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The effect of site-directed mutagenesis of two transmembrane serine residues on agonist-specific coupling of a cloned human α_{2A}-adrenoceptor to adenylyl cyclase

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1 The effects of substitution of the Ser^{200} and Ser^{204} residues with alanine on the signalling properties of the cloned human α_{2A}-adrenoceptor, stably expressed in Chinese hamster ovary (CHO) cell lines, have been investigated using noradrenaline and the structural isomers of octopamine.

2 The Ser→Ala^{200} or the Ser→Ala^{204} mutant forms of the α_{2A}-adrenoceptor, when expressed in cells in the absence of pertussis toxin pretreatment, are two orders of magnitude more sensitive to inhibition of cyclic AMP production by (±)-*para*-octopamine and (±)-*meta*-octopamine, respectively, than cells expressing the wild-type receptor. Binding studies indicate that the effects are not due to an increased agonist affinity for the mutant receptors and that they are likely to be due to agonist-mediated conformational changes in receptor structure.

3 After incubation with pertussis toxin, (±)-*meta*-octopamine (100 μM and above) produced a stimulation of cyclic AMP levels in cells expressing the Ser→Ala^{204} mutant form of the α_{2A}-adrenoceptor but showed no stimulation in cells expressing the Ser→Ala^{200} mutant receptor. Under these conditions (±)-*para*-octopamine did not produce any increases in cyclic AMP production in cells expressing either of the mutant receptor forms or the wild-type receptor.

4 The results emphasise the importance of the Ser^{200} and Ser^{204} residues of the α_{2A}-adrenoceptor in exerting an inhibitory influence on the ability of (±)-*para*-octopamine and (±)-*meta*-octopamine respectively, to induce a receptor-agonist conformation capable of inhibiting forskolin-stimulation of cyclic AMP levels.

5 It is clear that Ser^{204} also prevents *meta*-octopamine from generating a receptor-agonist conformation that can increase cyclic AMP levels, emphasising the importance of this residue in the agonist-specific coupling of this receptor to different second messenger systems.

Keywords: α_{2A}-Adrenoceptor; *in vitro* mutagenesis; octopamine; cyclic AMP; adenylyl cyclase; noradrenaline

Abbreviations: ADP, adenosine 5′-diphosphate; CHO, Chinese hamster ovary cells; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; EDTA, ethylenediaminetetraacetic acid; G-protein, guanosine 5′-triphosphate binding protein; GTPγS, guanosine 5′-0-(3-thiotriphosphate); HEPES, N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; IBMX, 3-isobutyl-1-methylxanthine; NA, (−)-Noradrenaline; NAD, β-nicotinamide adenine dinucleotide; PTX, pertussis toxin; Ser→Ala^{200}, alanine substitution of Ser^{200} in cloned human α_{2A}-adrenoceptor; Ser→Ala^{204}, alanine substitution of Ser^{204} in cloned human α_{2A}-adrenoceptor; TMII, transmembrane domain two of G-protein coupled receptor; TMIII, transmembrane domain three of G-protein coupled receptor; TMV, transmembrane domain five of G-protein coupled receptor; TMVII, transmembrane domain seven of G-protein coupled receptor; Tris, tris(hydroxymethyl)aminomethane

Introduction

Agonist-specific coupling (agonist trafficking) of G-protein coupled receptors to different second messenger systems has been demonstrated for a number of receptors (see Robb *et al*., 1994; Evans *et al*., 1995b; Kenakin, 1995). This can be seen as a development of both the extended ternary complex (see Samama & Lefkowitz, 1997) and the multi-state allosteric (see Gether *et al*., 1997) models for receptor conformation. In the former model the number of conformational states of the receptor is defined, but in the latter it is proposed that there can be an infinite number of states between which the receptor can oscillate, only some of which are capable of activating G-proteins. In agonist-specific coupling the receptor would adopt different conformations, either as a result of the induced fit between an agonist and the receptor, or as a result of the agonist stabilizing a particular conformation spontaneously adopted by the receptor (Gether & Kobilka, 1998). These different agonist-promoted conformations would then couple the receptor preferentially to specific second messenger pathways.

We have previously shown that the *meta*- and *para*-isomers of the biogenic amine, octopamine, which is a naturally occurring ligand of sympathetic α-adrenoceptors, can couple a cloned human α_{2A}-adrenoceptor to multiple second messenger systems when expressed in a Chinese hamster ovary (CHO) cell line (Evans *et al*., 1995a; Airriess *et al*., 1996). In contrast to the catecholamines which couple the α_{2A}-adrenoceptor to both a dose-dependent decrease and increase in the rate of cyclic AMP production, the structural isomers of octopamine were only able to couple the receptor to a dose-dependent decrease in cyclic AMP production (Airriess *et al*., 1997). These results suggest that the cloned human α_{2A}-adrenoceptor can be

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coupled selectively by different endogenous agonists to G-protein pathways mediating the regulation of adenylyl cyclase activity.

Previous studies have emphasized the importance of conserved serine residues in Transmembrane domain V (TMV) of adrenoceptors in possible hydrogen bond interactions with the *para*- and *meta*-hydroxyl groups of the phenyl ring of catecholamines in activation processes (e.g. Strader *et al*., 1989 for the β_2-adrenoceptor; Wang *et al*., 1991 for the α_{2A}-adrenoceptor; and Hwa & Perez, 1996 for the α_1-adrenoceptor). Studies on the β_2-adrenoceptor indicate that both Ser^{204} and Ser^{207} are required for binding and full agonist activity, Ser^{204} forming hydrogen bonds with the *meta*-hydroxyl group of the catecholamine ring and Ser^{207} forming hydrogen bonds with the *para*-hydroxyl group. Studies on the α_{2A}-adrenoceptor suggest that only Ser^{204} (equivalent to Ser^{207} of the β_2-adrenoceptor), which is thought to interact with the *para*-hydroxyl of the catecholamine ring, appears to contribute partially to agonist-binding and receptor activation. Ser^{200} (equivalent to Ser^{204} of the β_2-adrenoceptor) does not appear to be involved in receptor activation. Studies on the α_1-adrenoceptor activation suggest that it is the interaction between the *meta*-hydroxyl group of the catecholamine ring and Ser^{188} (equivalent to Ser^{204} of the β_2-adrenoceptor) that allows receptor activation and not the interaction between the *para*-hydroxyl group and Ser^{192} (equivalent to Ser^{207} of the β_2-adrenoceptor). Furthermore, since Ser^{188} and Ser^{192} are separated by three residues on the TMV α-helix, whereas Ser^{204} and Ser^{207} of the β_2-adrenoceptor are separated by only two residues, the orientation of the catecholamine ring in the α_1-adrenoceptor binding pocket appears to be rotated approximately 120° with respect to that in the β_2-adrenoceptor. Thus, since the structural isomers of octopamine differ from noradrenaline by the absence of one of the hydroxyl groups on the catecholamine ring, it seems very likely that their different effectiveness in coupling the α_{2A}-adrenoceptor to the regulation of adenylyl cyclase activity will be influenced by an interaction with the serine residues in TMV.

In the present study we report on the effect of substitution of the Ser^{200} and Ser^{204} residues of the α_{2A}-adrenoceptor with alanine on the ability of (±)*meta*- and (±)*para*-octopamine (see Figure 1 for comparison of structures with that of noradrenaline) to couple the α_{2A}-adrenoceptor expressed in CHO cells to the modulation of adenylyl cyclase activity. The ability to inhibit or stimulate forskolin-stimulated cyclic AMP levels in CHO cell lines expressing the Ser→Ala^{200} and Ser→Ala^{204} mutant receptors was compared with that of the wild-type receptor in the presence and absence of pertussis toxin. Parallel studies on agonist binding and on the agonist-mediated stimulation of [^{35}S]-GTPγS binding were also performed. The results are interpreted in terms of a model to explain the production of different agonist induced configurations of the α_{2A}-adrenoceptor. A brief account of some of this work has already been published in abstract form (Rudling *et al*., 1997).

Methods

Cell culture

Chinese hamster ovary cells transfected with the α_{2A}-adrenoceptor (11 pmol receptor mg^{-1} protein) were grown to ~90% confluence in cell culture at 37°C (Fraser *et al*., 1989). The culture medium consisted of 90% Ham's F-12 nutrient mixture (Gibco) and 10% bovine foetal calf serum (Gibco).

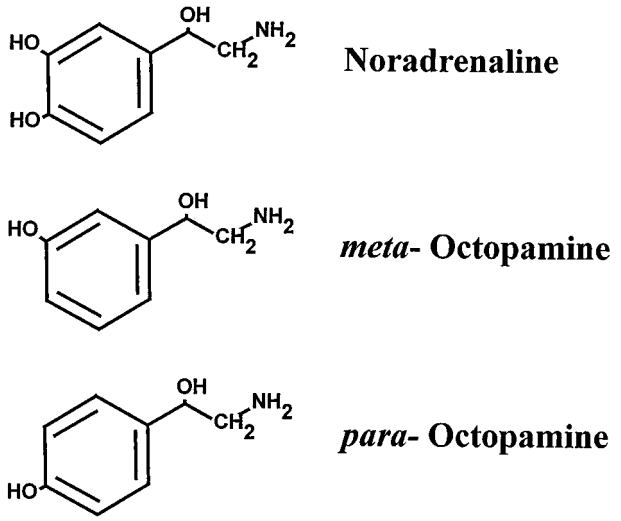

Figure 1 Structures of (−)-Noradrenaline, (±)-*meta*-Octopamine and (±)-*para*-Octopamine.

Penicillin and streptomycin were included in the culture medium at 50 u ml^{-1} and 50 μg ml^{-1}, respectively. G-418 sulphate (Geneticin; 50 μg ml^{-1}) was used in the medium to select for cells expressing the α_{2A}-adrenoceptor. Uncoupling of G_i-mediated inhibition of cyclic AMP production was achieved by 24 h pre-incubation of cells in growth medium containing 200 ng ml^{-1} pertussis toxin (Sigma).

Identical cell culture procedures were used for the CHO cell line expressing the mutant α_{2A}-adrenoceptor in which Ser^{200} had been replaced by Ala (Ser→Ala^{200}) (11 pmol receptor mg^{-1} protein) and for the CHO cell line expressing the mutant α_{2A}-adrenoceptor in which Ser^{204} had been replaced by Ala (Ser→Ala^{204}) (7 pmol receptor mg^{-1} protein). The production and characterization of the CHO cell lines expressing the Ser→Ala^{200} and the Ser→Ala^{204} mutant α_{2A}-adrenoceptors used in the present study has been described previously (Wang *et al*., 1991).

Membrane preparation

Cells were washed by rinsing 150 mm culture dishes with 5 ml of Dulbecco's phosphate buffered saline three times, to remove the culture medium. The cells were then scraped into 4 ml of ice-cold lysis buffer (50 mM sodium phosphate buffer, pH 7.4, 1 mM $MgSO_4$) and then washed with a further 4 ml of ice-cold lysis buffer. After incubation on ice for 15 min, the cells were lysed by performing 20 complete strokes with a Dounce homogenizer using the small clearance pestle. The cell lysate was centrifuged at 2300 r.p.m. to pellet whole cells and nuclei and the supernatant was centrifuged at 19,000 r.p.m. in a Sorval Superspeed (RC-5B) for 30 min at 4°C to pellet the crude membrane fraction. The resulting membrane pellet was resuspended in lysis buffer. Protein concentrations were determined using a protein assay kit (Merck) based on the method of Bradford (1976), with bovine serum albumin as a reference standard.

ADP-ribosylation

To ensure that the 24 h pertussis toxin pretreatment of the cells described above was sufficient to induce the ADP-ribosylation of all available pertussis toxin substrate, control experiments were carried out in which membranes were prepared from cells

that had been pretreated in the presence or absence of pertussis toxin (200 ng ml^{-1}). The membranes were then subjected to pertussis toxin catalyzed ADP-ribosylation in the presence of [^{32}P]-NAD (800 Ci $mmol^{-1}$, New England Nuclear) following the method described by Perez *et al.* (1993). The overnight pertussis toxin pretreatment of the cells completely blocked the incorporation of [^{32}P]-NAD into a 43-kDa protein, which is most likely to be a G_i-like G-protein. This result is identical to that found for other CHO cell lines expressing the different α_{2A}-adrenoceptor receptor subtypes when pretreated overnight with pertussis toxin at a concentration of 500 ng ml^{-1} (Eason *et al.*, 1992).

Ligand binding studies

Radioligand binding assays using [^{3}H]-yohimbine were carried out as follows. Membranes (20–25 μg protein) were incubated at 37°C for 20 min in the presence of 6 nM [methyl-^{3}H]-yohimbine (91 Ci $mmol^{-1}$, Amersham), in binding assay buffer (mM) NaCl 150, $MgCl_2$ 5, EDTA 20, Tris 50, pH 7.4, with varying concentrations of unlabelled yohimbine (expression level assays) or agonist (competitive displacement assays), in a final incubation volume of 500 μl. Non-specific binding was determined in the presence of 1 mM yohimbine. The reaction was terminated by dilution with 500 μl of ice-cold buffer and centrifugation at 4°C at 14,000 r.p.m. for 10 min. The pellets were washed with 1 ml ice-cold buffer and recentrifuged. The pellet was resuspended in 0.1 M NaOH and added to 7 ml of scintillation fluid for counting.

Radioligand binding curves were analysed using a non-linear regression program of GraphPAD software. The binding data was fitted to both one-site and two site binding models, however, in each case the best fit was obtained by a one site model with R^2 values ranging from 0.9–0.96. The B_{max} was then converted from c.p.m.s to pmol mg^{-1} membrane protein. Data represent the average of three separate experiments performed in duplicate.

[^{35}S]-GTPγS binding

50 μl of agonist (either (−)-noradrenaline, (±)-*m*-octopamine or (±)-*p*-octopamine), at 10× final concentration was added to 400 μl of membrane mix and preincubated at 37°C for 20 min. The membrane mix consisted of 25 μg of membrane protein per tube in 20 mM HEPES buffer, pH 7.5, 3 mM $MgCl_2$ and in the presence of 3 μM GDP. After the preincubation, 50 μl (0.138 μCi) of [^{35}S]-GTPγS (1103 Ci $mmol^{-1}$, Amersham) was added to each tube and incubated for 20 min at 37°C. The reaction was terminated by adding 500 μl of ice-cold buffer (20 mM HEPES, pH 7.5, 3 mM $MgCl_2$). The samples were centrifuged at 4°C at 14,000 r.p.m. for 10 min. The supernatant was removed and the pellet washed with 1 ml of buffer and recentrifuged. The membrane pellets were resuspended in 100 μl 0.1 M NaOH, added to 7 ml scintillation fluid and counted for the estimation of bound [^{35}S]-GTPγS. Non-specific binding was determined using 10 μM unlabelled GTPγS.

Cyclic AMP production

Cells were first washed, by rinsing culture plates (60 mm) with 3 ml of Dulbecco's phosphate buffered saline (PBS; Gibco), to remove culture medium. They were then incubated for 20 min at 37°C in PBS containing 100 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma), a phospho-diesterase inhibitor. The cells were then exposed to solutions of agonists at specific concentrations in the presence of 10 μM forskolin (Sigma), a membrane permeant adenylyl cyclase activator, plus 100 μM IBMX. Solutions of 10 μM forskolin plus 100 μM IBMX alone were used to determine the control rate of forskolin-stimulated cyclic AMP production.

Incubations were terminated after 20 min by removal of the PBS followed by the addition of 500 μl of ice-cold, acidified ethanol (60 ml absolute-EtOH : 1 ml 1 N HCl). The plates were scraped and pooled with two subsequent 250 μl washes with acidified ethanol. Cell debris was then removed by centrifugation at 13,000 r.p.m. for 5 min. The supernatant was evaporated to dryness by means of a vacuum centrifuge (Savant) and the residue was re-suspended in 150 μl of Tris/EDTA buffer. Cyclic AMP levels were determined in duplicate using the [8-^{3}H]-cyclic AMP assay kit of Amersham (Biotrak TRK 432).

Dose response curves for the various agonists, both with and without pertussis toxin pretreatment of the cells, were constructed for concentrations ranging from 1 nM to 1 mM. The concentration of cyclic AMP (pmol $plate^{-1}$) in experimental plates was expressed as a percentage of the [cyclic AMP] in plates from the same group which were exposed only to forskolin in the absence of any agonist. Four of these internal control plates were used in conjunction with each group of fourteen experimental plates.

Analysis of variance (ANOVA) was used to test for significant agonist-mediated effects in individual experiments. Significant ANOVA's were then further analysed using Tukey's HSD multiple comparison test, to determine at what concentration the levels of cyclic AMP production differed significantly from the forskolin-only control values. Unless otherwise stated, all data are shown as mean ± s.e.mean.

Pharmaceutical compounds

Racemic *m*-octopamine was from the Aldrich Chemical Company; racemic *p*-octopamine, IBMX and forskolin were from Sigma.

Results

Cyclic AMP production

Noradrenaline After 20 min of incubation with (−)-noradrenaline (NA) in the absence of pertussis toxin (PTX) pretreatment, maximum significant inhibition of cyclic AMP production ($F = 5.68$; $d.f. = 6,26$; $P < 0.001$) was seen in Chinese hamster ovary (CHO) cells expressing the wild-type α_{2A}-adrenoceptor at an agonist concentration of 100 nM (Figure 2). At higher concentrations of NA between 10 μM and 1 mM, the inhibitory effects of this agonist were not as great but still significant. The biphasic nature of the response to NA is due to the ability of the α_{2A}-adrenoceptor to couple to the stimulation of cyclic AMP production at high concentrations of NA when expressed in CHO cells (Fraser *et al.*, 1989; Eason *et al.*, 1992; Airriess *et al.*, 1997).

NA was almost two orders of magnitude less effective at inhibiting cyclic AMP production in CHO cells expressing the mutant α_{2A}-adrenoceptor form in which Ser^{200} had been replaced by Ala ($Ser \rightarrow Ala^{200}$) (Figure 2). A maximum significant inhibition of cyclic AMP production was seen at an agonist concentration of 100 μM ($F = 18.64$; $d.f. = 6,55$; $P < 0.001$) and in contrast with the cells expressing the wild-type receptor, no significant increases in the level of cyclic AMP production above the maximal inhibition were observed at concentrations up to 1 mM.

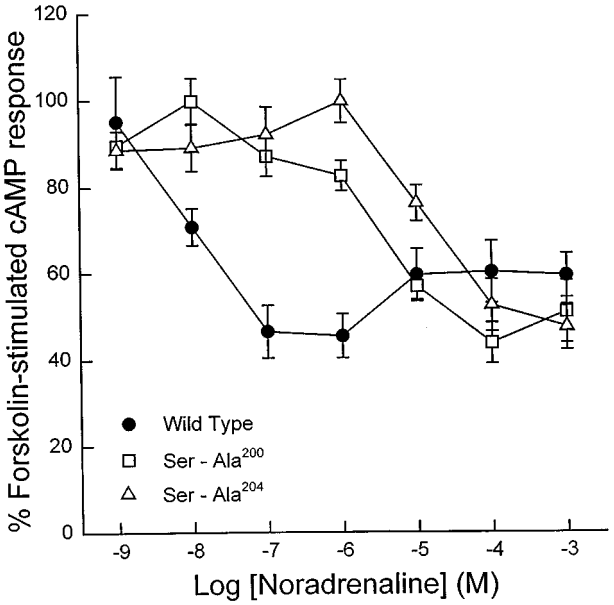

Figure 2 A comparison of the effects of (−)-noradrenaline on forskolin-stimulated cyclic AMP production in CHO cells stably expressing either the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. Noradrenaline is less effective at inhibiting forskolin-stimulated cyclic AMP production in cells expressing either of the mutant receptor forms compared to its effects on cells expressing the wild-type receptor. Noradrenaline was about an order of magnitude more potent at inhibiting forskolin-stimulated cyclic AMP production in cells expressing the Ser→Ala^{200} mutant receptor compared to its actions on cells expressing the Ser→Ala^{204} mutant receptor. Data represent means and vertical lines show s.e.mean, $n=4-14$.

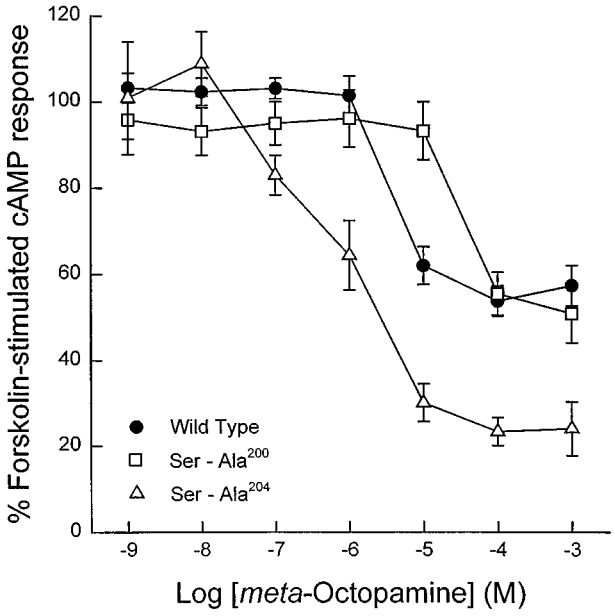

Figure 3 A comparison of the effects of *meta*-octopamine on forskolin-stimulated cyclic AMP production in CHO cells stably expressing either the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. *meta*-Octopamine was an order of magnitude less effective at inhibiting forskolin-stimulated cyclic AMP production in cells expressing the Ser→Ala^{200} mutant receptor compared to cells expressing the wild-type receptor. However, *meta*-octopamine was almost two orders of magnitude more effective at inhibiting forskolin-stimulated cyclic AMP production in cells expressing the Ser→Ala^{204} mutant receptor compared to cells expressing the wild-type receptor. The maximum inhibition obtained in cells expressing the Ser→Ala^{204} mutant receptor was 40% greater than that found for the cells expressing either the wild-type receptor or the Ser→Ala^{200} mutant receptor. Data represent means and vertical lines show s.e.mean, $n=4-12$.

Similarly, NA was about three orders of magnitude less effective at inhibiting cyclic AMP production in CHO cells expressing the mutant α_{2A}-adrenoceptor form in which Ser^{204} had been replaced by Ala (Ser→Ala^{204}) (Figure 2). A maximum inhibition of cyclic AMP production was seen at an agonist concentration of 1 mM ($F=14.73$; $d.f.=6,55$; $P<0.001$) and again in contrast to cells expressing the wild-type receptor, no significant increases in the level of cyclic AMP production above the maximal inhibition were observed up to this concentration.

meta-*Octopamine* The *meta*-isomer of (±)-octopamine produced a significant inhibition of the forskolin stimulated cyclic AMP levels in CHO cells expressing the wild-type α_{2A}-adrenoceptor at concentrations of 10 μM and above ($F=43.38$; $d.f.=6,65$; $P<0.001$) in the absence of pertussis toxin pretreatment (Figure 3). At concentrations of 100 μM and 1 mM the inhibition levelled off but no significant stimulation of cyclic AMP production was observed.

In CHO cells expressing the Ser→Ala^{200} mutant α_{2A}-adrenoceptor (±)-*m*-octopamine was an order of magnitude less potent at inhibiting cyclic AMP production compared with the wild-type receptor, with a significant inhibition of forskolin stimulated cyclic AMP levels occurring only at a concentration of 100 μM ($F=19.00$; $d.f.=6,32$; $P<0.001$) (Figure 3). However, the maximum inhibition of cyclic AMP production by (±)-*m*-octopamine was equal in CHO cells expressing either the wild-type receptor or its Ser→Ala^{200} mutant form within the concentration range tested. In contrast, in cells expressing the Ser→Ala^{204} mutant form of the α_{2A}-adrenoceptor (±)-*m*-octopamine was almost two orders of magnitude more potent at inhibiting cyclic AMP production compared with the wild-type receptor, with a significant inhibition of forskolin stimulation of cyclic AMP levels occurring at a concentration of 100 nM ($F=24.50$; $d.f.=6,36$; $P<0.05$) (Figure 3). In addition, the maximum inhibition of cyclic AMP production by (±)-*m*-octopamine was also greater in cells expressing the Ser→Ala^{204} mutant receptor than in those expressing the wild-type receptor in the concentration range tested.

para-*Octopamine* The *para*-isomer of (±)-octopamine was almost an order of magnitude less potent than the (±)-*m*-octopamine in producing a significant inhibition of forskolin stimulated cyclic AMP levels in CHO cells expressing the wild-type α_{2A}-adrenoceptor, with a significant inhibition occurring at a concentration of 100 μM and above ($F=24.25$; $d.f.=6,58$; $P<0.001$) in the absence of pertussis toxin pretreatment (Figure 4). No significant stimulation of cyclic AMP production was observed at any of the concentrations tested up to 1 mM.

In CHO cells expressing the Ser→Ala^{200} mutant α_{2A}-adrenoceptor (±)-*p*-octopamine was two orders of magnitude more potent at inhibiting cyclic AMP production compared with the wild-type receptor, with a significant inhibition of forskolin-stimulation of cyclic AMP levels occurring at a concentration of 1 μM ($F=14.74$; $d.f.=6,32$; $P<0.05$) (Figure 4). In addition, the maximum inhibition of cyclic AMP production by (±)-*p*-octopamine was greater for cells

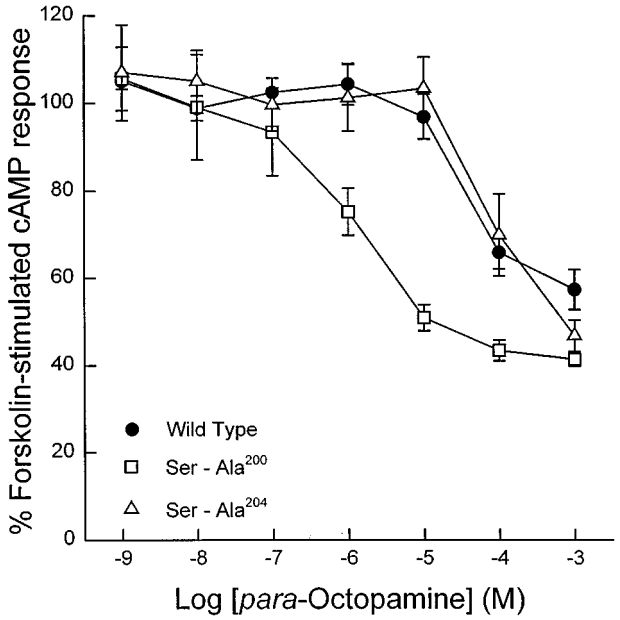

Figure 4 A comparison of the effects of *para*-octopamine on forskolin-stimulated cyclic AMP production in CHO cells stably expressing either the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. There is no significant difference in the inhibition of forskolin-stimulated cyclic AMP production mediated by *para*-octopamine in cells expressing either the Ser→Ala^{204} mutant receptor or the wild-type receptor. However, *para*-octopamine was about two orders of magnitude more effective at inhibiting forskolin-stimulated cyclic AMP production in cells expressing the Ser→Ala^{200} mutant receptor compared to cells expressing the wild-type receptor. The maximum inhibition obtained in cells expressing the Ser→Ala^{200} mutant receptor was 20% greater than that found for the cells expressing the wild-type receptor. Data represent means and vertical lines show s.e.mean, $n=6-12$.

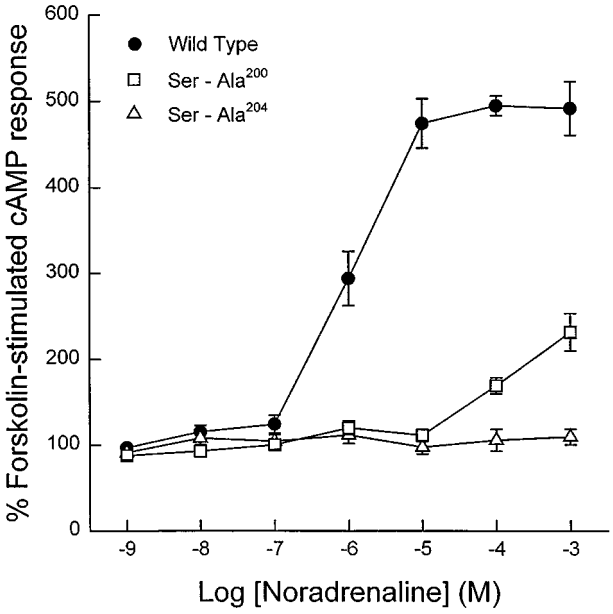

Figure 5 A comparison of the effects of (−)-noradrenaline on forskolin-stimulated cyclic AMP production in CHO cells stably expressing either the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor after pretreatment with pertussis toxin (24 h, 200 ng ml^{-1}) to inhibit receptor coupling to G_i. Noradrenaline produced a dramatic stimulation in cyclic AMP production in CHO cells expressing the wild-type human α_{2A}-adrenoceptor, but its ability to stimulate cyclic AMP production was severely depressed in CHO cells expressing either the Ser→Ala^{200} mutant form or the Ser→Ala^{204} mutant form of the α_{2A}-adrenoceptor. Data represent means and vertical lines show s.e.mean, $n=4-8$.

expressing the Ser→Ala^{200} mutant receptor compared with those expressing the wild-type receptor. In contrast, in cells expressing the Ser→Ala^{204} mutant α_{2A}-adrenoceptor the inhibition of cyclic AMP production produced by (±)-*p*-octopamine was not significantly different from that produced by cells expressing the wild-type receptor.

The effect of pertussis toxin pretreatment After 24 h of pre-incubation with pertussis toxin (200 ng ml^{-1}), which blocks the activation of the inhibitory G proteins (G_i and G_o) by ADP-ribosylation of the α-subunits, inhibition of cyclic AMP production by NA was abolished and was replaced by a dramatic stimulation ($F=85.52$; $d.f.=6,20$; $P<0.001$) with a maximum efficacy at a concentration of 1 mM (Figure 5) in CHO cells expressing the wild-type α_{2A}-adrenoceptor. However, in CHO cells expressing either the Ser→Ala^{200} or the Ser→Ala^{204} mutant forms of the α_{2A}-adrenoceptor, the ability of NA to stimulate the level of cyclic AMP production after pertussis toxin pretreatment was severely depressed. In cells expressing the Ser→Ala^{200} mutant α_{2A}-adrenoceptor a significant stimulation of cyclic AMP production was only seen at concentrations of 100 μM and above ($F=23.80$; $d.f.=6,47$; $P<0.001$). However, in cells expressing the Ser→Ala^{204} mutant α_{2A}-adrenoceptor no significant stimulation of cyclic AMP production was seen at any of the concentrations tested up to 1 mM.

In CHO cells expressing the wild-type α_{2A}-adrenoceptor (±)-*m*-octopamine did not produce a significant increase in cyclic AMP production after pertussis toxin pretreatment at any of the concentrations tested up to 1 mM (Figure 6). Similar results were obtained for CHO cells expressing the Ser→Ala^{200} mutant α_{2A}-adrenoceptor. Surprisingly, however, after pertussis toxin pretreatment CHO cells expressing the Ser→Ala^{204} mutant α_{2A}-adrenoceptor showed a significant stimulation of cyclic AMP production at a (±)-*m*-octopamine concentration of 100 μM and above ($F=33.06$; $d.f.=6,50$; $P<0.001$) (Figure 6).

CHO cells expressing either the wild-type α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant α_{2A}-adrenoceptor or the Ser→Ala^{204} mutant α_{2A}-adrenoceptor did not demonstrate any significant increases in cyclic AMP production, after pertussis toxin pretreatment when exposed to (±)-*p*-octopamine at any concentration up to 1 mM (Figure 7).

Ligand binding

The increased potencies of (±)-*m*-octopamine on the Ser→Ala^{204} mutant α_{2A}-adrenoceptor and of (±)-*p*-octopamine on the Ser→Ala^{200} mutant α_{2A}-adrenoceptor in the functional assays on cyclic AMP inhibition, could be explained by an increase in the affinities of the agonists for the mutant receptors. This possibility was tested directly by comparing the ability of (±)-*m*-octopamine, (±)-*p*-octopamine and (−)-noradrenaline to displace the binding of [^{3}H]-yohimbine, an α_{2A}-adrenoceptor antagonist (See Table 1) from the receptors.

Figure 8 indicates that Ala substitution of either of the serines (Ser^{200} or Ser^{204}), in TMV of the α_{2A}-adrenoceptor, thought to be involved with the binding of the catecholamine ring hydroxyls (Fraser *et al.*, 1989; Wang *et al.*, 1991), reduces

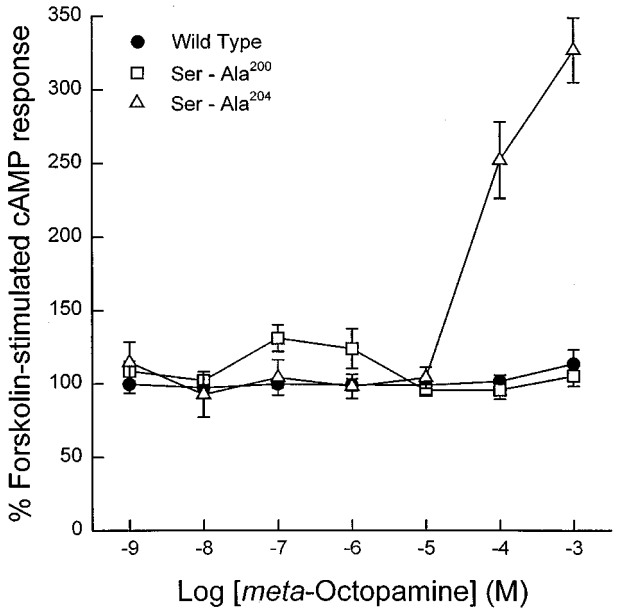

Figure 6 A comparison of the effects of *meta*-octopamine on forskolin-stimulated cyclic AMP production in CHO cells stably expressing either the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor after pretreatment with pertussis toxin (24 h, 200 ng ml^{-1}) to inhibit receptor coupling to G_i. *meta*-Octopamine, surprisingly, stimulates forskolin-stimulated cyclic AMP production at concentrations above 10 μM in cells expressing the Ser→Ala^{204} mutant receptor with a maximum stimulation of 325% of basal levels being observed at 1 mM. In contrast, after pertussis toxin pretreatment *meta*-octopamine did not induce any changes in forskolin-stimulated cyclic AMP production in cells expressing either the wild-type receptor or the Ser→Ala^{200} mutant receptor. Data represent means and vertical lines show s.e.mean, $n=5-13$.

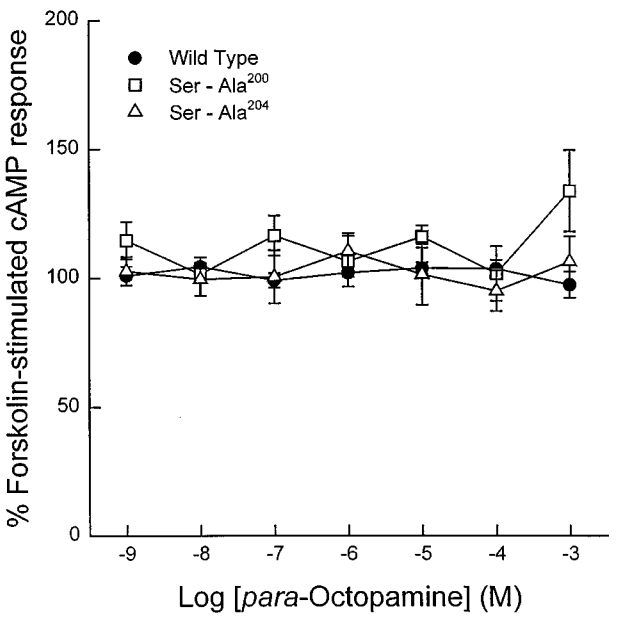

Figure 7 A comparison of the effects of *para*-octopamine on forskolin-stimulated cyclic AMP production in CHO cells stably expressing either the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor after pretreatment with pertussis toxin (24 h, 200 ng ml^{-1}) to inhibit receptor coupling to G_i. *para*-Octopamine produced no increases in forskolin-stimulated cyclic AMP levels under these conditions in cells expressing either the Ser→Ala^{204} mutant receptor, the Ser→Ala^{200} mutant receptor or the wild-type receptor. Data represent means and vertical lines show s.e.mean, $n=5-9$.

Table 1 Ligand-binding properties of Ser→Ala mutant α_{2A}-adrenoceptors

	Wild-type K_i (M)	*Ser→Ala^{200}* K_i (M)	*Ser→Ala^{204}* K_i (M)
B_{max} (pmol mg^{-1})	11	11	7
(−)-Noradrenaline	1.78×10^{-5}	7.14×10^{-5}	7.36×10^{-5}
(±)-*meta*-Octopamine	2.44×10^{-5}	3.06×10^{-4}	6.86×10^{-5}
(±)-*para*-Octapamine	1.06×10^{-4}	5.43×10^{-5}	1.28×10^{-4}

K_i values were determined in competition displacement experiments.

the ability of NA to displace [^{3}H]-yohimbine binding by 4 fold. In contrast, substitution of Ser^{204} with Ala did not change the binding affinity of (±)-*m*-octopamine, whereas substitution of Ser^{200} with Ala decreased it by 12 fold (Figure 9). Finally, neither Ala substitution changed the binding affinity of (±)-*p*-octopamine for the α_{2A}-adrenoceptors (Figure 10).

These results are essentially identical to those obtained by Wang *et al*. (1991) for the α_{2A}-adrenoceptor using a related set of agonists. The results suggest that the increased potencies of the various isomers of octopamine in functional assays on the inhibition of cyclic AMP production by the mutant α_{2A}-adrenoceptors are not likely to be due to an increased binding affinity of the agonists for the receptors.

[^{35}S]-GTPγS binding

The above cyclic AMP and ligand binding results suggest it is likely that the agonists induce specific receptor conformations in the mutant α_{2A}-adrenoceptors resulting in an increased effectiveness of interactions with the G-protein systems controlling the cyclic AMP producing system. This supposition can be tested more directly by assaying the ability of the agonists to stimulate the binding of [^{35}S]-GTPγS to membrane preparations of cells expressing the receptors (Lazareno & Birdsall, 1993).

Figure 11 shows that NA was almost two orders of magnitude less potent at stimulating [^{35}S]-GTPγS binding in membranes from CHO cells expressing either the Ser→Ala^{200} or Ser→Ala^{204} mutant forms of the α_{2A}-adrenoceptor compared with that observed in membranes expressing the wild-type receptor. In contrast, both the potency and the efficacy of (±)-*m*-octopamine stimulated [^{35}S]-GTPγS binding were increased in membranes from CHO cells expressing the Ser→Ala^{204} mutant form of the α_{2A}-adrenoceptor in comparison with the results obtained with the wild-type receptor (Figure 12). However, (±)-*m*-octopamine was less potent at stimulating [^{35}S]-GTPγS binding in membranes from CHO cells expressing the Ser→Ala^{200} mutant form of the α_{2A}-

adrenoceptor than in those expressing the wild-type receptor. In addition, in membranes from cells expressing the Ser→Ala^{200} mutant form of the α_{2A}-adrenoceptor, (±)-*p*-octopamine was equipotent but more efficacious at inducing GTPγS binding than in membranes from cells expressing the wild-type receptor (Figure 13). Further, (±)-*p*-octopamine was less potent at inducing GTPγS binding in membranes from cells expressing the Ser→Ala^{204} mutant form of the α_{2A}-adrenoceptor than in those expressing the wild-type receptor.

The results from the GTPγS binding studies confirm that the increased potencies of the isomers of octopamine in functional assays on the inhibition of cyclic AMP production are likely to be due to differences in agonist-induced or agonist-selected conformations of the different mutant α_{2A}-adrenoceptors.

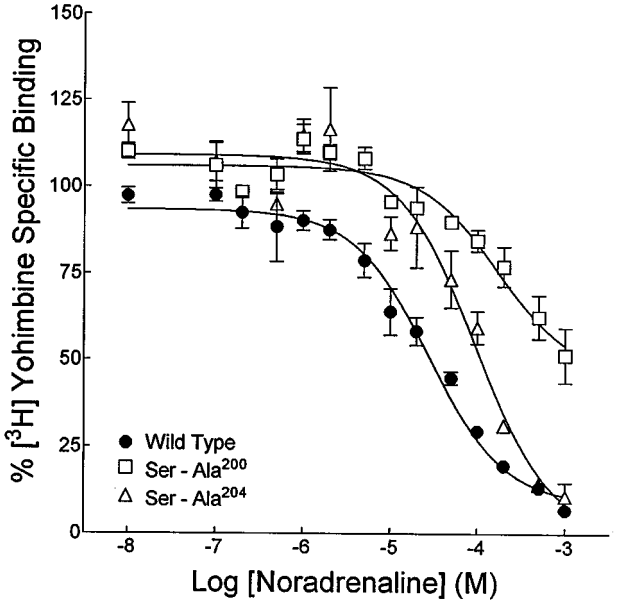

Figure 8 Competition binding curves of (−)-noradrenaline to the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. Competition displacement experiments were performed on membranes prepared from transfected CHO cells expressing wild-type or the indicated mutant α_{2A}-adrenoceptors. Data represent means of three experiments performed in duplicate and vertical lines show s.e.mean.

Discussion

Structure-function studies on expressed α_{2A}-adrenoceptors containing specific point mutations generated by *in vitro* site-directed mutagenesis have provided considerable information on many aspects of receptor activation and signalling. These include the identification of the likely sites of interaction between the receptor and catecholamine agonists, the amino acids necessary to prevent constitutive activation of the receptor, the amino acid sequences involved in interactions with specific G-proteins and the role of phosphorylation in the desensitization process (see Savarese & Fraser, 1992; Eason & Liggett, 1996; Hieble *et al*., 1997; Scheer & Cotecchia, 1997). Wang *et al*. (1991) demonstrated that Asp^{113} in TMIII was likely to be the site of interaction with the amino nitrogen of the catecholamines and that Ser^{204}, which is thought to interact with the *para*-hydroxyl on the catecholamine ring, appears to contribute partially to agonist-binding and receptor activation. Ser^{200} did not appear to be involved in receptor activation and no specific identification of the *meta*-hydroxyl interactions with the receptor were identified although Cys^{201} was suggested as a possibility.

In the present study substitution of Ser^{200} and Ser^{204} with Ala in the cloned human α_{2A}-adrenoceptor reduced the potency of noradrenaline in inhibiting forskolin-stimulated

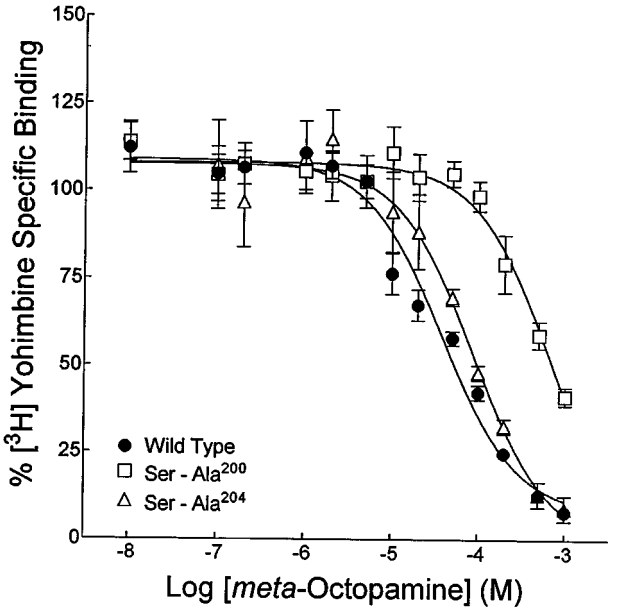

Figure 9 Competition binding curves of *meta*-octopamine to the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. Competition displacement experiments were performed on membranes prepared from transfected CHO cells expressing wild-type or the indicated mutant α_{2A}-adrenoceptors. Data represent means of three experiments performed in duplicate and vertical lines show s.e.mean.

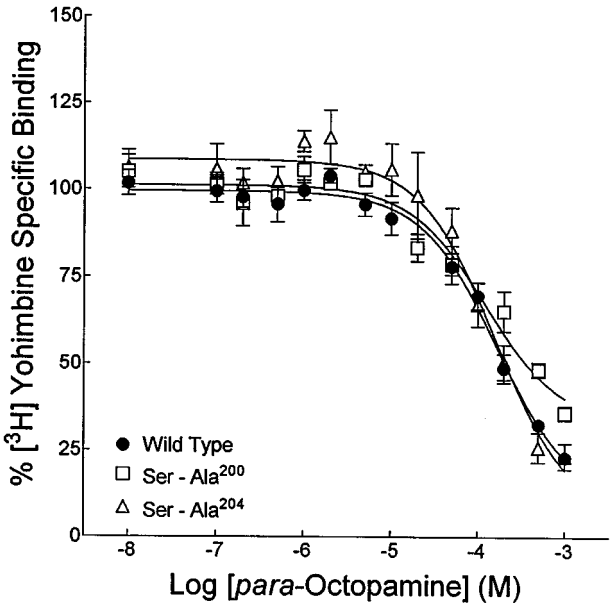

Figure 10 Competition binding curves of *para*-octopamine to the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. Competition displacement experiments were performed on membranes prepared from transfected CHO cells expressing wild-type or the indicated mutant α_{2A}-adrenoceptors. Data represent means of three experiments performed in duplicate and vertical lines show s.e.mean.

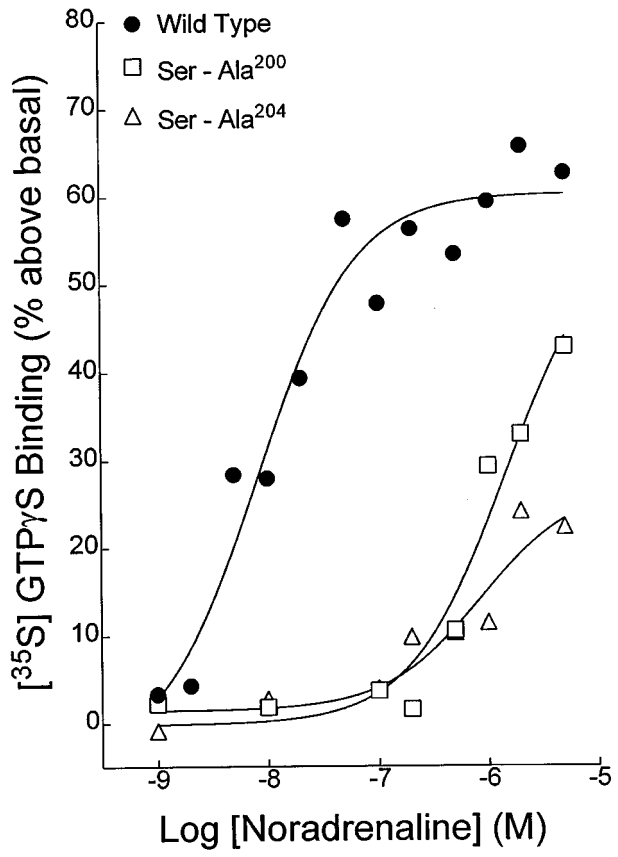

Figure 11 (−)-Noradrenaline stimulation of [^{35}S]-GTPγS binding to membranes of CHO cells expressing the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. Data represent means of three experiments performed in duplicate and s.e.means are less than 10% for all points.

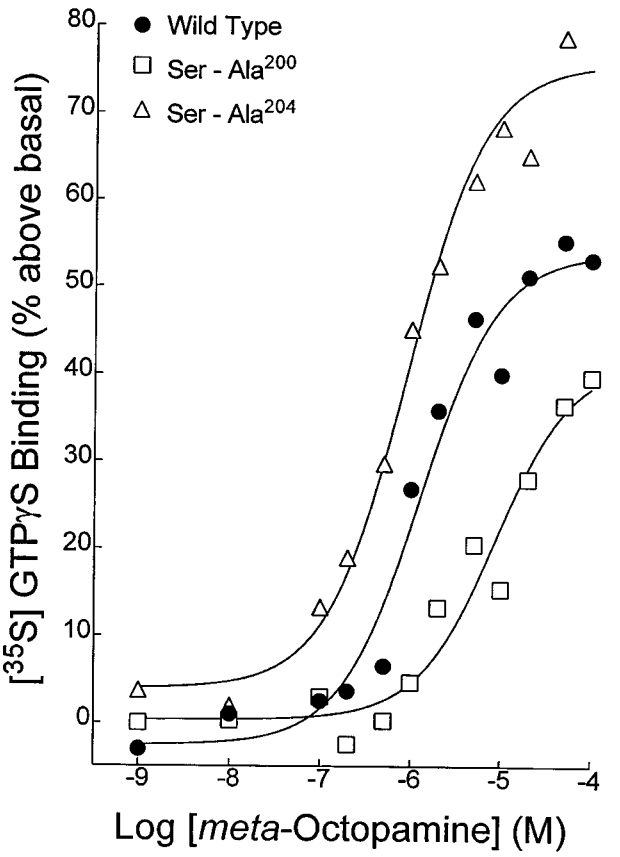

Figure 12 *meta*-Octopamine stimulation of [^{35}S]-GTPγS binding to membranes of CHO cells expressing the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. Data represent means of three experiments performed in duplicate and s.e.means are less than 10% for all points.

cyclic AMP production when the receptors were expressed in CHO cells, confirming the previous observations of Wang *et al.* (1991). The potency of noradrenaline was less affected in the Ala^{200} mutant than in the Ala^{204} mutant which has been suggested to indicate that the interaction of the Ser^{204} with the *para*-hydroxyl group on the catecholamine ring contributes more to the production of the active conformation of the receptor (Wang *et al.*, 1991). This contrasts with the situation observed for the cloned rat α_{1A}-adrenoceptor where Hwa & Perez (1996) suggested that the receptor was predominantly activated by an interaction between the *meta*-hydroxyl group on the catecholamine ring with Ser^{188}. It also contrasts with the findings of Strader *et al.* (1989) on the β_2-adrenoceptor where hydrogen bond interactions between both the *meta*- and the *para*-hydroxyl groups of the catecholamine ring with Ser^{204} and Ser^{207} respectively, are thought to underly receptor activation.

In the present study, *meta*-octopamine showed an increased efficacy at inhibiting forskolin-stimulated cyclic AMP production *via* the Ser→Ala^{204} mutant receptor. This result parallels the increased efficacy of phenylephrine (a homologue of adrenaline with only a single *meta*-hydroxyl group on its aromatic ring) at inhibiting forskolin stimulation of cyclic AMP levels with this mutant receptor which was observed by Wang *et al.* (1991), (see their Figure 6D) but not commented on. The present results suggest that in the absence of the Ser^{204} side chain, the *meta*-hydroxyl group of *meta*-octopamine can more effectively interact with the remaining Ser^{200} (or possibly another binding group) to induce a conformation of the receptor that is more effective at inhibiting the production of cyclic AMP. In contrast, the *para*-hydroxyl group of *para*-octopamine was unable to induce this conformation with increased activity in the Ala^{204} mutant. The Ser→Ala^{200} mutant receptor shows the converse effect. *para*-Octopamine shows an increased efficacy at inhibiting forskolin-stimulated cyclic AMP production *via* this receptor. This result parallels the increased efficacy of synephrine (a homologue of adrenaline with a single *para*-hydroxyl on its aromatic ring) at inhibiting forskolin-stimulation of cyclic AMP levels with this mutant receptor which was observed by Wang *et al.* (1991) (see their Figure 6F) but not commented on. The present results suggest that in the absence of Ser^{200} the *para*-hydroxyl group of *para*-octopamine can more effectively interact with the remaining Ser^{204} (or possibly another binding group) to induce a conformation of the receptor that is more effective at inhibiting the production of cyclic AMP. In contrast, the *meta*-hydroxyl group of *meta*-octopamine was unable to induce this conformation with increased activity in the Ala^{200} mutant.

The substitution of Ser^{204} with Ala, besides allowing a substantial increase in the inhibition of forskolin stimulated cyclic AMP production with *meta*-octopamine, also allowed *meta*-octopamine to generate a receptor-agonist conformation that can increase cyclic AMP levels after pertussis toxin pretreatment is used to inhibit coupling of the receptor to G_i.

However, the substitution of Ser^{200} by Ala does not produce a receptor conformation that can be efficiently coupled to cyclic AMP production under these circumstances by *para*-octopamine.

Hwa & Perez (1996) conclude that since the conserved serines in TMV of the α_{1A}-adrenoceptor are separated by three amino acids, rather than two as in the β_2-adrenoceptor (Strader *et al*., 1989) (see Table 2), then the orientation of the catechol ring in the α_{1A}-adrenoceptor binding pocket may be more parallel to the extracellular surface and rotated by approximately 120° to that in the β_2-adrenoceptor. Since the equivalent serines (Ser^{200} and Ser^{204}) in the human α_{2A}-adrenoceptor are also separated by three amino acids (Fraser *et al*., 1989) (see Table 2), it seems likely that the orientation of the catechol ring in the α_{2A}-adrenoceptor may be more like that in the α_{1A}-adrenoceptor than that in the β_2-adrenoceptor. This suggestion is compatible with the results obtained in the present study. The substitution of either Ser→Ala^{200} or Ser→Ala^{204} substantially reduces the potency of (−)-noradrenaline. This suggests that the presence of the two catecholamine ring hydroxyls does not allow the agonist to increase the effective interactions of either hydroxyl with the remaining serine in either receptor mutant. However, we propose that our results obtained with agonists with single ring hydroxyls can be explained by a better docking or an optimization of the interaction of the one hydroxyl for its corresponding serine in receptor mutants lacking either the Ser^{200} or Ser^{204} residues. In the Ser→Ala^{204} mutant *meta*-octopamine can optimize its receptor interactions such that the *meta*-hydroxyl can form a more efficient interaction with the remaining Ser^{200} (or another residue). Thus, the conformation the receptor assumes after *meta*-octopamine binding is able to inhibit forskolin-stimulated cyclic AMP production much better than that assumed by the wild-type receptor. Conversely, *para*-octopamine can not carry out such an optimization of its receptor interactions in the binding pocket to give an increased interaction with Ser^{200}. In the Ser→Ala^{200} mutant *para*-octopamine can optimize its receptor interactions such that the *para*-hydroxyl can form a more efficient interaction with the remaining Ser^{204} (or another residue). The conformation the receptor assumes after *para*-octopamine binding is able to inhibit forskolin-stimulation of cyclic AMP levels better than that assumed by the wild-type receptor. Conversely, *meta*-octopamine can not carry out such an optimization of receptor interactions in the binding pocket to give an increased interaction with Ser^{204}. The activated conformation of the Ser→Ala^{204} mutant receptor induced by the binding of *meta*-octopamine is different from that produced by the binding of *para*-octopamine to the Ser→Ala^{200} mutant receptor, since the former is also able to increase the interactions of the receptor agonist complex with G_s to produce a stimulation of forskolin-stimulated cyclic AMP production after pertussis toxin pretreatment, whilst the latter is not.

The increased effectiveness of the specific isomers of octopamine in the functional assays on the inhibition of cyclic AMP production by the mutant α_{2A}-adrenoceptors could be due solely to an increase in affinity of the agonists for the mutant receptors. Such an increased affinity has been suggested to underly the increased efficacy of phenylephrine on the production of inositol phosphate by the Ser→Ala^{192} mutant form of the rat α_{1A}-adrenoceptor (Hwa & Perez, 1996). However, this is unlikely to be the case for the human α_{2A}-

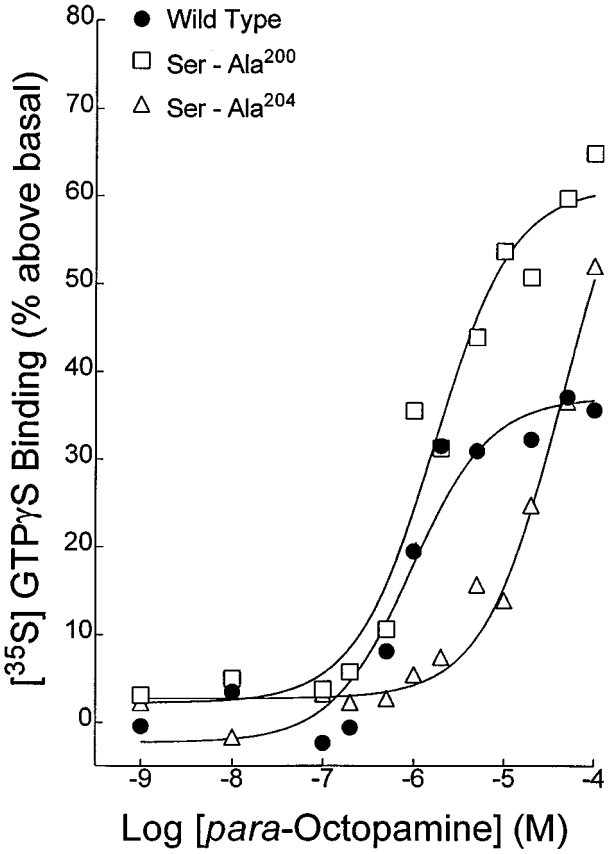

Figure 13 *para*-Octopamine stimulation of [^{35}S]-GTPγS binding to membranes of CHO cells expressing the cloned wild-type human α_{2A}-adrenoceptor, the Ser→Ala^{200} mutant receptor or the Ser→Ala^{204} mutant receptor. Data represent means of three experiments performed in duplicate and s.e.means are less than 10% for all points.

Table 2 Sequence comparison of adrenoceptor TMV regions to show relative positions of conserved serine residues

Species	*Receptor*		*Sequence*	
			188 192	
Rat	α_{1A}-AR	176	QINEEPGYVLF<u>S</u>ALG<u>S</u>FYVPLAIILVMYCR	206
			200 204	
Human	α_{2A}-AR	189	EINDQKWYVIS<u>S</u>CIG<u>S</u>FFAPCLIMILVYVR	217
			204 207	
Hamster	β_2-AR	192	DFFTNQAYAIAS<u>S</u>IV<u>S</u>FYVPLVVMVFVYSR	221

The conserved serines in TMV of the α_{1A}-adrenoceptor (Ser^{188} and Ser^{192}) (Hwa & Perez, 1996) are separated by three amino acids, rather than two as in the β_2-adrenoceptor (Ser^{204} and Ser^{207}) (Strader *et al*., 1989). The equivalent serines (Ser^{200} and Ser^{204}) in the human α_{2A}-adrenoceptor are also separated by three amino acids (Fraser *et al*., 1989).

adrenoceptor since in both the studies of Wang *et al*. (1991) with phenylephrine and synephrine and in the present study with (±)-*p*-octopamine and (±)-*m*-octopamine, no corresponding increases in ligand binding could be found to correlate with the increased efficacy and potency of the agonists in functional assays on the inhibition of cyclic AMP production. Further, the agonist specific increased efficacy and potency effects observed in the functional assays on the inhibition of cyclic AMP production in the present study were also observed in studies of agonist induced [^{35}S]-GTPγS binding to the mutant receptors. This strongly suggests that agonist-induced changes in receptor conformation underly the observed selective increases in potency and efficacy produced by the isomers of octopamine on the coupling of the mutant α_{2A}-adrenoceptors to the inhibition of cyclic AMP production.

In previous studies on ligand binding and activation of the α_{2A}-adrenoceptor only the interaction of the *para*-hydroxyl on the catecholamine ring with Ser^{204} on the receptor has been suggested to be of importance (Wang *et al*., 1991). Ser^{200} has been suggested not to participate in agonist-receptor interactions and the *meta*-hydroxyl group of the catecholamine ring has been suggested to interact with alternate residues on the receptor, such as Cys^{201}. However, the results of the present study strongly support a role for both Ser^{200} and Ser^{204} in exerting inhibitory influences on the ability of *para*- and *meta*-octopamine respectively, to induce a receptor-agonist conformation capable of inhibiting forskolin-stimulation of cyclic AMP levels. This effect occurs irrespective of whether Ser^{200} is directly involved in the binding of the *meta*-hydroxyl on the catecholamine ring. In addition, the presence of Ser^{204} also prevents *meta*-octopamine from generating a receptor-agonist conformation that can increase cyclic AMP levels after the inhibition of the coupling of the receptor to G_i, emphasizing the underlying importance of this residue in the agonist-specific coupling of this receptor to different second messenger systems.

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