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Intracellular Adenosine Inhibits IgE-Dependent Degranulation of Human Skin Mast Cells

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Abstract

Purpose—Adenosine (ADO) can enhance and inhibit mast cell degranulation. Potentiation of degranulation occurs at relatively low concentrations of ADO (10^{-6} – 10^{-5} M) through triggering of A3AR, whereas, inhibition occurs at higher concentrations of ADO reportedly through triggering of A2aAR. However, the discrepancy in the concentration of ADO that inhibits degranulation and that required to trigger ADORs suggests a different mechanism. The purpose of this study is to determine the mechanism by which ADO inhibits human mast cell degranulation.

Methods—We compare the effectiveness of A2aAR specific antagonist ZM241385 and equilibrative nucleoside transporter inhibitors Dipyridamole and NBMPR in preventing ADO-mediated inhibition of FcεRI-induced degranulation of human skin mast cells (hSMCs). Western blotting is done to analyze the effect of ADO on FcεRI-induced Syk phosphorylation.

Results—Dipyridamole and NBMPR completely and dose-dependently prevented ADO from inhibiting FcεRI-induced degranulation in all hSMC preparations. In contrast, ZM241385 at 10^{-5} M was effective in only 3 of 10 hSMC preparations. Moreover, NBMPR was effective even in those hSMC preparations not responsive to ZM241385. ADO inhibited degranulation induced by FcεRI crosslinking, but not that induced by complement component 5a (C5a), Substance P or

calcium ionophore. Accordingly, ADO significantly attenuated FcεRI-induced phosphorylation of Syk at the critical activating tyrosine (Y525).

Conclusion—Blocking the influx of ADO, but not A2aAR signals, is necessary and sufficient to prevent ADO from inhibiting FcεRI-induced mast cell degranulation. Thus, ADO specifically inhibits FcεRI-induced degranulation of hSMCs primarily by an intracellular mechanism that requires its influx via equilibrative nucleoside transporter 1 (ENT1).

Keywords

Adenosine; mast cells; equilibrative nucleoside transporter; adenosine receptors; syk; degranulation

Introduction

Adenosine, a purine nucleoside, is produced when the ecto-nucleotidases ecto-nucleoside triphosphate diphospho-hydrolase (E-NTDPase) (CD39) and ecto-5'-nucleotidase (CD73) sequentially dephosphorylate adenosine triphosphate (ATP) [1]. The dephosphorylation of ATP, which is produced in all cell types including mast cells, generates the energy required to propel cellular activities. Therefore, ADO production is intimately related to cellular metabolism. Accordingly, ADO concentrations are higher when cellular metabolism is increased, as with inflammation or hypoxia [2]. Whereas the concentration of circulating ADO in plasma is <1 μM under normal conditions, plasma levels in patients with sepsis have been reported to approach 10 μM [3]. In tissues, ADO levels in the range of 10–100 μM were found in synovial fluid from rheumatoid arthritis patients [4, 5], and near 200 μM in the epithelial lining fluid of asthmatic lungs [6]. Once produced, ADO can traverse plasma membranes through bi-directional equilibrative nucleoside transporters (ENTs, SLC29 gene family) and concentrative nucleoside transporters (CNTs, SLC28 gene family) [7, 8]. Whether in the extracellular or intracellular space, ADO is rapidly converted to inosine by adenosine deaminase (ADA), or phosphorylated intracellularly by adenosine kinase to re-enter the energy cycle [1, 9]. Extracellular ADO can also interact with four known G protein-coupled ADO receptors (ADORs) (A1AR, A2aAR, A2bAR and A3AR) to exert its physiological effects [9].

ADO modulates mast cell degranulation. Marquardt, et al. [10] first demonstrated that ADO could potentiate the degranulation of immunologically activated rodent mast cells. In studies with human mast cells from lung and skin, ADO at 10^{-6} – 10^{-5} M enhanced IgE-dependent degranulation, but inhibited it at 10^{-5} – 10^{-3} M [11–15]. Thus, IgE-dependent mast cell degranulation is enhanced by ADO at low concentrations, but inhibited with higher concentrations. Because administered ADO induces mast cell mediated (histamine-dependent) bronchospasm in asthmatics (but not non-asthmatics) [16, 17], identification of the ADORs involved in potentiating the release of allergic/ inflammatory mediators has been intensely studied. In mice and humans, A3AR has clearly been identified as a potentiating ADOR of mast cell degranulation [11, 18, 19], and is likely involved in ADO-mediated bronchoconstriction [20]. Interestingly, the inhibitory effect of ADO at higher concentrations appears to be a more consistent and robust observation than potentiation. For example, Peters et al. [13] reported a mere 19 ± 3 % increase in histamine release from 1 to 8

% pure human lung mast cells treated with 10 μ M ADO, and, similarly, we found a maximal increase of 25 ± 9 % in β -hexosaminidase release from purified hLMCs treated with 1 μ M ADO [11]. In another study, ADO completely failed to enhance, but dramatically inhibited, IgE-induced degranulation of hLMCs [14]. The inhibitory effect of ADO on mast cell degranulation has been attributed to A2aAR signals [14, 21]. Indeed, most anti-inflammatory actions of ADO are attributed to inhibitory signals from A2aAR. However, the concentrations of ADO required to inhibit IgE-dependent degranulation is reportedly much higher than that needed to trigger ADORs, including A2aAR; thus, suggesting that other mechanisms may be involved.

Herein, we investigate the inhibition of IgE-dependent mast cell degranulation by ADO. We show that ADO inhibits degranulation of hSMCs through an intracellular mechanism requiring its influx via equilibrative nucleoside transporter 1 (ENT1), rather than signaling through surface-expressed ADORs. We demonstrate that ADO inhibits Fc ϵ RI-induced degranulation, but not that induced by Complement Factor 5a (C5a), Substance P, or the calcium ionophore A23187. Thus, ADO-mediated inhibition of degranulation is specific for early Fc ϵ RI signals. Accordingly, we show that Fc ϵ RI-induced tyrosine phosphorylation of spleen tyrosine kinase (Syk) at the activation residue Y525, an essential step in IgE-dependent mast cell degranulation, is strongly attenuated in ADO-treated hSMCs. Together, these findings demonstrate a novel inhibitory role for intracellular ADO in the regulation of IgE-dependent degranulation of human mature mast cells.

Materials and Methods

Isolation and Purification of hSMCs

Fresh surgical samples of discarded human skin were obtained from the Department of Pathology at Virginia Commonwealth University, Cooperative Human Tissue Network of the National Cancer Institute or the National Disease Research Interchange, as approved by the Human Studies Internal Review Board at Virginia Commonwealth University. The tissues were digested at 37 °C with collagenase and hyaluronidase (Sigma, St. Louis, MO) 3 $\times$ 1 h in HBSS buffer (1X HBSS, 0.04 % NaHCO₃, 1 % fetal bovine serum, 1 % HEPES, 0.1 % CaCl₂) containing amphotericin B and Antibiotic/Antimycotic solution. After digestion, the samples were filtered through 70 μ m and 40 μ m nylon cell strainers, washed and separated on a Percoll gradient. The cells at the buffer/Percoll interface were collected, washed and re-suspended at 5×10^5 cells/ml in serum-free X-VIVO 15 medium (Lonza, Walkersville, MD)(also free of adenosine deaminase per manufacturer) containing stem cell factor (SCF) (100 ng/ml; a gift from Swedish Orphan Biovitrum, Stockholm, Sweden) in 24-well plates with weekly medium changes. Purity was assessed cytochemically by metachromatic staining with acidic toluidine blue and by flow cytometry with the anti-Fc ϵ RI α mAb, 22E7 (a gift from J. P. Kochan (Hoffman-LaRoche, Nutley, NJ) [22]. Typically, mature hSMCs were of 95–100 % purity by 6 weeks of culture and used experimentally between 8 and 12 weeks.

Mast Cell Activation

hSMCs (10^6 cells/ml) were activated by cross-linking Fc ϵ RI with the monoclonal antibody 22E7 (100 ng/ml unless otherwise indicated), which recognizes and binds the alpha subunit of Fc ϵ RI, at 37 °C for 30 min in Tyrode's Buffer (135 mM NaCl, 1 mM MgCl₂, 20 mM Hepes, 5 mM KCl, 1.8 mM CaCl₂, 5.6 mM glucose; pH 7.4) containing 0.05 % bovine serum albumin. Mast cell activation was also induced with complement factor 5a (C5a) (100 ng/ml) (Calbiochem, Gibbstown, NJ), Substance P (1 μ M) or the calcium ionophore A23187 (1 μ M) (both from Sigma-Aldrich, St. Louis, MO). Unless otherwise indicated, mast cells were pre-incubated for 10 min with ADO (Sigma-Aldrich, St. Louis, MO) before activation. ZM241385 (4-(2-[7-Amino-2-(2-furyl)[1, 2, 4]triazolo[2,3-a][1, 3, 5]triazin-5-ylamino]ethyl)phenol), MRS1220 (*N*-[9-Chloro-2-(2-furanyl)[1, 2, 4]triazolo[1,5-c]quinazolin-5-yl]benzene acetamide), PSB1115 (4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1*H*-purin-8-yl)-benzenesulfonic acid potassium salt), Dipyrindamole (2,6-*bis*(Diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine), or NBMPR (nitrobenzylmercaptapurine riboside) (all from Tocris-Cookson, Ellisville, MO) was added to the cells 15 min prior to ADO. Vehicle was DMSO (Sigma-Aldrich, St. Louis, MO).

Degranulation Assay

After the activation period, the mast cells and medium were separated by centrifugation. The cells were lysed with 1 % Triton X-100 and degranulation was assessed by measuring the secretion of β -hexosaminidase activity as described [23]. Absorbance values were read at 405 nm with a SpectraMax 384 Plus UV-VIS plate reader (Molecular Devices Corporation, Chicago, IL). Percent release values were calculated using the formula:

$$\% \text{Release} = \frac{A_{405} (\text{Releasate})}{A_{405} (\text{Releasate}) + A_{405} (\text{Lysate})} \times 100$$

Syk Immunoblotting

hSMCs (10^6 /ml) were pre-incubated without and with ADO (250 μ M) for 10 min in Tyrode's buffer, and then activated with 22E7 (100 ng/ml) for 5 min. The mast cells were then lysed with 2X SDS sample buffer containing Na₃VO₄ and β -mercaptoethanol and boiled for 5 min. Whole cell extracts were separated by SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were blocked with 5 % non-fat dry milk in 25 mM Tris, pH7.4, containing 0.15 M NaCl and 0.1 % Tween-20 (TNT buffer) for 1 h at RT, and then incubated overnight at 4 °C with a rabbit polyclonal antibody against total Syk (Cell Signaling Technology, Danvers, MA) or a mouse monoclonal antibody against human phosphoY525 Syk (R&D Systems, Minneapolis, MN) in 5 % milk/TNT. The blots were washed then incubated with HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) or goat anti-mouse IgG (R&D Systems, Minneapolis, MN) in 5 % milk/TNT for 1 h at RT. After washing, the blots were incubated for 5 min in SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL), exposed to BIO-MAX XAR film (Sigma-Aldrich, St. Louis, MO) and developed. Densitometry was performed with a Molecular Imager FX (Bio-Rad, Hercules, CA).

Intracellular Staining

hSMCs (2×10^5) were fixed and permeabilized with the staining buffer kit from eBiosciences (San Diego, CA) according to manufacturer's protocol. Permeabilized mast cells were resuspended in 200 μ l of permeabilization buffer containing 1 % human serum (Sigma-Aldrich, St. Louis, MO), and incubated for 10 min on ice with human FcR Blocking Reagent (Miltenyi Biotec, Auburn, CA). Rabbit anti-ENT-1 IgG or a rabbit polyclonal IgG isotype control (400 ng) (Santa Cruz Biotechnologies, Santa Cruz, CA) was added to the samples, which were then incubated an additional 30 min on ice. Labeled mast cells were washed, resuspended in 200 μ l of permeabilization buffer containing 500 ng of DyLight488-conjugated Donkey anti-rabbit IgG (Biolegend, San Diego, CA), incubated for 30 min on ice, washed and resuspended in PBS containing 1 % BSA. Flow cytometry was performed on a FacsCantoII (BD Biosciences).

Reverse-Transcriptase PCR

RNA from resting mast cells was isolated with the RNeasy Miniprep kit (Qiagen, GmbH, Germany), and cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. For PCR, 2 μ l of cDNA was combined with 1 μ l each of sense and antisense primers (10 μ M each) and 12.5 μ l of SYBR Green Supermix from the iScript™ SYBR Green RT-PCR kit (Bio-Rad, Hercules, CA) in a final volume of 25 μ l. A hot-start protocol (95 °C for 5 min, (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) x 35 cycles, 95 °C for 1 min, 55 °C for 1 min) was run on a CFX96 Real Time System from Bio-Rad (Hercules, CA). Oligonucleotide primers: ENT1/SLC29A1 (NM_001078177; 5'-gactgggg actggggactgccg-3'; 5'-cctgtccacgggtccagatcgat-3') and GAPDH (NM_002046.3; 5'-caatgacccttcattgacc-3'; 5'-ttgatttggaggatctcg-3'). PCR products were electrophoresed in a 1.5 % agarose gel with Tris-Borate-EDTA buffer. The stained gels were visualized on a Bio-Rad Molecular Imager FX.

Statistical Analysis

Statistical analysis was performed by one-way ANOVA with Bonferroni post test as described in figure legends using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego, CA, USA; www.graphpad.com.

Results

ADO Inhibits but Does not Potentiate FcεRI-Induced Degranulation of hSMCs

To confirm our earlier observation that ADO does not potentiate IgE-dependent degranulation of hSMCs [11], we assessed degranulation by the release of β -hexosaminidase from hSMCs activated with the anti-FcεRI α mAb 22E7 in the absence or presence of ADO under different conditions. As Fig. 1 shows, ADO has no potentiating effect on hSMC degranulation. In contrast to earlier reports [15, 24, 25], the timing of exposure to ADO was inconsequential to the overall effect since ADO failed to potentiate degranulation regardless of whether it was added 10 min before (A), 10 min after (B), or simultaneously with (C) 22E7 (100 ng/ml). The strength of the primary activating signal also was irrelevant since

ADO (added 10 min prior) did not enhance the degranulation of hSMCs activated with a suboptimal concentration (10 ng/ml) of 22E7 (D). However, ADO at concentrations 10^{-4} M significantly inhibited Fc ϵ RI-induced degranulation of hSMCs when added 10 min before (A and D) or simultaneously with (C) 22E7. As expected, given that degranulation is essentially completed within minutes of Fc ϵ RI cross-linking, β -hexosaminidase release was not inhibited when ADO was added 10 min after 22E7 (100 ng/ml) (B), and ADO alone did not induce β -hexosaminidase release (E). Importantly, prolonged exposure to ADO also did not potentiate degranulation of hSMCs, but resulted in a 10-fold increase in sensitivity to ADO. Whereas, 10^{-4} M ADO inhibited degranulation of hSMCs when incubated for 10 min (A), only 10^{-5} M ADO with 1 h incubation was required for significant inhibition (F). Altogether, these data confirm that ADO has no potentiating effect on Fc ϵ RI-induced degranulation of hSMCs, but potently inhibits mediator release. Therefore, we used hSMCs as a model to investigate the inhibitory effect of ADO on degranulation without interference from potentiating signals.

Inhibition of Fc ϵ RI-Induced Degranulation by ADO is not due to Acute Toxicity

To determine if the inhibition of degranulation by ADO was due to toxicity, we incubated hSMCs in serum- and ADA-free culture media (containing SCF) $\pm$ 0.125, 0.25, 0.5, 1, 1.25 and 12.5 mM ADO for 3 days and assessed membrane integrity daily by trypan blue exclusion (Fig. 2). Mean viabilities were 97 % at each time-point in the absence of ADO (day 1, 97.9 $\pm$ 0.3 %; day 2, 97.4 $\pm$ 0.5 %; day 3, 97.7 $\pm$ 0.4 %), and remained essentially unchanged in mast cells cultured with 0.125, 0.25 and 0.5 mM ADO. However, a gradual decrease in % viability was apparent with 1, 1.25 and 12.5 mM ADO, respectively, at each time-point: day 1 (93 $\pm$ 1 %, 95 $\pm$ 1 %, 80 $\pm$ 3 %); day 2 (81 $\pm$ 2 %, 61 $\pm$ 6 %, 18 $\pm$ 4 %); day 3 (69 $\pm$ 2 %, 41 $\pm$ 5 %, 3 $\pm$ 1 %). Thus, hSMCs cultured with ADO at concentrations up to 500 μ M remained completely viable as evidenced by the lack of appreciable loss in membrane integrity over 3 days. Based on these findings, we conclude that the inhibitory effect of ADO at concentrations shown to inhibit Fc ϵ RI-induced degranulation (up to 500 μ M) was not due to acute cytotoxicity.

A2aAR Signals are not Solely Responsible for ADO-Induced Inhibition of Fc ϵ RI-Mediated Degranulation

To determine if the inhibition of Fc ϵ RI-mediated mast cell degranulation by ADO was due to A2aAR signals as had been reported [14, 21], we analyzed the effect of the A2aAR-specific antagonist ZM241385 on this process (Fig. 3). In independent experiments, hSMCs from 10 different skin tissue preparations from different donors were pre-treated with 10^{-7} , 10^{-6} and 10^{-5} M ZM241385 for 15 min followed by a 10 min incubation period with 250 μ M ADO (the IC₅₀ for inhibition at 10 min), and then activated with 22E7 (100 ng/ml). No statistically significant effect of ZM241385 on inhibition of degranulation by ADO was observed or calculated when the data from the 10 different hSMC preparations was analyzed collectively (graph not shown). However, analysis of individual experiments revealed that hSMCs from 3 of the 10 different skin preparations were, in fact, somewhat responsive to ZM241385, whereas 7 of the 10 hSMC preparations were completely non-responsive to ZM241385. To demonstrate this, the degranulation data from both groups, which we termed “ZM-Responsive” and “ZM-Non-Responsive”, was plotted separately as shown in Fig. 3a (*n*

=3) and Fig. 3b ($n=7$). As demonstrated in Fig. 3a, 22E7-induced β -hexosaminidase release from the “ZM-Responsive” group of hSMCs pre-treated with 10^{-5} M ZM241385 and exposed to 250 μ M ADO was not statistically different ($p > 0.05$) from that of control cells activated with 22E7 alone (63 ± 5 %), indicating that 10^{-5} M ZM241385 effectively blocked the inhibitory effect of ADO. In contrast, ZM241385 at 10^{-7} M and 10^{-6} M concentrations was statistically ineffective at blocking the ADO-induced inhibition since the degranulation values were statistically different ($p < 0.05$) from control cells activated in the absence of ADO, although a slight preventative pattern is apparent. Mean % release of β -hexosaminidase $\pm$ S.E.M. values from the “ZM-Responsive” group of hSMCs treated with 10^{-7} , 10^{-6} , and 10^{-5} M ZM241385, respectively, were 40 ± 2 %, 45 ± 2 %, and 53 ± 4 %. In contrast, 22E7-induced β -hexosaminidase release from all “ZM-Non-Responsive” group samples treated with ZM241385 and ADO was significantly different than that from control hSMCs (Fig. 3b). Importantly, the ability to degranulate in response to 22E7 by the “ZM-Responsive” group was comparable to that of the “ZM-Non-Responsive” group (63 ± 5 % compared to 66 ± 2 %, respectively), and both groups were equally susceptible to ADO-mediated inhibition as indicated by the comparable 22E7-induced mean % degranulation values obtained in the presence of 250 μ M ADO (36 ± 1 % and 40 ± 2 %, respectively). Spontaneous release was 8 ± 2 % from “ZM-Responsive” hSMCs, and 8 ± 1 % from the “ZM-Non-Responsive” group. Moreover, ZM241385 alone (10^{-5} M) did not inhibit 22E7-induced degranulation, or affect spontaneous release. To determine if other ADORs could be involved, we performed similar independent experiments with different hSMC preparations ($n=3$) using antagonists specific for A2bAR (PSB1115) and A3AR (MRS1220) (Fig. 3c and d, respectively), but found no effect on ADO-mediated inhibition. These data indicate that A2aAR signals can contribute to ADO-mediated inhibition of degranulation in some cases, but does not account for the observed inhibition in the majority of cases.

Facilitated Influx of ADO via ENT1/SLC29A1 is Necessary and Sufficient for the Inhibition of Fc ϵ RI-Mediated Degranulation

The finding that ZM241385 was ineffective at blocking the inhibition by ADO of Fc ϵ RI-induced degranulation in a majority of hSMC preparations suggested the involvement of a mechanism other than A2aAR signals. To investigate the possibility that ADO was acting intracellularly, hSMCs ($n=5$ preparations) were pre-treated with the non-specific inhibitor of nucleoside transporters Dipyridamole (10 μ M) for 15 min, then incubated with 250 μ M ADO for 10 min, and activated with 22E7 (100 ng/ml). ADO significantly inhibited β -hexosaminidase release from control samples activated with 22E7 alone, as expected, but failed to do so in the presence of Dipyridamole (Fig. 4a). Mean % release of β -hexosaminidase $\pm$ S.E.M. values from hSMCs activated with 22E7 alone and 22E7 + ADO without Dipyridamole, respectively, were 59 ± 4 % and 33 ± 4 %; whereas, with Dipyridamole those values were 52.8 ± 5 % and 48.4 ± 6.1 %. Dipyridamole alone had no influence on spontaneous or 22E7-induced degranulation. Thus, blocking the influx of ADO significantly prevented the inhibition of Fc ϵ RI-induced degranulation induced by a high concentration of added ADO.

To characterize the requirement for facilitated transport of ADO for the inhibition of degranulation, we analyzed the sensitivity of this process to nitrobenzylmercaptapurine

riboside (NBMPR), a specific inhibitor of equilibrative nucleoside transporter 1 (ENT1/SLC29A1) (8). First, it was important to demonstrate that hSMCs express ENT1. Therefore, we used RT-PCR and flow cytometry to show the expression of ENT1 in hSMCs from 3 different preparations of skin from individual donors (Fig. 4b). For functional analysis, hSMCs from 11 different skin tissue preparations (including the 10 preparations used in Fig. 3) were pre-treated with 10^{-12} – 10^{-5} M NBMPR for 15 min, then exposed to 250 μ M for 10 min, and activated with 22E7 (100 ng/ml). As demonstrated in Fig. 4c, the inhibitory effect of ADO was prevented by NBMPR in a dose-dependent manner with 10^{-7} M concentrations and greater being most effective. The mean % release of β -hexosaminidase $\pm$ S.E.M values from hSMCs treated with 10^{-12} M– 10^{-8} M NBMPR+ADO were significantly lower than that of hSMCs activated with 22E7+ NBMPR (10 μ M) (58.9 ± 2.9 %), whereas those of hSMCs treated with 10^{-7} M, 10^{-6} M and 10^{-5} M NBMPR+ADO (51.1 ± 3 %, 54.1 ± 2.6 % and 52.5 ± 3 %, respectively) were statistically similar. Noteworthy, NBMPR (10 μ M) alone had no effect on 22E7-induced degranulation, or spontaneous release. Thus, NBMPR is highly effective at preventing ADO-mediated inhibition of Fc ϵ RI-induced degranulation.

To investigate further, we separated the NBMPR response data from Fig. 4c according to responsiveness of the hSMCs to ZM241385. As the graphs reveal, NBMPR was effective at blocking the ADO-mediated inhibition of degranulation in both “ZM-Responsive” and “ZM-Non-Responsive” groups (Fig. 5); thus, revealing a key role for ENT1 in this process. Overall, these data reveal that the influx of ADO via ENT1 is necessary and sufficient to inhibit Fc ϵ RI-mediated degranulation, whereas A2aAR signals are not necessarily required but could contribute to the overall inhibitory effect. Therefore, we conclude that ADO inhibits IgE-dependent degranulation of hSMCs primarily through an intracellular mechanism.

Intracellular ADO Specifically Inhibits Degranulation Induced by Fc ϵ RI Crosslinking

Although allergic responses are generally associated with Fc ϵ RI cross-linking, mast cell degranulation can be triggered by a variety of agents, including complement anaphylatoxins C5a and C3a [26, 27], neuropeptides [28] and calcium ionophores [29]. We questioned whether the inhibitory action of intracellular ADO was a global response, or if it was restricted to degranulation initiated by Fc ϵ RI. To address this, we analyzed the effect of ADO on degranulation induced with C5a, Substance P, or the calcium ionophore A23187 (Fig. 6). As a positive control, hSMCs were activated with 22E7 (100 ng/ml) $\pm$ ADO (250 μ M). As expected, ADO significantly inhibited Fc ϵ RI-induced degranulation in all experiments. In contrast, ADO failed to inhibit degranulation induced with C5a (100 ng/ml), Substance P (1 μ M), and A23187 (1 μ M). The mean % release of β -hexosaminidase $\pm$ S.E.M. induced by the various stimuli in the absence and presence of ADO, respectively, was: C5a (35 ± 3 % and 33 ± 2 %; $n = 3$); Substance P (59 ± 3 % and 55 ± 1 %; $n = 3$); calcium ionophore A23187 (61 ± 7 % and 60 ± 8 %; $n = 3$). Noteworthy, these findings showing that hSMCs treated with high concentrations of ADO are functionally responsive to C5a, Substance P and calcium ionophore further support our conclusion that the inhibition of degranulation by ADO is not due to acute toxicity. Moreover, they demonstrate that the inhibitory effect of intracellular ADO is specific for degranulation induced by Fc ϵ RI crosslinking.

Intracellular ADO Inhibits FcεRI-Induced Syk Activation

The finding that intracellular ADO inhibits the degranulation of hSMCs activated by FcεRI cross-linking, but not degranulation induced through receptors for Substance P, C5a, or by calcium ionophore revealed a specificity of ADO for FcεRI pathways. In addition, the fact that artificially-induced degranulation with calcium ionophore was not affected by ADO suggested that a membrane-proximal event rather than one downstream and common to multiple pathways could be targeted by intracellular ADO for inactivation. Therefore, early signaling events were examined as potential targets of intracellular ADO. Among the earliest events following FcεRI cross-linking is activation of spleen tyrosine kinase (Syk), a critical component of FcεRI-initiated mast cell degranulation [30–32]. To determine if Syk activation is affected by ADO, we analyzed the phosphorylation status of Syk in hSMCs activated with 22E7±ADO (250 μM) (Fig. 7). Significantly lower amounts of Syk phosphorylated at Y525 in the activation loop were detected in hSMCs activated in the presence of ADO. Based on density values of Western blot bands, the ratio of pY525 Syk to total Syk was: 0.2±0.1 (non-treated), 0.2±0.1 (ADO alone), 0.8±0.2 (22E7 alone), and 0.3±0.1 (22E7+ADO). Thus, FcεRI-induced Y525 phosphorylation (activation) of Syk in hSMCs is significantly inhibited by intracellular ADO.

Discussion

Herein, we identified a negative regulatory role for intracellular ADO in IgE-dependent mast cell degranulation. We show that high concentrations of, or prolonged exposure to, ADO inhibits FcεRI-induced degranulation of hSMCs by a mechanism that requires the influx of ADO from the extracellular space via ENT1/SLC29A1. We demonstrate that the inhibitory effect of ADO is specific for FcεRI-induced degranulation, and that it targets early FcεRI proximal signals. Moreover, we show that A2aAR signals can contribute to the overall inhibitory effect, but are not absolutely required for ADO to inhibit. Overall, this study demonstrates a potentially anti-allergic effect of intracellular ADO that is due to specific inhibition of FcεRI-proximal signaling events.

The finding that the influx of ADO from the extracellular space was required for ADO to inhibit mast cell degranulation was somewhat unexpected because of reported findings showing that A2aAR was responsible for inhibiting FcεRI-induced degranulation of human basophils and lung mast cells [14, 21, 33]. We demonstrate here, however, that signals from A2aAR (or other ADORs) are not required for ADO to inhibit degranulation but may contribute to the inhibitory response since only a minority (3 of 10) of hSMCs preparations responded to ZM241385. On the other hand, we show that the influx of ADO was necessary and sufficient for inhibition of hSMC degranulation. Why mast cells from some skin preparations but not others are responsive to ZM241385 is not known. One possible explanation is that the “ZM-Responsive” hSMCs express greater amounts of A2aAR than the “ZM-Non-Responsive” mast cells. Quantitative variability in A2aAR expression in hSMCs from different skin preparations was shown previously [11]. It is also interesting to speculate on whether the disparate response to A2aAR antagonism is related to allergic or atopic status of the skin donors. Unfortunately, specific donor information is not available for us to be able to correlate responsiveness to ZM241385 and atopy in this study. Further

arguing against A2aAR signaling as the inhibitory mechanism is that the reported concentrations of ADO required to inhibit IgE-dependent degranulation, including those in the present study, are much greater than that required to trigger A2aAR. Therefore, if A2aAR activation were solely responsible for inhibiting degranulation, the inhibitory effect would be expected to occur at significantly lower concentrations of ADO. Moreover, if the inhibitory effect of ADO on degranulation was due to broad-based effects like elevated cAMP or closing of K⁺ channels [14, 33], consequent to A2aAR signaling, ADO would be expected to impact many different degranulation-inducing signaling pathways. To the contrary, we demonstrate that ADO specifically inhibits de-granulation induced by FcεRI stimulation, and has no effect on that induced by other receptors. These observations, together with our finding that blockade of ADO influx, but not A2aAR signaling, was sufficient and necessary to prevent the inhibition by ADO of FcεRI-induced degranulation by hSMCs supports our conclusion that ADO inhibits degranulation predominantly by an intracellular mechanism.

Our finding that ADO specifically inhibits degranulation induced through FcεRI suggests that the intracellular targets of ADO are specific to FcεRI signaling pathways. Moreover, the fact that ADO has no effect on degranulation induced artificially with calcium ionophore, a downstream activator that bypasses the requirement for receptor stimulation, suggests that the target(s) of intracellular ADO are signaling molecules proximal to FcεRI. Supporting this notion is our finding that FcεRI-induced activation (phosphorylation) of Syk kinase, a key element in FcεRI-induced mast cell degranulation [30, 32, 34], is significantly attenuated in ADO-treated hSMCs. It is interesting to speculate on how intracellular ADO might inhibit Syk activation. One possibility is that ADO somehow induces changes at the membrane level that could inhibit the recruitment of Syk to phosphorylated γ chain ITAMs of FcεRI; thereby, preventing the formation macro-molecular signaling complexes. Interestingly, increased lipid methylation was reportedly associated with IgE-dependent degranulation of mast cells and basophils [35–39], and it has been postulated that ADO prevents IgE-dependent degranulation by inhibiting S-adenosylmethionine-dependent transmethylation of phospholipids [40]. Experiments are planned to determine if ADO treatment results in altered distribution of critical signaling molecules in membranes of hSMCs. Another possibility is that attenuation of Syk activation is an indirect consequence of other metabolic effects of intracellular ADO. For example, adenosine deaminase (ADA) deficiency in which ADO levels are elevated is associated with accumulation of dATP. dATP is a negative feedback regulator of ribonucleotide reductase, and it is believed to cause the lymphotoxic phenotype of adenosine deaminase-severe combined immunodeficiency (ADA-SCID) in humans [41]. However, studies have shown that high concentrations of ADO under conditions of ADA deficiency have no effect on ribonucleotide reductase in an in vitro system [42], and concentrations of ADO that inhibit degranulation are not toxic to hSMCs (Fig. 2). Therefore, the observed attenuation in Syk phosphorylation in the presence of ADO is not likely due to cytotoxic effects of accumulated dATP or impaired ribonucleotide reductase. Nevertheless, the possibility exists that other metabolic effects of high concentrations of intracellular ADO could be the cause of impaired signaling in mast cells.

One important question is whether concentrations of ADO that inhibit FcεRI-induced degranulation are achieved in vivo. As shown in Fig. 1, 10 μM ADO significantly inhibited degranulation when hSMCs were incubated for 2 h, whereas 100 μM ADO was required with a 10 min incubation period. The concentration of ADO in plasma under normal conditions is <1 μM. However, dramatic increases in ADO under conditions of high metabolic stress have been reported. For example, plasma levels of ADO in patients with sepsis reportedly approach 10 μM, and ADO levels in the range of 10–100 μM were found in synovial fluid from patients with rheumatoid arthritis [3–5]. Even more impressive, ~200 μM adenosine in the epithelial lining fluid of asthmatic lungs was reported [6]. Given the rapid rate at which ADO is metabolized, it is likely that actual tissue concentrations of ADO are much higher. Therefore, the concentrations of ADO required to inhibit FcεRI-induced degranulation reported here can be achieved in vivo, particularly under inflammatory conditions.

To summarize, this study shows that high concentrations of, or prolonged exposure to, ADO specifically inhibits IgE-dependent degranulation of hSMCs by an intracellular mechanism that targets FcεRI-proximal signaling events. We demonstrate that ADO-induced inhibition of degranulation requires the influx of ADO via ENT1/SLC29A1, but not A2aAR signals. Further, we show that the inhibitory action of ADO is specific to degranulation mediated by FcεRI since it has no effect on degranulation induced by C5a, Substance P or calcium ionophore. Accordingly, ADO significantly attenuates FcεRI-dependent phosphorylation of the critical tyrosine kinase Syk. Overall, these findings identify a novel role for intracellular ADO in the negative regulation of IgE-dependent release of allergic mediators from human mast cells.

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Fig. 1.

ADO inhibits, but does not potentiate, Fc ϵ RI-induced degranulation of hSMCs. Percent release of β -hexosaminidase $\pm$ S.E.M. from hSMCs activated with 100 ng/ml (**a**, **b**, **c**, **f**) or 10 ng/ml (**d**) of the anti-Fc ϵ RI α mAb 22E7 when ADO was added 10 min before (**a**, $n=10$; **d**, $n=5$), 10 min after (**b**, $n=4$), at the same time as (**c**, $n=4$), or 1 h before (**f**, $n=3$) 22E7. Capacity for degranulation was demonstrated by activating the mast cells with the calcium ionophore A23187 (1 μ M). ADO alone did not induce β -hexosaminidase release (**e**, $n=3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and #, $p < 0.0001$ by one-way ANOVA with Bonferroni post-test comparing to % release values induced by 22E7 alone (**a**, **b**, **c**, **d**, **f**) or those from the buffer control (**e**). Independent experiments were carried out with mast cells isolated from skin of different donors

Fig. 2.

Effect of ADO on viability of hSMCs. Trypan blue exclusion analysis of hSMCs cultured in serum- and ADA-free medium $\pm$ 12.5, 1.25, 1, 0.5, 0.25 and 0.125 mM ADO for 1, 2 and 3 days ($n = 6$). Best-fit curves were determined by non-linear regression analysis of data from independent experiments carried out with mast cells isolated from skin of different donors.

**, $p < 0.01$; #, $p < 0.0001$ by one-way ANOVA with Bonferroni post-test comparing to values of mast cells cultured without ADO at the same time-point

Fig. 3. ZM241385, an A2aAR-specific antagonist, blocks the inhibitory effect of ADO on some hSMC preparations but not others. β -Hexosaminidase release from hSMCs pre-incubated without and with antagonists specific for A2aAR (ZM241385) (**a**, $n = 3$ and **b**, $n = 7$), A2bAR (PSB1115, $n = 3$) (**c**), or A3AR (MRS1220, $n = 3$) (**d**) $\pm$ adenosine (250 μ M) then activated with 22E7 (100 ng/ml). ZM241385 at 10^{-5} M blocked the inhibitory effect of ADO in 3 of 10 hSMC preparations (**a**), whereas the other 7 preparations were completely non-responsive (**b**). Accordingly, these groups were termed “ZM-responsive” and “ZM non-responsive”. PSB115 and MRS1220 were completely ineffective. **, $p < 0.01$; ***, $p < 0.001$; and #, $p < 0.0001$ by one-way ANOVA with Bonferroni post-test comparing to % release values induced by 22E7 alone

Fig. 4.

Nucleoside transporter inhibitors Dipyridamole and NBMPR block ADO-mediated inhibition of FcεRI-induced hSMC degranulation. Effect of the non-specific nucleoside transporter inhibitor Dipyridamole (10 μM, $n=5$) (**a**), and ENT1/SLC29A1-specific inhibitor NBMPR (10⁻⁵ M–10⁻¹² M, $n=11$) (**c**), on % release of β-hexosaminidase ± S.E.M. from hSMCs activated with 22E7 (100 ng/ml)±adenosine (250 μM). **, $p < 0.01$; ***, $p < 0.001$; and #, $p < 0.0001$ by one-way ANOVA with Bonferroni post-test comparing to % release values from hSMCs activated with 22E7 alone. Independent experiments were carried out with mast cells isolated from skin of different donors. **b** Expression analysis of ENT1/SLC29A1 in hSMCs. Flow cytometric analysis of ENT1/SLC29A1 protein (open histogram) compared to isotype control (shaded histogram) in permeabilized hSMCs. Results representative of 3 separate experiments with mast cells isolated from skin of 3 different donors. RT-PCR products from RNA of resting hSMCs from 3 different skin preparations (insert)

Fig. 5.

NBMPR blocks ADO-mediated inhibition of FcεRI-induced degranulation of “ZM-responsive” and “ZM-non-responsive” hSMCs. hSMCs used in Fig. 4c were also used for Fig. 3 (a and b); therefore, we separated and plotted the NBMPR data from Fig. 4c according to responsiveness of hSMCs to ZM241385. Shown is the effect of NBMPR on % β-hexosaminidase release ± S.E.M. from “ZM-responsive” (a, $n = 3$) and “ZM-non-responsive” (b, $n = 7$) hSMC groups. Independent experiments were carried out with mast cells isolated from skin of different donors. The plots show that the inhibitory effect of ADO was blocked by NBMPR in both groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and #, $p < 0.0001$ by one-way ANOVA with Bonferroni post-test comparing to % release values induced by 22E7 alone

Fig. 6.

ADO does not inhibit degranulation of hSMCs activated with C5a, Substance P or calcium ionophore A23187. hSMCs were pre-treated with ADO (250 μ M) and activated with C5a (100 ng/ml) (**a**, $n=7$), Substance P (100 ng/ml) (**b**, $n=4$) or A23187 (1 μ M) (**c**, $n=3$). hSMCs activated with 22E7 (100 ng/ml) $\pm$ ADO served as a positive control for each independent experiment. Independent experiments were carried out with mast cells isolated from skin of different donors. ***, $p < 0.001$; #, $p < 0.0001$ by one-way ANOVA with Bonferroni post-test comparing to % release values induced by 22E7, C5a, Substance P or calcium ionophore in the absence of ADO

Fig. 7.

ADO inhibits FcεRI-induced phosphorylation of Syk. hSMCs were pre-treated for 10 min with ADO (250 μM) then activated for 1 min with 22E7 (100 ng/ml) and Syk phosphorylation was analyzed. **a** Representative Western blot of the effect of ADO on FcεRI-induced Y525 phosphorylation of Syk ($n=4$). **b** Ratio of Y525-phosphorylated Syk to total Syk based on densitometry values of band intensities from Western blots represented in (a). Independent experiments were carried out with mast cells isolated from skin of different donors. *, $p < 0.05$ ($n=4$) by one-way ANOVA with Bonferroni post-test comparing to values from non-activated hSMCs