311$ and 170 ± 12%, $n = 13$, $p = 0.0004$, respectively) and asthmatic (133 ± 14%, $n = 10$, $p = 0.0337$ and 146 ± 20%, $n = 10$, $p = 0.0357$, respectively) donors (Fig. 6C–F). The basal levels of expression of CD11b activated form were similar in healthy and asthmatic eosinophils (2111 ± 275 ΔGMFI, $n = 13$ and 2341 ± 289 ΔGMFI, $n = 10$, respectively) (Fig. 6G).

PAF and eotaxin also increased overall eosinophil CD11b cell surface expression in healthy (121 ± 4%, $n = 7$, $p = 0.0009$ and 116 ± 7%, $n = 8$, $p = 0.0423$, respectively) and asthmatic (129 ± 4%, $n = 12$, $p < 0.0001$ and 109 ± 3%, $n = 12$, $p = 0.0068$, respectively) donors. However, α,β-meATP or ATP had no effect (105 ± 5%, $n = 8$ and 97 ± 4%, $n = 7$, respectively, for healthy) (99 ± 3%, $n = 12$ and 93 ± 2%, $n = 12$, respectively, $p = 0.0078$, for asthmatics). Basal levels of CD11b expression were similar in healthy and asthmatic eosinophils (10427 ± 1037 ΔGMFI, $n = 8$ and 10926 ± 742 ΔGMFI, $n = 12$, respectively). These results show that P2X1 receptor activation upregulates the expression of CD11b activated form in healthy eosinophils.

P2X1 receptor activation increases healthy eosinophil adhesive properties

Because $\alpha_M\beta_2$ integrin mediates eosinophil adhesion to ICAM-1 and BSA (32), we investigated the contribution of the P2X1 receptor to eosinophil adhesion on BSA-coated plates. Cell adhesion was assessed as residual peroxidase activity of attached eosinophils, as previously described (32). α,β-meATP (10 μM) increased healthy (180 ± 23% of control response, $n = 6$, $p = 0.0060$), but not asthmatic (123 ± 13% of control, $n = 6$), eosinophil adhesion (Fig. 7). NF449 (1 μM) inhibited α,β-meATP–mediated healthy eosinophil adhesion (121 ± 14% of control, $n = 6$, $p = 0.0320$). It also significantly reduced the adhesion of asthmatic eosinophils treated with α,β-meATP (89 ± 12% of control, $n = 6$, $p = 0.0201$). The latter can be explained by the fact that P2X1 currents are dramatically decreased in eosinophils from asthmatic donors (Fig. 3B). Alone, NF449 did not alter eosinophil adhesion (135 ± 20% and 109 ± 17% of control for healthy [$n = 6$] and asthmatic [$n = 6$], respectively). IL-5 (10 ng/ml) similarly potentiated healthy and asthmatic eosinophil adhesion (189 ± 23% and 171 ± 22% of control for healthy [$n = 6$, $p = 0.0019$] and asthmatic [$n = 6$, $p = 0.0128$], respectively). There was no difference in the basal adhesion levels of healthy and asthmatic eosinophils (the raw percentage of cell adhesion was 10 ± 1% and 10 ± 0% for healthy [$n = 6$] and asthmatic [$n = 6$], respectively). These data suggest that P2X1 receptor regulates healthy eosinophil $\alpha_M\beta_2$ integrin–dependent adhesion.

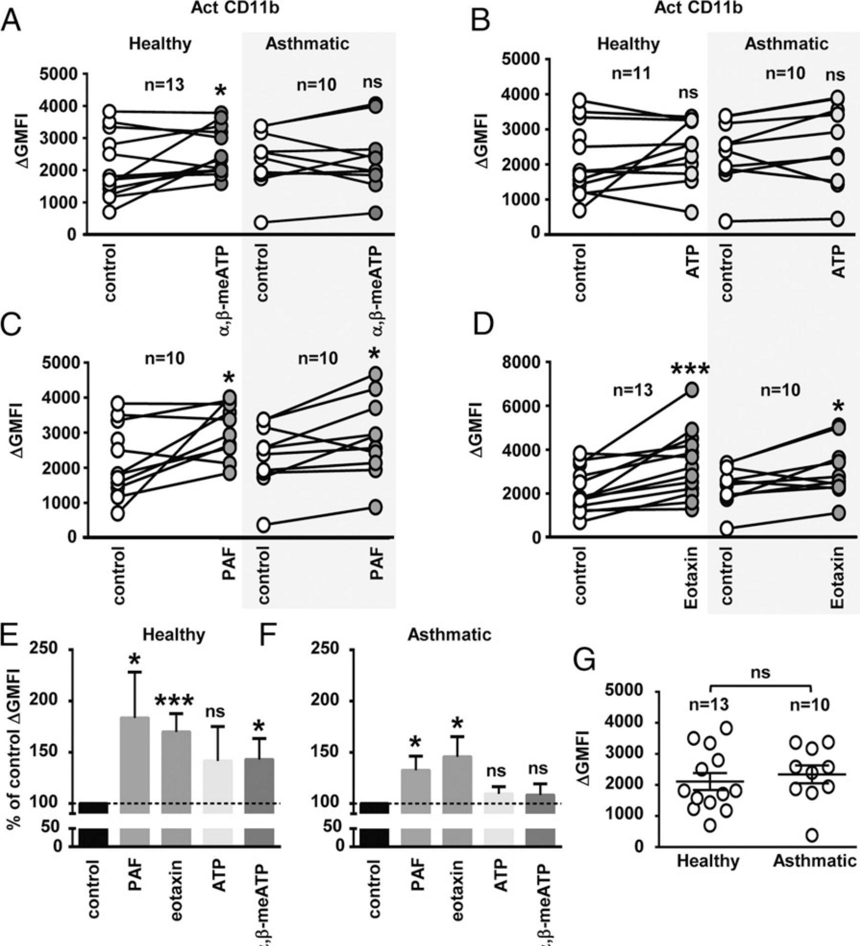

FIGURE 6. P2X1 receptor increases the expression of healthy eosinophil CD11b activated form. Effect of α,β-meATP (10 μM) (**A**), ATP (100 μM) (**B**), PAF (1 μM) (**C**), and eotaxin (100 ng/ml) (**D**) on CD11b activated form expression in healthy and asthmatic eosinophils. Summary of the effect of PAF (1 μM), eotaxin (100 ng/ml), ATP (100 μM), and α,β-meATP (10 μM) on eosinophil CD11b activated form expression level in healthy (**E**) and asthmatic (**F**) donors. (**G**) Basal level of CD11b activated form in healthy and asthmatic eosinophils. n = number of donors. *$p < 0.05$, ***$p < 0.005$. ns, not significant.

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Discussion

In addition to their antiparasitic activity and involvement in allergic reactions, eosinophils are associated with the development of airway diseases. A range of ligands regulates eosinophil function (3); among these, extracellular nucleotides are important. Extracellular nucleotides, acting at P2 receptors, control many cells functions, including platelet activation (38) and modulation of inflammation (39). In the current study, we show that healthy human eosinophils express functional P2X1 receptors, P2X1 activation leads to increased eosinophil $\alpha_M\beta_2$ integrin–dependent adhesion, and P2X1 receptor responses are reduced in asthmatic eosinophils.

We demonstrated the expression of P2X1, P2X4, and P2X5 receptor subtype transcripts in healthy donor eosinophils, as reported by other groups (18, 19). In agreement with Mohanty et al. (19), we could not detect P2X7 receptor mRNA in eosinophils, although it was reported by Ferrari et al. (18). This difference could be due to the high purity of our eosinophil preparation (99.4 ± 0.0%) containing a negligible proportion of other hematic cells, including platelets (Supplemental Fig. 2). The original full-length P2X7 receptor gene is composed of 13 exons and a plethora of splice variants, and polymorphisms have been identified (40). Our sets of primers amplify sequences ranging from exons 6 to 11 (27) and from exons 10 to 13. They target the known functional P2X7 splice isoforms, as well as nonfunctional variants (40), therefore ruling out the presence of functional P2X7 receptors in human eosinophils.

Although P2X5 receptor transcripts were the most abundant in human eosinophils (~35-fold more than P2X1), no P2X5 current feature was observed (33). This is consistent with P2X5 receptor splice variant in humans lacking the pore-forming domain of the channel (41). The absence of P2X4 currents (42) could be explained by the low levels of receptor mRNA (~10-fold less than P2X1). The presence of functional P2X/P2X4 (8) and P2X1/P2X5 (7) heteromeric receptors is also unlikely because they would display current characteristics different from those observed in human eosinophils. The currents elicited by the extracellular nucleotide ATP and its structural analog α,β-meATP exhibited kinetics (43) and sensitivity to NF449 (34) that are characteristic of the P2X1 receptor, confirming the functional presence of this receptor in human eosinophils. While our manuscript was under revision, a study by Alberto et al. (44), describing the presence of P2X1-like receptor currents in rat eosinophils, was published, further supporting our findings. This functional homomeric P2X1 receptor phenotype is not a trait unique to eosinophils; it is also observed in platelets (45) and neutrophils (46). However, it seems that divergence exists within the hematic lineage because mast cells (27) and macrophages (47) exhibit additional P2X receptor subtype activities (i.e., P2X4 and P2X7).

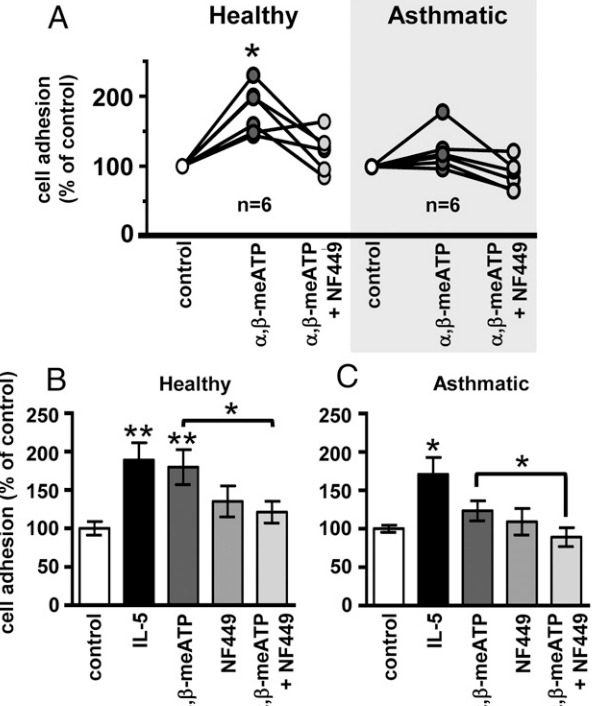

FIGURE 7. P2X1 receptor increases healthy eosinophil adhesion properties. (**A**) Effect of α,β-meATP (10 μM) and P2X1 antagonist NF449 (1 μM) on the adhesion of healthy and asthmatic eosinophils expressed as a percentage of the control response. Summary of the effect of IL-5 (10 ng/ml), α,β-meATP (10 μM), and NF449 (1 μM) on healthy (**B**) and asthmatic (**C**) eosinophil adhesion. n = number of donors. $*p < 0.05$, $**p < 0.01$.

P2X1 currents were 3.2-fold smaller in eosinophils from asthmatic donors compared with healthy donors. The diminished currents are unlikely to be due to defective transcriptional and/or translation event(s) because P2X1 transcript and protein expression levels were similar in cells from asthmatic and healthy donors, although the possibility that defective P2X1 receptors could originate from a compromised intracellular pool in asthmatics cannot be ruled out.

In contrast, P2X1 activity was similar in healthy and asthmatic platelets, indicating that P2X1-decreased activity in asthmatic eosinophils has a degree of specificity. Eosinophils in asthma are presumed to be exposed to increased concentrations of growth factors, such as IL-5, which prime eosinophils for enhanced function (48). However, IL-5 did not induce a reduction in P2X1 current amplitude, suggesting that other causal events are responsible for altering P2X1 responses in asthmatic eosinophils (Supplemental Fig. 3).

Under inflammatory conditions, cells can release nucleotides into the extracellular environment (12, 14, 16, 17, 49), desensitizing neighboring P2 receptors and causing aberrant regulation of P2 receptor functions (50). In asthmatic eosinophils, P2X1 activity was rescued when the cells were treated with high concentrations of apyrase. This is consistent with asthmatic eosinophil P2X1 receptors being desensitized as a result of exposure to increased amounts of extracellular ATP. An increase in extracellular nucleotides could be derived from eosinophils through an autocrine pathway (12) and/or neighboring cells (15). We found no difference in the basal ATP release level within the bulk extracellular medium of eosinophils from healthy and asthmatic donors. However, ATP could be released in extracellular subcompartments where it is degraded rapidly by locally expressed ectonucleotidases, as shown by Joseph et al. (51). Alternatively, an increase in extracellular ATP could be the result of a lower extracellular nucleotide clearance by ectonucleotidases. Interestingly, a recent report by Wang et al. (52) showed the downregulation of the transcripts for the ectonucleotidase CD39 in PBMCs from asthmatic patients, suggesting that CD39 mRNA deficiency could contribute to the asthmatic phenotype. Although we did not observe any change in the expression level of CD39 protein between healthy and asthmatic eosinophils (Supplemental Fig. 1C–E), we cannot exclude the involvement of a regulatory protein malfunction and/or other ectonucleotidases.

Idzko et al. showed that the P2X receptor agonist α,β-meATP, at concentrations up to 1 mM, induced Ca^{2+} transients, chemotaxis, ROS production, actin polymerization, IL-8 release, and increased CD11b cell surface expression in healthy human eosinophils (22, 23). In our study, using a submaximal concentration of

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α,β-meATP (53), ROS generation, cell shape change, upregulation of CD11b expression, and chemotaxis were not observed in healthy or asthmatic eosinophils (data not shown).

Like in human eosinophils, P2X1 is the only functional P2X receptor subtype in neutrophils (46) and platelets (54). Although P2X1 currents are also relatively small in these cells (<100 pA/pF) (46, 54), their physiological effects can be rather extensive. For example, P2X1 receptors promote neutrophil chemotaxis (46) and protect against endotoxemia by dampening neutrophil activation (35). In platelets, P2X1 amplifies the responses (e.g., aggregation and dense granule secretion) of many ligands (e.g., collagen, thrombin, and ADP) (55, 56). Platelet P2X1 receptor deficiency or inhibition dramatically reduces aggregation mediated by low concentrations of collagen (0.5–1.25 μg/ml) (54). However, α,β-meATP or NF449 had no effect on PAF- or eotaxin-induced eosinophil respiratory burst and shape change, suggesting that the physiological role of P2X1 in eosinophils is not to fine-tune the effect of these natural eosinophil ligands (data not shown).

P2X1 receptor regulates integrin activity in hematic cells, such as platelets (54), neutrophils, and monocytes (35), whereas in healthy eosinophils, P2X1 activation upregulates CD11b expression (23). The integrin $\alpha_M\beta_2$ is involved in eosinophil adherence-dependent function, including transmigration and degranulation (32, 37, 57). Our study showed that, although P2X1 activation had no effect on the overall expression of CD11b, it increased the expression of the activated state of the integrin in healthy, but not asthmatic, eosinophils. Zhu et al. (32) reported that eosinophils can bind to BSA and ICAM-1 in an $\alpha_M\beta_2$-dependent manner. Following their eosinophil-BSA–adhesion protocol, we found that P2X1 receptor activation caused an increase in healthy (but not asthmatic) eosinophil adhesion, revealing the contribution of P2X1 to eosinophil $\alpha_M\beta_2$ integrin–dependent adherence. This could represent a mechanism for retention of eosinophils in a tissue compartment. Because P2X1 receptors are sensitive to low ATP concentrations (e.g., due to constitutive release from neighboring cells), and consequently desensitization (5), a subtle equilibrium needs to be reached between the release of ATP and the activity of the local ectonucleotidases. This delicate balance seems to be lost in asthmatic patients; therefore, the rehabilitation of P2X1 receptor activity could be a new therapeutic target for asthma.

In summary, healthy human eosinophils express functional P2X1 receptors whose activation leads to an increased eosinophil $\alpha_M\beta_2$-dependent adhesion that is absent in asthmatic eosinophils. P2X1 responses are constitutively reduced in asthmatic eosinophils compared with healthy eosinophils, likely as the result of an increase in extracellular nucleotide concentration. Our work suggests that P2X1 receptor regulates eosinophil homeostasis because the receptor function is compromised in asthmatic eosinophils.

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Disclosures

The authors have no financial conflicts of interest.

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