0026-895X/94/050978-05$03.00/0

MOLECULAR PHARMACOLOGY, 45:978-982

^{125}I-4-Aminobenzyl-5′-*N*-methylcarboxamidoadenosine, a High Affinity Radioligand for the Rat A_3 Adenosine Receptor

MARK E. OLAH, CAROLA GALLO-RODRIGUEZ, KENNETH A. JACOBSON, and GARY L. STILES

Departments of Medicine and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710 (M.E.O., G.L.S.), and Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892 (C.G.-R., K.A.J.)

Received November 12, 1993; Accepted February 11, 1994

SUMMARY

The rat A_3 adenosine receptor (AR) is a recently characterized AR subtype cloned from testis and brain cDNA libraries. N^6-2-(4-Amino-3-[^{125}I]iodophenyl)ethyladenosine, a high affinity A_1AR agonist, has served as the only radioligand available for study of the A_3AR. The relatively low affinity of N^6-2-(4-amino-3-[^{125}I]iodophenyl)ethyladenosine for the A_3AR and its greater A_1AR selectivity necessitate the development of more appropriate radioligands for A_3AR analysis. This report characterizes ^{125}I-4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine (^{125}I-AB-MECA), a high affinity radioligand for the A_3AR, in two cell lines that express this AR subtype. Membranes from Chinese hamster ovary (CHO) cells expressing the rat A_3AR and from the rat mast cell line RBL-2H3 bound ^{125}I-AB-MECA with K_d values of 1.48 ± 0.33 nM and 3.61 ± 0.30 nM, respectively. As determined by ^{125}I-AB-MECA binding, levels of A_3AR expresssion in the A_3AR-CHO cell line and RBL-2H3 cells were 3.06 ± 0.21 pmol/mg and 1.02 ± 0.13 pmol/mg, respectively. Binding of ^{125}I-AB-MECA was characterized in competition assays. In the A_3AR-CHO cell line a potency order of cyclohexyl-5′-*N*-ethylcarboxamidoadenosine (cyclohexyl-NECA) = benzyl-NECA > (−)-N^6-[(*R*)-phenylisopropyl]adenosine = NECA was observed, and in RBL-2H3 cells (−)-N^6-[(*R*)-phenylisopropyl]adenosine and NECA were equipotent. Xanthine amine congener (XAC) and 8-cyclopentyl-1,3-dipropylxanthine did not significantly inhibit ^{125}I-AB-MECA binding. The parent compound, AB-MECA, dose-dependently inhibited forskolin-stimulated adenylyl cyclase activity in A_3AR-CHO cell membranes. ^{125}I-AB-MECA bound to the rat A_1AR and canine A_{2a}AR expressed in COS-7 cells with K_d values of 3.42 ± 0.43 nM and 25.1 ± 12.6 nM, respectively. This binding was significantly reduced in the presence of 1 μM XAC. In RBL-2H3 cells, XAC had no effect on ^{125}I-AB-MECA affinity and reduced the level of radioligand binding by ~5%.

Many of the varied physiologic effects of adenosine are the result of its activation of specific cell surface ARs. Classification of ARs into subtypes has been based on the biochemical effects of receptor activation, the pharmacologic properties of the ARs, and, more recently, cDNA structures (1, 2). Presently, based on these criteria, four AR subtypes have been described, the A_1AR, A_{2a}AR, A_{2b}AR, and A_3AR. The entire cDNA for the A_3AR was originally isolated from a rat testis cDNA library (3), and characterization of this cDNA was performed after its isolation from a rat brain cDNA library (4). The classification of the protein encoded by this cDNA as a previously unrecognized AR subtype was based on 1) the fact that in its putative transmembrane domain regions the protein has 58% and 57% amino acid identity to the A_1AR and A_{2a}AR, respectively, and 2) the ability of the expressed protein to specifically bind adenosine and several of its agonist analogs, including ^{125}I-APNEA, (*R*)-PIA, and NECA, with relatively high affinity (4). Several properties of the expressed A_3AR are unique to this subtype, compared with the A_1AR and A_2ARs, including its 15 nM affinity for ^{125}I-APNEA, the potency series of (*R*)-PIA = NECA > (*S*)-PIA in displacing ^{125}I-APNEA binding, and its insensitivity to the methylxanthine class of compounds, several of which are high affinity AR antagonists (4).

PCR analysis of reverse transcribed rat mRNA revealed the highest level of A_3AR expression to be in the testis, with moderate expression in the kidney, lung, and heart (4). Lower levels of expression were observed in brain regions such as cerebral cortex, striatum, and olfactory bulb (4).

Recently, a cDNA coding for a protein of identical size (317 amino acids) and 72% overall amino acid identity, compared

G.L.S. was supported by National Heart, Lung, and Blood Institute Specialized Center of Organized Research Grant P50HL17670 in Ischemic Disease, in part by National Heart, Lung, and Blood Institute Grant RO1HL35134, and by Grant-in-Aid 91008200 from the American Heart Association.

ABBREVIATIONS: AR, adenosine receptor; APNEA, N^6-2-(4-aminophenyl)ethyladenosine; (*R*)-PIA, (−)-N^6-[(*R*)-phenylisopropyl]adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; (*S*)-PIA, (+)-N^6-[(*S*)-phenylisopropyl]adenosine; AB-MECA, 4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; CHO, Chinese hamster ovary; XAC, xanthine amine congener; benzyl-NECA, N^6-benzyl-5′-*N*-ethylcarboxamidoadenosine; cyclohexyl-NECA, N^6-cyclohexyl-5′-*N*-ethylcarboxamidoadenosine; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate.

with the rat A_3AR, was isolated from a sheep pars tuberalis library (5). Based on distinct pharmacologic differences of the expressed protein and significant variations in tissue expression, relative to the rat A_3AR, it is unclear whether this sheep AR represents a species homolog of the A_3AR or a distinct receptor subtype.

With the recent recognition of the existence of the A_3AR, this receptor has been implicated as the AR involved in various physiologic responses to adenosine. Ramkumar *et al.* (6) demonstrated that the ability of adenosine to enhance antigen-induced secretory responses in the rat mast cell line RBL-2H3 is the result of A_3AR activation. Findings also suggest that the A_3AR mediates the hypotensive response produced by APNEA, (*R*)-PIA, and NECA administration in the pithed rat (7).

Currently, ^{125}I-APNEA is the preferred ligand for the study of the rat A_3AR (4, 6). However, the affinity of ^{125}I-APNEA (K_d of 15 nM and 34 nM for CHO cells expressing the A_3AR and RBL-2H3 cells, respectively) for the A_3AR requires dilution of the specific activity of the radioligand with unlabeled iodinated APNEA to obtain a valid determination of A_3AR binding parameters in saturation analyses. Furthermore, the use of ^{125}I-APNEA to study the A_3AR in native tissues containing multiple AR subtypes may be hampered by the approximately 10–20-fold greater affinity of this radioligand for the A_1AR, compared with the A_3AR. This paper describes the characterization of ^{125}I-AB-MECA as an agonist radioligand for the A_3AR, demonstrating approximately 10-fold greater affinity for this receptor subtype, compared with ^{125}I-APNEA.

Experimental Procedures

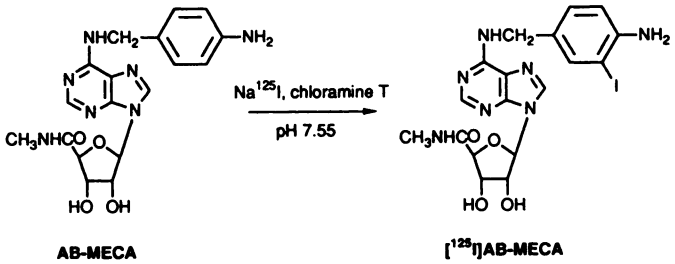
Materials. All cell culture supplies and G418 (Geneticin) were from GIBCO-BRL (Gaithersburg, MD). (*R*)-PIA and papaverine were from Sigma Chemical Co. (St. Louis, MO). XAC and CPX were purchased from Research Biochemicals International (Natick, MA). NECA, adenosine deaminase, and all restriction enzymes were from Boehringer-Mannheim (Indianapolis, IN). Forskolin was obtained from Calbiochem (La Jolla, CA). Na^{125}I and [α-^{32}P]ATP were from DuPont-New England Nuclear (Boston, MA). The synthesis of cyclohexyl-NECA (8) and benzyl-NECA (9) was as described. ^{125}I-APNEA was prepared as described previously (10).

Cell culture and DNA transfection. CHO cells were maintained in Ham's F12 nutrient mixture containing 10% fetal bovine serum. COS-7 cells and RBL-2H3 cells (supplied by Dr. L. A. Kindman, Duke University) were both grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All media were supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). A CHO cell line stably expressing the rat A_3AR cDNA (4) subcloned into the *Hin*dIII/*Sma*I sites of the expression vector pCMV5 (Dr. D. Russell, University of Texas Southwestern) was created by co-transfection of the expression vector-cDNA construct with the neomycin resistance vector pSVneo (Pharmacia, Piscataway, NJ) via a calcium phosphate precipitation and glycerol shock technique (11), as described previously for the bovine A_1AR (12). Cell colonies resistant to G418 (400 μg/ml) were selected, expanded, and screened for A_3AR expression by ^{125}I-APNEA binding. The rat A_1AR cDNA and sheep A_3AR (both kindly provided by Dr. S. Reppert, Harvard Medical School) subcloned into pCMV5 (rat, *Eco*RI/*Xba*I sites; sheep, *Hin*dIII/*Xba*I sites) and canine A_{2a}AR cDNA subcloned into pBC12BI (provided by Dr. G. Vassart, Université Libre de Bruxelles, Brussels, Belgium) were transiently transfected into COS-7 cells by a DEAE-dextran procedure (13). COS-7 cells were used for binding assays approximately 72 hr after transfection.

Synthesis and radioiodination of AB-MECA. A detailed synthesis of AB-MECA is described elsewhere (9) and followed the general method of Olsson *et al.* (8). The compound was characterized by NMR spectroscopy, mass spectroscopy, and elemental analysis. The melting point was determined to be 135° (decomposition). A genuine sample of 3-[^{127}I]iodo-AB-MECA (m.p. 132°, decomposition) was prepared by an unambiguous synthetic method, starting from 4-amino-3-iodobenzylamine (9), as a reference standard for identification of the iodinated product and for structural assignment of the product as shown in Fig. 1. Radioactive (see below) and nonradioactive iodinated AB-MECA coeluted upon HPLC separation, using two solvent systems. The solvents used were isocratic 60% (v/v) methanol/40% (v/v) 20 mM ammonium formate, pH 8.0, and 50 mM $NH_4H_2PO_4$, pH 5.2/9% (v/v) methanol/1% (v/v) acetonitrile, to give retention times of 9.2 min and 3.9 min, respectively (Waters μBondapak C18 column; 25 × 0.46 cm; flow rate, 0.75 ml/min).

Radioiodination of AB-MECA and separation of the labeled ligand by HPLC was essentially as described previously for ^{125}I-APNEA (10). Briefly, 0.2 mg of AB-MECA was dissolved in 1 ml of methanol, and 10 μl of this solution were taken to dryness under vacuum. The dried material was resuspended in 40 μl of 0.3 M NaH_2PO_4, pH 7.55. To this 1.5 mCi of Na^{125}I was added, followed by 10 μl of a 1 mg/ml chloramine T solution. After 4 min at room temperature, the reaction was stopped by addition of 15 μl of a 0.5 mg/ml sodium metabisulfite solution. The entire solution was applied to a Waters 501 HPLC system. Products were separated using an isocratic protocol and a Waters μBondapak C18 column equilibrated with a mobile phase of 60% (v/v) methanol/40% (v/v) 20 mM ammonium formate, pH 8.0. The ^{125}I-AB-MECA peak was defined by both UV absorbance and incorporation of ^{125}I.

Radioligand binding assays. Radioligand binding assays for the rat A_3AR and A_1AR, using both transfected CHO and COS-7 cells, were performed as described previously (12). Briefly, cells were washed twice with 10 ml of 10 mM Tris, 5 mM EDTA, pH 7.4 at 5°, and then scraped into 5 ml of the same buffer. The cells were disrupted by 20 strokes by hand in a glass homogenizer on ice. The suspension was centrifuged at 43,000 × *g* for 10 min and the resulting pellet was resuspended in 50 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.26 at 5° (50/10/1 buffer), to yield a protein concentration of 0.02 mg/ml. Adenosine deaminase was added to give a final concentration of 2 units/ml. Assays were conducted at 37° for 60 min and terminated by filtration using a Brandel cell harvester and rapid washing with 50/10/1 buffer containing 0.01% CHAPS, over glass fiber filters that had been pretreated with 0.3% polyethylenimine. Radioligand binding assays with membranes prepared from COS-7 cells transfected with the A_{2a}AR cDNA were performed in a similar manner except that the binding buffer consisted of 50 mM HEPES, 10 mM $MgCl_2$, pH 6.8 (14). Saturation binding assays were performed using 10 μM (*R*)-PIA to define nonspecific binding and typically used concentrations of ^{125}I-AB-MECA ranging from 0.25 nM to 3.0–6.0 nM. Additionally, a set of experiments were performed with RBL-2H3 cell membranes in which nonradioactive ^{127}I-AB-MECA was used to dilute the specific activity of the radioligand, to enable concentrations of ligand of 100–150 nM to be used. Competition binding assays were performed with a concentration of ^{125}I-AB-MECA approximately equal to the K_d value for the radioligand in the particular cell type. For ^{125}I-AB-MECA binding experiments conducted in the presence of XAC, the membrane suspension was treated with the antagonist and then immediately added to



AB-MECA [^{125}I]AB-MECA

Fig. 1. Structure of ^{125}I-AB-MECA. The radioiodination of the compound is described in the text.

assay tubes and the incubation was started. Data were analyzed by a previously described computer modeling system (15). For all competing ligands, Hill coefficients were not significantly different from unity. IC_{50} values obtained from computer analysis of competition curves were converted to K_i values by using the Cheng-Prusoff equation (16). Protein concentrations were determined by the method of Bradford (17).

Adenylyl cyclase assays. Assays of adenylyl cyclase activity of membranes prepared from CHO cells stably expressing the A_3AR were performed by the method of Salomon *et al.* (18). Briefly, cell membranes were prepared and pelleted as described for radioligand binding assays. Membranes were resuspended in 75 mM Tris, 200 mM NaCl, 1.25 mM $MgCl_2$, pH 8.12 at 4° (TNM buffer), to give a final concentration of 0.1 mg/ml, and 2 units/ml adenosine deaminase was added. Adenylyl cyclase assays consisted of 40 μl of membrane suspension, 40 μl of cyclase mixture (TNM buffer supplemented with 140 μM dATP, 5 μM GTP, 30 units/ml creatine kinase, 5 mM creatine phosphate, 2.2 mM dithiothreitol, 100 μM papaverine, and 1.5 μCi of [α-^{32}P]ATP), and 20 μl of test compounds. Assays were conducted at 30° for 15 min and terminated by addition of a stop solution containing 20,000 cpm/ml [^{3}H]cAMP. Labeled cAMP was isolated by sequential chromatography over Dowex and alumina columns, and quantities were determined by liquid scintillation counting. For all assays, adenylyl cyclase was activated with 1 μM forskolin, which typically induced a 6–8-fold stimulation of the enzyme.

Results and Discussion

An analysis of ligand-receptor structure-activity relationships for the rat A_3AR has revealed that, of the compounds examined, N^6,5′-disubstituted adenosine analogs demonstrate the highest affinity for this AR subtype.[1] One such compound, AB-MECA, was radioiodinated (Fig. 1) and its binding to membranes prepared from CHO cells stably expressing the rat A_3AR was examined. Initial experiments using approximately 1 nM ^{125}I-AB-MECA demonstrated specific binding of the radioligand by the A_3AR. Specific binding of ^{125}I-AB-MECA to membranes obtained from nontransfected CHO cells was negligible. When conducted at 37°, ^{125}I-AB-MECA binding to stably transfected CHO cells reached equilibrium at approximately 25 min of incubation and remained relatively stable over 90 min (data not shown). Saturation binding analysis demonstrated that binding parameters (K_d and B_{max} values) did not vary if the incubation was conducted for 30 min or 60 min at 37°. An incubation of 60 min was used for all subsequent assays. As shown in Fig. 2A, ^{125}I-AB-MECA binding was saturable, specific, and of high affinity. In four experiments, K_d and B_{max} values of 1.48 ± 0.33 nM and 3.06 ± 0.21 pmol/mg, respectively, were obtained. In contrast, ^{125}I-APNEA bound with a K_d of approximately 15 nM (data not shown). To further analyze the binding properties of ^{125}I-AB-MECA, this radioligand was used in competition binding assays with a series of adenosine analogs (Fig. 2B; Table 1). The rank order of potencies for displacement of ^{125}I-AB-MECA from CHO cell membranes was cyclohexyl-NECA = benzyl-NECA > (*R*)-PIA = NECA. The relatively high affinities of the N^6,5′-disubstituted analogs and the equal potencies of (*R*)-PIA and NECA are in agreement with previous studies of the A_3AR using ^{125}I-APNEA as radioligand.[1] It was found that, among N^6 substituents, the benzyl group was best suited for A_3AR selectivity, and the group was compatible with the A_3AR-enhancing effect of the 5′-uronamides (9). Additionally, ^{125}I-AB-MECA binding was insensitive to xanthine antagonist compounds, inasmuch as the potent A_1AR antagonist CPX, at a concentration of 10 μM, inhibited binding by 8.5 ± 2.7% (three experiments).

The A_3AR has previously been shown to be coupled to the inhibition of adenylyl cyclase via a pertussis toxin-sensitive G protein (4). To demonstrate the agonist activity of AB-MECA, the ability of the noniodinated compound to inhibit forskolin-stimulated adenylyl cyclase activity in membranes prepared from stably transfected CHO cells was examined. As shown in Fig. 3, AB-MECA inhibited forskolin-stimulated adenylyl cyclase activity in a dose-dependent fashion, with inhibition of approximately 25% at 10 μM. The agonist nature of ^{125}I-AB-MECA was also indicated by the effect of guanine nucleotides on binding of the radioligand to membranes prepared from the stably transfected CHO cells. In three experiments, the inclusion of 10 μM Gpp(NH)p in the binding assay did not affect the affinity of the binding interaction (K_d values of 1.25 ± 0.16 nM and 1.70 ± 0.18 nM in control and Gpp(NH)p-treated membranes, respectively). However, the population of receptors in this high affinity state was reduced by 49.7 ± 4.3% by Gpp(NH)p treatment.

To characterize ^{125}I-AB-MECA binding in a native tissue expressing the A_3AR, the rat mast cell line RBL-2H3 was selected. The presence of the A_3AR in these cells was recently demonstrated pharmacologically in ligand binding assays, functionally in antigen-induced secretion assays, and by Northern blotting and PCR analysis of reverse-transcribed mRNA obtained from the cells (6). Furthermore, PCR analysis indicated that these cells do not express A_1ARs or A_2ARs (6). As shown in Fig. 4A, ^{125}I-AB-MECA binding to membranes prepared from RBL-2H3 cells was similar to that observed for the stable CHO cell line. In five experiments, the calculated K_d and B_{max} values were 3.61 ± 0.30 nM and 1.02 ± 0.13 pmol/mg, respectively. Dilutional saturation curves using maximum concentrations of ligand of 100–150 nM yielded K_d and B_{max} values of 3.04 ± 0.33 nM and 0.87 ± 0.08 pmol/mg, respectively (four experiments). Interestingly, the approximately 2.5-fold lower affinity of the RBL-2H3 cell A_3AR, compared with the receptor in the stable A_3AR-CHO cell line, is similar to the difference observed for ^{125}I-APNEA affinities in the two cell types (34 nM versus 15 nM). Again, to demonstrate the pharmacologic characteristics of ^{125}I-AB-MECA and A_3AR binding, competition binding assays were performed (Fig. 4B; Table 1). As described previously (4, 6), (*R*)-PIA and NECA were equipotent at the A_3AR and 10 μM CPX displaced only 4.65 ± 0.3% of the ^{125}I-AB-MECA binding (two experiments).

To ascertain the utility of ^{125}I-AB-MECA in the study of A_3ARs in tissues containing a mixed population of AR subtypes, the binding of the radioligand to membranes prepared from COS-7 cells transfected with either the rat A_1AR or canine A_{2a}AR cDNAs was examined. ^{125}I-AB-MECA bound to the rat A_1AR with a K_d of 3.42 ± 0.43 nM (eight experiments), compared with a K_d of 1.32 ± 0.35 nM (four experiments) for ^{125}I-APNEA. Binding of ^{125}I-AB-MECA to the A_{2a}AR was of lower affinity (K_d = 25.1 ± 12.6 nM, six experiments) and a much lower level of total binding was obtained, thus resulting in a substantially lower percentage of specific binding, compared with the rat A_3AR and A_1AR. The lack of binding of xanthine antagonists by the A_3AR (4) enables the use of these compounds

[1] P. J. M. van Galen, A. H. Van Bergen, C. Gallo-Rodriguez, N. Melman, M. E. Olah, A. P. IJzerman, G. L. Stiles, and K. A. Jacobson. A binding site model and structure-activity relationships for the rat A_3 adenosine receptor. Manuscript in preparation.

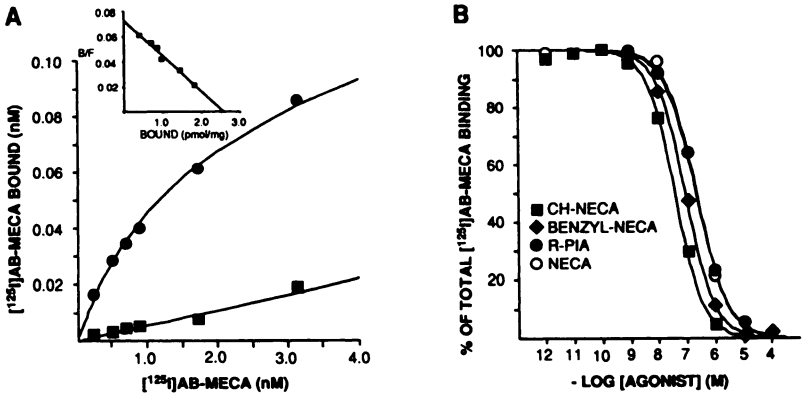


Fig. 2. Binding of ^{125}I-AB-MECA to CHO cells stably expressing the rat A_3AR. Membrane preparation and radioligand binding procedures are described in the text. A, Binding isotherm of ^{125}I-AB-MECA. ●, Total binding; ■, nonspecific binding, which was defined with 10 μM (*R*)-PIA. *Points* represent the mean of duplicate samples. Computer analysis of this experiment yielded K_d and B_{max} values of 1.29 nM and 2.57 pmol/mg, respectively. *Inset*, Scatchard transformation of the same data, with *symbols* representing specific binding. This experiment was repeated three times with similar results. B, Agonist competition for ^{125}I-AB-MECA binding. Concentration of agonist is shown on the *x*-axis, and ^{125}I-AB-MECA was present at a concentration of 1.1 nM. The amount of ^{125}I-AB-MECA binding was normalized and expressed as percentage. The best fit lines for (*R*)-PIA and NECA were superimposable. In this experiment, K_i values for cyclohexyl-NECA (*CH-NECA*), benzyl-NECA, (*R*)-PIA, and NECA were 21.8 nM, 50.9 nM, 123.3 nM, and 130.6 nM, respectively. Similar experiments were performed five to eight times.

TABLE 1

Agonist competition for ^{125}I-AB-MECA binding

Binding was performed using two different cell types that contain the A_3AR. A_3-CHO represents CHO cells stably transfected with the rat A_3AR cDNA. K_i values represent the mean ± standard error. The number of times each experiment was performed is given in parentheses.

Ligand	K_i	
	A_3-CHO	RBL-2H3
	nM	
Cyclohexyl-NECA	33.1 ± 10.1 (5)	ND[a]
Benzyl-NECA	43.7 ± 3.6 (5)	ND
(*R*)-PIA	217.9 ± 42.7 (8)	166.0 ± 40.0 (3)
NECA	260.5 ± 36.1 (8)	223.3 ± 37.0 (3)

[a] ND, not determined.

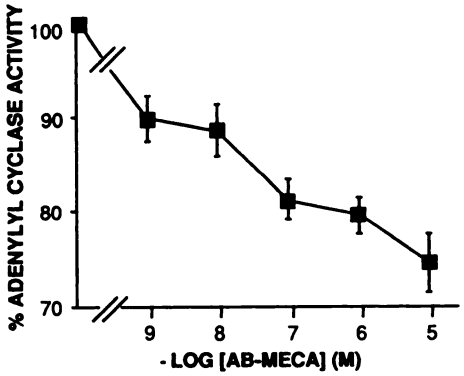


Fig. 3. Inhibition of forskolin-stimulated adenylyl cyclase activity by AB-MECA. The preparation of membranes from CHO cells stably expressing the rat A_3AR and conditions of the assay are described in the text. Forskolin was present at a concentration of 1 μM, which typically produced a 6–8-fold stimulation of adenylyl cyclase activity (100% response). Each *point* represents the mean ± standard error of three experiments, each performed in duplicate.

to mask ^{125}I-AB-MECA binding by other AR subtypes, while A_3AR binding of the radioligand is unaffected. K_i values for XAC of 4.6 nM and 50 nM for the purified rat brain A_1AR (19) and rat striatal A_{2a}AR (20), respectively, have been reported. Therefore, ^{125}I-AB-MECA saturation curves were constructed in the presence of 1 μM XAC using membranes prepared from

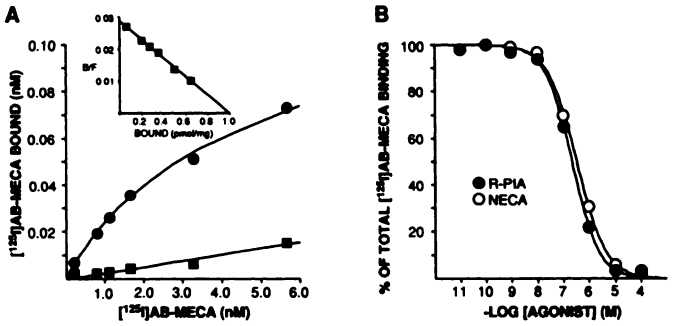


Fig. 4. Binding of ^{125}I-AB-MECA to membranes prepared from RBL-2H3 cells. Membrane preparation and radioligand binding procedures are described in the text. A, Binding isotherm of ^{125}I-AB-MECA. ●, Total binding; ■, nonspecific binding, which was defined with 10 μM (*R*)-PIA. *Points* represent the mean of duplicate samples. Computer analysis of this experiment yielded K_d and B_{max} values of 2.99 nM and 0.99 pmol/mg, respectively. *Inset*, Scatchard transformation of the same data, with the *symbols* representing specific binding. The experiment was repeated four times with similar results. B, Agonist competition for ^{125}I-AB-MECA binding. Concentration of agonist is shown on the *x*-axis, and ^{125}I-AB-MECA was present at a concentration of 1.1 nM. The amount of ^{125}I-AB-MECA binding was normalized and expressed as percentage. In this experiment, K_i values for (*R*)-PIA and NECA were 140.0 nM and 240.0 nM, respectively. This experiment was performed three times.

COS-7 cells expressing either the A_1AR or the A_{2a}AR and from RBL-2H3 cells. In three experiments, binding of ^{125}I-AB-MECA to the A_1AR was virtually abolished by coincubation with 1 μM XAC. In general, coincubation of the A_{2a}AR with XAC resulted in complete inhibition of ^{125}I-AB-MECA binding at lower concentrations of the radioligand, whereas binding was reduced ~50–75% at the maximum concentration (~4 nM) used. Conversely, in the presence of 1 μM XAC the affinity of ^{125}I-AB-MECA for the RBL-2H3 cell A_3AR was unchanged and the B_{max} value was decreased by only 5.7 ± 0.9% (three experiments).

To extend the characterization of ^{125}I-AB-MECA as a high affinity A_3AR ligand and to further compare the rat A_3AR with the recently cloned sheep A_3AR (5), the binding of the radioligand to membranes from COS-7 cells transiently expressing the sheep A_3AR was examined. ^{125}I-AB-MECA bound with K_d

and B_{max} values of 4.36 ± 0.48 nM and 1.35 ± 0.10 pmol/mg, respectively (six experiments). Thus, ^{125}I-AB-MECA has an affinity for the sheep A_3AR similar to that of N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine (K_d = 6 nM), which was the radioligand used to initially characterize this receptor (5). The high affinity binding of ^{125}I-AB-MECA does not appear to be limited to the rat A_3AR, and this compound may be a radioligand of choice for receptors of this subtype. Although it possesses slightly higher affinity for the rat versus sheep A_3AR, ^{125}I-AB-MECA would not appear useful for the further definition of these two receptors as unique AR subtypes or as species homologs. The lack of discrimination demonstrated by ^{125}I-AB-MECA is certainly not apparent for other classical AR ligands such as cyclopentyladenosine and several members of the xanthine antagonist series. Thus, these compounds would be preferred for the characterization of ARs constituting the A_3AR subtype.

In summary, this report characterizes ^{125}I-AB-MECA as an agonist radioligand for the rat A_3AR. ^{125}I-AB-MECA has much greater affinity for the A_3AR than does ^{125}I-APNEA, which had previously been the most useful radioligand for the study of this recently described AR subtype. The availability of ^{125}I-AB-MECA should permit better analysis of the tissue distribution of the A_3AR via radioligand binding assays and autoradiography. The high affinity of ^{125}I-AB-MECA for the A_3AR will be useful in future studies of receptor regulation and in the study of the structural requirements of AR-ligand interactions.

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Send reprint requests to: Gary L. Stiles, Duke University Medical Center, Division of Cardiology, P.O. Box 3444, Durham, NC 27710.