Abstract

Rat serosal mast cell adenosine receptors were characterized by $^3$H]adenosine binding to cell membrane particulates, and functional changes in mast cell mediator release and cyclic AMP levels were assessed, utilizing various adenosine analogs. $^3$H]adenosine binding to sonicated mast cell membrane preparations at $0^\circ$C in the presence of deoxycoformycin is linear with initial cell number, rapid and reversible. The cells display 16,400 $\pm$ 1600 high affinity $^3$H]adenosine binding sites/cell, equivalent to 118 fmol bound/mg protein, with an equilibrium dissociation constant of 27.97 $\pm$ 3.0 nM. Competition studies reveal that adenosine > 2-chloroadenosine > NECA > L-PIA > D-PIA in competing for $^3$H]adenosine binding sites and that aminophylline and cromolyn sodium also bind to the putative receptor. Adenosine and its analogs, NECA, and L-PIA, appear to activate adenylate cyclase in resting mast cells by raising cyclic AMP, suggesting an $R_a$ cell surface adenosine receptor subtype; these same analogs potentiate mast cell $\beta$-hexosaminidase release stimulated by specific antigen. The identification of rat mast cell $^3$H]adenosine binding sites whose stimulation augments resting cell cyclic AMP levels and antigen-induced mediator release suggests that these receptors may be important in the biochemical mechanisms of allergic diseases. The ability to assess the number and affinity of mast cell adenosine receptors will enable one to monitor receptor alterations during pharmacologic manipulation and in disease states.

Introduction

Adenosine is an endogenous mediator of numerous physiologic and biochemical responses in a variety of cells and tissues [1, 2]. It has been shown in general to interact with adenylate cyclase [3], regulate phospholipid turnover [4], and activate cyclic AMP phosphodiesterase [5] as well as to specifically inhibit lipolysis [6], block platelet aggregation [7], dilate coronary vessels [8], stimulate steroidogenesis [9], and alter immune responsiveness [10]. Relevant to immediate hypersensitivity reactions is the ability of exogenous adenosine to potentiate histamine release from rat peritoneal mast cells [11] and, depending on the time of addition of the nucleoside, from dispersed human lung mast cells as well [12]. The nature of the enhancement of histamine release remains poorly understood, but functional evidence suggests that adenosine binds to cell surface ‘$R$’ receptors, resulting in changes in cAMP metabolism and subsequent mediator release [13]. Plasma adenosine levels in man approximate 0.3 $\mu$M [14] and increase up to ten fold in lung tissue exposed to hypoxic conditions [15] or antigen challenge [16]. Endogenous adenosine may have a physiologic importance in asthma and other allergic diseases in general [2].

Radioligand binding techniques have been used to directly assess adenosine receptors in brain [17], placenta [18], vascular smooth muscle [19], and fat cells [20], using various labeled adenosine analogs including $^3$H]adenosine, 2-chloro$^3$H]adenosine, and $N^6$-cyclohexyl$^3$H]adenosine. Direct binding studies have been used to subtype adenosine cell surface receptors, determine receptor density and binding affinity, and observe the potency of competitors for adenosine binding sites. To directly assess adenosine receptors on a purified mast cell population and correlate these findings with effects upon cell function, we performed $^3$H]adenosine binding to rat peritoneal mast cell membranes and evaluated the ability of adenosine and analogs of this nucleoside to alter $^3$H]adenosine binding, to change mast cell cyclic AMP levels, and to modulate mediator release.

Methods

Chemicals

The following were purchased from the manufacturer: heparin, aminophylline, p-nitrophenyl $N$-acetyl $\beta$-D-glucos-
aminide, 2-chloroadenosine, adenosine, inosine, hypoxanthine, dipyridamole (Sigma, St Louis, MO), cAMP \[^{125}\text{I} \text{-radioimmunoassay kit, 2,8,9-}\left[^{3}\text{H}\right]\text{adenosine 52 Ci/mmol (New England Nuclear, Boston, MA)}, \text{calcium ionophore A23187, DNAse (Calbiochem, La Jolla, CA)}, \text{metrizamide, analytical grade (Accurate Chemical and Scientific Corporation, Hicksville, NY)}, \text{(--)-N}\text{\textsubscript{6}}\text{-[(D)-I-1-methyl-2-phenylethyl]-adenosine, (--)-N}\text{\textsubscript{6}}\text{-[(L)-1-methyl-2-phenylethyl]-adenosine (Boehringer Mannheim, Indianapolis, IN), BetaPhase liquid scintillation fluid (Westchem, San Diego, CA)}, \text{Whatman GF/C 2.4-cm glass fiber filters (Fisher Scientific, Pittsburgh, PA)}.

The following were generously donated: mouse hybridoma anti-DNP IgE antibody and DNP-BSA antigen (Dr Futong Liu and Dr David Katz, La Jolla, CA), cromolyn sodium (Fisons Corporation, Bedford, MA), 5'-N-ethylcarboxamideadenosine (Dr Ian Skidmore, Glaxo Group Research Ltd, Ware, England), deoxycoformycin (Developmental Therapeutics Program, Chemotherapy Division, NCI, NIH, Bethesda, MD).

Mast cell purification
Mast cells were obtained by lavage of the pleural and peritoneal cavities of Sprague-Dawley rats (250-300 g) with heparinized Tyrode's buffer lacking divalent cations and containing 0.1% gelatin. Mast cells with greater than 95% purity and viability were isolated using metrizamide gradients by a method described previously [21].

Radioligand binding experiments
Mast cell membranes were prepared by homogenization with a Brinkman Polytron at setting 8 for 15 seconds at 0°C in 50 mM Tris buffer containing 1 mM Mg\(^{++}\) pH 7.5. The resulting suspension was centrifuged at 4°C at 40,000 xg for 15 minutes, washed with the same buffer, centrifuged again, and the pellet was resuspended in the Tris buffer with 20 pulses from a Branson sonifier at setting 4 at 0°C. Membranes from 4.0 x 10\(^5\) mast cells, equivalent to 92 \mu g protein as assayed by the method of Lowry [22], were incubated in Tris buffer in propylene tubes with various concentrations of \[^{3}\text{H}\text{adenosine and 1 \mu M deoxycoformycin in the absence or presence of 10 \mu M adenosine. Incubations were carried out for 20 minutes unless otherwise stated. At the end of the incubation period, 5 ml of buffer at room temperature were added to each tube, and the binding was terminated by rapid vacuum filtration through Whatman GF/C 2.4-cm glass fibre filters. The filters were washed twice more with 5 ml of buffer, dried briefly, and placed in scintillation vials to which 4 ml BetaPhase was added. After at least one hour of equilibration, the radioactivity was counted in a Beckman LS 230 liquid scintillation counter with 40% efficiency. Specific binding is defined as the difference in counts per minute in binding in the absence and presence of 10 \mu M adenosine and was generally 40-60% of total binding.

\[^{3}\text{H}\text{adenosine stability}
The purity of the stock \[^{3}\text{H}\text{adenosine was assessed by thin-layer chromatography in a 65:22.5:12.5 ethyl acetate, isopropyl alcohol, water system, demonstrating a 92% purity of the radioligand. To quantify the metabolism of the radioligand under the experimental conditions described, binding experiments were carried out and following the vacuum filtration step, filters were placed in 1 M formic acid, shaken, and the filter removed. The solution was lyophilized and reconstituted with 10 \mu l water and spotted onto a cellulose thin-layer chromatography plate in parallel with adenosine, hypoxanthine, and inosine standards. In the solvent system described above, these three compounds separate well with \textit{R}_f values of 0.57, 0.44, and 0.31, respectively, and the nucleotides remain at the origin [23]. In the presence of 1 \mu M deoxycoformycin, less than 7% of the \[^{3}\text{H}\text{adenosine was metabolized in this radioligand binding assay.}

B-Hexosaminidase release
Intact mast cells (10\(^5\)/ml) were sensitized with 1 \mu g anti-DNP IgE antibody for 30 minutes at 37°C, washed three times in Tyrode's buffer, aliquoted into polypropylene tubes (3 x 10\(^5\) cells/400 \mu l) containing varying concentrations of adenosine analogs and immediately challenged with 100 ng DNP-BSA antigen for ten minutes at 37°C. Tubes were centrifuged at 200 x g for 10 minutes at room temperature, and the supernatants were decanted into separate tubes on ice. The cell pellets were resuspended in Tyrode's buffer, sonicated, and b-hexosaminidase activity was assessed by quantitating the absorbance at 410 nm for the p-nitrophenyl N-acetyl-B-D-glucosaminide substrate.

Cyclic AMP experiments
Resting cell cAMP levels were assessed by incubating 3 x 10\(^5\) mast cells with varying concentrations of adenosine analogs in a volume of 180 \mu l Tyrode's buffer for 20 seconds at 37°C followed by the immediate addition of 20 \mu l of 100% TCA and freezing in dry ice and acetone. The reaction mixtures were thawed, sonicated at 4°C, and centrifuged at 250 x g for 20 minutes. The supernatants were extracted three times with water-saturated ether, lyophilized, and resuspended in 100 \mu l of sodium acetate buffer. Cyclic AMP levels in the absence or presence of adenosine analogs were quantitated using a \[^{125}\text{I}\text{-radioimmunoassay.

Statistical analysis
Statistical significance was assessed utilizing the Student's \textit{t}-test. Results are presented as the mean ± standard error (SE) unless otherwise indicated.

Results
\[^{3}\text{H}\text{adenosine binding to rat mast cell membranes
In order to assess the number and binding