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ACCELERATED COMMUNICATION

^{125}I-4-(2-[7-Amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol, a High Affinity Antagonist Radioligand Selective for the A_{2a} Adenosine Receptor

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SUMMARY

The A_{2a} adenosine receptor (AR) mediates several important physiological effects of adenosine, including vasodilation and inhibition of platelet aggregation. Until recently, no antagonist radioligand of sufficient selectivity or affinity was available. We describe the synthesis and characterization by radioligand binding of ^{125}I-4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}-{1,3,5}triazin-5-yl-amino]ethyl)phenol (^{125}I-ZM241385) in membranes from two cell types that express A_{2a} ARs. Membranes from Chinese hamster ovary (CHO) cells expressing a recombinant canine A_{2a} AR bound ^{125}I-ZM241385 with high affinity, and agonist competition experiments with 2-(*p*-carboxyethyl)-phenylamino-5′-*N*-carboxamidoadenosine, 5′-*N*-ethylcarboxamidoadenosine, and (−)-N^6-[(*R*)-phenylisopropyl]adenosine revealed a potency order characteristic of an A_{2a} AR binding site. Membranes from bovine striatum, which contain a native A_{2a} AR, also bound ^{125}I-ZM241385 with similarly high affinity and also displayed a pharmacological profile for displacement of radioligand binding that was consistent with that of an A_{2a} AR. Also, under conditions in which ^{125}I-ZM241385 bound with high affinity to a recombinant rat A_{2a} AR expressed in CHO cells, no specific binding was detectable in membranes from CHO cells expressing functional rat A_1, A_{2b}, or A_3 ARs, indicating that over the range of concentrations used in radioligand binding assays, ^{125}I-ZM241385 is a highly selective antagonist radioligand for study of A_{2a} ARs within a given species.

The multiple physiological effects of adenosine are mediated by its binding to specific cell-surface receptors. Biochemical and molecular cloning studies have identified four such receptors, termed A_1, A_{2a}, A_{2b}, and A_3. The A_2 ARs are distinguished by their ability to interact with G_s and stimulate adenylyl cyclase activity (1, 2). However, just as characterization and purification of A_1 ARs have been greatly facilitated by the development of specific antagonist ligands, characterization of the A_2 ARs has been hindered by the dearth of similarly useful compounds. Only in the past 5 years have any selective agonist radioligands been developed for the study of A_{2a} ARs (3–5) and, until recently, high-affinity selective antagonists did not exist. Some A_{2a}-selective antagonists have been developed, such as the triazoloquinoxaline 4-amino-8-chloro-1-phenyl[1,2,4]triazolo[4,-3a]quinoxaline (6) and 8-styryl-substituted 1,3,7-alkylxanthines (7, 8). One of the latter class of compounds, KF17837, has been produced in a tritiated form and can bind with reasonable affinity to A_{2a} ARs in rat striatum (9). However, this compound has been reported to be light sensitive, and the resulting product exhibits a reduced affinity for the A_{2a} AR (8, 9). Also, for the study of A_{2a} ARs in tissues expressing low levels of functional receptors, such as platelets or cells of myocardial origin (10), an iodinated radioligand with high specific activity would be desirable. Therefore, we used the recently developed, highly A_{2a} AR-selective

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ABBREVIATIONS: AR, adenosine receptor; CHO, Chinese hamster ovary; ZM241385, 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol; CGS21680, 2-(*p*-carboxyethyl)phenylamino-5′-*N*-carboxamidoadenosine; KF17837, 1,3-dipropyl-7-methyl-(3,4-dimethoxystyryl)xanthine; HPLC, high performance liquid chromatography; NECA, 5′-*N*-ethylcarboxamidoadenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

antagonist ZM241385 (11) to synthesize, purify, and characterize the first high affinity, iodinated antagonist radioligand selective for A_{2a} ARs.

Experimental Procedures

Materials. NECA was the generous gift of Dr. Ray Olsson (University of South Florida, Tampa, Florida). (−)-N^6-[(*R*)-phenylisopropyl]adenosine and adenosine deaminase were obtained from Boehringer-Mannheim. CGS21680 was purchased from Research Biochemicals International. Carrier-free $Na^{125}I$ (specific activity = 2200 Ci/mmol) was from Amersham International. Sources of other materials have been described previously (12, 13).

Receptor cDNAs and expression. Recombinant ARs were expressed in CHO cells; these cells have since been demonstrated to be devoid of ARs as determined by radioligand binding and adenylyl cyclase assays (12–14). CHO cells stably expressing rat A_3 ARs under the control of a cytomegaloviral promoter have been described and characterized previously (12, 13). The rat A_{2a} AR cDNA (15) was subcloned as a *Hin*dIII/*Xba*I fragment into similarly digested pCMV5, and the resulting construct was used to transiently transfect CHO cells using a DEAE-Dextran procedure. For expression of the rat A_1 and A_{2b} ARs, CHO cells were transiently transfected with pcDNA/rat A_{2b} AR and pCMV5/rat A_1 AR constructs that have been previously described (12, 16). For expression of the canine A_{2a} AR, a *Hin*dIII/*Xba*I fragment encoding the cDNA was subcloned into the polylinker region of similarly digested pM_2N expression vector (17), which was generously donated by Dr. Simon Cook, Onyx Pharmaceuticals, Richmond, California. This vector contains both a *neo* gene, to facilitate selection of stable clones, and a modified murine metallothionin promoter with several additional metal-responsive elements, to provide optimal heavy metal inducibility of any introduced gene. Generation of cell lines with stably incorporated pM_2N/A_{2a} AR was achieved by transfection of CHO cells using a modified calcium phosphate precipitation-glycerol shock procedure followed by selection in G418-containing media. Resistant clones were isolated, expanded, and assayed for heavy metal induction of [^{3}H]CGS21680 binding in isolated membrane preparations. One cell line (CHOΔA_{2a}) was expanded and used for further experiments. A control cell line (CHOΔ*neo*) containing the pM_2N cDNA with no insert was also generated. Optimal induction of A_{2a} AR expression was achieved by incubating transfected CHO cells at ~80% confluence with 100 μM $ZnCl_2$ and 2 μM $CdCl_2$ for 48 hr, with replacement of the media ~16 hr before cell harvest.

Synthesis and radioiodination of ZM241385. The characterization of ZM241385 as an A_{2a} AR-selective antagonist has been described previously (11). ZM241385 was synthesized by the addition of 2.74 g of 4-(aminoethyl)phenol to a stirred suspension of 1.4 g of 7-amino-2-(2-furyl)-5-methylsulfonyl-[1,2,4]triazolo-[1,5-a][1,3,5]triazine in acetonitrile and being mixed overnight. After evaporation of the solvent, the residue was purified by chromatography over silica (100 g), eluting with dichloromethane containing 50% (v/v) methanol. The resulting solid (1.23 g) was crystallized from ethyl acetate to give the pure final product (m.p. 225–227°; elemental analysis for $C_{16}H_{15}N_7O_2$: formula weight, 337; calculated, C, 57.0; H, 4.5; N, 29.1%; found, C, 56.7; H, 4.6; N, 29.4%). Nuclear magnetic resonance spectra of ZM241385 solutions were consistent with the predicted structure (Fig. 1).

For iodination, 0.1 mg of ZM241385 was dissolved in 1 ml of methanol and 10 μl was taken to dryness under nitrogen. After resuspension in 40 μl of 0.3 M NaH_2PO_4, pH 7.5, 1.5 mCi $Na^{125}I$ was added, followed by 10 μl of 1 mg/ml chloramine T. After incubation at room temperature for 2 min, the reaction was stopped by the addition of 25 μl of 2 mg/ml sodium metabisulfite. Separation of ^{125}I-ZM241385 from the parent compound was achieved by application of the iodination mixture to a Waters 501 HPLC system. Resolution was achieved by reverse-phase HPLC using a 60% (v/v) methanol/40% (v/v) 20 mM ammonium formate, pH 8.0, mobile phase, and a C18 μBondapak column at a flow rate of 0.75 ml/min. The ^{125}I-ZM241385 peak was defined by measurement of UV absorbance and γ radiation. Under these conditions, ^{125}I-ZM241385 (elution time = 14.8 min) was completely resolved from the starting material (elu-

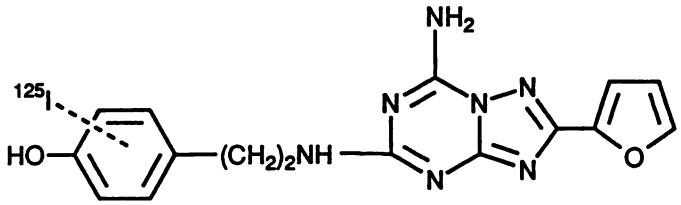

Fig. 1. Chemical structure of ^{125}I-ZM241385. The iodination of the parent compound and purification of the radioligand are described in Experimental Procedures.

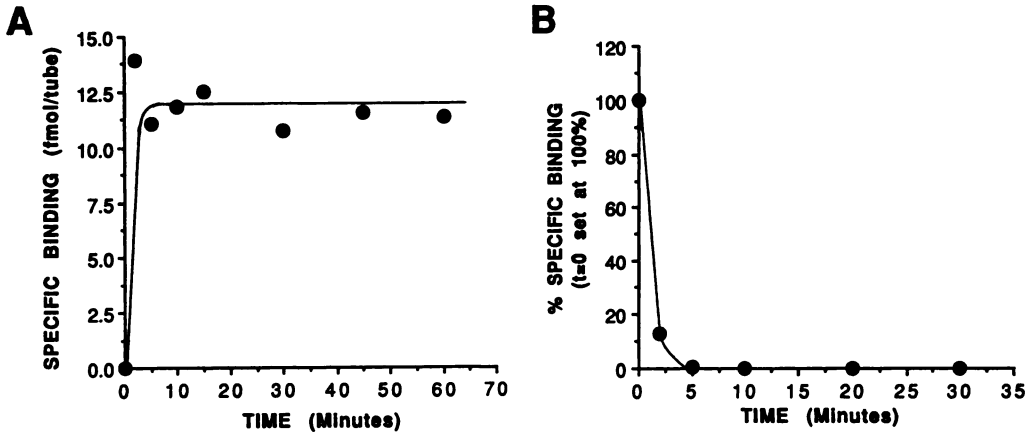

Fig. 2. Association and dissociation kinetics of ^{125}I-ZM241385 binding to membranes from CHO cells expressing the canine A_{2a} AR. A, Membranes from CHO cells expressing the canine A_{2a} AR were incubated with 1 nM ^{125}I-ZM241385 at 37° for the indicated times before separation of bound radioligand from free by vacuum filtration over glass-fiber filters as described in Experimental Procedures. Nonspecific binding was determined using 50 μM NECA. This represents one of two experiments that produced quantitatively similar results. B, Membranes from CHO cells expressing the canine A_{2a} AR were incubated with 1 nM ^{125}I-ZM241385 at 37° for 30 min, at which time NECA was added to a concentration of 50 μM. Samples were then processed at the indicated times after the addition by vacuum filtration as described in A. Data are expressed as a percentage of the specific binding observed before the addition of NECA at t = 0. This represents one of two experiments that produced quantitatively similar results.

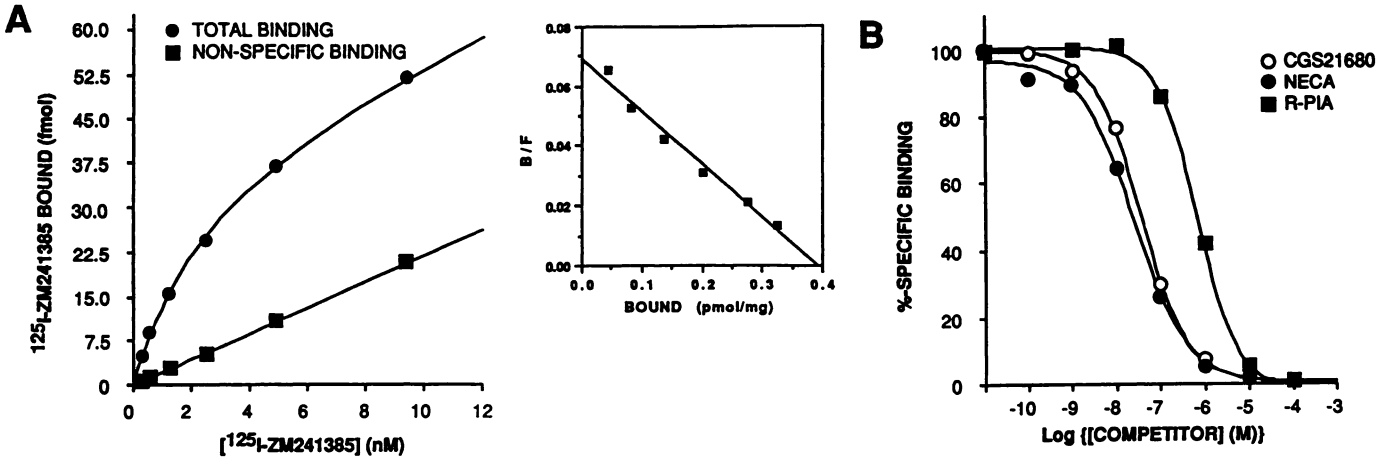

Fig. 3. Binding of ^{125}I-ZM241385 to membranes from CHO cells expressing the canine A_{2a} AR. Membranes were prepared from CHOΔA_{2a} cells after induction with $ZnCl_2$/$CdCl_2$ for use in radioligand binding assays as described in Experimental Procedures. A, Saturation isotherm for ^{125}I-ZM241385 binding. *Inset*, Scatchard transformation of the specific binding data from the same experiment. This represents one of three experiments, data from which are given in Results and Discussion. B, Agonist competition for ^{125}I-ZM241385 binding. ^{125}I-ZM241385 was present at a concentration of 0.5 nM. Specific binding of radioligand at this concentration accounted for 85% of the total binding. The amount of specific binding has been normalized and expressed as a percentage. Pooled data from three such experiments are given in Table 1.

tion time = 8.9 min). Therefore, the specific activity of ^{125}I-ZM241385 was assumed to be 2200 Ci/mmol.

Membrane preparation and radioligand binding. Membranes were prepared from bovine striatum and stored at −80° in 1-ml aliquots as previously described (4). For use in radioligand binding, an aliquot of membranes was thawed and added to 9 ml of binding buffer (50 mM HEPES, pH 6.8, 10 mM $MgCl_2$) containing 0.3 unit/ml adenosine deaminase and incubated at 37° for 15 min. After centrifugation, membranes were resuspended in 9 ml of binding buffer containing 0.1 unit/ml adenosine deaminase with a motor-driven Teflon pestle in a Potter-Elvehjem homogenizer for use in radioligand binding assays. Membranes were prepared from CHO cells by scraping of the cells into 5 ml of lysis buffer (10 mM HEPES, pH 7.5, 5 mM EDTA) after cell monolayers were washed several times with ice-cold buffer. After Dounce homogenization on ice (20 strokes), membranes were pelleted by centrifugation and similarly resuspended in binding buffer supplemented with 0.3 unit/ml adenosine deaminase for immediate use in radioligand binding assays.

Binding studies were performed in a 250-μl reaction volume containing 150 μl of membrane suspension, 50 μl of radioligand, and 50 μl of water or competing ligand. Incubations were carried out for 1 hr at 37° with agitation and were terminated by vacuum filtration over 0.3% (v/v) polyethylimine-treated glass-fiber filters and rapid washing with ice-cold binding buffer containing 0.03% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate using a Brandel cell harvester. For saturation analysis of ^{125}I-ZM241385 binding, radioligand concentrations ranging from 0.25 nM to 4–8 nM were used. Nonspecific binding was defined by the inclusion of 50 μM NECA. Saturation and competition curves were analyzed by a previously validated computer-assisted curve-fitting program (18). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng-Prusoff equation (19). Data are presented as mean ± standard error for the number of experiments indicated.

Adenylyl cyclase assays were performed on isolated membranes as previously described (12).

Results and Discussion

ZM241385 has been demonstrated previously to be a highly selective antagonist ligand for blockade of A_{2a} ARs(11). Specifically, it exhibits 80-fold selectivity for A_{2a} ARs versus A_{2b} ARs in functional assays, as well as 500-1000-fold and 500,000-fold selectivities for A_{2a} ARs versus A_1 and A_3 ARs, respectively, in radioligand binding assays (11). Inspection of the chemical structure also revealed that it was amenable to radioiodination, as shown in Fig. 1, due to the presence of a phenolic functional group. Such a radioligand would potentially be of great use for the study of A_{2a} ARs. Therefore, ^{125}I-ZM241385 was synthesized, purified by HPLC (see Experimental Procedures), and characterized with respect to its ability to bind to recombinant AR subtypes. Fig. 2A demonstrates that ^{125}I-ZM241385 bound very rapidly to membranes from CHO cells expressing a recombinant canine A_{2a} AR. Steady state appeared to be reached as early as the first time point examined (2 min) and was sustained for at least 60 min (Fig. 2A). Binding was rapidly reversed on the addition of NECA to a concentration of 50 μM, with >80% dissociation being evident as early as a 2-min incubation (Fig. 2B). Fig. 3A demonstrates that ^{125}I-ZM241385 bound to a single saturable high affinity site in membranes from CHO cells expressing a recombinant canine A_{2a} AR, exhibiting K_d and B_{max} values of 1.62 ± 0.49 nM and 0.47 ± 0.07 pmol/mg protein, respectively (three experiments). In addition, competition experiments demonstrated that ^{125}I-ZM241385 binding was displaced by agonist ligands

TABLE 1

Competition for ^{125}I-ZM241385 binding

Competition binding was performed as described in Experimental Procedures on membranes prepared from bovine striatum and from CHOΔA_{2a} cells after induction of receptor expression by incubation with 100 μM $ZnCl_2$ and 2 μM $CdCl_2$ for 48 hr. Data are presented from three separate experiments.

Competing ligand	Bovine striatum		CHOΔA_{2a}	
	K_i	n_H	K_i	n_H
	nM		nM	
Agonist				
CGS21680	41.5 ± 10.9	0.73 ± 0.05	33.0 ± 6.2	0.82 ± 0.04
NECA	116.1 ± 18.0	0.79 ± 0.04	28.7 ± 7.2	0.83 ± 0.10
R-PIA	2244 ± 889	0.97 ± 0.27	725 ± 135	1.01 ± 0.02
Antagonist				
XAC	153.5 ± 63.9	1.16 ± 0.04	53.0 ± 22.2	0.99 ± 0.01
BW1433	86.3 ± 8.2	0.88 ± 0.07	79.9 ± 25.0	1.02 ± 0.05

BW1433, 1,3-dipropyl-8-(4-acrylate)phenylxanthine; XAC, xanthine amine congener.

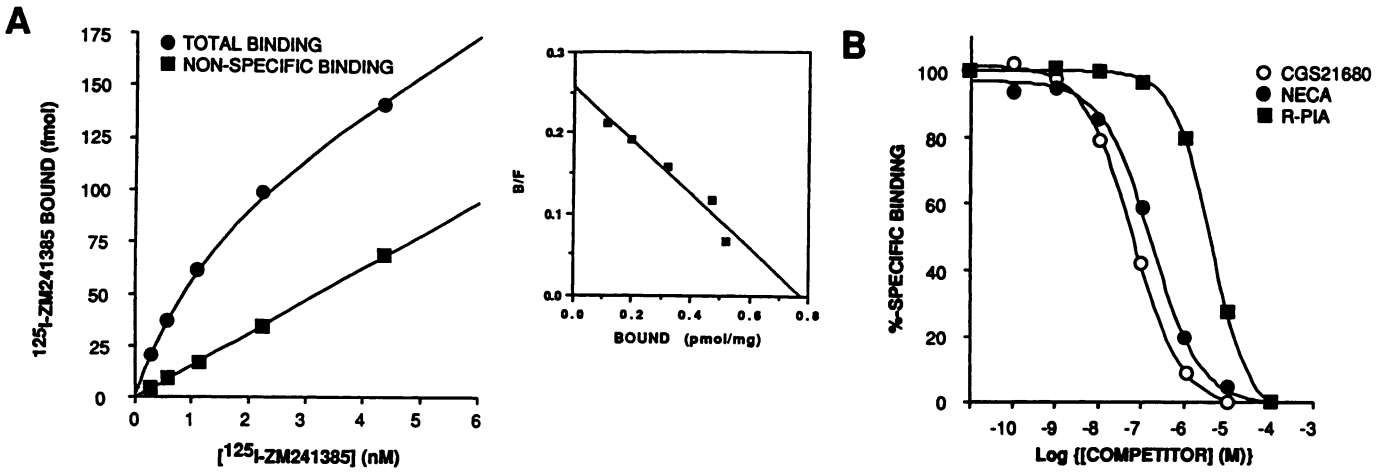

Fig. 4. Binding of ^{125}I-ZM241385 to membranes from bovine striatum. Membranes were prepared from bovine striatum for use in radioligand binding assays as described in Experimental Procedures. A, Saturation isotherm for ^{125}I-ZM241385 binding. *Inset*, Scatchard transformation of the specific binding data from the same experiment. This represents one of three experiments, data from which are given in Results and Discussion. B, Agonist competition for ^{125}I-ZM241385 binding. ^{125}I-ZM241385 was present at a concentration of 0.5 nM. Specific binding of radioligand at this concentration accounted for 70% of the total binding. The amount of specific binding has been normalized and expressed as a percentage. Pooled data from three such experiments are given in Table 1.

with a pharmacological profile consistent with that of an A_{2a} AR, i.e., CGS21680 and NECA were significantly more potent than (−)-N^6-[(*R*)-phenylisopropyl]adenosine, and that the receptor exhibited a lower affinity for xanthine antagonists compared with A_1 ARs (1, 2) (Fig. 3B and Table 1). No specific binding was observed in membranes from the negative control CHOΔ*neo* cell line (data not shown).

To determine the use of ^{125}I-ZM241385 in identifying A_{2a} ARs in cells that may express multiple AR subtypes, radioligand binding was performed in membranes from CHO cells expressing rat AR subtypes. Similar to the results obtained with the expressed canine A_{2a} AR, CHO cells transiently transfected with a rat A_{2a} AR cDNA bound ^{125}I-ZM241385 with high affinity (K_d = 0.66 ± 0.03 nM, B_{max} = 2.67 ± 0.10 pmol/mg protein; three experiments). However, over the range of ^{125}I-ZM241385 concentrations capable of labeling A_{2a} ARs (0.25–8 nM), no specific binding to rat A_1 or A_3 ARs could be detected despite the ability to detect these receptors by [^{3}H]1,3-dipropyl-8-cyclopentylxanthine and ^{125}I-4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine binding, respectively (data not shown). In addition, we failed to detect any specific binding of a similar range of concentrations of ^{125}I-ZM241385 to membranes from CHO cells transiently transfected with a rat A_{2b} AR cDNA (data not shown). This was not due to the lack of expression of functional receptor as parallel adenylyl cyclase assays on the same membranes preparations demonstrated that 50 μM NECA could produce a 2.6 ± 0.1-fold stimulation of activity above basal (three experiments). Therefore, under our assay conditions, no specific binding of ^{125}I-ZM241385 could be detected to A_1, A_{2b}, or A_3 ARs, indicating that this radioligand is highly selective for identification of A_{2a} ARs within a given species.

To be useful as a radioligand, ^{125}I-ZM241385 should be capable of labeling A_{2a} ARs expressed endogenously by a given cell type as well as recombinant proteins. Therefore, ^{125}I-ZM241385 binding was performed on membranes from bovine striatum, the A_{2a} AR of which has previously been well characterized by ^{125}I-2-(4-[2-[(4-aminophenyl)methylcarbonyl]ethyl]phenyl)ethylamino-5′-*N*-ethylcarboxamidoadenosine and [^{3}H]CGS21680 binding (4, 20). Saturation analysis revealed that ^{125}I-ZM241385 bound to a single saturable high affinity site, with K_d and B_{max} values of 1.39 ± 0.39 nM and 0.72 ± 0.08 pmol/mg protein, respectively (three experiments) (Fig. 4A). Therefore, the K_d values exhibited by the native bovine A_{2a} AR and the recombinant canine receptor are essentially identical. Also, agonist competition for ^{125}I-ZM241385 binding exhibited the characteristic profile of an A_{2a} AR (Fig. 4B and Table 1) and was very similar to that of the recombinant canine A_{2a} AR (Fig. 3B and Table 1). The slight differences in the K_i values for some of the competing ligands between the bovine and canine receptors probably reflect species-dependent differences that have also been observed for displacement of agonist radioligand binding at A_{2a} ARs (21).

In conclusion, we synthesized and characterized a selective antagonist radioligand for the study of the A_{2a} AR. Although another antagonist radioligand, [^{3}H]KF17837S, has been described for this receptor (9), its usefulness in detecting the low levels of A_{2a} ARs expressed in many tissues may be limited due to its lower specific activity and light sensitivity. Because of its excellent selectivity and high affinity and the facile nature of the synthesis, which results in its high specific activity, ^{125}I-ZM241385 would be more suitable for these purposes. In addition, the availability of an antagonist radioligand can facilitate a more detailed investigation of the atypical coupling of the A_{2a} AR to G_s that we and others have noted (5, 22, 23).

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