Activation of A₃ Adenosine Receptors on Human Eosinophils Elevates Intracellular Calcium

By Yutaka Kohno, Xiao-duo Ji, Steve D. Mawhorter, Masahiro Koshiba, and Kenneth A. Jacobson

Adenosine (ADO) is a potent bronchoconstrictor in allergic patients and has been shown to increase the release of histamine from human lung tissues. Antagonists of AÒ and A₂a receptors are not effective in attenuating these effects. Therefore, involvement of ADO A₃ receptors in the bronchoconstrictor and/or inflammatory effects have to be considered. Eosinophils also play a pivotal role in allergic diseases such as asthma, thus it is natural to consider a link between the A₃ receptor and eosinophils. Human peripheral blood eosinophils express the ADO A₃ receptor as indicated by detection of the transcript for A₃ receptors in polymerase chain reaction-amplified cDNA derived from the cells. A₃ receptors on eosinophil membranes were characterized using the A₃ receptor agonist radioligand 125I-labeled AB-MECA, which yielded Bₘₐₓ and Kᵢ values of 1.31 pmol/mg protein and 3.19 nmol/L, respectively. Treatment of eosinophils with the highly potent and selective A₃ receptor agonist CHB-MECA clearly induced Ca²⁺ release from intracellular Ca²⁺ pools followed by Ca²⁺ influx, suggesting the presence of phospholipase C-coupled A₃ receptors. In contrast, the ADO receptor agonists CPA and CGS 21680, selective for A₁ and A₂a receptors, respectively, at concentrations of ≤30 μmol/L did not elevate the intracellular Ca²⁺ level. These results attest to the existence of ADO A₃ receptors on eosinophils and suggest that ADO stimulates these cells to release Ca²⁺ from intracellular stores via the activation of A₃ receptors.

© 1996 by The American Society of Hematology.
mancia, Uppsala, Sweden) density gradient centrifugation and negative selection with anti-CD16 monoclonal antibody (MoAb) and immunomagnetic beads coated with goat antimouse IgG (Dynabeads M450; Dynal AS, Oslo, Norway) as previously described.21 The purity of eosinophils based on light microscopic examination of cytocentrifugation preparations stained by Diff-Quik (American Scientific Products, McGraw Park, IL) was >99%.

Reverse transcription polymerase chain reaction (PCR). The eosinophils used in this assay were carefully isolated with particular attention given to purity. The purity based on light microscopic examination was 100%, based on random counting of 500 cells. Total RNA was prepared by the single-step method21 with slight modifications (RNA STAT-60; TEL-TEST “B”, INC, Friendswood, TX). One microgram of total RNA was incubated at room temperature for 15 minutes with 1 U of RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN), and the first strand cDNA was synthesized with 0.5 μg of oligo (dT)12-14 primer and 200 U of the cloned M-MLV reverse transcriptase (SUPERSCRIPT II preamplification system; Life Technologies, Gaithersburg, MD).

The human A1 ADO receptor sequence was amplified with 5′ primer sequence (ACCCCATGTTTGGCCTG) and 3′ primer sequence (GCACAACTGTGGTACCTCA) giving a 361 bp product.22,24 The PCR was carried out in 50 μL using the following conditions: the initial denaturing step at 95°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, except the last elongation at 72°C for 10 minutes. Subsequently, 10 μL of PCR products were run on the 4% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) and examined by ethidium bromide staining.

Membrane preparation and radioligand binding. Binding studies using the high affinity ADO A1 receptor radioligand [3H]N6-(3-iodo-4-aminobenzyl)-adenosine-5′-N-methyluronamide ([3H]AB-MECA; Amersham Life Science, Inc, Arlington Heights, IL) were performed and analyzed as described previously.23 For binding studies, eosinophils were centrifuged at 1,000 rpm for 10 minutes on a desktop centrifuge. The pellet was washed with a lysis buffer (10 mmol/L Tris, 5 mmol/L EDTA, pH 7.4) at 5°C. The resulting pellet was resuspended in 50 mmol/L Tris, 10 mmol/L MgCl2, 1 mmol/L EDTA, pH 8.26 at 5°C (50/10/1 buffer) and disrupted by Dounce homogenization on ice (20 strokes by hand). After centrifugation at 43,000g and 5°C for 10 minutes, the crude membrane pellet was resuspended in lysis buffer to yield a protein concentration of 0.2 mg/mL (corresponding to ~8 × 106 cells). ADO deaminase was added to provide a final concentration of 3 μmol/L in the lysis buffer for 40 minutes at room temperature. The cells were centrifuged at 1,200 rpm for 10 minutes to remove extracellular dye and were resuspended in HBSS. Two milliliters of cell suspension in a 1:1 mixture of RPMI medium and HBSS buffer at approximately 106 cells/mL. Ten milliliters of a dimethylsulfoxide (DMSO) solution containing 10 μg of fluo-3 AM (Molecular Probes, Inc, Eugene OR) was added to 10 μL of the cell suspension, and cells were left in the dark for 40 minutes at room temperature. The cells were centrifuged at 1,200 rpm for 10 minutes to remove extracellular dye and were resuspended in HBSS. Two milliliters of cell suspension was added to each cuvette with a magnetic stir bar, and the fluorescence intensity of fluo-3 AM was quantified using a Deltascan fluorescence spectrophotometer (Photon Technology International, Inc, Brunswick, NJ) with the excitation wavelength set at 506 nm and emission wavelength monitored at 526 nm. The maximum fluorescence intensity of fluo-3 AM was quantified using a Deltascan fluorescence spectrophotometer (Photon Technology International, Inc, Brunswick, NJ) with the excitation wavelength set at 506 nm and emission wavelength monitored at 526 nm. The maximum fluorescence was determined by adding Triton-X (0.025%) or 4-bromoA23387 (1 μmol/L) in the presence of 1 mmol/L Ca2+. The minimum fluorescence (Fmin) was determined following quenching of fluo-3 with 0.5 mmol/L ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA).

RESULTS

Expression of A ADO receptors on eosinophils. Amplification of a A1 receptor-specific sequence was performed from reverse-transcribed mRNA obtained from eosinophils. The agarose-gel electrophoresis revealed the single band of the expected 361-bp PCR product, indicating the existence of ADO A1 receptor on eosinophils (Fig 1).

Radioligand binding. The level of expression of the AOD A1 receptor in human eosinophils was also determined using the high affinity radioligand [3H]AB-MECA25 in satu-
ration binding experiments (Fig 2). This ligand bound to a single, saturable high affinity site in membranes from eosinophils, with $K_d$ and $B_{max}$ values of $3.19 \pm 0.21$ nmol/L and $1.31 \pm 0.15$ pmol/mg membrane protein, respectively (four experiments). At cloned human A$_3$ receptors membranes of HEK293 cells a $K_d$ value of 0.59 nmol/L for $[^{125}]$AB-MECA saturation was determined.26 Xanthines selected for competition experiments included an amine congener, XAC, and two carboxylic acid derivatives, 8-[4-[[carboxy]methyl]oxy]phenyl]-1,3-dipropylxanthine (XCC) and 1,3-dipropyl-8-[4-(z-carboxyethenyl)phenyl]xanthine (BWA 1433). Selected xanthine analogues competed for $[^{125}]$AB-MECA binding with a rank order of potency of BWA 1433 $>$ XCC $=$ XAC, indicative of A$_3$ receptor pharmacology (Fig 3). The $K_d$ values of $5.23 \pm 1.46$, $20.0 \pm 8.5$, and $22.6 \pm 7.0$ pmol/L were obtained, respectively. These values for $[^{125}]$AB-MECA are comparable with those obtained in CHO cells stably transfected with the A$_3$ receptor cDNA derived from rat brain.18 At cloned human A$_3$ receptors membranes of HEK293 cells the $K_d$ values versus $[^{125}]$AB-MECA for XAC and BWA1433 were determined to be $0.600 \pm 0.053$ and $0.664 \pm 0.031$ pmol/L, respectively, assuming a $K_d$ value of 0.59 nmol/L for the radioligand at this receptor.26 At A$_1$ receptors, these xanthines bind with considerably greater affinity.28

The level of A$_3$ receptor binding was much higher than levels of A$_1$ and A$_2A$ receptors present in the same membranes. Binding assays at A$_1$ and A$_2A$ receptors using single concentrations of radioligand were performed as described.26 At 0.6 nmol/L of $[^3H]$R-N$^6$-phenylisopropyladenosine, specific binding representing 0.059 pmol/mg protein of A$_1$ receptors was detected. At 3 nmol/L of $[^3H]$2-[4-[(2-carboxyethyl)phenyl]ethyl-amino]-5'-N-ethylcarbamoyladenosine, specific binding representing 0.063 pmol/mg protein of A$_2A$ receptors was detected.

**Cytosolic Ca$^{2+}$ concentration.** The highly selective A$_3$ receptor agonist, CI-IB-MECA (10 and 30 pmol/L), produced increases in concentrations of intracellular free Ca$^{2+}$ ([Ca$^{2+}$]). (Fig 4). It produced a rapid rise followed by a sustained increase in [Ca$^{2+}$]. Among the donors selected, those with more significant eosinophilia had a more pronounced [Ca$^{2+}$] response, consistent with minimally activated (primed) eosinophils compared with normals. In separate experiments these patients showed increased expression of activation markers, including CD69 and CD25, on their peripheral blood eosinophils compared with normal donors (S. Mawhoret, personal communication, March 1996). Chelation of extracellular Ca$^{2+}$ with EGTA (0.5 mmol/L) did not abolish the initial rise in [Ca$^{2+}$]. Thus, the first rapid increase in [Ca$^{2+}$], was due to Ca$^{2+}$ release from intracellular Ca$^{2+}$ pools, while the second phase appeared to result from an influx of extracellular Ca$^{2+}$ (Fig 5). Although NECA (10 pmol/L) induced an increase in intracellular Ca$^{2+}$ levels, the intensity of the elevation of [Ca$^{2+}$] was clearly lower than
in eosinophils are very similar in intensity and pharmacology to those observed in HL-60 cells, for which we have also proposed a functional A3 receptor.29

The role of the ADO A3 receptor on the eosinophils is unclear at present. However, when the eosinophils were exposed to a relatively high concentration of an A3 agonist, synthesized in our laboratory,27 that is ~2,000-fold selective in binding assays at rat adenosine receptors, an increase in [Ca^{2+}]_{i} was observed. Upon chelation of extracellular Ca^{2+}, the initial rapid rise in [Ca^{2+}]_{i} produced by CI-IB-MECA was still present, and the later prolonged elevation was absent. These results are consistent with the presence of phospholipase C-coupled ADO A3 receptors on eosinophils. In a previous study of the effects of A3 receptor agonists on HL-60 (human promyelocytic leukemia) cells, very similar effects on [Ca^{2+}]_{i} were seen.29 The calcium ionophore A23187 stimulates these cells to induce the release of granule substances and intact granules by a cytolytic mechanism.31 Some substances are known to stimulate eosinophil function, associated with mobilization of Ca^{2+} from intracellular stores and secretion of granule substances. Prostaglandin D_{2}31 and PAF32,33 one of the most potent chemoattractants for eosinophils, are potent stimuli for calcium mobilization. Secretion and other functions induced by these substances appear to depend on both the release of Ca^{2+} from intracellular stores and on G proteins.32,33 Therefore, it is thought that ADO stimulates these cells via the activation of A3 receptors, resulting in the release of Ca^{2+} from intracellular stores. It is well-documented that in certain types of cells elevation of the intracellular Ca^{2+} level induced by various agents can play a crucial role in triggering apoptosis. Also in thymocytes, an antibody against the CD3-T cell receptor complex,34 ATP35 and ADO36 were reported to induce apoptosis by Ca^{2+}-mediated mechanisms, and their effects are mimicked by treatment of the thymocytes with a calcium ionophore. In addition to thymocytes, ADO induced apoptosis in chick embryonic sympathetic neurons.37 Thus, there may be a close relationship between apoptosis and activation of ADO A3 receptor.29

Recent studies have identified a novel ADO receptor subtype, A3, in the rat38 and similar receptors have been cloned from both human34 and sheep brain cDNA libraries.39 Few
We proved that A2 receptors are present on eosinophils, and our data are consistent with the possibility that the A2 receptors on the cells are involved in the etiology of various allergic diseases although the possibility that other adenosine receptors or other cells may be involved in these diseases has not been ruled out.

New selective A3 receptor antagonists are currently under development in our laboratory. It will be of great interest to determine if a potent antagonist would prevent the elevation of intracellular calcium concentration evoked by an A3 receptor agonist and the activation or apoptosis of the cells. It will be necessary to follow this study with an investigation on how A3 receptor activation affects eosinophil function. A3 receptor antagonists may prove to inhibit infiltration or transmigration of eosinophils to tissues including airway lumen and thus be useful therapeutically in allergic diseases.

ACKNOWLEDGMENT

We thank Dr Bruce S. Bochner (Johns Hopkins Asthma and Allergy Center, Baltimore, MD) for the gift of eosinophils and for helpful discussions and Dr Mark E. Olah and Prof Gary L. Stiles (Duke University Medical Center, Durham, NC) for preparation of [3H]AB-MECA used in this study.

REFERENCES

7. Filley WV, Holley KE, Kephart GM, Gleich GJ: Identification by immunofluorescence of eosinophil granule major basic protein in lung tissue of patients with bronchial asthma. Lancet 2:11, 1982


Activation of A3 adenosine receptors on human eosinophils elevates intracellular calcium

Y Kohno, X Ji, SD Mawhorter, M Koshiba and KA Jacobson