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A_1 Adenosine Receptors in Human Neutrophils: Direct Binding and Electron Microscope Visualization

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Abstract By occupying specific surface receptors, adenosine and adenosine analogues modulate neutrophil functions; in particular, functional and biochemical studies have shown that A_1 adenosine receptors modulate chemotaxis in response to chemotactic peptides. Until now, the characteristics of the specific agonist binding and the visualization of A_1 receptors in human neutrophils have not been investigated. In the present study, we used the agonist [^{3}H] CHA for radioligand binding studies and a CHA-biotin XX probe in order to visualize the A_1 binding sites in human neutrophils, ultrastructurally, by conjugation with colloidal gold-streptavidin. [^{3}H] CHA bound A_1 adenosine receptors with selectivity and specificity, although with a low binding capacity. Scatchard analysis showed a Kd value of 1.4 ± 0.08 nM and a maximum density of binding sites of 7.1 ± 0.37 fmol/mg of proteins. The good affinity and selectivity of the CHA-biotin XX probe for A_1 adenosine receptors allowed us to visualize them, after conjugation with colloidal gold-streptavidin, as electron-dense gold particles on the neutrophil surface and inside the cell. The internalization of the ligand-receptor complex was followed in a controlled temperature system, and occurred through a receptor-mediated pathway. The kinetics of the intracellular trafficking was fast, taking less than 5 min. These data suggest that the CHA-biotin XX-streptavidin-gold complex is a useful marker for the specific labelling of A_1 binding sites and to follow the intracellular trafficking of these receptors. J. Cell. Biochem.75:235–244, 1999. © 1999 Wiley-Liss, Inc.

Key words: human neutrophils; adenosine receptors; agonist binding; electron microscopy

Inflammation is the primary response to tissue injury or microbial invasion, and is characterized by a local accumulation of neutrophils. Although they are essential to limit the spread of infection, activated neutrophils are capable of damaging injured tissues while en route to sites of infection or inflammation. Adenosine release is a mechanism by which normal cells may protect themselves from activated neutrophils [Cronstein et al., 1986; Schrier and Imre, 1986]. Extracellular adenosine affects neutrophil functions in a paradoxical fashion: it diminishes generation of toxic oxygen products (O_2^- or H_2O_2) and surprisingly promotes neutrophil chemotaxis [Cronstein et al., 1983; Roberts et al., 1985; Rose et al., 1988]. Thus adenosine may protect the vasculature from damage produced by migrating activated neutrophils, both reducing generation of toxic oxygen products and hastening the arrival of neutrophils at sites of tissue damage or infection. It has been demonstrated [Cronstein et al., 1985] that high nucleoside concentrations inhibit stimulated neutrophil adhesion and O_2^- generation, acting as a brake on neutrophil-mediated tissue destruction at ischemic or inflamed sites, while a low concentration of adenosine promotes stimulated neutrophil adhesion to cultured endothelial cells and chemotaxis toward the inflamed sites. These different effects of adenosine are consistent with the presence of two distinct neutrophil receptors which respond to different concentrations of adenosine [Roberts et al., 1985; Rose et al., 1988]. The occupancy of A_1 adenosine receptors promotes phagocytosis and chemotaxis (EC_{50} in the picomolar range), whereas the occupancy of A_2 receptors inhibits O_2^- generation and neutrophils adhesion (EC_{50}

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in the nanomolar range) [Cronstein et al., 1990, 1992].

On the basis of the above considerations, we undertook a [^{3}H] ^{6}N-cyclohexyladenosine (CHA) binding study of A_1 adenosine receptors in human neutrophils, subsequently visualizing these receptors by means of a macromolecular probe, exploiting avidin-biotin technology. For this purpose we used a CHA-biotin XX probe as a tool; interacting with streptavidin colloidal gold, this allowed us to visualize A_1 adenosine receptors at an ultrastructural level. In addition, to improve knowledge of the molecular mechanisms of A_1 adenosine receptors in the regulation of neutrophil responses, the intracellular trafficking of these receptors was studied in a rate-controlled and dependent manner. It is noteworthy that receptor-mediated endocytosis depletes the plasma membrane of high-affinity receptors, thus contributing to cellular responses to agonists both by desensitization and resensitization of signalling [Lohse, 1993]. It is of great importance to understand the mechanisms of signal attenuation, because defects in the regulation of intercellular signalling result in uncontrolled stimulation and disease, and several of the regulatory steps are attractive therapeutic targets.

MATERIALS AND METHODS

Radioligand Binding Assay

Neutrophil preparation. Human neutrophils were isolated from heparinized whole blood of healthy donors in accordance with the method of Boyum [1968]. Blood, containing heparin as an anticoagulant, was diluted 1:1 with plasma substitute (Emagel, Boehring) for 40 min at room temperature to obtain a preliminary erythrocyte sedimentation. The supernatant was layered (5:3) over Lymphoprep and centrifuged at 800*g* for 20 min at room temperature in a swing-out rotor. After centrifugation, neutrophils were sedimented at the bottom of the tube. The contaminating erythrocytes were lysed by 1% ammonium oxalate and 0.01% sodium azide solution. The cells, which were essentially neutrophils (95% ± 2), were washed twice with PBS buffer at pH 7.4 containing 0.5% serum albumin (BSA) and 0.13% EDTA.

Neutrophil membrane preparation. For membrane preparations, cells were washed twice with 50 mM Tris-HCl, 2 mM $MgCl_2$ buffer, pH 7.5 (T_1) in the presence of protease inhibitors (200 µg/ml bacitracine, 160 µg/ml benzamidine, 20 µg/ml trypsin inhibitor) and centrifuged at 48,000*g* for 15 min at 4°C. The resulting pellets were incubated for 60 min at 37°C in the same buffer, following the addition of adenosine deaminase (2 U/ml), and then the neutrophils were washed in T_1 buffer before the binding assay.

Radioligand binding assay. The A_1 adenosine receptor binding assay was performed on human neutrophil membranes, using [^{3}H] CHA as a radiolabelled ligand. Briefly, aliquots of neutrophil membrane suspensions (0.3–0.4 mg of proteins) were incubated in 250 µl of T_1 buffer with [^{3}H] CHA ranging from 0.2 to 12 nM, in the presence and in the absence of 16 µM (-) N^6-(2-phenylisopropyl)-adenosine (R-PIA) for non-specific binding determination. After 45 min incubation at 25°C, the binding reaction was stopped by filtration through Whatman GF/C fiber filters, and then two washes with 4 ml of cold T_1 buffer were performed. Statistical analysis and curve-fitting were carried out on an IBM-compatible personal computer using the non-linear multipurpose curve-fitting program Ligand, from which the dissociation constant (Kd) and the maximum number of receptor sites (Bmax) were generated [McPherson, 1985].

Visualization of A_1 Adenosine Receptors

Synthesis of the biotinilated probe. Oxidized CHA (10.1 µmol), prepared as previously reported by periodate oxidation of CHA [Dalpiaz et al., 1995], was dissolved in 80 mM acetate buffer solution pH 5.5, and reacted with 6-([6-(biotinoyl) amino] hexanoyl) amino hydrazide (biotin XX hydrazide; Molecular Probes, Leiden Netherlands; 9.1 µmol), previously solubilized in dimethylformamide (DMF): 80 mM acetate buffer (1:1) overnight at 5°C. The resulting mixture was analyzed by HPLC/electrospray mass spectrometry (ES-MS) on a triple quadrupole mass spectrometer (QUATTRO II, MICROMASS, UK) equipped with an electrospray (ES) ion source and coupled to an HP1100 HPLC pump. The HPLC conditions were: Supelcosil ABZ 5µ column (4.6 mm × 15 cm); eluant A: 10 mM ammonium acetate buffer solution, pH 5; eluant B: acetonitrile; gradient from 15% to 80% B over 35 min; flow 1.5 ml/min; UV detection 254 nm. The eluent was split in the ratio of approximately 20:1 resulting in 50 µl/min effective flow into the ion source.

Preparative HPLC of the resulting mixture was performed on a Beckman Ultrasphere Ods

5μ column (10 mm × 25 cm) in a Beckman System Gold apparatus under the conditions reported above. Eluted fractions, corresponding to the peaks shown by the HPLC/ES-MS chromatogram, were collected, evaporated, lyophilized, and assayed for their ability to displace 1 nM [^{3}H]CHA from A_1 adenosine receptors in bovine cortical membranes and 5 nM [^{3}H]CGS21680 from A_{2A} adenosine receptors in bovine striatal membranes. Membrane preparations and binding assays were carried out as previously described [Martini et al., 1996; Franchetti et al., 1998]. The IC_{50} values were calculated from the displacement curves by log-probit analysis with four to six concentrations of the collected fractions, each performed in triplicate. Ki values were calculated from the Cheng and Prusoff [1973] equation.

Electron microscope localization. Neutrophils were incubated with 7.8 μM of the biotinilated probe in 500 μl of 10 mM Hepes, 145 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose buffer (T_2), pH 7.4, either in the absence (total binding) or in the presence (non-specific binding) of 16 μM R-PIA for specific binding determination. In order to visualize A_1 adenosine receptors on the neutrophil surface, the incubation was performed at 0°C for 60 min. For receptor internalization studies, the incubation was performed at 37°C for different time intervals (5–60 min). Cells were then centrifuged for 1 min in an Eppendorf centrifuge and rinsed three times with T_2 buffer.

Samples were processed in accordance with the pre-embedding and post-embedding techniques. For the pre-embedding method, samples were incubated in 1:50 diluted streptavidin gold for 60 min at 4°C, centrifuged for 1 min in an Eppendorf centrifuge and rinsed three times with T_2 buffer. Pellets were cut into small pieces (1 mm^3), fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at 4°C and postfixed in 1% cacodylate buffered osmium tetroxide for 120 min at room temperature. Following dehydratation in a graded series of ethanol, samples were briefly transferred to propylene oxide and embedded in Epon-Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on formvar-carbon coated copper grids (200 mesh), and stained with uranyl acetate and lead citrate. For the post-embedding method, neutrophil pellets were fixed with 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 min at 4°C and post-fixed with 1% osmium tetroxide in the same buffer for 60 min at room temperature. After rinsing in cacodylate buffer, samples were dehydrated in a graded series of ethanol, infiltered with Unicryl resin following the manufacturer's instructions (British Bio-Cell International, Cardiff, UK), placed in transparent gelatin capsules and allowed to polymerize for 3 days at 4°C under UV light. Ultrathin sections, mounted on formvar-carbon coated nickel grids, were incubated face down on a drop (50 μl) containing 0.5% BSA and 0.1% fish gelatin in PBS for 30 min at room temperature. They were then incubated on a drop (50 μl) of diluted (1:50) streptavidin-colloidal gold (10–15 nm in diameter) for 15 min at room temperature, fixed with 2% glutaraldehyde in PBS for 10 min, washed with deionized water, and finally stained with uranyl acetate and lead citrate. Ultrathin sections were observed with a Jeol 100 SX trasmission electron microscope (TEM).

RESULTS

Radioligand Binding Assay

In human neutrophil membrane preparations, [^{3}H] CHA binding was saturable and reversible. Scatchard analysis of the saturation curve yielded a single straight line, suggesting the presence of a homogeneous population of binding sites with an affinity constant (Kd) of

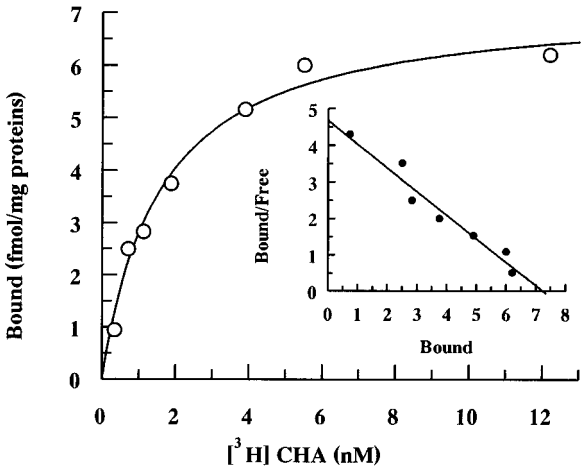

Fig. 1. Saturation curve of [^{3}H] CHA binding to A_1 adenosine receptors in human neutrophil membranes. Membranes were incubated with increasing concentrations of [^{3}H] CHA (0.2–12 nM) in T_1 buffer for 45 min at 25°C. The curve is representative of a single experiment performed three additional times with similar results. Saturation binding data were transformed using the EBDA/LIGAND and Grafit Version 3.0 computer programs. Inset: Scatchard plot of the saturation curve of [^{3}H] CHA binding to human membranes.

1.8 ± 0.15 nM and a very low maximal number of binding sites (Bmax) of 7.2 ± 0.62 fmol/mg of proteins (Fig. 1).

Visualization of A_1 Adenosine Receptors

The CHA-biotin XX hydrazide derivative was found to be a complex equilibrium mixture of various compounds: the total ion current (TIC) chromatogram and the related positive ES mass spectra resulting from HPLC/ES-MS are shown in Figure 2. Three major partially resolved TIC peaks (retention time 6.47 min, 7.02 min, and 7.30 min) were all dominated by m/z 832 $[M+H]^+$ and two minor peaks (retention time 10.21 min, 10.66 min) by m/z 814$[M+H]^+$ as the main ion.

Preparative HPLC was carried out on the mixture reaction, and the eluted fractions corresponding to the molecular weight of 832 and 814, were assayed for their ability to interact with A_1 and A_{2A} adenosine receptors. Of these fractions, only the one corresponding to a m/z 832 [M+H], indicated by us as the biotin XX hydrazide probe, maintained a good selectivity and affinity for A_1 adenosine receptors (inibition of [^{3}H] CHA binding = 78 ± 5 nM) and the inability to displace [^{3}H] CGS 21680 binding from A_{2A} adenosine receptors up to 20 μM. The bifunctional probe with a high affinity and

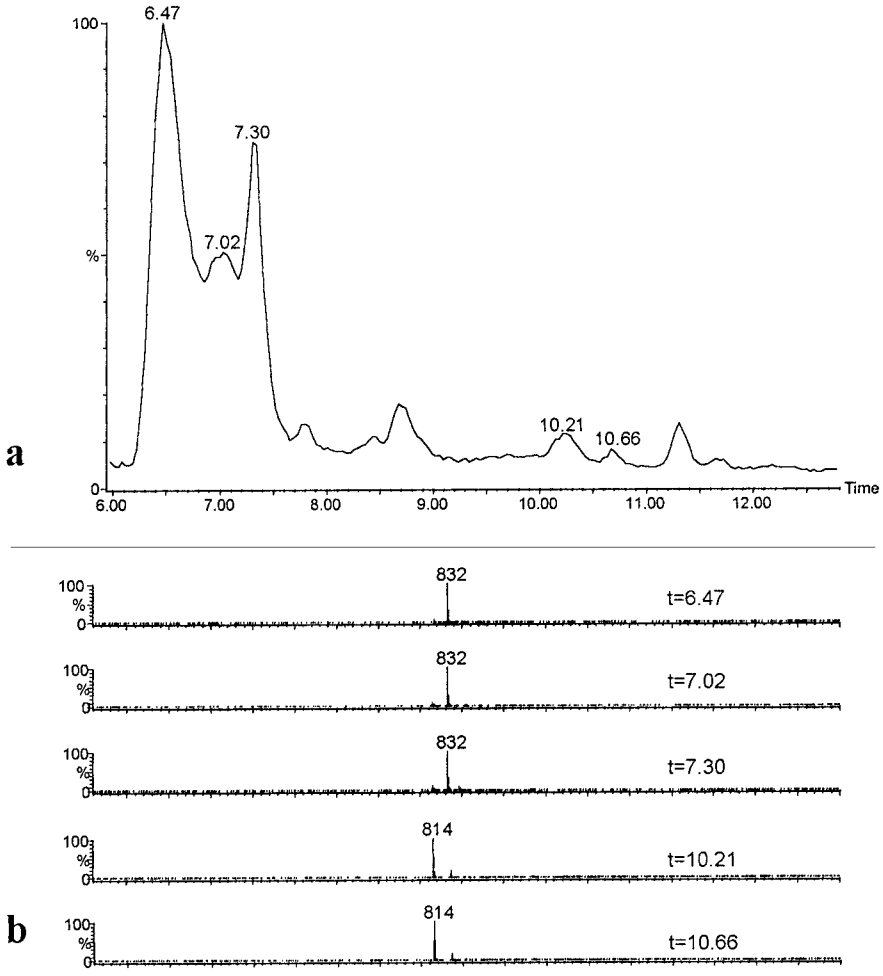

Fig. 2. **a**: Total ion current chromatogram of the mixture resulting from treatment of oxidized CHA with biotin XX hydrazide after HPLC/ES-MS. **b**: Positive ES mass spectra associated with the peaks representative of the CHA-biotin XX hydrazide derivative, i.e., the three major peaks centered at retention times of 6.47, 7.02, and 7.30 min and two major peaks centered at the retention times of 10.21 and 10.66.

selectivity for A_1 adenosine receptors was used for the ultrastructural visualization of these binding sites on the neutrophil plasma membranes and inside the cells.

Ultrastructural Analysis of CHA-Biotin XX Binding to A_1 Adenosine Receptors

Under the TEM, neutrophils appeared as roundish cells (9–10 μm in diameter) with a polylobed nucleus (Fig. 3). The heterochromatin was characteristically massed on the inner nuclear membrane. The cytoplasm contained some Golgi complexes and granules of high (azurophil granules) and medium (specific granules) electron-density. In the cortical cytoplasm some vesicles depicting an endocytotic activity were visible.

The ligand-receptor complex (CHA-biotin XX-streptavidin-gold) was visualized as electron-dense gold particles localized on the neutrophil surface and within membrane-bound cytoplasmic structures. The distribution of CHA-biotin XX on the surface of neutrophils, incubated at 0°C, was diffuse, with both single particles and small clusters of particles (Figs. 4 and 5). Following incubation at 37°C, the internalization of the CHA-biotin XX probe occurred after 5 min by means of pinocytosis. Electron-lucent pits and vesicles measuring up to 0.5 μm in diameter were visible at the cortical cytoplasm, and commonly contained one to several gold particles (Fig 6). In the deeper cytoplasm, the complex was found within small endosomes, and on the contents of the specific granules after 30–60 min of incubation in the probe (Fig. 7 and

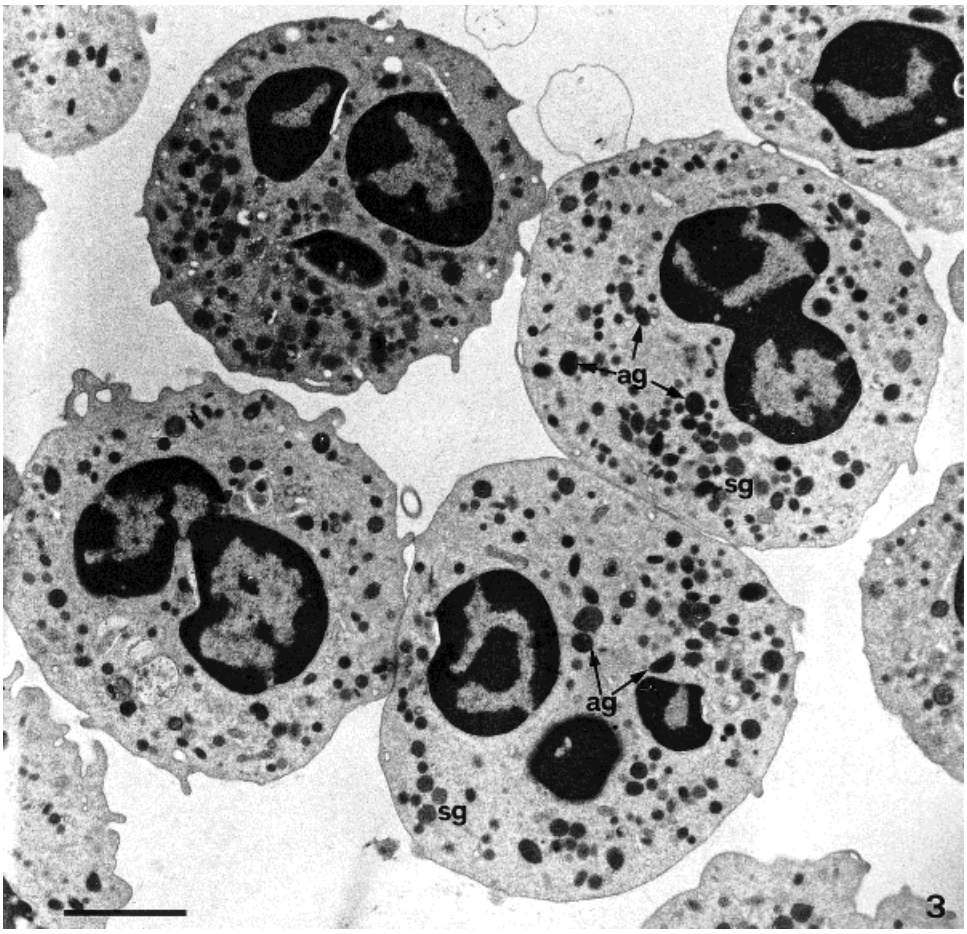

Fig. 3. Cluster of untreated neutrophils showing a polylobed nucleus. Some large azurophil granules (ag) and many smaller specific granules (sp) are visible in the cytoplasm. Arrowheads point to micropinocytotic vesicles beneath the plasma membrane. Scale bar = 2.5μm.

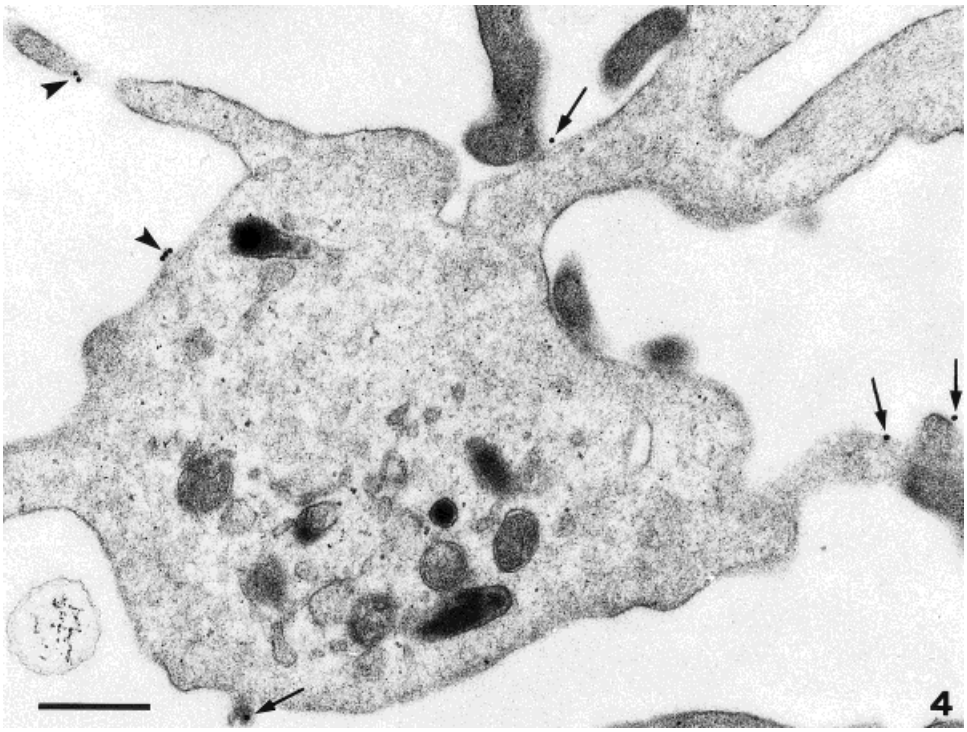

Fig. 4. CHA-biotin XX-streptavidin-gold treated neutrophils. Anucleate portion of a neutrophil showing pseudopodia. Single (arrow) and clustered (arrowhead) gold particles are visible on the plasma membrane. Thirty min incubation in CHA-biotin XX probe at 0°C, pre-embedding technique. Scale bar = 0.5μm.

inset). Neutrophils incubated with an excess of R-PIA added to CHA-biotin XX probe (non-specific binding), showed appreciably less (minus that 5%) or no surface binding for any time of incubation.

DISCUSSION

It has been demonstrated that occupancy of A_1 adenosine receptors promotes neutrophil adherence to the endothelium and chemotaxis (a proinflammatory role), whereas occupancy of A_{2A} adenosine receptors inhibits adherence and generation of toxic oxygen metabolites (an anti-inflammatory role) [Cronstein et al., 1983; Roberts et al., 1985; Rose et al., 1988].

Recent investigations using a monoclonal antibody have confirmed the presence of A_1 adenosine receptors on human neutrophils [Salmon et al., 1993]. However, no direct binding studies or ultrastructural investigations have been performed on A_1 adenosine receptors in these cells, to date.

In our study, [^{3}H] CHA labelled A_1 adenosine receptors in neutrophil membranes with kinetic properties corresponding to A_1 subtypes (Kd = 1.4 ± 0.08 nM) with a low binding capacity (Bmax = 7.1 ± 0.37 fmol/mg of proteins). We demonstrated the surface localization of A_1 adenosine receptors by electron microscopy using the selective CHA-biotin XX macromolecular probe. This probe was designed on the basis of the "functionalized congener" methodology, previously used with success by Jacobson [1990] for probing adenosine receptors: both adenosine and xanthine analogues, derivatized with a functional group, were coupled to biotin, forming bifunctional probes to serve as non-covalent cross-linkers between adenosine receptors and avidin. In our case, cyclohexyladenosine was derivatized by oxidation with sodium periodate and coupled with a long-chained biotin-containing hydrazide, according to Bayer et al. [1988] methodology described for sucrose periodate oxidation and biotin condensation. HPLC/ES-MS analysis of the reaction products suggests the presence of isomeric forms, as reported by McCornick [1966] for similar condensation reactions of periodate-oxidized saccharides with hydrazides. Comparing our data with those reported in literature [Khym, 1963; McCornick, 1966; Hansske and Cramer, 1976; Senapathy et al., 1985; Bayer et al., 1988] the

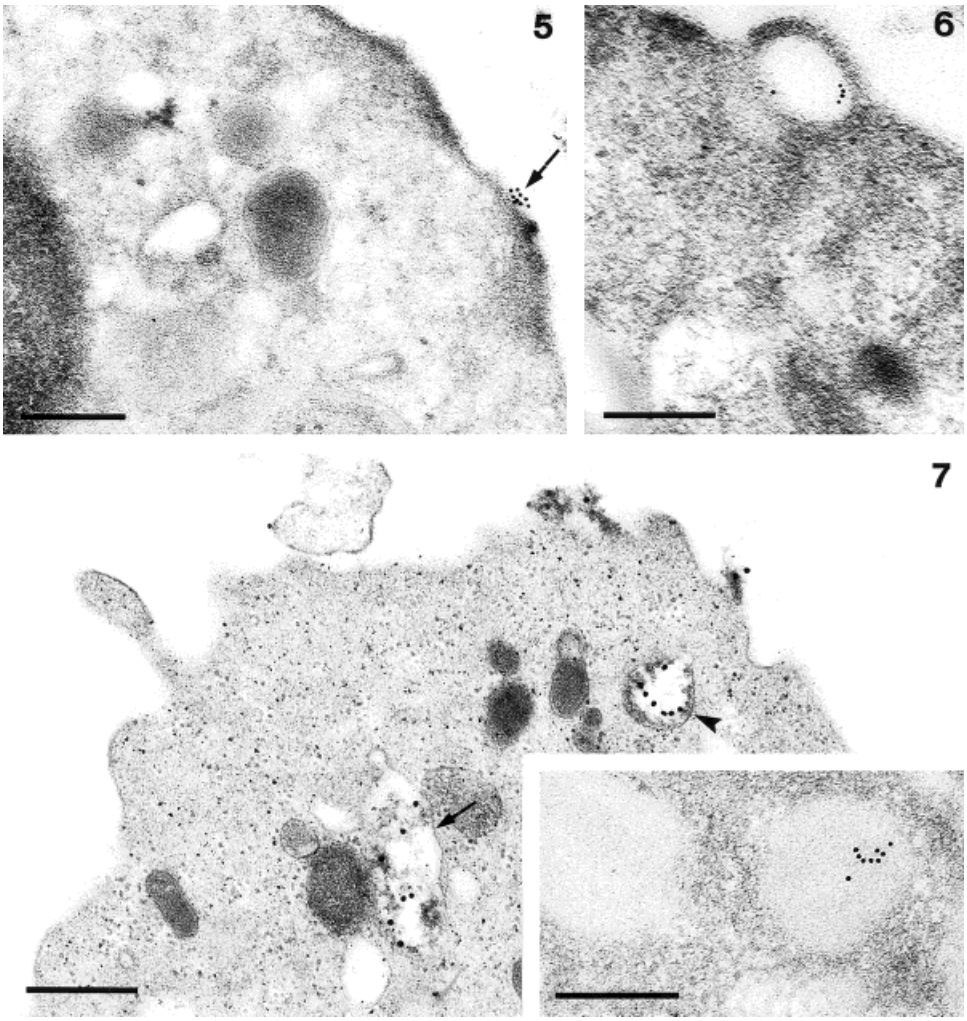

Fig. 5. CHA-biotin XX-streptavidin-gold treated neutrophils. A small aggregate of gold particles (arrow) on the cell surface. Sixty min incubation in CHA-biotin XX probe at 0°C, pre-embedding technique. Scale bar = 0.25μm.

Fig. 6. CHA-biotin XX-streptavidin-gold treated neutrophils. A pinocytotic vesicle with several gold particles inside is visible in the cortical cytoplasm. Five min incubation in CHA-biotin XX probe at 37°C, post-embedding technique. Scale bar = 0.25μm.

Fig. 7. CHA-biotin XX-streptavidin-gold treated neutrophils. A small vesicular endosome (arrow) containing gold particles. The arrowhead points to a pinocytotic vesicle. Thirty min incubation in CHA-biotin XX probe at 37°C, post-embedding technique. Scale bar = 0.25μm. Inset: Gold particles on a specific granule. Sixty min incubation in CHA-biotin XX probe at 37°C, post-embedding technique. Scale bar = 0.25μm.

MW 832, which corresponds to the three major peaks, was attributed to the cyclic morpholine type I stereo-isomers while the two minor peaks with MW 814 were found to be representative of the acyclic monohydrazone II and the cyclic hemiacetal III (as reported in Fig. 8). The morpholine stereo-isomers, which maintain a good affinity and selectivity for A_1 adenosine receptors, were in equilibrium with the other forms, too: after HPLC injection of the separated fraction corresponding to the molecular weight of 832, the presence of all five peaks, as in the original chromatogram, was observed (data not shown). However, the percentage of MW 814 compounds, compared with the morpholine stereo-isomers, was negligible. The availability of the morpholine stereo-isomers allowed us, after coupling with streptavidin-colloidal-gold, to vi-

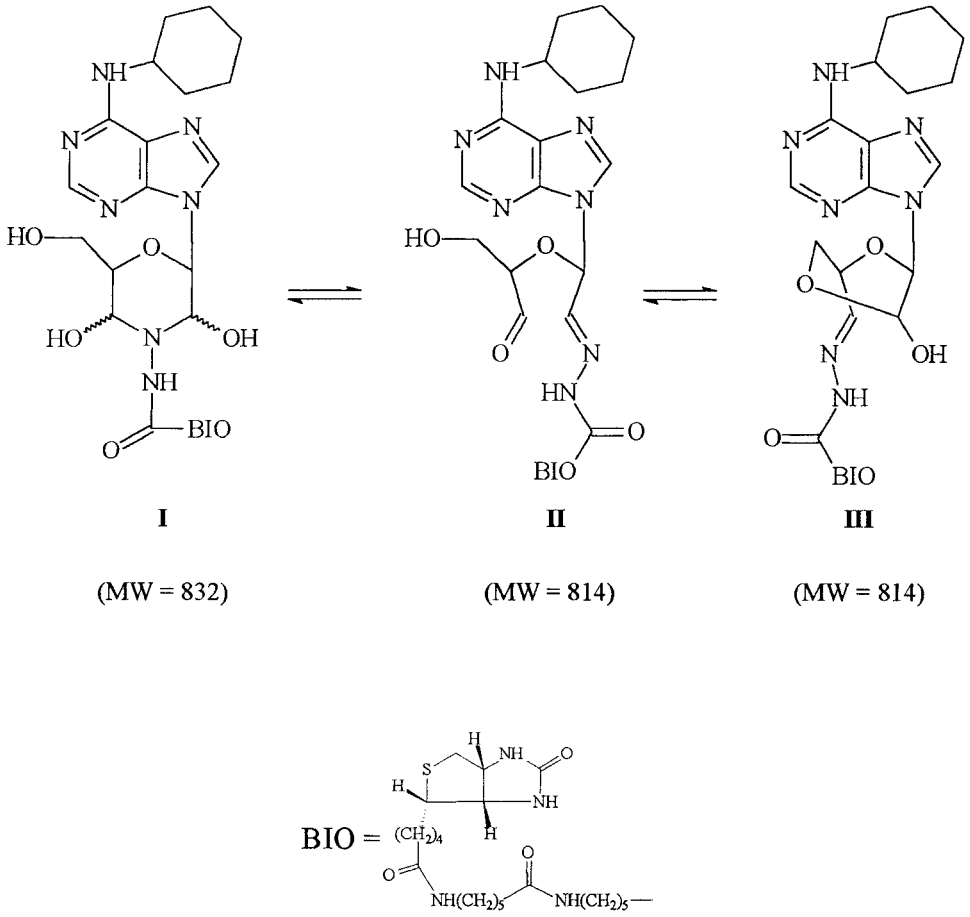

Fig. 8. Possible structure of the CHA-biotin XX derivatives. I: General cyclic morpholine stereo-isomers structure. II: Acyclic monohydrazone. III: Cyclic hemiacetal.

sualize the distribution of A_1 receptors in human neutrophils.

Receptor internalization or sequestration has already been described for several G-protein coupled receptors [Bohm et al., 1997]. Our data showed that at 4°C, A_1 binding sites were localized on neutrophil surfaces as single particles or small clusters. At 37°C, a temperature that induces the normal receptor turnover, the activation of A_1 adenosine receptors induced a fast disappearance of the receptors from the cell surface and their internalization by micropinocytotic vesicles. A similar finding has been reported for immunochemical studies in the smooth muscle (DDT1 MT-2 line), where the chronic activation of A_1 adenosine receptors induces a rapid receptor aggregation on the cell surface (5–15 min), followed by receptor internalization [Cirulea et al., 1997].

In addition, our study showed that after 60 min of incubation in the probe, several gold particles were visible in the deeper cytoplasm in the specific granules of medium electron-density. Neutrophil preincubation with the agonist R-PIA showed scarce or absent surface binding, suggesting that a specific A_1 receptor-mediated endocytosis was involved in the internalization of the macromolecular probe. No significant differences were observed with the pre-embedding and post-embedding techniques.

However, the techniques used in the present investigation did not allow us to evaluate the definitive fate of the A_1 receptors themselves. It is generally assumed that during ligand internalization by receptor-mediated endocytosis, the segment of membrane containing the receptor is internalized with the ligand. Studies in which both receptors and ligands have been labelled suggest that sorting of a receptor destined for recycling to the plasma membrane occurs after the uncoupling of the receptor from the ligand, in an endosome structure referred to as CURL (compartment of uncoupling of receptor from ligand) [Geuze et al., 1983]. Sorting is thought

to occur by accumulation of the ligand within the large vesicular portion of the endosome and migration of receptors to a thinner tubular portion (CURL). Following separation from one another, the tubular portion is thought to transport receptors to the cell surface, while the ligand-containing vesicle fuses with lysosomes [Salmon et al., 1993]. Investigations to ascertain the fate of A_1 receptors are in progress in our laboratory.

The intracellular transport of the CHA-biotin XX probe and its localization at the level of specific granules which contain lysozime, alkaline phosphatase, collagenase and specific receptors for chemotactic factors such as N-Formyl peptides [Leffell and Spitznagel, 1972; Bretz and Baggiolini, 1974; Kane and Peters, 1975; Fletcher and Gallin, 1983] could suggest a direct modulatory effect of adenosine at the intracellular level. In this connection, a modulatory effect of adenosine on FMLP binding to its receptor has been also demonstrated [Cronstein et al., 1990; Cronstein and Haines, 1992]. Furthermore, the presence on the specific granule membrane of FMLP binding sites which are biochemically similar to those on the cell surface suggests that they may be mobilized to the cell surface following activation and play a modulatory role [Fletcher and Gallin, 1983; Cronstein et al., 1990]. In this way, cellular trafficking may allow neutrophils to maintain functional responsiveness during chemotaxis.

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