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Phenoxybenzamine and benextramine, but not 4-DAMP mustard, display irreversible non-competitive antagonism at G protein-coupled receptors

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Irreversible non-competitive antagonism

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Non-standard abbreviations

4-DAMP mustard, 4-diphenylacetoxy-*N*-[2-chloroethyl]piperidine hydrochloride; 5-HT, 5-hydroxytryptamine; 5-HT_{2A}-SH-SY5Y, cultured SH-SY5Y cells expressing 5-HT_{2A} receptors; α_{2A}-AH, α_{2A}-adrenoceptors expressed at relative high numbers; α_{2A}-AL, α_{2A}-adrenoceptors expressed at relative low numbers; CHO, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMEM, minimum essential medium (Earle's base); GPCR, G protein-coupled receptor; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid]; IBMX, 3-isobutyl-1-methylxanthine; IP_x, total inositol phosphates; mACh receptor, muscarinic acetylcholine receptor; PBS, phosphate buffered saline; PLC, phospholipase C; SH-SY5Y, cultured human neuroblastoma cell line; PTX, pertussis toxin; SQ 30,741, [1*S*-[1α,2α(5*Z*),3α,4α]]-7-[[[[[(oxaheptyl)amino]acetyl]amino]-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; TCA, trichloroacetic acid; U-73122, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione; UK 14,304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (brimonidine); UltraMEM, reduced serum minimum essential medium.

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ABSTRACT

Many irreversible antagonists have been shown to inactivate G protein-coupled receptors (GPCRs) and employed to study agonists and spare receptors. Presumably, they bind to primary (agonist) binding sites on the GPCR, although non-competitive mechanisms of antagonism have been demonstrated, but not thoroughly investigated. We studied non-competitive antagonism by phenoxybenzamine and benextramine at α_{2A}-adrenoceptors in stably transfected Chinese hamster ovary cells, benextramine and 4-DAMP mustard at endogenous muscarinic acetylcholine (mACh) receptors in human neuroblastoma SH-SY5Y cells, and benextramine at serotonin 5-HT_{2A} receptors in stably transfected SH-SY5Y cells. Primary binding sites were protected by reversible competitive antagonists during pre-treatment with irreversible antagonists. We conducted appropriate radioligand binding assays by measuring remaining primary binding sites and agonist affinity, functional assays to evaluate agonist-induced responses, and constitutive [^{35}S]GTPγS-Gα_o binding assays to determine remaining G protein activity. Phenoxybenzamine (100 μM; 20 minutes) and benextramine (10 μM or 100 μM; 20 minutes) at α_{2A}-adrenoceptors, but not 4-DAMP mustard (100 nM; 120 minutes) at mACh receptors, displayed irreversible non-competitive antagonism in addition to their known irreversible competitive antagonism. While agonist binding affinity is not influenced, signal transduction is modulated in a G protein-dependent manner via allotopic interactions. Benextramine non-competitively inhibits agonist-induced responses at three different GPCR types (α_{2A}-, mACh and 5-HT_{2A}-receptors), that signal via three families of G proteins ($G_{i/o}$, G_s and $G_{q/11}$). We conclude that, where irreversible antagonists are utilized to study drug-receptor interaction mechanisms, the

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presence of significant irreversible non-competitive antagonism may influence the interpretation of results under the experimental conditions employed.

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INTRODUCTION

Irreversible competitive antagonists are generally accepted to bind pharmacological receptors irreversibly, presumably by forming strong covalent bonds with the receptor. Thereby they prevent other ligands from binding to the primary (orthosteric) binding site(s) on these receptors. [The reader may consult Neubig et al. (2003) regarding specific terminology]. The classical irreversible α-adrenoceptor blocking drug phenoxybenzamine (Dibenzyline®) has been used since the 1960's in the clinical setting to treat pheochromocytoma (Crago et al., 1967). Most irreversible competitive antagonists, however, found application in experimental pharmacology to investigate and eliminate spare receptors, implementing the Furchgott analysis (Furchgott, 1966) to estimate the relative intrinsic efficacy of agonists and the apparent equilibrium dissociation constants of agonist-receptor complexes (Adham et al., 1993; Agneter et al., 1993; Herepath and Broadley, 1990; Kenakin, 1997; Koek et al., 2000; Morey et al., 1998; Tian et al., 1996; Zhu, 1993). Irreversible antagonists have also been employed to label and count receptor subtypes, to investigate drug and receptor specificity and receptor structure (Jenkinson, 2003), to investigate receptor trafficking (McKernan et al., 1988; Taouis et al., 1987), and to unravel drug action mechanisms (Timmermans et al., 1985). Selective inactivation of 'unwanted' pharmacological receptor subtypes, while protecting the receptors of interest with a highly selective reversible competitive antagonist, has also been implemented to study and characterize receptors (Eglen et al., 1994; Hieble et al., 1985).

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Phenoxybenzamine is known to irreversibly bind to α-adrenergic, H_1-histamine and muscarinic acetylcholine (mACh) receptors (Timmermans et al., 1985; Eglen et al., 1994; Amobi and Smith, 1995; Frang et al., 2001; Giardinà et al., 1995; Giardinà et al., 2002; Ruffolo and Hieble, 1999; Van der Graaf and Danhof, 1997; Van der Graaf and Stam, 1999). Benextramine has been known to irreversibly bind to α_2-adrenoceptors (Timmermans et al., 1985; Hieble et al., 1985; Belleau et al., 1982b; Brink et al., 2000; Lew and Angus, 1984; McKernan et al., 1988; Melchiorre, 1981; Taouis et al., 1987; Umland et al., 2001), 5-HT_{1A}-serotonergic receptors (Stanton and Beer, 1997), H_2-histaminergic receptors (Belleau et al., 1982a), and neuropeptide Y-receptors (Melchiorre et al., 1994). Present knowledge regarding the mechanism of action of benextramine is limited to the observations from predominantly radioligand binding studies and it is generally assumed that benextramine irreversibly inactivates the ligand (primary or syntopic) binding sites at these receptors. 4-Diphenylacetoxy-*N*-(2-chloroethyl)piperidine (4-DAMP mustard) is known to bind to mACh receptors (Eglen et al., 1994; Umland et al., 2001; Ehlert and Griffin, 1998; Ragheb et al., 2001; Sawyer and Ehlert, 1999; Thomas et al., 1992), with moderate selectivity for M_3-mACh receptors. It does not, however, discriminate between M_1, M_2 or M_4-mACh receptors (Eglen et al., 1994).

The elimination of pharmacological receptors by an irreversible competitive antagonist depends on both the concentration used and the incubation time (Furchgott, 1966; Kenakin, 1997). Phenoxybenzamine has been typically used *in vitro* at concentrations of up to 10 μM for 30 minutes (Piascik et al., 1988), benextramine at

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concentrations of up to 100 μM for 120 minutes (Van der Graaf et al., 1996), and 4-DAMP mustard at concentrations of up to 40 nM for 4 hours (Sawyer and Ehlert, 1999).

Reports in literature suggest that some irreversible competitive antagonists may also display irreversible allosteric interactions (non-competitive antagonism) (Brink et al., 2000; Van der Graaf et al., 1996) by altering agonist binding affinity, modifying agonist-receptor binding kinetics (Van Ginneken, 1977) or even influencing signal transduction mechanisms. However, this has been difficult to prove (Jenkinson, 2003). The irreversible antagonism of benextramine at prostanoid TP-receptors cannot be prevented by the selective prostanoid TP-receptor antagonist [1*S*-[1α,2α(5*Z*),3α,4α]]-7-[[[[[(oxaheptyl)amino]acetyl]amino]-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ 30,741) (Van der Graaf et al., 1996), suggesting that benextramine and SQ 30,741 bind to different binding sites (i.e. benextramine exhibits allotopic interactions at prostanoid TP-receptors). The underlying mechanism, however, remains elusive.

In the present study, we examined three classical irreversible competitive antagonists, namely benextramine at α_{2A}-adrenoceptors and 5-HT_{2A} receptors, phenoxybenzamine at α_{2A}-adrenoceptors, and 4-DAMP mustard at mACh receptors. For any observed non-competitive interactions, the mechanisms were investigated, including possible allosteric interactions, where agonist affinity at the syntopic binding site of the GPCR is altered, or signal transduction is modulated downstream. We have been able to show that benextramine displays irreversible non-competitive antagonism by binding at a site affecting the α_{2A}-adrenoceptor-G_i protein coupling or G protein function, without affecting the GTP-binding capacity of the G_i-related $G\alpha_o$ protein. In

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addition, we have been able to show that benextramine also irreversibly and non-competitively inhibits the signaling of α_{2A}-adrenoceptors through G_s proteins, as well as the signaling of mACh receptors and serotonin 5-HT_{2A} receptors through G_q proteins.

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METHODS

Cell Lines. In this study, cultured cell lines expressing α-adrenoceptors and muscarinic acetylcholine (mACh) receptors, respectively, were employed. Two Chinese hamster ovary (CHO-K1) cell lines, stably transfected to express the wild-type porcine α_{2A}-adrenoceptor at high numbers (cell line denoted α_{2A}-H) and low numbers (cell line denoted α_{2A}-L) respectively, were kindly provided by Dr. Richard Neubig (Department of Pharmacology, University of Michigan, Ann Arbor, MI, U.S.A.). The pharmacological profiles and receptor expression characteristics of the α_{2A}-H and α_{2A}-L cell lines have been previously characterized. The determined α_{2A}-adrenoceptor concentrations were reported as 19 ± 2 pmol/mg membrane protein for α_{2A}-H and as about 1 pmol/mg membrane protein for α_{2A}-L (Brink et al., 2000). We confirmed the high receptor expression for α_{2A}-H in our laboratory, determined as 46 ± 5 pmol/mg membrane protein. These cell lines were used to investigate the mechanisms of antagonism of the irreversible α-adrenoceptor blocking drugs phenoxybenzamine and benextramine.

We also used human neuroblastoma cells (SH-SY5Y, from American Type Culture Collection), that endogenously express predominantly M_3-mACh receptors (Slowiejko et al., 1996), with some evidence for M_1 and M_2-mACh receptors (Kukkonen et al., 1992). The K_D value of [^{3}H]N-methyl scopolamine binding at the endogenous mACh receptors in intact cells was previously reported as 0.2 nM and the B_{max} as 100-150 fmol/mg membrane protein (Lambert et al., 1989). This cell line was employed to investigate the mechanisms of antagonism of the irreversible mACh receptor blocking drug 4-diphenylacetoxy-*N*-(2-chloroethyl)piperidine (4-DAMP mustard). The fourth cell line

employed was SH-SY5Y cells stably transfected to express the human 5-hydroxytryptamine-2A (5-HT_{2A}) receptor, denoted 5-HT_{2A}-SH-SY5Y cells (see transfection protocol below). The human 5-HT_{2A} plasmid cDNA in the pIRES (Neo^r) mammalian expression vector was kindly provided by Dr. Bryan Roth (Department of Biochemistry, Case Western Reserve University, Cleveland, OH, U.S.A.). Human neuroblastoma SH-SY5Y cells were transfected with this vector using DOTAP liposomal transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Since the plasmid encodes for G-418 resistance, the cells were subjected to G-418 (400 μg/ml; Gibco Life Technologies, Gaithersburg, MD, U.S.A.) treatment after 48 hours and the surviving colony was harvested and implemented in this study. Transfected cells were not cloned, but the transfection mix used. Successful transfection was confirmed pharmacologically by dose-response curves with the 5-HT_{2A} receptor agonist serotonin (with control SH-SY5Y cells showing no serotonin-induced response, data not shown) and by the observed K_D value of [^{3}H]spiperone at 5-HT_{2A} receptors (K_D = 8.3 ± 3.0 nM, B_{max} = 3,166 ± 421 receptors/cell) as determined from saturation binding curves, using 10 μM ritanserin to define non-specific binding. This K_D value corresponds with reported K_i values for [^{3}H]spiperone at 5-HT_{2A}-receptors against various radioligands, ranging between 0.12 nM (Roth et al., 1987) and 50.11 nM (Boess et al., 1994). The 5-HT_{2A}-SH-SY5Y cells were employed to determine the effect of benextramine pre-treatment on 5-HT_{2A}-receptors by determining agonist-stimulated, G_q-mediated activation of phospholipase C (PLC), by measuring [^{3}H]IP_x accumulation. These cells were also employed for radioligand binding studies to investigate the effect of benextramine on 5-HT_{2A}-receptor numbers.

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The α_{2A}-L and α_{2A}-H cells were maintained in a humidified environment and grown to 95% confluency in 150 cm^2 cell culture flasks with Ham's F-12 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. The SH-SY5Y cells were similarly maintained but the growth medium used was a 1:1 ratio mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM; Highveld Biological, Gauteng, South Africa) containing 10% bovine serum albumin. The 5-HT_{2A}-SH-SY5Y cells were maintained as the SH-SY5Y cells, but with 400 μg/ml G-418.

Preparation and pre-treatment with irreversible competitive antagonists. Cells were seeded in 24-well plates in preparation for the [^{3}H]cAMP, [^{3}H]IP_x, or ligand binding assays as described below, and maintained for at least 18 hours. The α_{2A}-H and α_{2A}-L cells were seeded at a density of approximately 3×10^5 cells/ml, while SH-SY5Y and 5-HT_{2A}-SH-SY5Y cells were seeded at a density of 6×10^5 cells/ml. When cell pre-treatments were intended for membrane preparation, the pre-treatments were performed directly in the 150 cm^2 culture flasks. Cells attached adequately to the well bottoms, allowing several aspirations and new additions of medium without significant cell loss (confirmed by microscopic observation).

When the experiments were performed, the pre-treatments were initiated by incubating the cells with an appropriate concentration (0 M or a concentration > 1000 × K_D value) of the reversible antagonist (allowing equilibrium of ligand-receptor binding), whereafter the cells were exposed to different concentrations of the appropriate irreversible antagonist plus the reversible antagonist. This was followed by several

rinsing and incubation steps (washing procedure) with phosphate buffered saline (PBS; containing as w/v 0.8% NaCl, 0.02% KCl, 0.09% Na_2HPO_4 and 0.02% KH_2PO_4) and DMEM to remove all unbound and reversibly bound drugs. The following pre-treatment steps were employed:

(i) α_{2A}-L or α_{2A}-H cells were incubated with either 0 or 10 μM of the reversible competitive α_2-adrenoceptor antagonist (Becker et al., 1999) yohimbine hydrochloride (Sigma Chemical, St Louis, MO, U.S.A.) in DMEM for 30 minutes at 37 °C in 5% CO_2, to allow equilibrium of ligand-receptor binding. Likewise, SH-SY5Y cells were incubated with either 0 or 10 μM of the reversible competitive non-selective mACh receptor antagonist (Zwart and Vijverberg, 1997) atropine sulfate (Sigma Chemical, St Louis, MO, U.S.A. – reported average K_i = 0.50 nM for M_3-mACh receptors (Hirose et al., 2001)) in DMEM for 30 minutes at 37 °C in 5% CO_2, to allow equilibrium of receptor binding. 5-HT_{2A}-SH-SY5Y cells were incubated with either 0 or 10 μM of the reversible competitive 5-HT_2-receptor antagonist ritanserin (Sigma Chemical, St Louis, MO, U.S.A.; reported average K_i = 0.25 nM for 5-HT_{2A}-receptors by Bonhaus et al., 1997) in DMEM for 30 minutes at 37 °C and 5% CO_2.

(ii) Thereafter, α_{2A}-L or α_{2A}-H cells were correspondingly incubated with either 0 or 10 μM yohimbine plus the indicated concentration of freshly prepared phenoxybenzamine hydrochloride (Sigma Chemical, St Louis, MO, U.S.A.) or benextramine tetrahydrochloride (Sigma Chemical, St Louis, MO, U.S.A.) (0, 1, 10 or 100 μM) for 20 minutes at 37 °C in 5% CO_2. SH-SY5Y cells were correspondingly incubated with either 0 or 10 μM atropine plus the indicated concentration of 4-DAMP

mustard (Sigma Chemical, St Louis, MO, U.S.A.) (0, 10 or 100 nM) for 20 minutes at 37 °C in 5% CO_2. Since 4-DAMP mustard is completely converted to its corresponding active aziridinium ion only after about 30 minutes in aqueous solution (Thomas et al., 1992), it was kept for at least 30 minutes at 37 °C in DMEM before use in the assay. 5-HT_{2A}-SH-SY5Y cells were correspondingly incubated with either 0 or 10 μM ritanserin plus the indicated concentration of freshly prepared benextramine (0, 10 or 100 nM) for 20 minutes at 37 °C in 5% CO_2.

(iii) Cells were then rinsed twice with PBS and incubated twice with DMEM for 20 minutes at 37 °C in 5% CO_2 to allow dissociation of any reversibly bound drugs, whereafter the cells were used for the functional or ligand-binding assays as described below.

Measurement of whole-cell [^{3}H]cAMP accumulation. [^{3}H]cAMP accumulation was determined in whole α_{2A}-L cells in 24-well plates as described previously (Wade et al., 1999; Wong, 1994). Briefly, cells were radiolabeled by adding 1 μCi per well [2-^{3}H]adenine (19-23 Ci/mmol; Amersham Pharmacia Biotech, U.K.), plus 100 ng/ml pertussis toxin (PTX; Sigma Chemical, St Louis, MO, U.S.A.) when indicated, at least 18 hours before the assay. After the washing procedure (as described above), the assay was initiated by adding DMEM with 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical, St Louis, MO, U.S.A.) and 30 μM forskolin (Sigma Chemical, St Louis, MO, U.S.A.) and the appropriate concentration of the full α_{2A}-adrenoceptor (Brink et al., 2000) agonist 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK 14,304; Sigma Chemical, St Louis, MO, U.S.A.) to construct appropriate

semilogarithmic dose-response curves. After a 20 minute incubation time at 37 °C in 5% CO_2, the medium was aspirated and the reaction terminated with 1 ml ice-cold 5% (w/v) trichloroacetic acid (TCA; Sigma Chemical, St Louis, MO, U.S.A.) containing 1 mM ATP (Sigma Chemical, St Louis, MO, U.S.A.) and 1 mM cAMP (Sigma Chemical, St Louis, MO, U.S.A.) and allowed to stand for 30 minutes at 4 °C to lyse the cells. The acid soluble nucleotides were separated on Dowex and Alumina columns as described before (Salomon et al., 1974) and radioactivity determined by liquid scintillation counting. The cAMP accumulation was normalized by dividing the [^{3}H]cAMP counts by the total [^{3}H]nucleotide counts. This value was then divided by the corresponding value obtained in the presence of IBMX and forskolin, without agonist (to calculate percentage of control).

Measurement of whole-cell [^{3}H]IP$_x$ accumulation. [^{3}H]IP$_x$ accumulation was determined in whole SH-SY5Y and 5HT$_{2A}$-SH-SY5Y cells in 24-well plates according to the principles described before (Godfrey, 1992; Casarosa et al., 2001), but with minor modifications. Briefly, cells were labeled by adding 1 μCi per well *myo*-[2-^{3}H]inositol (17 Ci/mmol; Amersham Pharmacia Biotech, U.K.) in inositol-free medium (EMEM, Earle's base + bovine serum albumin) at least 18 hours before the assay. After the pre-treatment and washing procedure (as described above), the [^{3}H]IP$_x$ assay was initiated by adding a mixture of DMEM, 20 mM LiCl (Sigma Chemical, St Louis, MO, U.S.A.), 25 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulphonic acid] (HEPES) (Sigma Chemical, St Louis, MO, U.S.A.) and the appropriate concentration of the full mACh receptor agonist (Olianas and Onali, 1991) methacholine chloride (Sigma Chemical, St Louis, MO, U.S.A.) or the endogenous 5HT$_{2A}$ receptor agonist serotonin creatinine

sulfate (Sigma Chemical, St Louis, MO, U.S.A.) to construct appropriate semilogarithmic dose-response curves. After a 60-minute incubation at 37 °C and 5% CO_2 the medium was aspirated and the reaction terminated with 1 ml ice-cold 10 mM formic acid (Saarchem-Holpro Analytic, Krugersdorp, Gauteng, South Africa) and let to stand for at least 90 minutes at 4 °C to lyse the cells. The [^{3}H]IP_x was separated on Dowex columns (250 µl Dowex 1 × 8-400, 200-400 mesh, 1-chloride form per 2 ml, Bio-Rad Poly-Prep column) and radioactivity determined by liquid scintillation counting. The [^{3}H]IP_x accumulation was expressed as the percentage of the control value measured in the absence of agonist.

Ligand binding assays. We determined the K_D value of [*O*-methyl-^{3}H]yohimbine from radioligand saturation binding experiments in whole α_{2A}-H cells. Non-specific binding was defined with 10 µM yohimbine. We report the K_D value of [*O*-methyl-^{3}H]yohimbine at α_{2A}-adrenoceptors in α_{2A}-H cells as 2.67 nM.

To determine relative receptor numbers in α_{2A}-H, SH-SY5Y and $5HT_{2A}$-SH-SY5Y cells after drug pre-treatments as described above, we performed single dose saturation binding assays. Cells were plated and incubated for at least 18 hours at 37 °C in 5% CO_2 as before, but without a radioligand. The cells were then rinsed once with reduced serum minimum essential medium (UltraMEM), whereafter the assay was initiated by adding UltraMEM with 0 or 10 µM reversible competitive antagonist (to define non-specific binding) plus the appropriate concentration of radioligand. After a 30 minute incubation at 37 °C in 5% CO_2, the medium was aspirated, the cells rinsed twice with ice-cold PBS and the reaction terminated with 1 ml ice-cold 5% (w/v) TCA and let to

stand for at least 30 minutes at 4 °C to lyse the cells. The TCA from each well was then transferred directly into scintillation vials and the radioactivity counted. The relative α_{2A}-adrenoceptor concentrations in α_{2A}-H cells (after the various pre-treatments with the appropriate irreversible competitive antagonists and washing procedure as described above) were determined from specific binding of 5 nM [*O*-methyl-^{3}H]yohimbine (83-92 Ci/mmol; Amersham Pharmacia Biotech, U.K.). Non-specific binding was defined with 10 μM yohimbine. Likewise, the mACh receptor relative concentrations in SH-SY5Y cells were determined from specific binding by 5 nM [*N*-methyl-^{3}H]4-DAMP (80.5 Ci/mmol; NEN Life Science Products, Boston, MA, U.S.A.). Non-specific binding was defined with 10 μM atropine. The 5-HT_{2A}-receptor relative concentrations in 5-HT_{2A}-SH-SY5Y cells were determined from specific binding by 5 nM [^{3}H]ketanserin (63.3 Ci/mmol; NEN Life Science Products, Boston, MA, U.S.A.). Non-specific binding was defined with 10 μM ritanserin.

To determine the pK_i value of the α_{2A}-adrenoceptor agonist UK 14,304 at α_{2A}-adrenoceptors in α_{2A}-H cells after pre-treatment with 10 μM yohimbine and 100 μM phenoxybenzamine or 100 μM benextramine as described above, we performed competition binding assays in whole α_{2A}-H cells against 5 nM [*O*-methyl-^{3}H]yohimbine. In preparation for the competition binding experiments the cells were plated and incubated as before, but without a radioligand. Cells were then rinsed once with UltraMEM, whereafter the assay was initiated by adding UltraMEM with 5 nM [*O*-methyl-^{3}H]yohimbine and different concentrations of UK 14,304. After a 30 minute incubation at 37 °C in 5% CO_2, the medium was aspirated, the washing procedure as described

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above were followed and the reaction terminated with 1 ml of 5% (w/v) TCA and let to stand for at least 30 minutes to allow the cells to lyse. The TCA from each well was then transferred directly into scintillation vials and the radioactivity (bound [*O*-methyl-^{3}H]yohimbine) counted.

Preparing membranes from α_{2A}-H cells. After the appropriate pre-treatment of whole α_{2A}-H cells (see above), the cells were washed twice with PBS, the cell monolayer was loosened with ethylenediaminetetraacetic acid (EDTA) in PBS (0.02% w/v), and the cells scraped from the culture flask surface with a cell scraper. The cell suspension was centrifuged in a bench top centrifuge (5,411 × *g*, 4 °C, 15 minutes), the supernatant discarded and the pellet washed twice with ice-cold PBS, whereafter the pellet was re-suspended in 1 mM Tris buffer (pH 7.4). The cell suspension was tumbled for 15 minutes at 4 °C, homogenized with a Teflon homogenizer, and centrifuged at 1,000 × *g* in a Beckman ultracentrifuge at 4 °C for 15 minutes. The supernatant was collected and kept on ice, while the pellet was re-suspended in the Tris buffer and the preceding procedure repeated to collect all protein. The resulting supernatants were centrifuged at 40,000 × *g* in a Beckman ultracentrifuge at 4 °C for 60 minutes. The resulting pellet was re-suspended and homogenized in TME buffer (50 mM Tris, 10 mM $MgCl_2$ and 1 mM EDTA, pH 7.4). Protein concentrations were determined with the Bradford method (Bradford, 1976), using bovine serum albumin as standard and determining absorbance with a 96-well plate reader and a 560 nm filter (Labsystems Multiskan RC). Snap-frozen aliquots were stored at -86 °C for up to 4 weeks.

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Measuring [^{35}S]GTPγS binding in α_{2A}-H cell membranes. The [^{35}S]GTPγS binding assay to α_{2A}-H membranes was based on the procedures previously described (Sternweis and Robishaw, 1984; Yang and Lanier, 1999), but adapted for the present study. Immediately before the [^{35}S]GTPγS binding assays, the membranes were thawed on ice and the protein concentrations adjusted to 1.4 μg/μl by adding the appropriate volume of TME dilution buffer. Freshly prepared assay buffer (0.5 nM [^{35}S]GTPγS, 1 μM GDP, 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol (DTT), pH 7.4, at 4 °C) was used to prepare a concentration range of UK 14,304. In each test tube 90 μl of the assay buffer containing the appropriate concentration of UK 14,304 was added and heated for 5 minutes at 25 °C in a water bath. Thereafter 10 μl membrane was added and incubated for 40 minutes at 25 °C in the water bath. After the incubation, 3 ml ice-cold TMN washing buffer (20 mM Tris, 25 mM $MgCl_2$, 100 mM NaCl, pH 7.4) was added to each membrane sample and it was immediately filtered through Whatman GF/C filters (Kent, U.K.), using a Hoefer filtration rig under vacuum. Each sample was washed thrice with ice-cold TMN buffer. Non-specific binding of [^{35}S]GTPγS was defined by samples with assay buffer but no membrane protein. Filters were air-dried, whereafter radioactivity was determined by liquid scintillation counting. The specific UK 14,304-induced binding of [^{35}S]GTPγS to the membranes was determined by subtracting the non-specific binding from the total binding.

Assessment of binding of [^{35}S]GTPγS to Gα_o protein. The [^{35}S]GTPγS binding assay to Gα_o protein was based on the procedures previously (Graber et al., 1992;

Sternweis and Robishaw, 1984), but adapted to accommodate the pre-treatment with benextramine. The $G\alpha_o$ protein was diluted to a concentration of 8 ng/µl with a sample dilution buffer (10 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.1% w/v nonaethylene glycol monododecyl ether, pH 8.0) and kept on ice. Before measuring the binding of [^{35}S]GTPγS to $G\alpha_o$, the protein was pre-treated with either 0 M or 100 µM benextramine for 2 hours at 4 °C, or for 30 minutes at 25 °C. Immediately after pre-treatment, the benextramine pre-treatment groups were divided into 10 µl samples in test tubes and 10 µl dilution buffer was added on ice. Thereafter, 20 µl binding cocktail (0.8 nM [^{35}S]GTPγS, 2 µM GTPγS in 50 mM HEPES, 1 mM EDTA, 40 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT, pH 8.0) was added to each test tube at the indicated temperature (4 °C or 25 °C). After incubation, 3 ml ice-cold TMN washing buffer (pH 8.0) was added to each sample, and the bound [^{35}S]GTPγS separated from the free fraction by rapid filtration through type HAWP nitrocellulose membrane filters (Millipore, Bedford, MA, U.S.A.), placed on a Hoefer filtration rig under vacuum. Each sample was washed thrice with ice-cold TMN buffer. Non-specific binding of [^{35}S]GTPγS was defined by samples with assay buffer but no $G\alpha_o$ protein. Filters were air-dried, whereafter radioactivity was determined by liquid scintillation counting.

Data analysis. Data from all studies were obtained from triplicate observations from at least 3 separate, comparable experiments, and results are expressed as SEM. Semilogarithmic dose-response curves were constructed as least square non-linear fits, utilizing the computer software GraphPad Prism (version 3.03 for Microsoft Windows, GraphPad Software, San Diego, CA, U.S.A.). Where data of dose-response curves are expressed as percent of control without drug, no statistical significant differences were

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found in the control values of second messenger accumulation among the different pre-treatments with each irreversible antagonist. One-site competition binding curves were constructed as least square non-linear fits, and the K_i values calculated from the IC_{50} value, applying the K_D value of the radioligand into the built-in Cheng-Prusoff correction of the software. Student's two-tailed, unpaired t test was implemented to compare the E_{max}, pEC_{50} and pK_i values. All reported p values are after the Bonferroni correction for multiple comparisons (when appropriate), and a value of $p < 0.05$ was taken as statistically significant.

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RESULTS

Specific binding before and after pre-treatment with the irreversible antagonists, with or without receptor protection. Figure 1*A* and Figure 1*B* depict the specific binding of [*O*-methyl-^{3}H]yohimbine to α_{2A}-adrenoceptors in α_{2A}-H cells before and after pre-treatment with different concentrations of phenoxybenzamine for 20 minutes, either without protection of the α_{2A}-adrenoceptors (0 M yohimbine) or with protection of the α_{2A}-adrenoceptors (10 μM yohimbine). It can be seen in Figure 1*A* that increasing concentrations of phenoxybenzamine progressively decreased specific binding (i.e. decreased α_{2A}-adrenoceptor concentration) ($p < 0.001$ for comparison of all bars) in the absence of receptor protection. This decrease in specific binding is not seen in Figure 1*B* ($p > 0.05$) when the α_{2A}-adrenoceptors are adequately protected from phenoxybenzamine by yohimbine. A small reduction in the specific binding of [*O*-methyl-^{3}H]yohimbine could be noted in bar b_1 (84 ± 1.5%) as compared to bar a_1 (100 ± 1.1%), which may represent some residual cold yohimbine after the washing steps and of which the negligible effect on receptor-mediated functional response is illustrated further below (Figure 2).

Likewise, Figure 1*C* and Figure 1*D* depict the specific binding of [*O*-methyl-^{3}H]yohimbine to α_{2A}-adrenoceptors in α_{2A}-H cells before and after pre-treatment with different concentrations of benextramine for 20 minutes, either without protection of the α_{2A}-adrenoceptors (0 M yohimbine), or with protection of the α_{2A}-adrenoceptors (10 μM yohimbine). It can be seen in Figure 1*C* that increasing concentrations of benextramine progressively decreased specific binding ($p \leq 0.01$ for comparison of all bars, except for

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comparison of bars c_3 and c_4 where $p = 0.07$) in the absence of receptor protection. This prominent decrease in specific binding is not seen in Figure 1*D* ($p > 0.05$ for comparison of bars when the α_{2A}-adrenoceptors are protected from benextramine by yohimbine, except for comparison of bars d_1 and d_3 or of bars d_1 and d_4 where $p = 0.02$ or $p = 0.01$, respectively). As with phenoxybenzamine, a small reduction in the specific binding of [*O*-methyl-^{3}H]yohimbine should be noted in bar d_1 (87 ± 3.4%) as compared to bar c_1 (100 ± 1.5%), which may represent some residual cold yohimbine after the washing steps and of which the negligible effect on receptor-mediated functional response is illustrated further below (Figure 2). The reductions in the specific binding of [*O*-methyl-^{3}H]yohimbine observed in bars d_3 (73 ± 2.9%) and d_4 (71 ± 2.8%) as compared to bar d_1 (87 ± 3.4%) may be due to incomplete receptor protection by 10 μM yohimbine, but are practically insignificantly small in terms of its expected effect on receptor-mediated response (illustrated further below, Figure 2). From the bars in Figure 1*B* and Figure 1*D* (when compared to the corresponding bars in Figure 1*A* and Figure 1*C*) it follows that 10 μM yohimbine prevented the irreversible antagonist from reducing receptor binding at α_{2A}-adrenoceptors.

Similarly, Figure 1*E* and Figure 1*F* depict the specific binding of [*N*-methyl-^{3}H]4-DAMP to mACh receptors in SH-SY5Y cells before and after pre-treatment with different concentrations of 4-DAMP mustard for 20 minutes, either without protection of the mACh receptors (0 M atropine), or with protection of the mACh receptors (10 μM atropine). It can be seen in Figure 1*E* that 4-DAMP mustard decreased specific binding ($p < 0.01$ for comparison of bars e_1 and e_2 or of bars e_1 and e_3) in the absence of receptor protection. The difference in specific binding between 10 nM and 100 nM 4-

DAMP mustard was not statistically significant (p = 0.11 for comparison of bars e_2 and e_3). As with phenoxybenzamine and benextramine, a small reduction in the specific binding of the radioligand should be noted in bar f_1 (84 ± 2.5%) as compared to bar e_1 (100 ± 3.1%), which may represent some residual cold atropine after the washing steps and of which the negligible effect on receptor-mediated functional response is illustrated further below (Figure 2). From the bars in Figure 1*F* (when compared to the corresponding bars in Figure 1*E*) it can be seen that increasing concentrations of 4-DAMP mustard with receptor protection by atropine did not cause a similar prominent decrease in receptor number ($p > 0.05$ for comparison of bars f_1 and f_2, of bars f_1 and f_3, and bars f_2 and f_3) and it follows that 10 μM atropine prevented the irreversible antagonist from reducing receptor binding at mACh receptors.

Specific binding and second messenger formation before and after pre-treatment with the reversible antagonists. Figure 2*A* depicts the specific binding of [*O*-methyl-^{3}H]yohimbine to α_{2A}-adrenoceptors in α_{2A}-H cells after pre-treatment with 0 or 10 μM yohimbine, but no irreversible antagonist. Although there was a difference in specific binding of [*O*-methyl-^{3}H]yohimbine of about 13.4% (p = 0.01) between the 0 or 10 μM yohimbine pre-treatment groups, this difference appears to be functionally insignificant (i.e. negligible effect on receptor-mediated functional response) as shown by the corresponding functional data in Figure 2*B*, depicting the dose-response curves of the α_{2A}-adrenoceptor full agonist UK 14,304 in α_{2A}-H cells after the pre-treatments with 0 or 10 μM yohimbine. These dose-response curves were practically superimposed with no statistically significant difference between the pEC_{50} values (p = 0.78) or E_{max} values (p = 0.37). This is predicted also by theory, where a reduction in

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receptor concentration of 13.4% in a system with significant spare receptors, should not significantly alter the EC_{50} value of a dose-response curve.

Likewise, Figure 2*C* depicts the specific binding of [*N*-methyl-^{3}H]4-DAMP to mACh receptors in SH-SY5Y cells after pre-treatment with 0 or 10 μM atropine, but no irreversible competitive antagonist. Although there was a difference in specific binding of [*N*-methyl-^{3}H]4-DAMP of about 16% (p = 0.02) between the 0 or 10 μM atropine pre-treatment groups, this difference appears to be functionally insignificant (i.e. negligible effect on receptor-mediated functional response) as shown by the corresponding functional data in Figure 2*D*, depicting the dose-response curves of the mACh receptor full agonist methacholine in SH-SY5Y cells after pre-treatments with 0 or 10 μM atropine. The dose-response curves were practically superimposed with no statistically significant difference between the pEC_{50} values (p = 0.50) or E_{max} values (p = 0.76). Again, this is in line with theoretical predictions, as mentioned above.

A similar approach was intended for 5-HT_{2A} receptors, by measuring the specific binding of [^{3}H]ketanserin to 5-HT_{2A} receptors in 5-HT_{2A}-SH-SY5Y cells after pre-treatment with 0 or 10 μM ritanserin, but no irreversible competitive antagonist. However, results from radioligand binding studies showed that, under the experimental conditions employed, 5-HT_{2A}-receptors could not be significantly protected against benextramine with ritanserin. Therefore, none of the dose-response curves in this cell line, as presented further below in this manuscript, were constructed in the presence of ritanserin and there was no need to include such curves in Figure 2.

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Agonist-mediated responses before and after pre-treatment with the irreversible antagonists with or without receptor protection. Figure 3*A* depicts dose-response curves of the α_{2A}-adrenoceptor full agonist UK 14,304 in α_{2A}-L cells after pre-treatment with different concentrations of phenoxybenzamine for 20 minutes. As the concentration of phenoxybenzamine was increased, the dose-response curves of UK 14,304 shifted to the right (1 and 10 μM), and at the highest concentration (100 μM) the E_{max} value was also suppressed (E_{max} a_4/a_1 ratio = 0.37, $p < 0.05$). Similarly, Figure 3*C* depicts dose-response curves of UK 14,304 in α_{2A}-L cells after pre-treatment with different concentrations of benextramine for 20 minutes. As the concentration of the benextramine was increased, the dose-response curves of UK 14,304 initially shifted to the right (1 μM benextramine: EC_{50} shift c_2/c_1 ratio = 23, $p < 0.05$), and at higher concentrations (10 and 100 μM) the E_{max} value was suppressed (E_{max} c_3/c_1 ratio = 0.56, $p < 0.05$; E_{max} c_4/c_1 ratio = 0.29, $p < 0.05$). However, when the α_{2A}-adrenoceptors were protected from binding to the irreversible competitive antagonist, the antagonism by the irreversible competitive antagonists were not abolished, as illustrated in Figure 3*B* and Figure 3*D*. For phenoxybenzamine, the highest concentration (100 μM) showed a statistically significant, although small, rightward shift of the dose-response curve (EC_{50} shift b_4/b_1 ratio = 3.1, $p < 0.05$). For benextramine, the highest concentration (100 μM) showed a relatively large rightward shift of the dose-response curve (EC_{50} shift d_4/d_1 ratio = 138, $p < 0.05$) and a 10 μM concentration benextramine produced a small rightward shift of the dose-response curve (EC_{50} shift d_3/d_1 ratio = 6.3, $p < 0.05$), but at 1 μM the rightward shift of the dose-response curve was not statistically significant (EC_{50} shift d_2/d_1 ratio = 1.9; $p > 0.05$). The EC_{50} values of all other curves differed

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statistically significantly (EC_{50} shift d_3/d_2 ratio = 3.4, $p < 0.05$; EC_{50} shift d_4/d_2 ratio = 74, $p < 0.05$; EC_{50} shift d_4/d_3 ratio = 22, $p < 0.05$).

Figure 3*E* depicts dose-response curves of the mACh receptor full agonist methacholine in SH-SY5Y cells after pre-treatment with different concentrations of 4-DAMP mustard for 20 minutes. The E_{max} value was statistically significantly suppressed at 100 nM 4-DAMP mustard for 20 minutes (E_{max} e_3/e_1 ratio = 0.75, $p < 0.05$), but not at 10 nM (E_{max} e_2/e_1 ratio = 0.89, $p > 0.05$). When the mACh receptors were protected from binding to the irreversible competitive antagonist, as presented in Figure 3*F*, the dose-response curves were practically superimposed with no apparent changes in the antagonism.

Affinity of UK 14,304 for α_{2A}-adrenoceptors before and after pre-treatment with phenoxybenzamine or benextramine. Since Figure 3 indicated that irreversible antagonism by benextramine or phenoxybenzamine was not abolished by α_{2A}-adrenoceptor protection, it was investigated whether or not the irreversible non-competitive antagonism by phenoxybenzamine or benextramine (i.e. observed rightward shift of dose-response curves of UK 14,304 after receptor protection) was due to an altered affinity of the α_{2A}-adrenoceptor for the full agonist UK 14,304. The pK_i value of UK 14,304 was determined from competition binding curves against 5 nM [*O*-methyl-^{3}H]yohimbine in α_{2A}-H cells before and after pre-treatment with 100 μM phenoxybenzamine (Figure 4*A*) or benextramine (Figure 4*B*) for 20 minutes, with α_{2A}-adrenoceptor protection by 10 μM yohimbine. Irreversible antagonist pre-treatment did not alter the α_{2A}-adrenoceptor receptor affinity for UK 14,304 and apparently also not

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the binding profile, as the competition-binding curves before and after the irreversible antagonist were practically superimposed. The pK_i values were determined as pK_i = 6.64 ± 0.05 versus pK_i = 6.77 ± 0.06 after 0 μM or 100 μM phenoxybenzamine respectively (p = 0.16), and pK_i = 6.91 ± 0.20 versus pK_i = 6.82 ± 0.13 after 0 μM or 100 μM benextramine respectively (p = 0.73).

[^{35}S]GTPγS binding to Gα_i proteins in α_{2A}-H membranes after benextramine pre-treatment, with or without α_{2A}-adrenoceptor protection. Since competition binding studies with UK 14,304 above revealed that benextramine does not affect agonist affinity, it follows that the non-competitive irreversible antagonism should involve inhibition of the α_{2A}-adrenoceptor signal-transduction. We therefore conducted G_i protein-[^{35}S]GTPγS binding studies to investigate whether the non-competitive antagonism by benextramine is due to irreversible binding to a site at the receptor and/or G protein level, or whether the binding is located downstream to the G protein in the signal transduction system. As before, α_{2A}-H cells were pre-treated with 0 or 100 μM benextramine for 20 minutes at 37 °C, plus 0 M yohimbine, or 10 μM yohimbine to protect the α_{2A}-adrenoceptors from binding to benextramine. After washing procedures to remove all unbound and reversibly bound drugs (as described above), membranes were prepared. Figure 5 depicts dose-response curves of the full α_{2A}-adrenoceptor agonist UK 14,304, measuring [^{35}S]GTPγS binding to G proteins in these membranes. In Figure 5*A* a concentration-dependent response of UK 14,304 was observed in membranes pre-treated with drug-free medium (i.e. control curve a_1, pre-treated with 0 μM yohimbine plus 0 μM benextramine). As expected, when the membranes were pre-

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treated with benextramine (i.e. curve a_2, pre-treated with 0 μM yohimbine plus 100 μM benextramine), the response to UK 14,304 was totally abolished (from E_{max}, $p < 0.01$). When the membranes were pre-treated with yohimbine alone (Figure 5*B*, curve b_1, pre-treated with 10 μM yohimbine plus 0 μM benextramine), a dose-response curve was obtained similar to curve a_1, confirming previous results that residual yohimbine after the washing procedure did not affect receptor function, as evidenced by Figure 2. Importantly, however, when the α_{2A}-adrenoceptors were protected from binding to benextramine during the pre-treatment (Figure 5*B*, curve b_2, pre-treated with 10 μM yohimbine plus 100 μM benextramine), the dose-response curve of UK 14,304 was completely suppressed as was found without receptor protection, as represented in curve a_2 (from E_{max}, $p < 0.01$). Results suggest that the protection of α_{2A}-adrenoceptors with yohimbine does not prevent benextramine from inhibiting the binding of [^{35}S]GTPγS to the membranes and therefore G_i mediated signaling through the receptor.

Binding of [^{35}S]GTPγS to Gα_o before and after incubation with benextramine at different incubation times and temperatures. Gα_o is a relatively stable GTP-binding protein of the $G_{i/o}$ family and has been shown to constitutively (in the absence of receptor and agonist) bind guanine nucleotides (Sternweis and Robishaw, 1984). To investigate whether benextramine directly binds to the G protein nucleotide binding site to inhibit GTP binding, Gα_o was pre-treated with 0 or 100 μM benextramine for 120 minutes at 4 °C, or for 30 minutes at 25 °C, whereafter the [^{35}S]GTPγS binding was measured, as presented in Figure 6. After pre-treatment with 0 or 100 μM benextramine for 120 minutes at 4 °C, the specific binding obtained is depicted in Figure

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6*A*. As can be seen from Figure 6*A*, benextramine has not significantly reduced the binding of [^{35}S]GTPγS to Gα$_o$ (*p* = 0.80) and the amount of [^{35}S]GTPγS bound after pre-treatment with 0 or 100 μM benextramine was measured as 17.2 ± 3.0 and 16 ± 3.1 fmol/ng membrane protein respectively. Likewise, after pre-treatment with 0 or 100 μM benextramine for 30 minutes at 25 °C, the specific binding obtained is depicted in Figure 6*B*. As can be seen from Figure 6*B*, benextramine has not significantly reduced the binding of [^{35}S]GTPγS to Gα$_o$ (*p* = 0.54) and the amount of [^{35}S]GTPγS bound after pre-treatment with 0 or 100 μM benextramine was measured as 2.9 ± 0.6 and 3.7 ± 1.1 fmol/ng membrane protein respectively. Although pre-treatment with benextramine at both incubation times and temperatures did not significantly decrease the binding of [^{35}S]GTPγS to Gα$_o$, overall binding is significantly lower at the shorter incubation time but higher temperature. These results suggest that incubation of Gα$_o$ at higher temperatures with shorter incubation times reduce the binding of [^{35}S]GTPγS to the nucleotide binding site of the G protein. According to Sternweis and Robishaw (Sternweis and Robishaw, 1984), purified G proteins (such as Gα$_o$), could be stored at -80 °C or even on ice (4 °C) for several weeks with little or no loss of binding activity. Thus the observed overall significant reduction in binding of [^{35}S]GTPγS to Gα$_o$ after pre-treatment with benextramine for 30 minutes at 25 °C may be due to reduced biological activity of the Gα$_o$ protein, since it is well-known that higher temperatures cause conformational changes that may modulate or destroy its biological activity.

G$_s$-mediated [^{3}H]cAMP accumulation in α$_{2A}$-H cells after pre-treatment with benextramine, with or without α$_{2A}$-adrenoceptor protection. Since our previous

observations investigated the non-competitive antagonism by benextramine at α_{2A}-adrenoceptors via a G_i-mediated response only, it was important to investigate whether similar non-competitive antagonism by benextramine can be observed via a G_s protein-mediated response, but with the same receptors and effector (adenylyl cyclase). It has been shown before that after PTX treatment of α_{2A}-H cells, the α_{2A}-adrenoceptors couple to G_s proteins to stimulate adenylyl cyclase activity (Brink et al., 2000). Figure 7 displays dose-response curves of UK 14,304 in PTX treated whole α_{2A}-H cells after pre-treatment with 0 or 100 μM benextramine plus 0 M (Figure 7*A*) or 10 μM yohimbine (Figure 7*B*) for 20 minutes at 37 °C. Without protection of the α_{2A}-adrenoceptors (Figure 7*A*), 100 μM benextramine abolished the agonist-induced response (E_{max} for a_1 = 100 ± 13.1% and E_{max} for a_2 = -3.6 ± 7.5%, $p < 0.01$). When the α_{2A}-adrenoceptors were protected by 10 μM yohimbine, the G_s-mediated response was only partially inhibited (E_{max} of curve b_1 = 100 ± 18.9% and E_{max} of curve b_2 = 53.0 ± 5.8%, $p < 0.05$) and the EC_{50} value remained unchanged.

Agonist-induced, G_q-mediated [^{3}H]IP_x accumulation in SH-SY5Y and 5-HT_{2A}-SH-SY5Y cells after pre-treatment with benextramine, with or without receptor protection. We investigated whether benextramine pre-treatment would antagonize the response in a signal transduction system with a receptor type, G protein type and effector totally different from those in previous observations. In SH-SY5Y cells mACh receptors signal through G_q proteins to activate PLC. Likewise, in 5-HT_{2A}-SH-SY5Y cells, 5-HT_{2A}-receptors signal through G_q proteins to activate PLC. Figure 8 depicts dose-response curves of methacholine in SH-SY5Y cells and of serotonin in 5-HT_{2A}-SH-

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SY5Y cells, measuring agonist-stimulated [^{3}H]IP$_x$ accumulation, after pre-treatment of the cells with 0 or 100 μM benextramine for 20 minutes at 37 °C.

In SH-SY5Y cells, benextramine partially suppressed the methacholine-mediated response (E_{max} values were 100 ± 17.2% for curve a_1 and 25.2 ± 8.6% for curve a_2, $p < 0.05$). The EC_{50} value, however, remained unchanged. Likewise, in 5-HT$_{2A}$-SH-SY5Y cells benextramine partially suppressed the serotonin-mediated response (E_{max} values were 100 ± 23.2% for curve b_1 and 34.7 ± 8.3% for curve b_2, $p < 0.05$). Again, the EC_{50} value remained unchanged.

Binding data for [^{3}H]4-DAMP at mACh receptors and [^{3}H]ketanserin at 5-HT$_{2A}$-receptors after pre-treatment with benextramine, with or without receptor protection. Since benextramine inhibits the [^{3}H]IP$_x$ accumulation in both SH-SY5Y and 5-HT$_{2A}$-SH-SY5Y cells (Figure 8), it was important to determine whether this inhibition could be ascribed to a reduction in receptor number, or whether non-competitive antagonism is displayed by benextramine.

After pre-treatment of SH-SY5Y whole cells with 0 M or 100 μM benextramine for 20 minutes at 37 °C, plus 0 M or 10 μM atropine (>1,000 × K_i value, to protect mACh receptors from binding to benextramine), radioligand binding assays were conducted to determine the relative number of receptors. Figure 9*A* and Figure 9*B* depict the specific binding of [^{3}H]4-DAMP to mACh receptors in SH-SY5Y cells after pre-treatment with 0 M or 100 μM benextramine. In the absence of receptor protection by atropine (Figure 9*A*), benextramine pre-treatment significantly reduced the specific binding of [^{3}H]4-DAMP from 100 ± 4.4% (bar a_1) to 35.6 ± 3.3% (bar a_2) ($p < 0.001$). However, after pre-

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treatment with 10 μM atropine to protect the mACh receptors from benextramine (Figure 9*B*), the specific binding was also significantly decreased from 76.8 ± 3.2% (bar b_1) to 42.3 ± 2.2% (bar b_2) ($p < 0.001$).

Likewise, after pre-treatment of 5-HT_{2A}-SH-SY5Y whole cells with 0 M or 100 μM benextramine for 20 minutes at 37 °C, plus 0 M or 10 μM ritanserin (>1,000 × K_i value, to protect 5-HT_{2A}-receptors from binding to benextramine), radioligand binding assays were conducted to determine the relative number of receptors. Figure 9*C* and Figure 9*D* depict the specific binding of [^{3}H]ketanserin to 5-HT_{2A}-receptors in 5-HT_{2A}-SH-SY5Y cells after pre-treatment with 0 M or 100 μM benextramine. In the absence of receptor protection by ritanserin (Figure 9*C*), benextramine pre-treatment significantly reduced the specific binding of [^{3}H]ketanserin from 100 ± 12.3% (bar c_1) to 31.1 ± 4.9% (bar c_2) ($p < 0.01$). However, after pre-treatment with 10 μM ritanserin to protect the 5-HT_{2A} receptors from benextramine (Figure 9*D*), the specific binding was also significantly decreased from 85.4 ± 19.3% (bar d_1) to 30.0 ± 6.0% (bar d_2) ($p < 0.05$).

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DISCUSSION

The experimental conditions and pre-treatments are suitable for the evaluation of non-competitive mechanisms by the irreversible competitive antagonists. As expected, phenoxybenzamine (Figure 1*A*) and benextramine (Figure 1*C*) pre-treatments decrease the α_{2A}-adrenoceptor number in α_{2A}-H cells and that 4-DAMP mustard (Figure 1*E*) decreased the mACh receptor number in SH-SY5Y cells. The binding of phenoxybenzamine (Figure 1*B*) and benextramine (Figure 1*D*) to α_{2A}-adrenoceptors were effectively prevented by yohimbine, while atropine effectively prevented the binding of 4-DAMP mustard to mACh receptors (Figure 1*F*). Therefore, the irreversible antagonists were used at sufficient concentrations and incubation times to eliminate a significant fraction of the operational receptors and the concentrations of the reversible antagonists employed were sufficient to protect these receptors from significant binding by the irreversible antagonists.

In addition, the washing procedure after the pre-treatments was sufficient to remove the reversibly bound antagonists. Results in Figure 2*A* suggest that the small decrease in the specific binding of [*O*-methyl-^{3}H]yohimbine to α_{2A}-adrenoceptors in α_{2A}-H cells after yohimbine pre-treatment is functionally insignificant, since dose-response curves of UK 14,304 at α_{2A}-adrenoceptors in α_{2A}-L cells before and after yohimbine pre-treatment were practically superimposed (Figure 2*B*). Likewise, a small reduction in the specific binding of [*N*-methyl-^{3}H]4-DAMP to mACh receptors in SH-SY5Y cells after atropine pre-treatment (Figure 2*C*), is functionally insignificant (Figure 2*D*). The pre-treatment procedures are therefore suitable for further investigation of any irreversible

non-competitive mechanisms of antagonism by the respective irreversible competitive antagonists.

Importantly, it was shown previously (Brink et al., 2000) and confirmed (data not shown) that in control Neo cells (control cell line, stably transfected with the empty vector without the α_{2A}-adrenoceptor) UK 14,304 does not elicit any change in [^{3}H]cAMP accumulation, suggesting that all observed UK 14,304-mediated responses in α_{2A}-H and α_{2A}-L cells are mediated via α_{2A}-adrenoceptors.

Benextramine and phenoxybenzamine, but not 4-DAMP mustard, display irreversible non-competitive antagonism after 20 minutes incubation time. When receptor number in a system with spare receptors is progressively reduced by an irreversible antagonist, theory predicts that the dose-response curve of the agonist should progressively shift to the right until all spare receptors are eliminated, whereafter the maximal response is reduced (Furchgott, 1966; Kenakin, 1997). The results with phenoxybenzamine and benextramine in the current study followed this pattern (Figure 3*A* and Figure 3*C*). In the absence of spare receptors, 4-DAMP mustard only suppressed the maximal response to methacholine (Figure 3*E*). If the primary binding sites could be sufficiently protected by a reversible competitive antagonist, theory predicts that the dose-response curves of the agonist before and after antagonist pre-treatment (plus sufficient washout so that any residual competitive antagonist does not influence receptor-mediated function) should superimpose. When dose-response curves do not superimpose under these conditions, it suggests non-competitive antagonism by the irreversible antagonist. Results from the present study therefore

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suggest that benextramine, and to a lesser degree phenoxybenzamine, display irreversible non-competitive antagonism at α_{2A}-adrenoceptors (Figures 3*B* and 3*D*). 4-DAMP mustard, however, does not display irreversible non-competitive antagonism at mACh receptors (Figure 3*F*) under the specific experimental conditions employed.

The non-competitive antagonism by phenoxybenzamine and benextramine does not alter the affinity of α_{2A}-adrenoceptors for UK 14,304. The affinity (pK_i values) of UK 14,304 at α_{2A}-adrenoceptors in α_{2A}-H cells were not affected by the pre-treatments with phenoxybenzamine or benextramine (Figure 4). A logical alternative explanation for the observations in Figure 3 could be that both drugs may modulate the signal transduction by exhibiting allotopic interactions at the α_{2A}-adrenoceptor macromolecule or by inhibiting G_i proteins or adenylyl cyclase downstream in the signal transduction pathway.

The non-competitive irreversible antagonism by benextramine at α_{2A}-adrenoceptors can be explained by the inhibition of signaling at the receptor-G_i protein level. Non-competitive antagonism by benextramine at α_{2A}-adrenoceptors is also observed earlier in the signal transduction system at the G protein level, when measuring agonist-induced increase in [^{35}S]GTPγS binding in cell membranes (Figure 5). Since GTPγS is a non-hydrolyzable GTP analogue, it is not likely that altered GAP function (enhanced GTP hydrolysis to GDP with G protein inactivation) may explain this observation. α_{2A}-Adrenoceptors have previously been shown to couple with high efficiency to PTX-sensitive G_i proteins (Chabre et al., 1994; Eason et al., 1992), as is present in the CHO-K1 cells employed (Eason et al., 1992), and with much lower

efficiency to G_s and G_q proteins (Brink et al., 2000; Wade et al., 1999; Chabre et al., 1994). It is therefore reasonable to assume that the observed [^{35}S]GTPγS binding results predominantly from G_i protein activation, as confirmed by unpublished data from the laboratory of Dr. Richard Neubig (Department of Pharmacology, University of Michigan, MI, U.S.A. – personal communications) that the α_{2A}-adrenoceptor-stimulated [^{35}S]GTPγS binding to α_{2A}-H cell membranes is completely abolished by PTX.

Non-competitive antagonism by benextramine does not involve direct inhibition of GTP binding to the G protein. From the results in Figure 6 it follows that the pre-treatment of purified $G\alpha_o$ with benextramine at a relatively high concentration and at two extreme and distinct temperatures and incubation times does not inhibit the constitutive binding of [^{35}S]GTPγS to $G\alpha_o$. $G\alpha_o$ and $G\alpha_i$ belong to the same G protein family (i.e. $G_{i/o}$ family) (Sternweis and Robishaw, 1984), are PTX-sensitive but cholera toxin-insensitive and display similar agonist-mediated α_{2A}-adrenoceptor signaling properties as measured by [^{35}S]GTPγS binding (Yang and Lanier, 1999). Therefore it can be reasonably assumed that benextramine will also not modulate [^{35}S]GTPγS-binding to $G\alpha_i$ and we propose that the observed non-competitive antagonism by benextramine at α_{2A}-adrenoceptors most likely results from an inhibition of receptor-$G\alpha_i$ protein coupling. Importantly, however, the current data do not exclude the possibility that G proteins may be affected in other ways (e.g. altered GDP binding, modifications at the receptor or effector coupling regions) or that different G protein types (i.e. $G\alpha_i$, $G\alpha_s$, or $G\alpha_q$ proteins) may be affected differently. The data also do not exclude the possibility that other signaling molecules or down-stream signaling entities, such as

adenylyl cyclase or PLC, could also be affected by benextramine. Further clarification of the mechanisms of action of benextramine and related drugs may form the basis for future studies.

Benextramine also irreversibly inhibits signaling of α_{2A}-adrenoceptors via G_s, but to a lesser extent than via G_i. α_{2A}-Adrenoceptors also signal through G_s proteins to activate adenylyl cyclase, although with 1,000 times lower coupling efficiency than to G_i (Chabre et al., 1994). This is evident after PTX treatment in systems with a high α_{2A}-adrenoceptor, concentration, measuring G_s-mediated increase in cAMP accumulation (Wade et al., 1999). The partial inhibition of the agonist-induced increase in [^{3}H]cAMP accumulation after receptor protection in PTX treated cells suggest that benextramine displays irreversible non-competitive antagonism at α_{2A}-adrenoceptors also when measuring G_s mediated responses (Figure 7).

Taken together, it can be postulated that benextramine most likely exhibits allotopic interactions at the α_{2A}-adrenoceptor macromolecule or binds to a common GPCR coupling site on the G_i and G_s proteins. The non-competitive antagonism by benextramine at α_{2A}-adrenoceptors is substantially less with G_s- than with G_i-mediated responses, suggesting that this action is dependent on the G protein type involved in the GPCR signaling. This complies with data suggesting distinct basic residues of the α_{2A}-AR that mediate G_i and G_s activation respectively (Wade et al., 1999). Also, whereas classical irreversible antagonists such as the haloalkylamines are believed to form uncomplicated covalent bonds with the receptor, it has been proposed that benextramine inactivates receptors at distinct sites via a disulfide-thiol interchange

reaction (Brasili et al., 1980; Brasili et al., 1986; Giardinà et al., 1996; Melchiorre, 1981; Melchiorre and Gallucci, 1983; Melchiorre et al., 1979;), thereby affecting the stereochemical properties of cysteine amino acid residues and probably disrupting the coupling of the α_{2A}-AR to G_i more than to G_s.

Benextramine irreversibly inhibits signaling of both muscarinic acetylcholine (mACh) receptors and 5-HT$_{2A}$-receptors via G$_q$. Both mACh receptors (Sorensen et al., 1999) and 5-HT$_{2A}$ receptors (Berridge, 1993) have been shown to signal through PTX-insensitive G_q proteins to activate PLC. We have also confirmed that the agonist-induced, mACh receptor-mediated PLC activation in SH-SY5Y cells is abolished by the PLC inhibitor 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U-73122) (Bleasdale and Fisher, 1993) and also that this mACh receptor-response is PTX insensitive (data not shown). Data from the present study show that benextramine irreversibly inhibits the methacholine-induced G_q-mediated signaling of mACh receptors in SH-SY5Y cells (Figure 8A), but that these receptors could not be protected by 10 μM atropine (i.e. >1,000 × K_D value) (Figure 9*B*). These results suggest that benextramine exhibits allotopic interactions at mACh receptors. In contrast, the antagonism by benextramine at mACh receptors in isolated guinea-pig atrium and ileum was reversible when measuring smooth muscle contraction (Benfey et al., 1980). Species and tissue differences may explain this apparent contradiction.

Benextramine also irreversibly inhibits the serotonin-induced, G_q-mediated signaling of 5-HT$_{2A}$-receptors (Figure 8*B*), which cannot be prevented by ritanserin at a relative high concentration of 10 μM (>1,000 × K_D value) (Figure 9*D*). These results suggest

that benextramine exhibits allotopic interactions, displaying irreversible non-competitive antagonism at 5-HT_{2A}-receptors. Antagonism by benextramine at 5-HT_{1A}-receptors has been shown before (Stanton and Beer, 1997) and it therefore does not seem to act selectively at a particular 5-HT receptor subtype.

These data suggest that benextramine binds to sites distinct from the primary binding sites (i.e. non-overlapping) at mACh and 5-HT_{2A}-receptors. These allotopic interactions may cause sterical changes in the receptor macromolecule, hindering binding of the ligand to the primary binding site of the receptor.

Final conclusions and implications of this study. We provide here evidence of irreversible non-competitive antagonism by phenoxybenzamine and benextramine at α_{2A}-adrenoceptors in addition to their known irreversible specific antagonism. Agonist affinity is not influenced, but signal transduction may be modulated via allotopic interactions. Irreversible non-competitive antagonism by benextramine at α_{2A}-adrenoceptors is G protein-dependent. Benextramine inhibits agonist-induced responses at three different GPCR types, involving signaling via three of the four main families of G proteins (i.e. $G_{i/o}$, G_s and $G_{q/11}$ families) in different pharmacological systems, presumably by binding to distinct binding sites at the various receptor types.

Under the conditions specified, 4-DAMP mustard does not display irreversible non-competitive antagonism at mACh receptors, while benextramine exhibits allotopic interactions at mACh receptors and 5-HT_{2A}-receptors, preventing ligand binding to the agonist binding site. However, the current data does not exclude the possibility that receptor-G protein coupling is also inhibited at mACh receptors and 5-HT_{2A}-receptors,

as was suggested for α_{2A}-adrenoceptors. Further investigation is needed to clarify the exact mechanisms of action of benextramine.

Benextramine may prove to be a useful experimental tool in investigating the signaling mechanisms of G protein-coupled receptors. Also, the mere confirmation of syntopic interactions (e.g. by receptor-radioligand binding studies), does not rule out irreversible non-competitive antagonism. Irreversible competitive antagonists should be utilized with due care for the implementation of any procedure where irreversible competitive antagonism is desired or intended (e.g. the Furchgott analysis). In such cases pure irreversible competitive antagonism should be verified under the experimental conditions applied. Finally, we propose that irreversible non-competitive mechanisms of antagonism could potentially contribute to the therapeutic response of phenoxybenzamine in the treatment of pheochromocytoma, but the specificity, scope or clinical significance of this mechanism needs further investigation.

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LEGENDS FOR FIGURES

Figure 1. Radioligand binding studies with (*A* – *D*) 5 nM [*O*-methyl-^{3}H]yohimbine in α_{2A}-H cells or (*E & F*) 5 nM [*N*-methyl-^{3}H]4-DAMP in SH-SY5Y cells. Specific binding of the radioligand was measured after pre-treatment with phenoxybenzamine (0, 1, 10 or 100 μM; 20 minutes) (*A*) without yohimbine or (*B*) with 10 μM yohimbine to protect the α_{2A}-adrenoceptors. Similarly, specific binding of the radioligand was measured after pre-treatment with benextramine (0, 1, 10 or 100 μM; 20 minutes) (*C*) without yohimbine or (*D*) with 10 μM yohimbine. Specific binding was also measured after pre-treatment with 4-DAMP mustard (0, 10 or 100 nM; 20 minutes) (*E*) without atropine or (*F*) with 10 μM atropine to protect the mACh receptors. In all conditions, the amounts of non-specific binding remained similar for the radioligand and its concentration used. The data are averages ± SEM of triplicate measurements from at least three experiments and are expressed as percent of control without drug.

Figure 2. Radioligand binding and functional studies after pre-treatment with the appropriate reversible antagonist, followed by the described washing procedure. (*A*) Specific binding of 5 nM [*O*-methyl-^{3}H]yohimbine in α_{2A}-H cells after pre-treatment with yohimbine (0 or 10 μM). (*B*) Semilogarithmic dose-response curves of UK 14,304 in α_{2A}-L cells by measuring whole-cell [^{3}H]cAMP accumulation after pre-treatment with yohimbine (0 or 10 μM). (*C*) Specific binding of 5 nM [*N*-methyl-^{3}H]4-DAMP in SH-SY5Y cells after pre-treatment with atropine (0 or 10 μM). (*D*) Semilogarithmic dose-response curves of methacholine in SH-SY5Y cells by measuring whole-cell [^{3}H]IP_x accumulation after pre-treatment with atropine (0 or 10 μM). The data are averages ±

SEM of triplicate measurements from at least three experiments and are expressed as percent of control without drug. The dose-response curves (*B & D*) are non-linear least square fits.

Figure 3. Semilogarithmic dose-response curves of (*A – D*) UK 14,304 in α_{2A}-L cells or (*E & F*) methacholine in SH-SY5Y cells. Whole-cell [^{3}H]cAMP accumulation measurements were performed after pre-treatment of α_{2A}-L cells with phenoxybenzamine (0, 1, 10 or 100 μM; 20 minutes) (*A*) without yohimbine or (*B*) with 10 μM yohimbine. Similarly, whole-cell [^{3}H]cAMP accumulation measurements were performed after pre-treatment of α_{2A}-L cells with benextramine (0, 1, 10 or 100 μM; 20 minutes) (*C*) without yohimbine or (*D*) with 10 μM yohimbine. Whole-cell [^{3}H]IP$_x$ accumulation measurements were also performed after pre-treatment of SH-SY5Y cells with 4-DAMP mustard (0, 10 or 100 nM; 20 minutes) (*E*) without atropine or (*F*) with 10 μM atropine. The data are averages ± SEM of triplicate measurements from at least three experiments and are expressed as percent of control without drug. Dose-response curves are non-linear least square fits.

Figure 4. Competition binding curves of UK 14,304 in α_{2A}-H cells against 5 nM [*O*-methyl-^{3}H]yohimbine. Specific binding of the radioligand was measured after pre-treatment of the cells with 10 μM yohimbine and (*A*) phenoxybenzamine (0 or 100 μM; 20 minutes), or (*B*) benextramine (0 or 100 μM; 20 minutes). The data are represented as the mean ± S.E.M and expressed as the percentage of the control radioligand binding without UK 14,304. Data represent the average of triplicate observations of three experiments. Curves are one-site competition non-linear least square fits.

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Figure 5. Semilogarithmic dose-response curves of UK 14,304 in α_{2A}-H cell membranes as measured by [^{35}S]GTPγS binding to endogenous G proteins in membranes. α_{2A}-H cell membranes were prepared after whole-cell pre-treatments for 20 minutes with *(A)* 0 or 100 μM benextramine plus 0 M yohimbine, or *(B)* 0 or 100 μM benextramine plus 10 μM yohimbine. [^{35}S]GTPγS binding in all curves is presented as the mean ± S.E.M. and expressed as percentage of the control E_{max} of curve a_1. Data represent the average of triplicate observations of three experiments. Curves a_1 and b_1 are non-linear least square fits.

Figure 6. Constitutive [^{35}S]GTPγS binding to purified $G\alpha_o$ protein (fmol/ng). The $G\alpha_o$ protein was pre-treated with 0 or 100 μM benextramine at *(A)* 4 °C for 120 minutes, or *(B)* 25 °C for 30 minutes before [^{35}S]GTPγS binding. The bar graphs represent the mean specific binding ± S.E.M and data represents the average of triplicate observations of three experiments.

Figure 7. Semilogarithmic dose-response curves of UK 14,304 in α_{2A}-H cells treated with pertussis toxin to observe G_s-mediated responses, by measuring whole-cell [^{3}H]cAMP accumulation. The α_{2A}-H cells were pre-treated with benextramine (0 or 100 μM, 20 minutes) plus *(A)* 0 M yohimbine, or *(B)* 10 μM yohimbine. The data are represented as the mean ± S.E.M and expressed as percentage of the control E_{max} of curve a_1. Data represent the average of triplicate observations of three experiments. Dose-response curves are non-linear least square fits.

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Figure 8. Semilogarithmic dose-response curves of *(A)* methacholine in SH-SY5Y cells, and *(B)* serotonin in 5-HT_{2A}-SH-SY5Y cells. The cells were pre-treated with benextramine (0 or 100 μM, 20 minutes), whereafter whole-cell total [^{3}H]IP_x accumulation was measured with increasing concentrations agonist. The data are represented as mean ± S.E.M. and curves a_1 and a_2 are expressed as percentage of the E_{max} of curve a_1, while curves b_1 and b_2 are expressed as percentage of the E_{max} of curve b_1. Data represent the average of triplicate observations of three experiments. The curves are non-linear least square fits.

Figure 9. Specific binding of *(A & B)* 5 nM [^{3}H]4-DAMP in SH-SY5Y cells, or *(C & D)* 5 nM [^{3}H]ketanserin in 5-HT_{2A}-SH-SY5Y cells. The cells were pre-treated with benextramine (0 or 100 μM, 20 minutes) and *(A)* 0 M atropine, or *(B)* 10 μM atropine to protect mACh receptors, and *(C)* 0 M ritanserin, or *(D)* 10 μM ritanserin to protect 5-HT_{2A} receptors. Thereafter, whole-cell specific binding was determined. The bar graphs represent the mean specific binding ± S.E.M. and are expressed as percent of control samples without benextramine and atropine or ritanserin. Data represent the average of triplicate observations of three experiments in *(A & B)* and four experiments in *(C & D).*

Figure 1

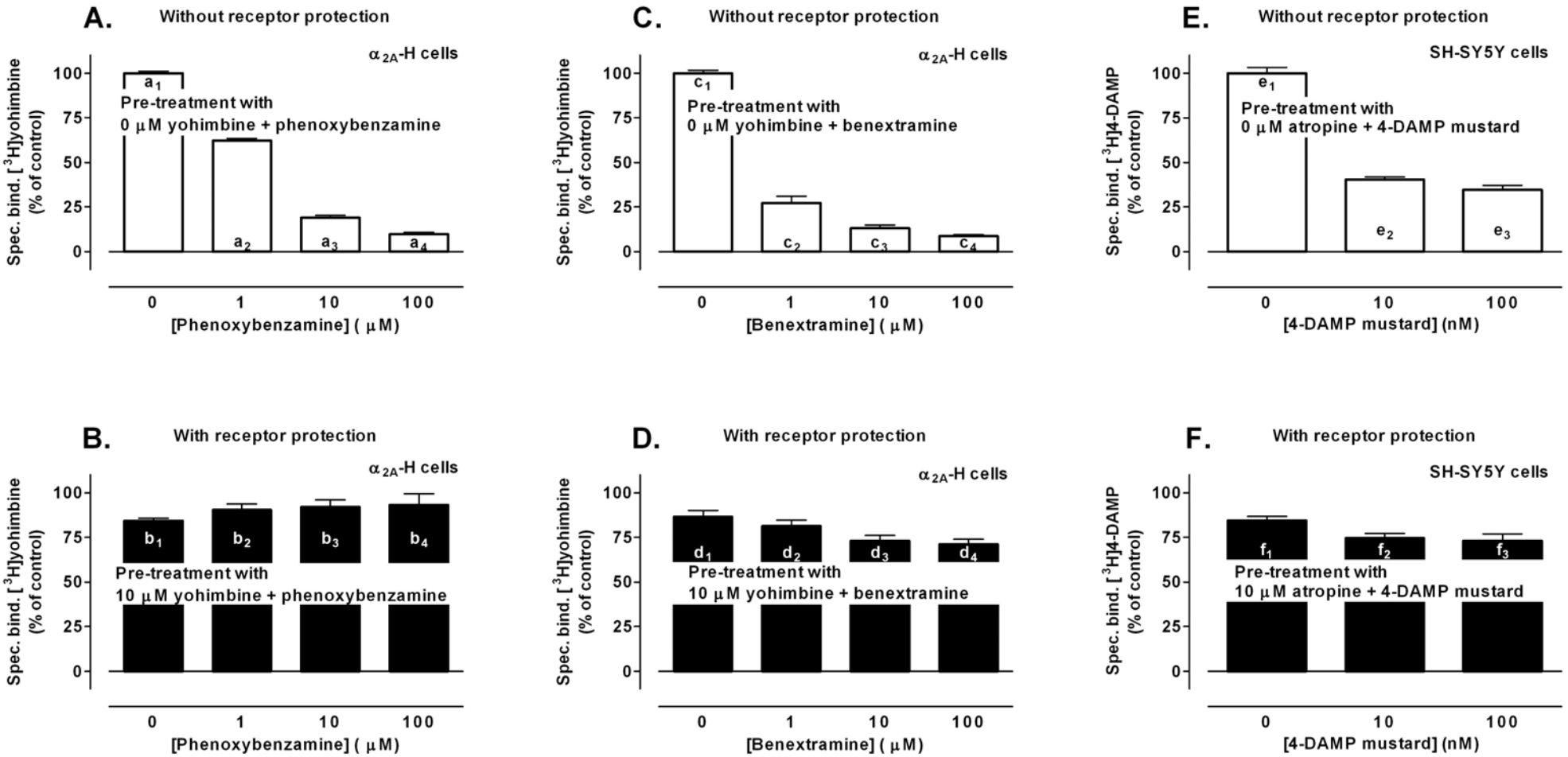

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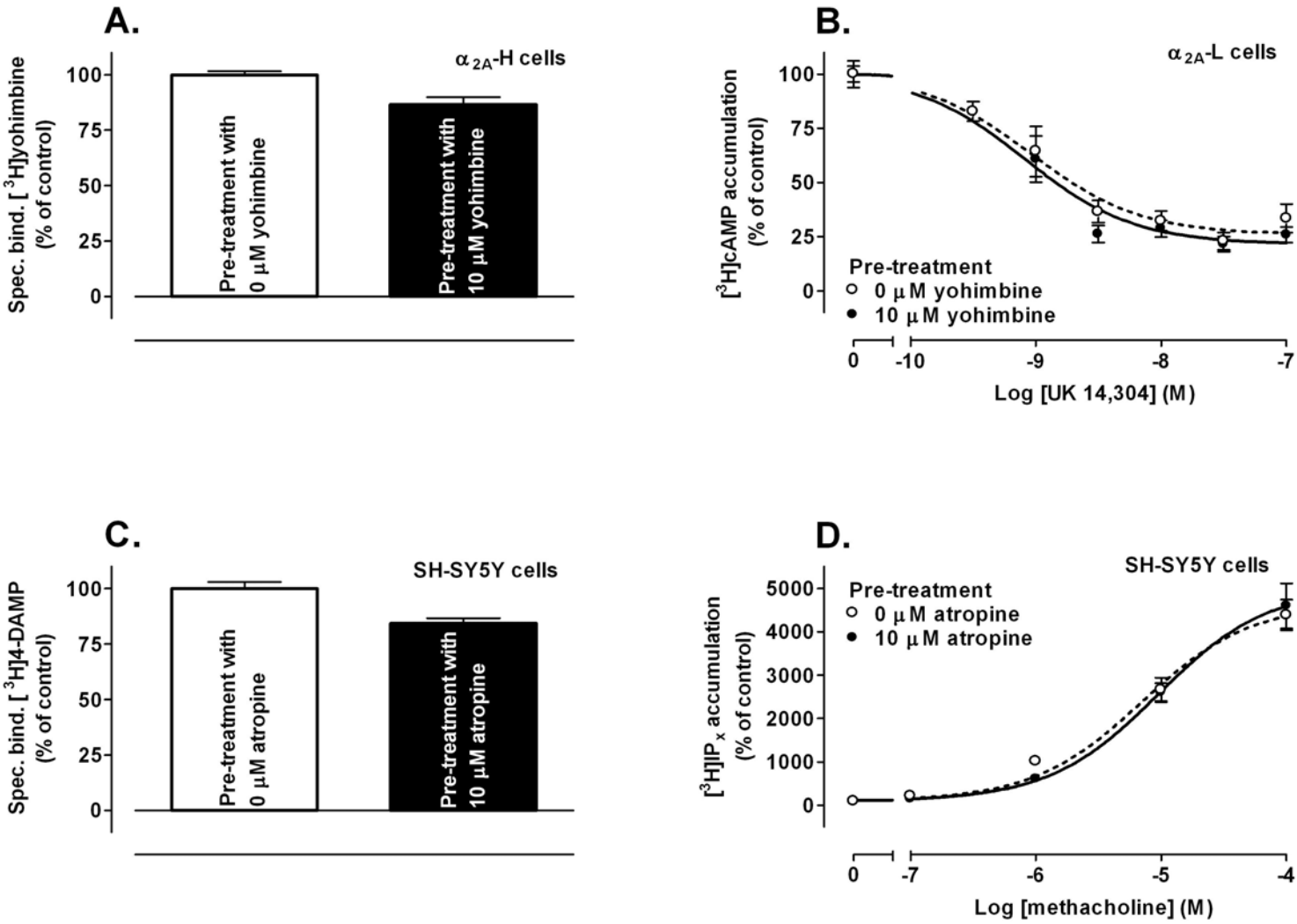
Figure 2



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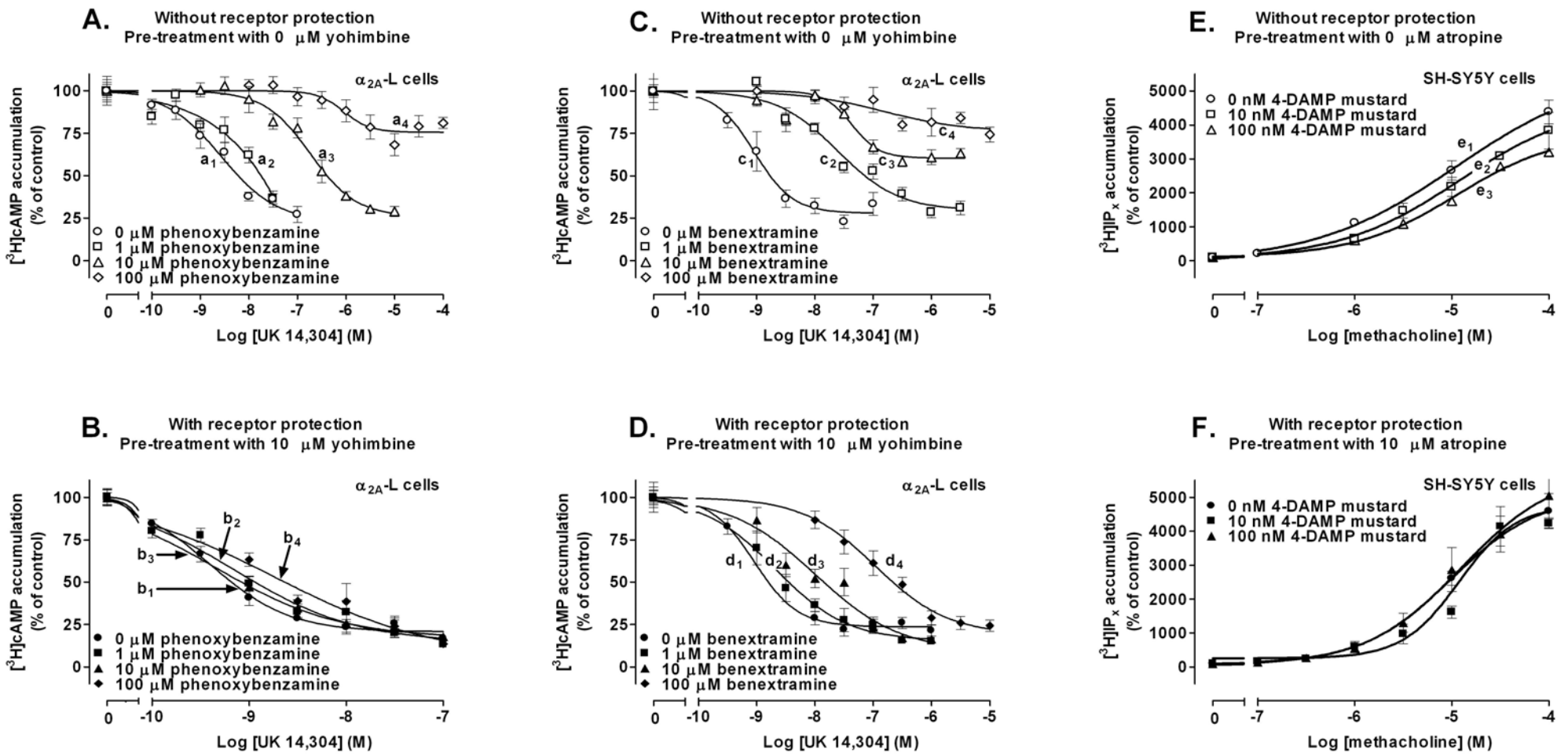

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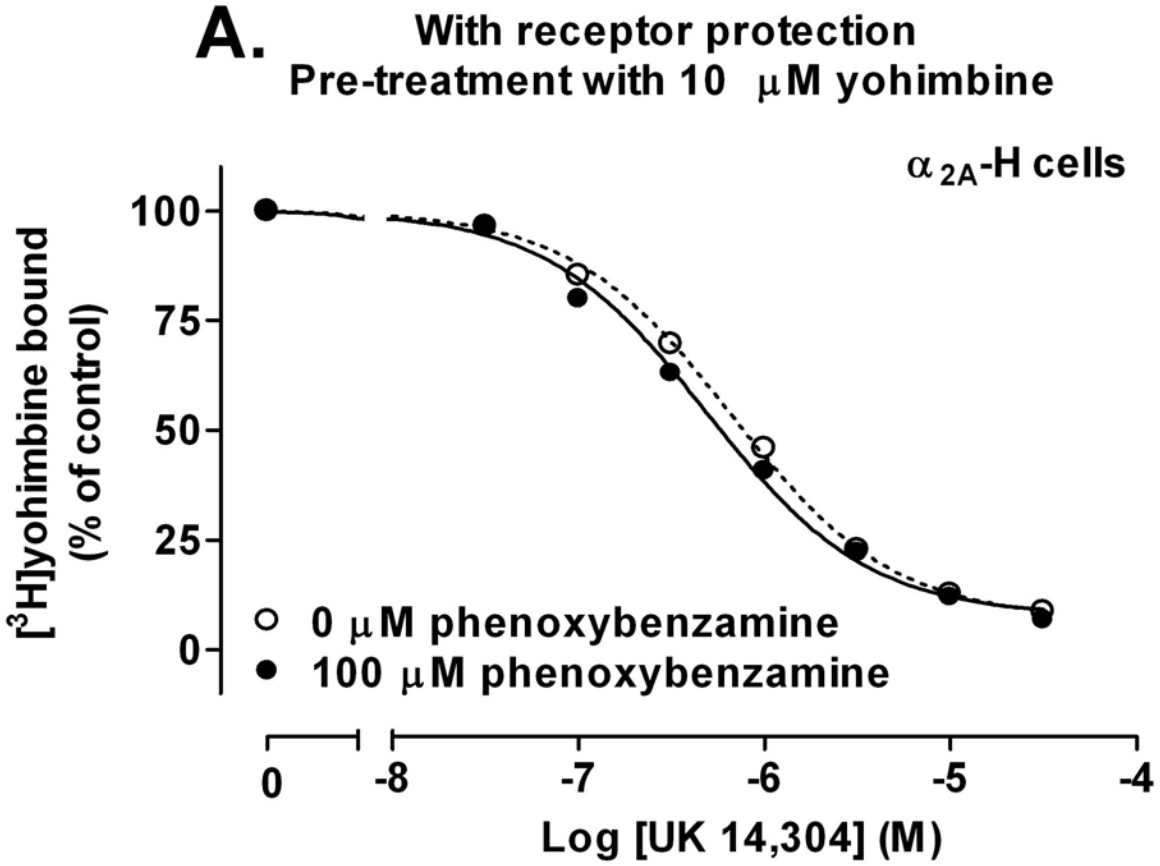
Figure 4



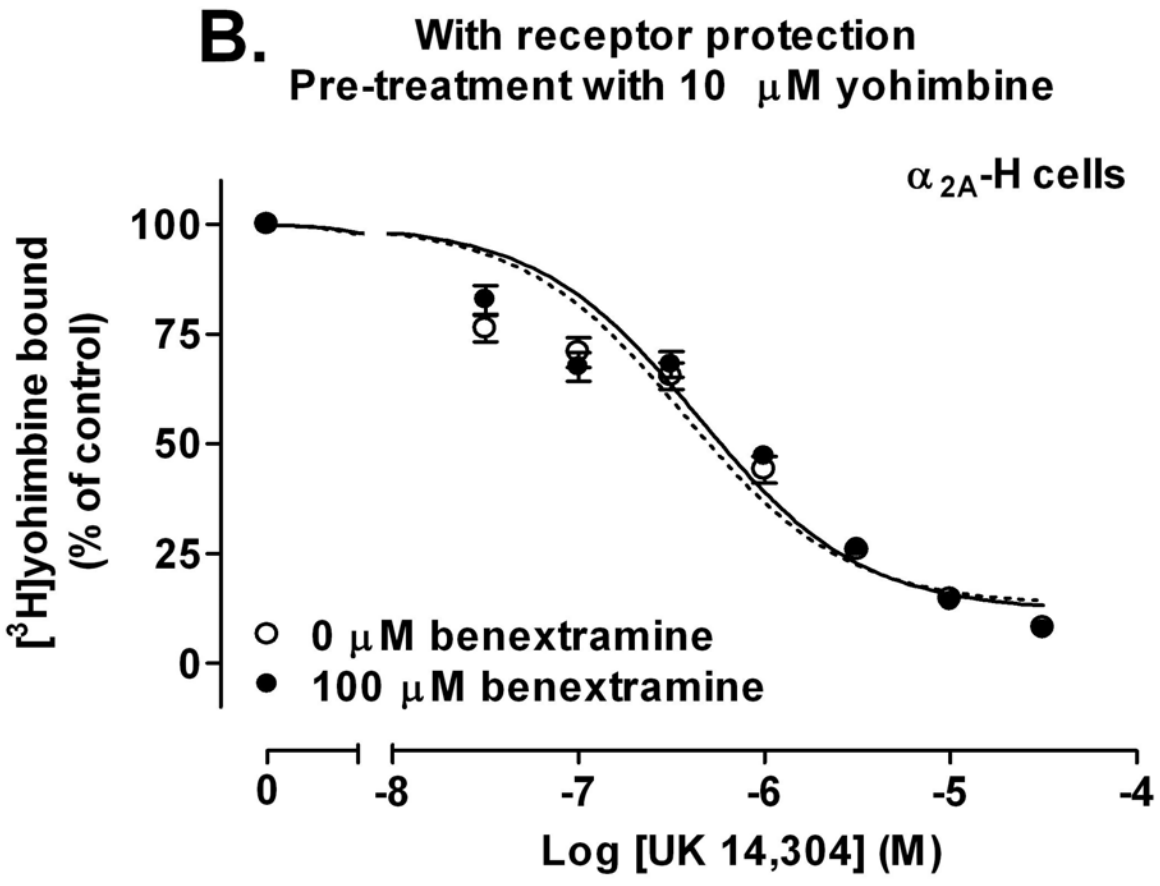


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Figure 5

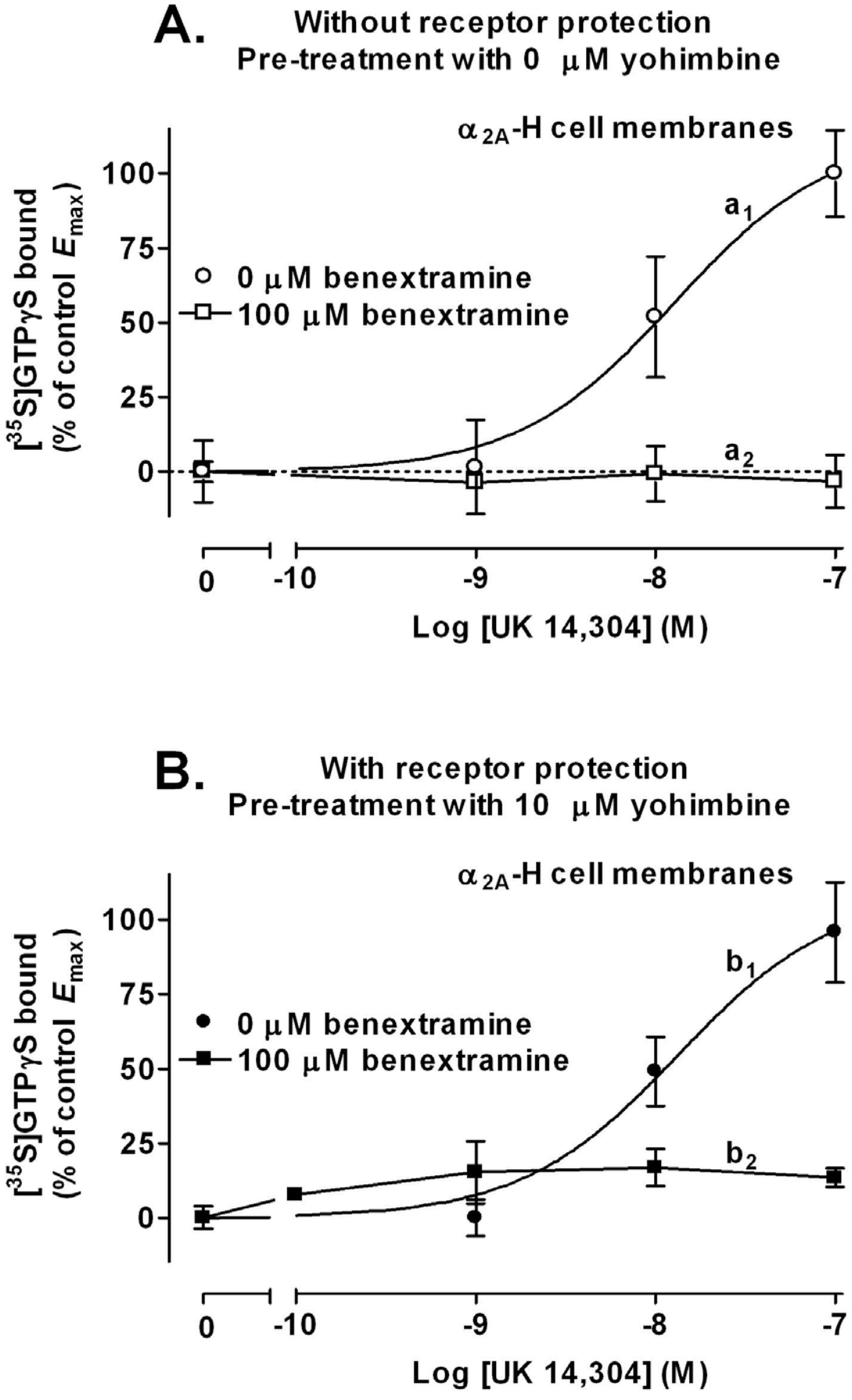

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Figure 6

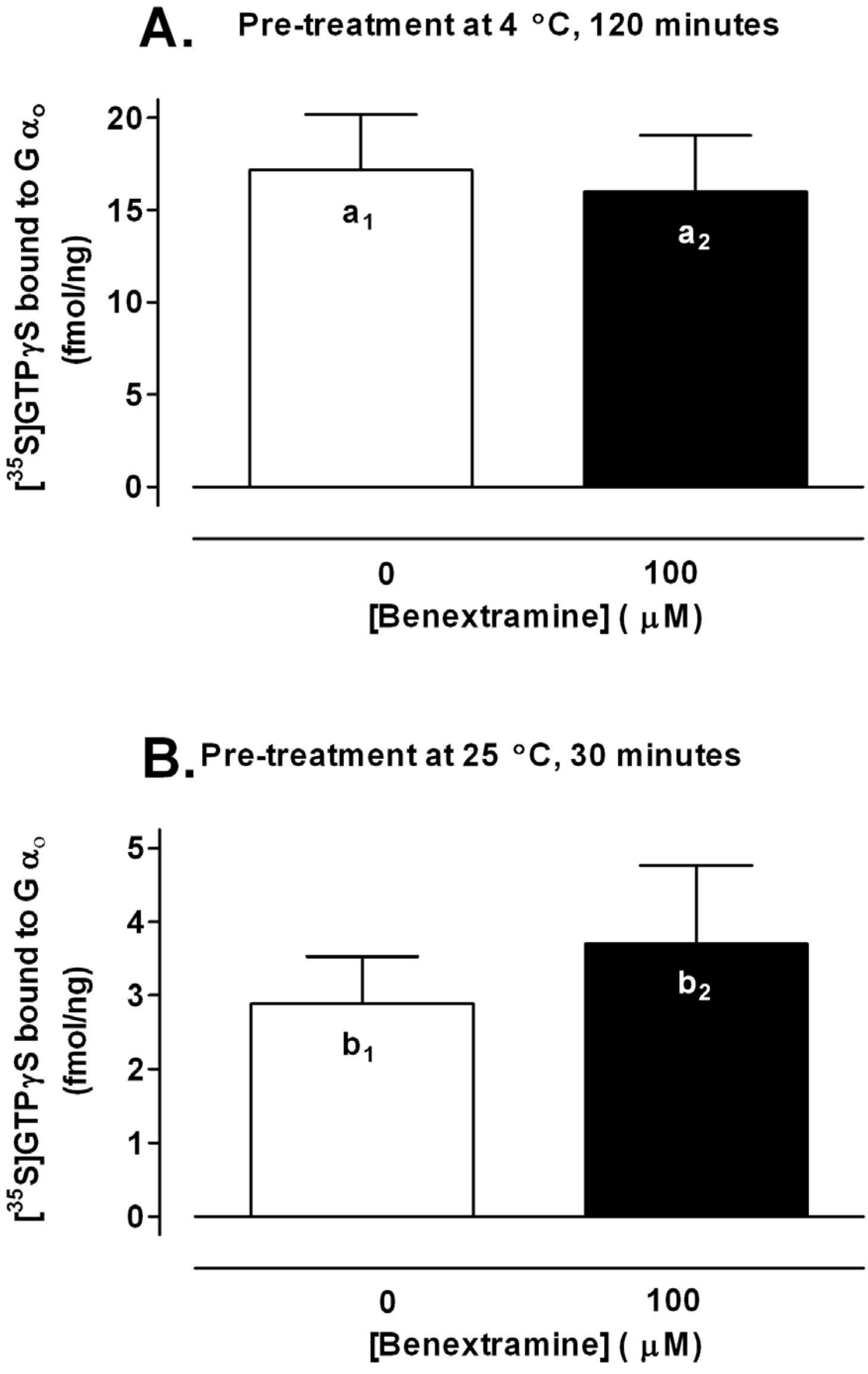

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Figure 7

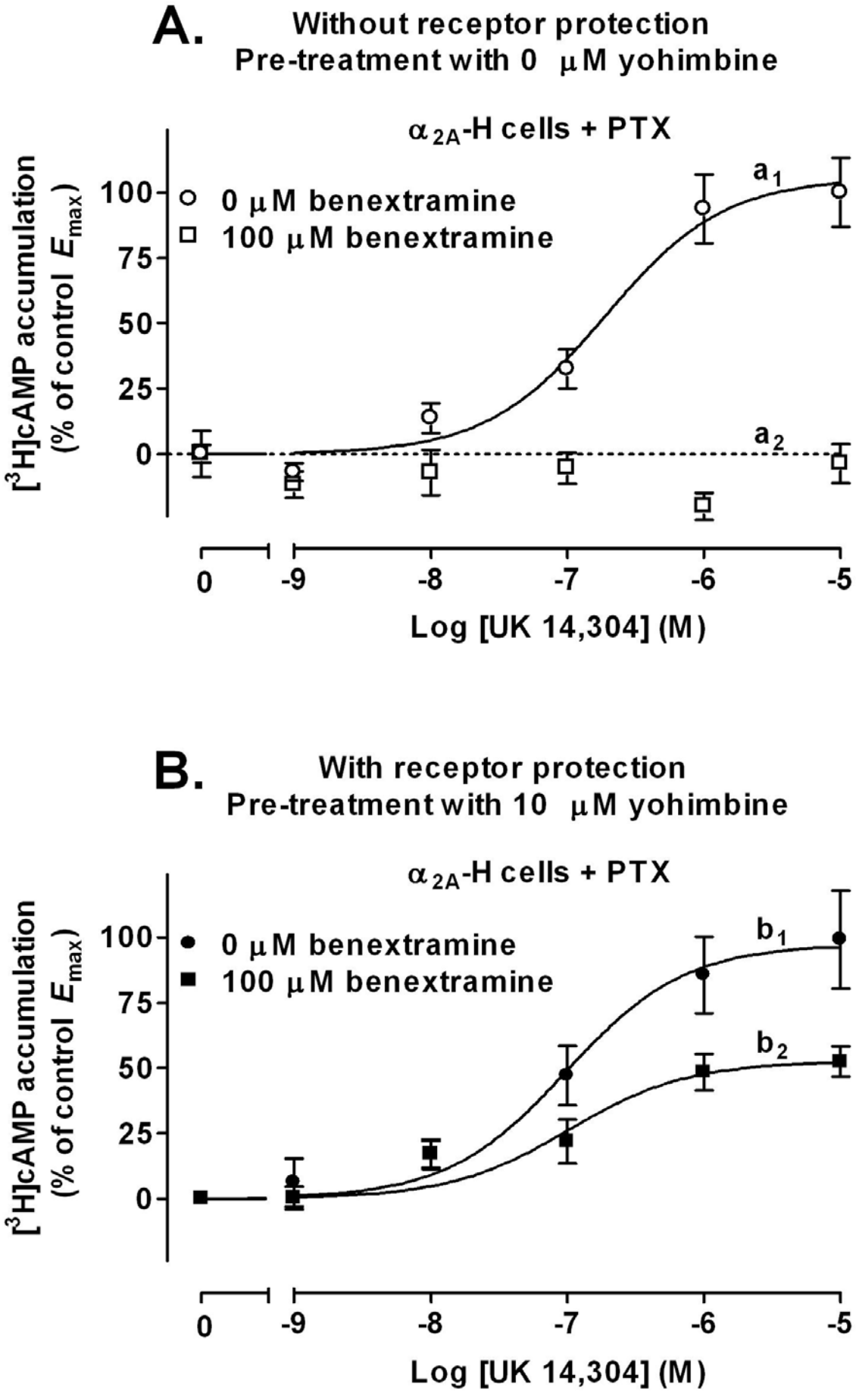

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Figure 8

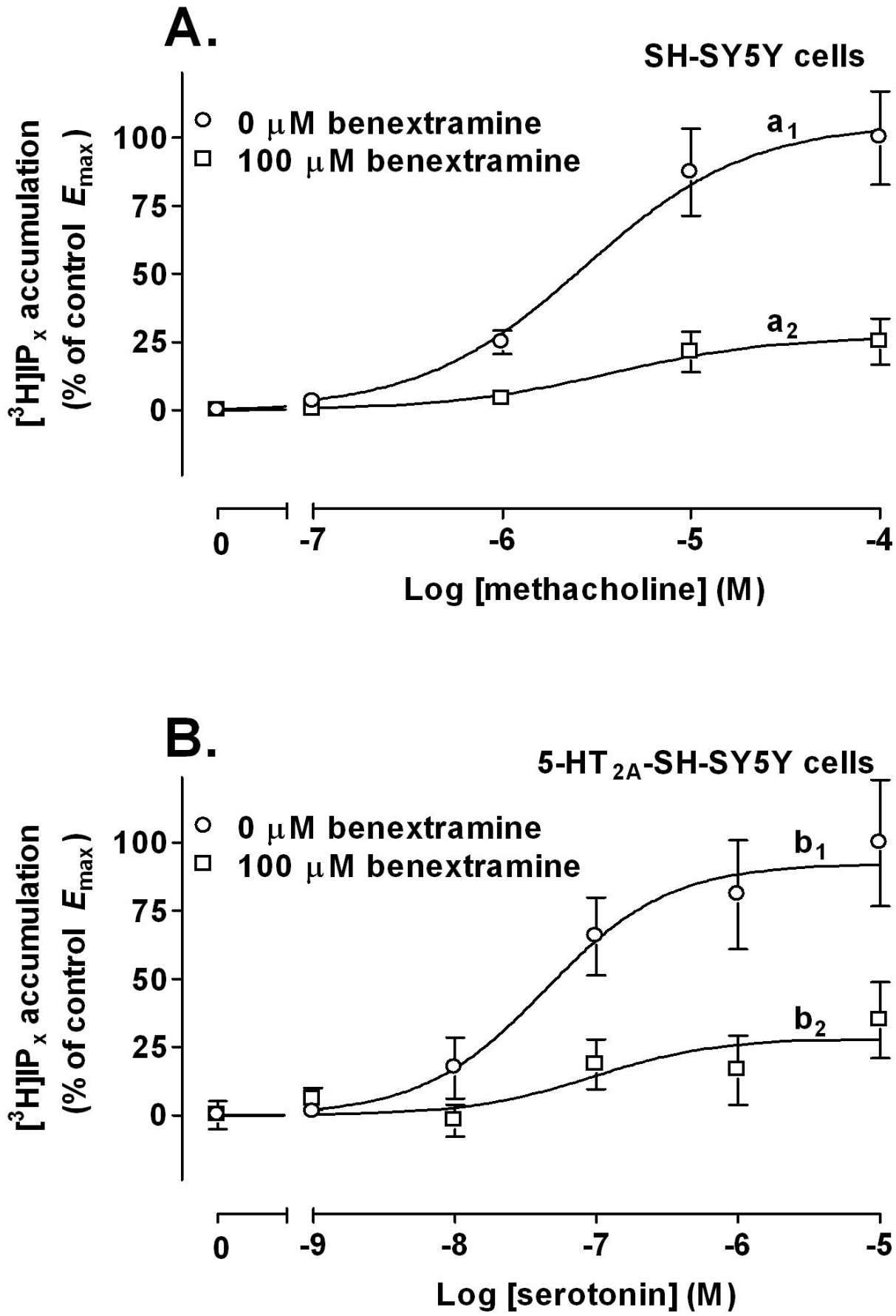

Figure 9