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# $^{125}$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



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(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 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 (CHOΔ$A_{2a}$) 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 $\mu$M $ZnCl_2$ and 2 $\mu$M $CdCl_2$ 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$\mu$l was taken to dryness under nitrogen. After resuspension in 40$\mu$l of 0.3 M $NaH_2PO_4$, pH 7.5, 1.5 mCi $Na^{125}I$ was added, followed by 10 $\mu$l of 1 mg/ml chloramine T. After incubation at room temperature for 2 min, the reaction was stopped by the addition of 25 $\mu$l 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 $\mu$Bondapak column at a flow rate of 0.75 ml/min. The $^{125}$I-ZM241385 peak was defined by measurement of UV absorbance and $\gamma$ radiation. Under these conditions, $^{125}$I-ZM241385 (elution time = 14.8 min) was completely resolved from the starting material (elution 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-$\mu$l reaction volume containing 150 $\mu$l of membrane suspension, 50 $\mu$l of radioligand, and 50 $\mu$l 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 $\mu$M 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 $\mu$M, 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 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-amino-benzyl-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 $\mu$M 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)methyl-carbonyl)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).

## Acknowledgments

We thank Drs. Scott Rivkees and Steven Reppert for rat $A_1$ $A_{2a}$, and $A_{2b}$ adenosine receptor cDNAs; Dr. Simon Cook for providing p$M_2$N; Mary Pound for technical assistance; and Linda Scherich for preparation of the manuscript.

## 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 |

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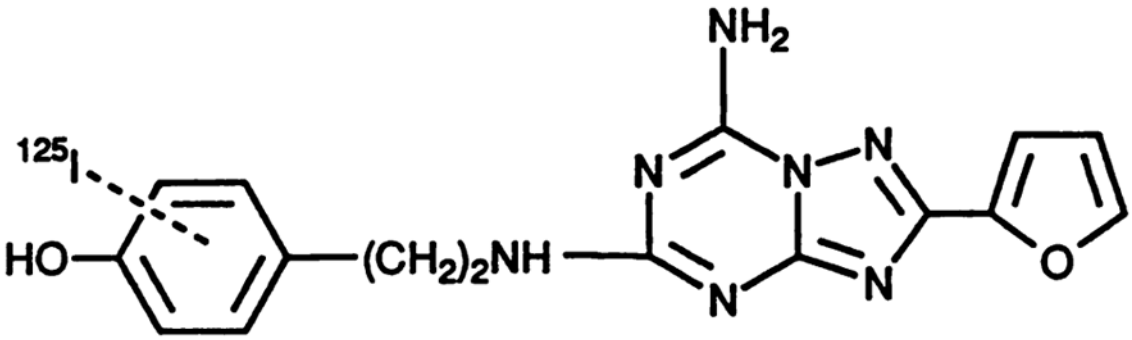


**Fig. 1.**
Chemical structure of $^{125}$I-ZM241385. The iodination of the parent compound and purification of the radioligand are described in Experimental Procedures.

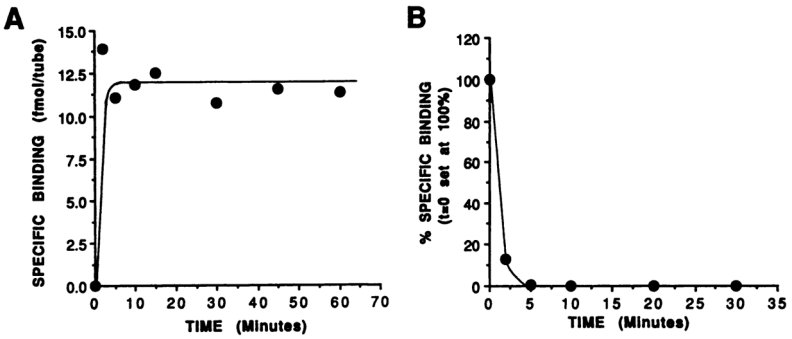


**Fig. 2.**

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 $\mu$M 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 $\mu$M. 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.

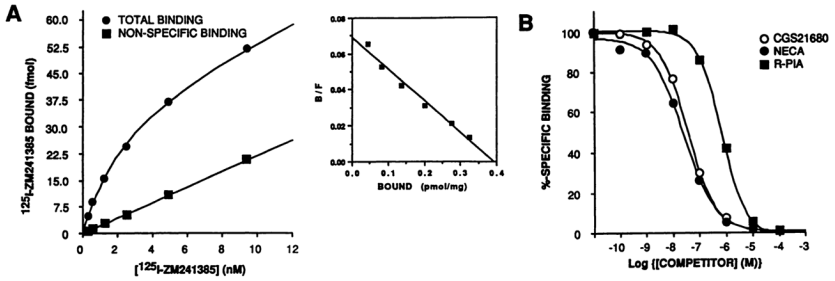


**Fig. 3.**

Binding of $^{125}$I-ZM241385 to membranes from CHO cells expressing the canine $A_{2a}$ AR. Membranes were prepared from CHO$\Delta A_{2a}$ 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.

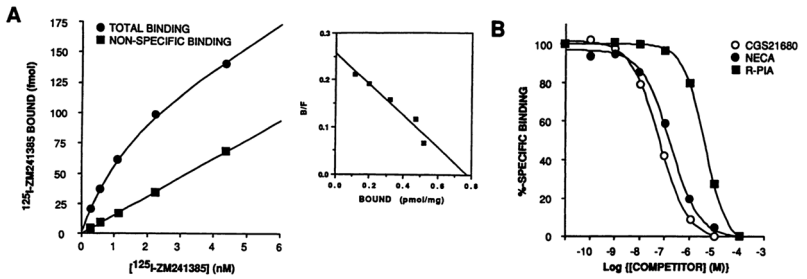


**Fig. 4.**

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.

**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 CHOΔ$A_{2a}$ cells after induction of receptor expression by incubation with 100 $\mu$M $ZnCl_2$ and 2 $\mu$M $CdCl_2$ for 48 hr. Data are presented from three separate experiments.

| Competing ligand | Bovine striatum | | CHOΔ$A_{2a}$ | |
|---|---|---|---|---|
| | $K_i$ | $n_M$ | $K_i$ | $n_M$ |
| | *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.