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Stimulation of P2 purinergic receptors induces the release of eosinophil cationic protein and interleukin-8 from human eosinophils

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1 Extracellular nucleotides are the focus of increasing attention for their role as extracellular mediators since they are released into the extracellular environment in a regulated manner and/or as a consequence of cell damage.

2 Here, we show that human eosinophils stimulated with different nucleotides release eosinophil cationic protein (ECP) and the chemokine interleukin 8 (IL-8), and that release of these two proteins has a different nucleotide requirement.

3 Release of ECP was triggered in a dose-dependent manner by ATP, UTP and UDP, but not by 2′-&3′-o-(4-benzoyl-benzoyl)adenosine 5′-triphosphate (BzATP), ADP and α,β-methylene adenosine 5′ triphosphate (α,β-meATP). Release of IL-8 was triggered by UDP, ATP, α,β-meATP and BzATP, but not by UTP or ADP. Pretreatment with pertussis toxin abrogated nucleotide-stimulated ECP but not IL-8 release.

4 Release of IL-8 stimulated by BzATP was fully blocked by the $P2X_7$ blocker KN-62, while release triggered by ATP was only partially inhibited. IL-8 secretion due to UDP was fully insensitive to KN-62 inhibition.

5 Priming of eosinophils with GM-CSF increased IL-8 secretion irrespectively of the nucleotide used as a stimulant.

6 It is concluded that extracellular nucleotides trigger secretion of ECP by stimulating a receptor of the P2Y subfamily (possibly $P2Y_2$), while, on the contrary, nucleotide-stimulated secretion of IL-8 can be due to activation of both P2Y ($P2Y_6$) and P2X ($P2X_1$ and $P2X_7$) receptors.
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Abbreviations: BzATP, 2′-&3′-O-(4-benzoyl-benzoyl)adenosine 5′-triphosphate; α,β-meATP, α,β-methylene adenosine 5′ triphosphate; ECP, eosinophil cationic protein; IL-8, interleukin 8; PTX, pertussis toxin

Introduction

Eosinophils are terminally differentiated effector cells involved in the defensive response against parasites (Butterworth *et al.*, 1975) as well as in the pathogenesis of allergic diseases and immunological disorders (Leung, 1999). Upon recruitment at the site of inflammation, they release a broad panel of different mediators that can provoke tissue damage and contribute to the pathophysiology of diseases such as asthma (Bousquet *et al.*, 1990; Gleich, 1990), atopic dermatitis (Leung, 1999) and rheumatoid arthritis (Mertens *et al.*, 1993). Among granule-associated mediators produced by these cells, eosinophil cationic protein (ECP) is one of the most investigated. It is a single-chain, zinc-containing protein with a molecular weight ranging from 16 to 22 kDa, depending on the glycosylation level. ECP is also named "ribonuclease 3" because of its ribonuclease activity and homology (Olsson & Venge, 1972; Olsson *et al.*, 1977). It is used as a marker of eosinophil activation in diseases since its level correlates with eosinophil counts (Marguet *et al.*, 2001; Pronk-Admiraal & Bartels, 2001). Cytotoxic or cytostatic effects of ECP have been widely described (Maeda *et al.*, 2002). Recently, *in vitro* degradation of cytoskeletal proteins by ECP was also shown, suggesting a role for the protein in the pathogenesis of "eosinophilic myopathies" (Sugihara *et al.*, 2001).

Eosinophils have been reported to synthesize and release different cytokines such as interleukin (IL)-1α (Weller *et al.*, 1993), IL-3 (Kita *et al.*, 1991), IL-5 (Dubucquoi *et al.*, 1994),

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IL-8, (Kita *et al*., 1995; Simon *et al*., 1995), granulocyte/macrophage colony-stimulating factor (GM-CSF) (Kita *et al*., 1991), IL-16 and RANTES (Lim *et al*., 1996). An increased secretion of IL-8 has been described in eosinophils from patients with bronchial asthma or atopic dermatitis (Yousefi *et al*., 1995). Furthermore, IL-8 concentration in BAL fluids from asthmatic patients is significantly increased in comparison to that of healthy subjects (Yousefi *et al*., 1995). Since IL-8 is a chemotaxin for neutrophils and CD16+ NK cells (Teran *et al*., 1996; Campbell *et al*., 2001), the increased IL-8 level in BAL fluids from asthmatic patients suggests the involvement of IL-8 in amplification of the inflammatory reaction.

Nucleotides play a role not only inside but also outside the cell as they can be released through different mechanisms. Extracellular nucleotides exert their effects by stimulating two subfamilies of plasma membrane receptors named P2 receptors: the metabotropic G-protein-coupled P2Y and the ionotropic ligand-gated ion channels P2X (Dubyak & El-Moatassim, 1993; Ralevic & Burnstock, 1998; Di Virgilio *et al*., 2001). ATP binds to both subfamilies with high affinity, while ADP activates $P2Y_1$ and $P2Y_{12}$, UTP primarily interacts with $P2Y_2$ and $P2Y_4$, and UDP binds to the $P2Y_6$ subtype (Von Kuegelgen & Wetter, 2000). In addition, α,β-meATP and BzATP preferentially activate P2X subtypes (Dubyak & El-Moatassim, 1993; Ralevic & Burnstock, 1998; North & Surprenant, 2000). It has been recently shown that human eosinophils express both P2Y and P2X receptor subtypes (Ferrari *et al*., 2000; Mohanty *et al*., 2001) whose stimulation induces intracellular Ca^{2+} transients, oxygen radical production and expression of the adhesion molecule CD11b (Dichmann *et al*., 2000; Ferrari *et al*., 2000; Idzko *et al*., 2001).

As ATP is released upon tissue damage and/or in response to inflammatory stimuli (Cook & Mccleskey, 2002) such as bacterial products (Ferrari *et al*., 1997) or salivary histatin 5 (Edgerton & Koshlukova, 2000), we focused our interest on the question whether stimulation of P2 receptors expressed by human eosinophils could induce the release of cytotoxic granular mediators such as the ECP and IL-8.

Methods

Reagents

ATP, UTP, UDP, ADP, BzATP, α,β-meATP, suramin, Ficoll, and Triton X-100, were obtained from Sigma (Deisenhofen, Germany); pertussis toxin from Calbiochem (La Jolla, CA, U.S.A.); the calcium indicator (1-[2-(5-carboxy-oxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methyl-phenoxy)- ethane-*N*,*N*,*N*,*N*′-tetraacetic acid, pentaacetoxymethyl-ester) (Fura-2/AM) was obtained from Molecular Probes (Leiden, Netherlands); immunomagnetic beads (Dynabeads M-450) were purchased from Dianova (Hamburg, Germany).

Preparation of human eosinophils

Human eosinophil granulocytes from healthy nonatopic volunteers were isolated from heparin-treated ($10\,U\,ml^{-1}$) blood by Ficoll separation, and negatively selected with anti-CD16 antibody-coated Dynabeads. Eosinophil purity was $\geqslant 96\%$ as judged by Pappenheim staining. Viability of purified eosinophils was measured by trypan blue exclusion and was more than 98%.

Cell viability

Survival of cultured eosinophils was assessed by propidium iodide staining and FACS analysis of at least 5000 cells. Briefly, cells were washed once in PBS plus 2% FCS and resuspended in $200\,\mu l$ of a propidium iodide solution ($0.5\,\mu g\,ml^{-1}$ dissolved in PBS).

Intracellular Ca^{2+} measurements

Ca^{2+} transients were measured in eosinophils loaded with the Ca^{2+} indicator Fura-2/AM (Calbiochem, La Jolla, CA, U.S.A.) by using the digital fluorescence microscope unit Attofluor (Zeiss, Oberkochen, Germany). Briefly, cells were incubated with $2\,\mu M$ Fura-2/AM for 30 min at 37°C in a Ca^{2+}- and Mg^{2+}-free Hanks' BSA solution. Cells were then washed twice and finally resuspended in the same buffer containing 1.5 mM $CaCl_2$ and $MgCl_2$. Traces were followed fluorospectrometrically and Ca^{2+} transients were determined by multiple cell acquisitions with the 340/380 nm wavelength excitation ratio at an emission wavelength of 505 nm. Curves shown are representative of the whole cell population.

Permeabilization to YO-PRO

Human eosinophils were incubated in a saline solution containing $10\,\mu M$ YO-PRO and $250\,\mu M$ sulfinpyrazone, in the presence or absence of 3 mM ATP for 30 min at 37°C. At the end of the incubation time, 5 mM $MgSO_4$ was added. Cells were centrifuged and resuspended in a YO-PRO, ATP-free solution. Fluorescence intensity was then measured in a luminescence spectrometer. Data are expressed as means ± s.e.m. of experiments performed in triplicate.

ECP and IL-8 determination

ECP and IL-8 secretion were measured by ELISA. Kits were purchased from Amersham and R&D Systems, respectively.

Statistical analysis

Unless otherwise stated, data are expressed as the means ± s.e.m. Analysis of variance (ANOVA) was used to compare experimental groups and control values. When the global test of differences was significant at the 5% level, pairwise tests of differences between groups were applied (Tukey's multiple comparison test).

Results

Nucleotides induce ECP release from human eosinophils

To study the involvement of P2 receptors in ECP release, eosinophils were stimulated with different nucleotides and secretion of the mediator into the supernatants was evaluated

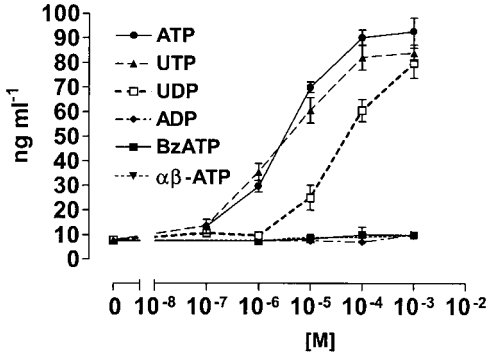

Figure 1 Effect of nucleotides on ECP secretion. Human eosinophils (10^6 cells ml^{-1}) were stimulated in culture medium for 2 h, with the indicated concentrations of ATP, UTP, UDP, ADP, BzATP or α,β-meATP. ECP release was evaluated by ELISA. Data are expressed as mean ± s.e.m. ($n = 5$).

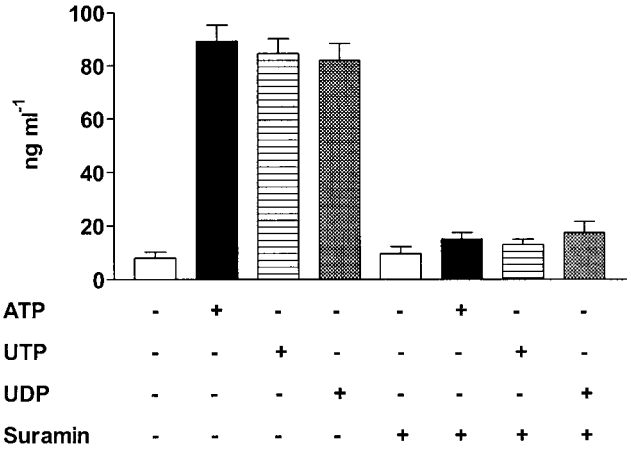

Figure 3 Effect of suramin on nucleotide-induced ECP secretion. Eosinophils were pretreated for 30 min with 100 μM suramin or medium alone. Thereafter, cells were stimulated with 1 mM ATP, UTP or UDP. Data are expressed as mean ± s.e.m. ($n = 3$).

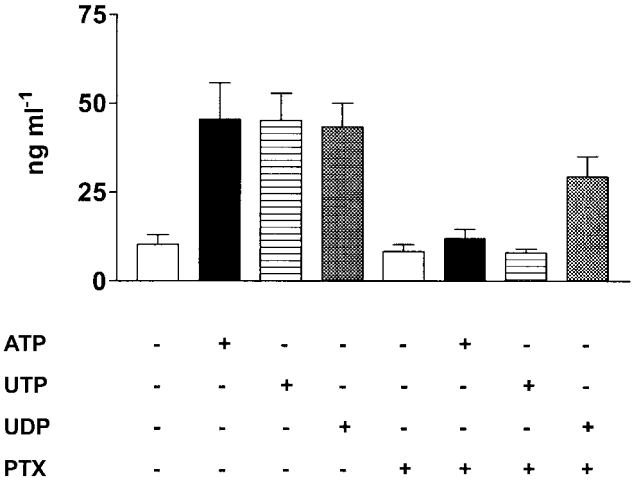

Figure 2 Effect of pertussis toxin on nucleotide-induced ECP secretion. Eosinophils were incubated in the presence or absence of 4 μg ml^{-1} PTX for 2 h. Cells were then stimulated for 2 h with 1 mM ATP, UTP or UDP and ECP release was evaluated. Data are expressed as mean ± s.e.m. ($n = 3$).

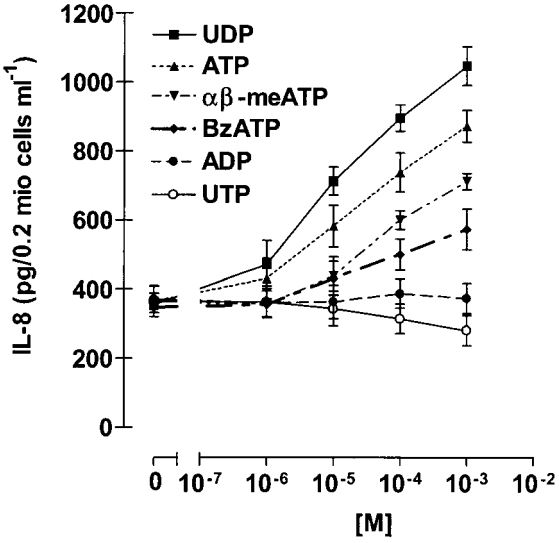

Figure 4 Effect of nucleotides on IL-8 release. Eosinophils were stimulated with the indicated concentrations of ATP, UTP, UDP, ADP, α,β-meATP and BzATP. After 24 h, supernatants were collected and IL-8 content was measured by ELISA. Data are expressed as mean ± s.e.m. ($n = 6$).

by ELISA. Figure 1 shows that the P2 agonist ATP induced a dose-dependent secretion of ECP. To characterize the involvement of P2Y receptors in this response, eosinophils were stimulated with UTP and UDP. These nucleotides activate different P2Y subtypes: UTP acts at $P2Y_2$ and $P2Y_4$ receptors, while UDP preferentially activates the $P2Y_6$ subtype (Warny *et al*., 2001). Both nucleotides were active in stimulating ECP secretion from eosinophils, although with a different dose-dependency. UTP dose-dependency was similar to that of ATP, while UDP stimulated release of ECP at concentrations starting from 10^{-5} M. Interestingly, ADP which is an agonist at $P2Y_1$ and $P2Y_{12}$ receptors did not stimulate ECP release. To evaluate the contribution of the P2X subtypes in ECP secretion, eosinophils were stimulated with increasing concentrations of two ATP derivatives, α,β-meATP and BzATP, known to stimulate P2X receptors. In contrast to UTP and UDP, these agonists did not induce secretion of ECP at any of the concentrations tested.

Nucleotide-stimulated-ECP release is pertussis toxin sensitive

Stimulation of P2Y receptors induce activation of different G proteins (Ralevic & Burnstock, 1998) and regulates Ca^{2+} mobilization from intracellular stores as well Ca^{2+} influx from the extracellular medium. To dissect the intracellular signaling pathway of P2Y-mediated ECP release, eosinophils were incubated either with the $G_{i/o}$ proteins inhibitor pertussis toxin (PTX) or in the presence of the Ca^{2+} chelator EGTA. As shown in Figure 2, pretreatment with PTX completely abrogated ATP- and UTP-induced ECP release while UDP-mediated response was reduced, showing that activation of $G_{i/o}$ proteins was a step of purinergic-mediated ECP secretion. Release of ECP by ATP, UTP or UDP was also blocked by the

Table 1 Effect of nucleotides on viability of human eosinophils

	Control	*ATP*	*UDP*	*α,β-meATP*	*BzATP*
0 h	96.52±3.81	97.25±2.68	95.90±2.63	97.56±3.34	94.15±2.23
2 h	94.79±2.84	93.92±4.20	93.07±3.12	95.13±2.59	92.55±3.42
6 h	92.55±5.02	92.21±3.12	94.81±4.84	94.34±3.35	89.79±5.74
12 h	89.49±4.46	92.05±4.31	92.22±3.79	92.34±2.78	88.09±4.32
24 h	82.79±5.84	89.92±4.20	90.07±4.89	88.55±4.66	85.69±3.84

Eosinophils (10^6 cells ml^{-1}) were cultured in the absence or presence of the different nucleotides (1 mM) for the indicated time. Viability was detected as described in the Methods section. Data are shown as percentage of viable eosinophils±s.e.m. obtained in five independent experiments. No significant difference between control and nucleotide-stimulated cells was detected.

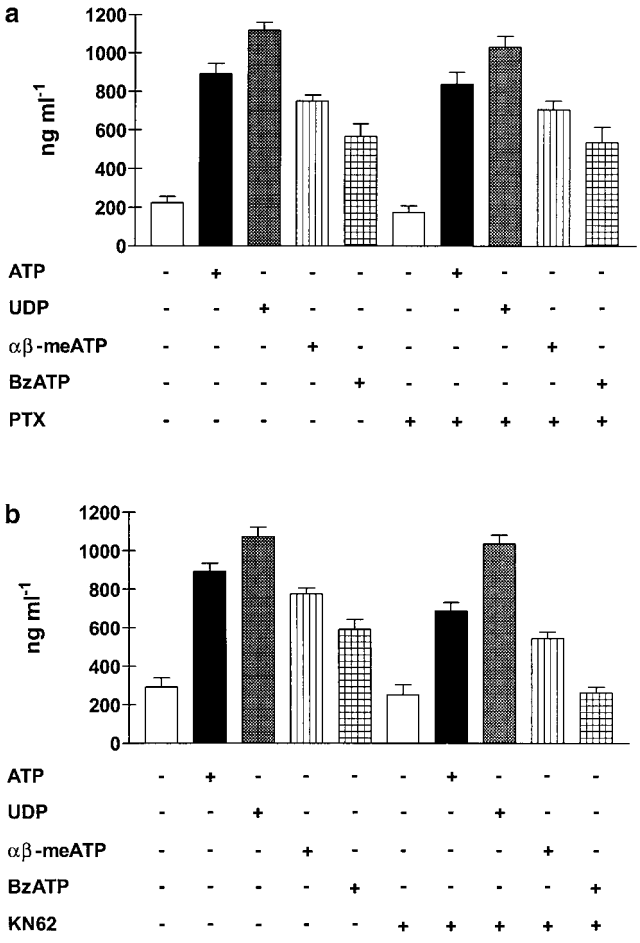

Figure 5 Effect of pertussis toxin (PTX) and KN-62 on nucleotide-induced IL-8 release. Eosinophils were preincubated in the presence or in the absence of 4 μg ml^{-1} PTX for 2 h (**a**); KN-62 (25 nM) was added to eosinophils 10 min prior to stimulation (**b**). Cells were then stimulated for 24 h with the indicated nucleotides and IL-8 release was measured by ELISA. Data are expressed as mean±s.e.m. ($n=3$).

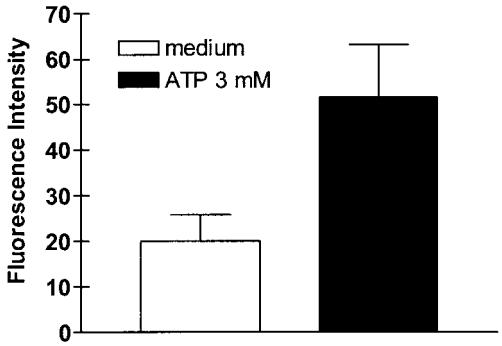

Figure 6 ATP-dependent YO-PRO uptake by human eosinophils. Cells were incubated in a 10 μM YO-PRO-containing saline solution and stimulated with 3 mM ATP for 30 min (see Methods for details). Fluorescence intensity was then measured as reported in the Methods. Data are expressed as means±s.e.m. of experiments performed in triplicate.

broad-spectrum P2Y inhibitor suramin (Figure 3). Addition of EGTA prior to stimulation with the nucleotides did not significantly affect ECP release (not shown).

Nucleotides induce IL-8 secretion from human eosinophils

IL-8 is an important chemotactic factor and a central mediator in inflammation, eliciting various responses in different cells (Mukaida, 2000; Baggiolini, 2001). Stimulation of eosinophils with the P2 agonist ATP, as well as with the P2X agonists α,β-meATP and BzATP, dose-dependently induced secretion of the cytokine. Interestingly, in addition to α,β-meATP and BzATP, only the $P2Y_6$ agonist UDP was able to induce the production of IL-8, while UTP and ADP were ineffective (Figure 4). To exclude the possibility that release of the cytokine was due to the cytotoxic effect of nucleotides, we performed a viability test. Eosinophils were incubated with the different nucleotides and vitality measured by propidium iodide and FACS analysis. No significant difference between control and nucleotide-stimulated cells was detected (Table 1).

Pretreatment of eosinophils with PTX did not significantly affect ATP-, UDP-, α,β-meATP- or BzATP-induced IL-8 production (Figure 5a). In contrast, preincubation of eosinophils with KN-62 (25 nM) for 10 min, completely abrogated BzATP- and reduced ATP- and α,β-meATP-induced IL-8 production; however, it had no influence on the UDP-mediated response (Figure 5b). Stimulation by BzATP and inhibition by KN-62 (Figure 5b) suggested that IL-8 production also involved stimulation of the $P2X_7$ subtype. We previously showed that human eosinophils expressed mRNA for $P2X_7$ receptor, but it is yet unknown whether the receptor is functional in these cells. To test this hypothesis, we performed the YO-PRO uptake assay. Figure 6 shows that eosinophils stimulated with ATP became permeable to the extracellular dye.

Virtually all cell types express ecto-nucleotidases (Zimmermann, 1992; Mizumoto *et al*., 2002); therefore, responses to extracellular nucleotides could also be due to stimulation of adenosine receptors by nucleotide degradation products. Table 2 shows that incubation of eosinophils with the A_1-receptor antagonist DPCPX, the A_{2a}-receptor antagonist CSC

Table 2 Lack of effect of adenosine receptor antagonists on nucleotide-induced IL-8 secretion

		DPCPX	*CSC*	*MRS-1220*
Control	332±48	357±38	292±68	360±56
ATP	925±43	893±53	932±73	1025±73
UDP	1073±52	1002±63	1127±59	999±102
$\alpha\beta$-meATP	774±35	743±29	704±53	798±86
BzATP	610±53	595±59	621±47	600±34

Cells were pretreated for 30 min with the A_1-receptor antagonist DPCPX or the A_{2a}-receptor antagonist CSC or the A_3-receptor antagonist MRS-1220 at a concentration of 1 μM, and were then stimulated for 24 h with 1 mM of the indicated nucleotide. Supernatants were then collected and IL-8 content was determined by ELISA. Data are expressed as mean±s.e.m. ($n=3$).

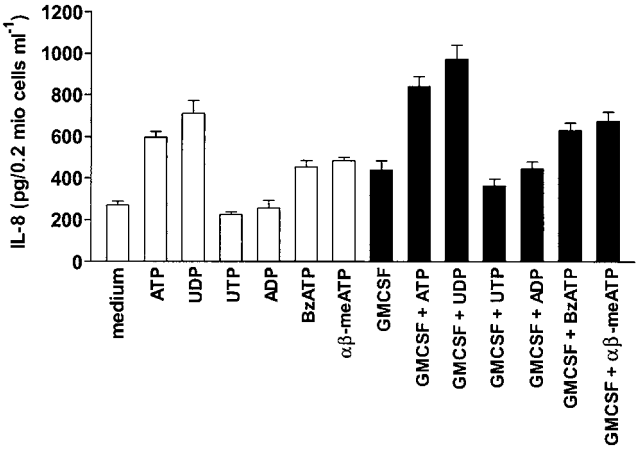

Figure 7 Nucleotide-induced IL-8 production is potentiated by GM-CSF. Cells were incubated for 30 min in the presence or absence of 10 ng ml^{-1} GM-CSF. Eosinophils were then stimulated with 1 mM of the indicated nucleotides and cultured for 24 h. Supernatants were then collected and IL-8 content was determined by ELISA. Data are expressed as mean±s.e.m. ($n=4$).

or A_3-receptor antagonist MRS-1220, did not affect nucleotide-stimulated IL-8 secretion.

GM-CSF has been recently shown to modulate eosinophil functions and survival by reducing spontaneous apoptosis (Esnault & Malter, 2002; Hoontrakoon *et al*., 2002). We asked whether GM-CSF would also modulate nucleotide-induced IL-8 secretion. Pretreatment of eosinophils with GM-CSF increased nucleotide-induced IL-8 production without changing nucleotide stimulation pattern (Figure 7).

Discussion

ECP released by eosinophils can cause respiratory, epithelial and cardiovascular tissue damage and consequently inflammation (Venge *et al*., 1999). Cell cytoplasm contains huge amounts (5 – 10 mM) of ATP, which can be released into the extracellular milieu as a consequence of shear stress forces, membrane stretching or following cell injury/death. However, ATP can also be accumulated into specific intracellular granules or vesicles such as platelets, dense granules or neuronal vesicles and secreted in a regulated manner (Jo & Role, 2002; Vonend *et al*., 2002). Many inflammatory cells (e.g. macrophages, dendritic cells, microglia) chemotact to ATP. Thus, it is conceivable that ATP and/or other intracellular nucleotides could function as an early alarm signal alerting the immune cells of damage to tissue. ATP release could also represent an amplification system spreading the initial alarm by generating additional mediators such as the proinflammatory cytokines IL-1, IL-6, IL-8, IL-18 (Ferrari *et al*., 1997; Perregaux *et al*., 2000; Shigemoto-Mogami *et al*., 2001; Warny *et al*., 2001). ATP is a chemotactic factor for eosinophils (Burgers *et al*., 1993) and purinergic stimulation of eosinophils elicit activatory responses such as production of reactive oxygen species and CD11b expression (Idzko *et al*., 2001). These findings suggest that ATP could function as an amplification signal to potentiate the killing capacity of eosinophils and recruitment of inflammatory cells.

Here we show that stimulation of eosinophils with nucleotides induced the release of ECP and secretion of IL-8. The two processes had different nucleotide requirements and involved different P2 receptor subtypes. Release of ECP was triggered by stimulation of P2Y receptors, possibly of the $P2Y_2$ subtype. In contrast to ECP, IL-8 secretion was due to activation of both P2X and P2Y receptors, as in addition to UDP, the P2X agonists α,β-meATP and BzATP also were able to induce this response. Interestingly, UDP (a $P2Y_6$ agonist) but not the other P2Y agonists UTP and ADP also induced IL-8 secretion. Further support to involvement of both P2 receptor families in IL-8 secretion came from the experiments with KN-62. This reversible $P2X_7$ inhibitor, originally described as a calmodulin blocker and subsequently shown to powerfully inhibit the human $P2X_7$ subtype (Blanchard *et al*., 1995; Gargett & Wiley, 1997; Idzko *et al*., 2001) completely abrogated BzATP-mediated response, while it did not affect the UDP-induced IL-8 release. Partial inhibition by KN-62 of ATP- and α,β-meATP-stimulated IL-8 secretion indicated that the $P2X_1$ and $P2X_7$ receptor subtypes play a role in stimulating this response; this observation also shows that the $P2X_7$ receptor in eosinophils, at variance with neutrophils (Gu *et al*., 2000), is functional.

GM-CSF has been involved in the pathogenesis of chronic allergic inflammation since it reduces eosinophil apoptosis; therefore, inhibition of GM-CSF-mediated effects would reduce eosinophil inflammation (Allam & Renzi, 2001). Here we demonstrated that GM-CSF was able to upregulate responses of eosinophils to extracellular nucleotides, showing that GM-CSF could act as a proinflammatory cytokine also by inducing the release of ECP and IL-8.

In summary, our data provide evidence that nucleotides modulate eosinophil functions, showing a novel role for these extracellular mediators in the activation of human eosinophils.

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