British Journal of Pharmacology (1997) 122, 191–198

Selective inhibition of adenylyl cyclase by octopamine via a human cloned α_{2A}-adrenoceptor

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1 In this study we have compared the abilities of the enantiomers of the structural isomers of the phenolamines, octopamine and synephrine, and the catecholamines, noradrenaline and adrenaline, to couple selectively a human cloned α_{2A}-adrenoceptor, stably expressed in a Chinese hamster ovary (CHO) cell line, to G-protein linked second messenger pathways mediating an increase and a decrease in cyclic AMP production.

2 The catecholamines couple the α_{2A}-adrenoceptor to both an increase and a decrease in the rate of cyclic AMP production. In the absence of pertussis toxin pretreatment both catecholamines tested showed a dose-dependent decrease with a maximum at 100 nM. After pertussis toxin pretreatment they both produced a dose-dependent increase in cyclic AMP production with a maximum at 10 μM.

3 The phenolamines, octopamine and synephrine were only able to couple the α_{2A}-adrenoceptor to a dose-dependent decrease in cyclic AMP production at concentrations up to 1 mM, with the synephrine isomers being more potent than the corresponding octopamine isomers. The *meta*-isomers of both phenolamines were more potent than the corresponding *para*-isomers and the (−)-enantiomers were more potent than the (+)-enantiomers. Thus, (−)-*meta*-synephrine [(−)-phenylephrine] was the most effective isomer tested with an observable decrease occurring between 100 nM and 1 μM.

4 The effects of octopamine and the catecholamines on the decrease in cyclic AMP production were additive at submaximal concentrations, whilst octopamine reduced the stimulant effect of submaximal concentrations of noradrenaline on cyclic AMP production after pertussis toxin pretreatment.

5 The time courses of the inhibitory effects of both *meta*-octopamine and noradrenaline were parallel and peaked after a 1 min exposure to the agonist. In contrast, the stimulant effects of noradrenaline after pertussis toxin pretreatment were of a much slower time course with a maximum effect occurring after a 5 min incubation period.

6 Since octopamine and synephrine occur naturally in, and are co-released with catecholamines from, mammalian tissues, the results of the present study suggest that the human cloned α_{2A}-adrenoceptor can be coupled selectively by different endogenous agonists to G-protein pathways mediating the regulation of adenylyl cyclase activity.

Keywords: α_{2A}-Adrenoceptor; octopamine; synephrine; cyclic AMP; adenylyl cyclase; adrenaline; noradrenaline

Introduction

Vertebrate α_2-adrenoceptors regulate many important physiological processes, such as blood pressure, heart rate, lipolysis, platelet aggregation, insulin release and electrolyte secretion (see Ruffolo *et al*., 1993). Pharmacologically they can be subdivided into three defined subtypes, α_{2A}, α_{2B} and α_{2C} which correspond to the three cloned human receptor subtypes, α_2C10, α_2C2 and α_2C4 receptors, respectively (Bylund *et al*., 1994). They are all members of the seven transmembrane spanning superfamily of G-protein coupled receptors.

Until recently, all of the α_2-receptor subtypes were thought to mediate their actions predominantly through a G_i-mediated inhibition of adenylyl cyclase activity. However, it is now apparent that not all of their physiological actions can be adequately described by this effect. Additional modes of action that are now being considered, include activation of phospholipase C activity, suppression of voltage-activated Ca^{2+}-channels, activation of receptor-operated K^+-channels and also stimulation of adenylyl cyclase activity (Isom & Limbird, 1988; Limbird, 1988; Lakhlani *et al*., 1996). The latter effect has been observed in a range of tissues, including cerebral cortical brain slices (Duman *et al*., 1986) and pancreatic islet cells (Ullrich & Wollheim, 1984). It has also been demonstrated for the cloned receptor subtypes expressed in a range of transfected cell lines, including Chinese hamster ovary (CHO) cells, COS-7 cells, HEK-293 cells, PC-12 cells, JEG-3 cells and the S115 mouse mammary tumour cell line (see Eason & Liggett, 1996). In many of these cell types the relative ability of the α_2-adrenoceptor to couple to either adenylyl cyclase stimulation or inhibition exhibits both cell type and receptor subtype differences. In CHO cells the α_2-adrenoceptor-mediated inhibition and stimulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production occurs as a complex biphasic concentration-dependent process (Fraser *et al*., 1989; Eason *et al*., 1992) and the magnitude of the two components varies for the different α_2-adrenoceptor subtypes (Eason *et al*., 1992; 1994). It has also been shown that the relative ability of the receptor to activate these two independent G-protein coupled pathways depends on the nature of the agonist used to activate the receptor (Eason *et al*., 1994). Thus, whilst receptor activation by the catecholamines, noradrenaline and adrenaline, appears to be equally effective in coupling the receptor to both pathways in a concentration-dependent manner, a range of synthetic agonists appears to show a differential ability to couple the receptor to these two pathways. Similar results have been obtained for the effects of synthetic agonists on cloned muscarinic (Gurwitz *et al*., 1994) and 5-hydroxytryptamine$_{1C}$ (5-HT$_{1C}$) and 5-HT$_{2A}$ receptors (Berg *et al*., 1995; 1996).

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The above phenomenon has many similarities to the recently described examples of agonist-specific coupling of G-protein coupled receptors to different second messenger systems by structurally similar endogenous agonists (see Evans *et al*., 1995b; Kenakin, 1995; 1996). For example, a cloned *Drosophila* octopamine/tyramine receptor expressed in a CHO cell line can be differentially coupled to the inhibition of adenylyl cyclase and the production of an intracellular Ca^{2+} signal by the biogenic amine, octopamine, and its metabolic precursor, tyramine (Robb *et al*., 1994). Similarly, the two naturally occurring forms of pituitary adenylyl cyclase activating peptide (PACAP), namely PACAP-28 and PACAP-35, can differentially couple a PACAP type 1 receptor to adenylyl cylase stimulation and inositol phosphate production (Spengler *et al*., 1993)

Adrenaline and noradrenaline are the currently accepted physiological ligands that mediate activation of the α_2-adrenoceptors. However, the *meta*- and *para*-isomers of octopamine and synephrine are also found in mammalian organs and sympathetic nerve fibres along with the above catecholamines (Williams *et al*., 1984; 1987; Ibrahim *et al*., 1985). Structurally the octopamines are very similar to noradrenaline having only one, rather than two, hydroxyl group on the aromatic ring, whilst the synephrines have the equivalent structural difference with adrenaline. Previous studies have shown that the (+)- and (−)-enantiomers of the positional isomers of octopamine and of synephrine can effect physiological changes via the activation of α-adrenoceptors (Brown *et al*., 1988). However, depending on the tissue assayed (−)-*m*-octopamine, the most potent of the structural isomers of octopamine, was between 4 and 150 times less potent than noradrenaline (Brown *et al*., 1988). Thus, Brown *et al*. (1988) concluded that, if *m*- and *p*-octopamine were co-released with noradrenaline in amounts proportional to their concentration, their activities on α-adrenoceptors were too low to be physiologically significant.

In view of the current interest in the phenomenon of agonist-specific coupling of G-protein coupled receptors to different second messenger systems by structurally related endogenous agonists (see Evans *et al*., 1995b; Kenakin, 1995; 1996), we have begun a study of the relative abilities of the octopamines and the catecholamines to couple a human cloned α_{2A}-adrenoceptor expressed in a CHO cell line (Fraser *et al*., 1989) to different second messenger systems. In the present study we have undertaken a detailed comparison of the abilities of the enantiomers of the structural isomers of octopamine and synephrine and the catecholamines, noradrenaline and adrenaline, to couple the α_{2A}-adrenoceptor to an increase and decrease in cyclin AMP production. A brief account of some of this work has already been published in abstract form (Evans *et al*., 1995a).

Methods

Cell culture

Chinese hamster ovary cells, transfected with the α_{2A}-adrenoceptor at an intermediate density (400 fmol receptor mg^{-1} protein; Fraser *et al*., 1989), were grown to ~90% confluence in cell culture at 37°C. The culture medium consisted of 90% Ham's F-12 nutrient mixture (Gibco) and 10% bovine foetal calf serum (Gibco). 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 against cells which did not express 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).

Experimental protocol

Cells were first washed, by rinsing culture plates 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-methyl-xanthine (IBMX; Sigma), a phosphodiesterase inhibitor. Next, the cells were 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 the addition of 500 μl of ice-cold, acidified ethanol (60 ml absolute-EtOH : 1 ml 1 N HCl). Cell debris was scraped from each plate after this and pooled with two subsequent 250 μl washes with acidified ethanol. It was then removed by centrifugation at 13,000 r.p.m. for 5 min. The supernatant was evaporated to dryness by means of centrifuge (Savant) and the residue was re-suspended in 150 μl of Tris/EDTA buffer. Cyclic AMP levels were determined in duplicate with the [8-^{3}H]-cyclic AMP assay kit of Amersham (Biotrak TRK 432).

Data analysis

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 100 μM. To investigate the interactions of the octopamines and noradrenaline, solutions of these agonists were mixed at concentrations reflecting their maximum and sub-threshold potencies. Concentrations having maximal effects on the rate of cyclic AMP production were used to determine the time course of agonist activity.

For dose-response and agonist-interaction experiments, the concentration of cyclic AMP (pmol/plate) 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. Two of these internal control plates were used in conjunction with each group of six experimental plates.

In studies of the time course of agonist-induced modulation of adenylyl cyclase activity, the rate of cyclic AMP production following incubation in the presence of agonist/forskolin solutions for defined intervals was compared with that following incubation in the presence of forskolin alone for the same intervals. Agonist effects were expressed as the difference between forskolin + agonist and forskolin-only [cyclic AMP] at the mid-point of each incubation interval.

Analysis of variance (ANOVA) was used to test for significant agonist-mediated effects in individual experiments. Significant ANOVA's were then further analysed by Tukey's HSD multiple comparison test, to determine at what concentration or after what duration of incubation the rates 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 obtained from the Aldrich Chemical Company; racemic *p*-octopamine, IBMX and forskolin were from Sigma. Rauwolscine was obtained from Research Biochemicals Incorporated. Enantiomers of the structural isomers of octopamine and synephrine were prepared by the Midgley laboratory at the Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow, Scotland (Midgley *et al*., 1989).

Results

Dose response

Following 20 min of incubation with (−)-noradrenaline (NA) in the absence of pertussis toxin (PTX) pretreatment, maxi-

mum, significant, inhibition of cyclic AMP production ($F=7.27$; d.f. = 5,18; $P<0.001$) was seen in α_{2A}-adrenoceptor-expressing CHO cells exposed to agonist concentrations of 100 nM (Figure 1a). At higher concentrations of (−)-NA the inhibitory effects of this agonist were not as great but still significant. Exposure to (−)-adrenaline over the same concentration range also revealed a similar pattern, with maximum inhibition of cyclic AMP production ($F=8.70$; d.f. = 5,18; $P<0.001$) at 100 nM in the absence of pertussis toxin pretreatment (Figure 1b). 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 their α-subunits, inhibition of cyclic AMP production by the catecholamines was abolished and was replaced by dramatic stimulation (NA: $F=94.30$; d.f. = 5,18; $P<0.001$; adrenaline: $F=49.90$; d.f. = 5,18; $P<0.001$) with maximum efficacy at an agonist concentration of 10 μM (Figure 1a,b). Adrenaline was almost an order of magnitude more potent than noradrenaline at stimulating this response under these conditions.

Racemic (i.e. ±)-*m*-octopamine caused significant inhibition in the rate of cyclic AMP production when administered at concentrations of 10 μM and higher ($F=18.41$; d.f. = 5,60; $P<0.001$) in the absence of pertussis toxin pretreatment (Figure 2a). However, after 24 h of pre-incubation with pertussis toxin, no catecholamine-style stimulation of cyclic AMP levels was revealed with exposure to (±)-*m*-octopamine ($P<0.05$) over this concentration range (Figure 2a) or after exposure to a concentration of 1 mM (113.8 ± 9.0% of control values; $n=10$). The addition of rauwolscine, an α_2-adrenoceptor antagonist, to the incubation medium abolished the

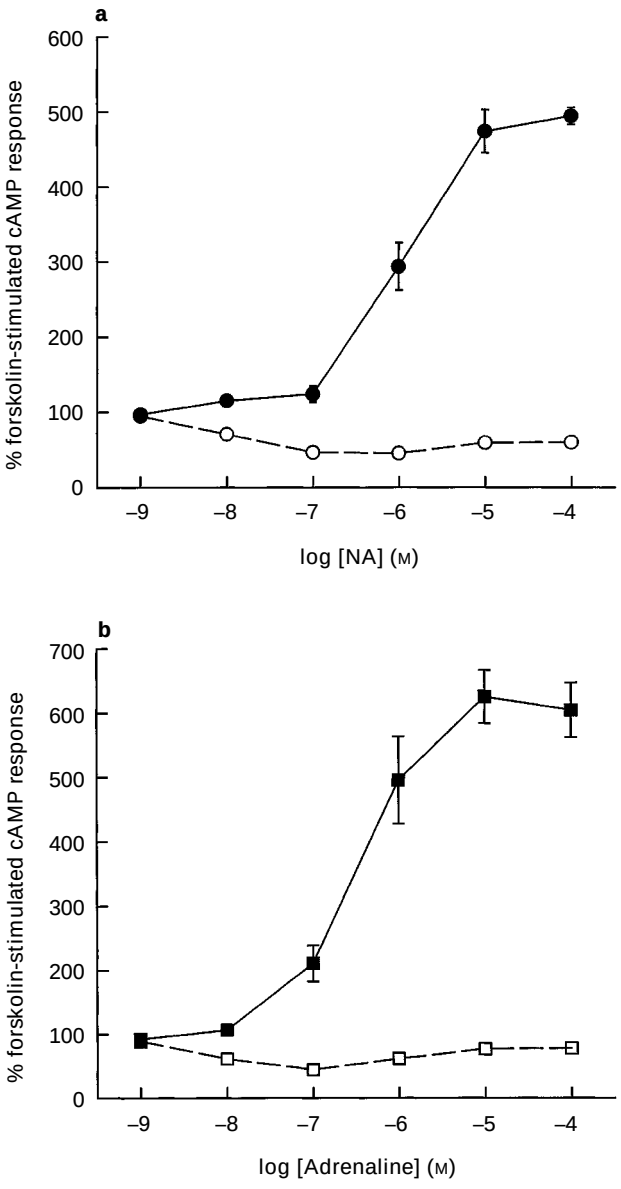

Figure 1 The effects of the catecholamines noradrenaline (NA) (a) and adrenaline (b) on cyclic AMP production in Chinese hamster ovary cells stably expressing the human cloned α_{2A}-adrenoceptor. In the absence of pertussis toxin (PTX) pretreatment (open symbols), both catecholamines caused inhibition of cyclic AMP production, while pretreatment with PTX (solid symbols) revealed a dramatic stimulation of cyclic AMP production. Data represent means and vertical lines show s.e.mean, $n=4$.

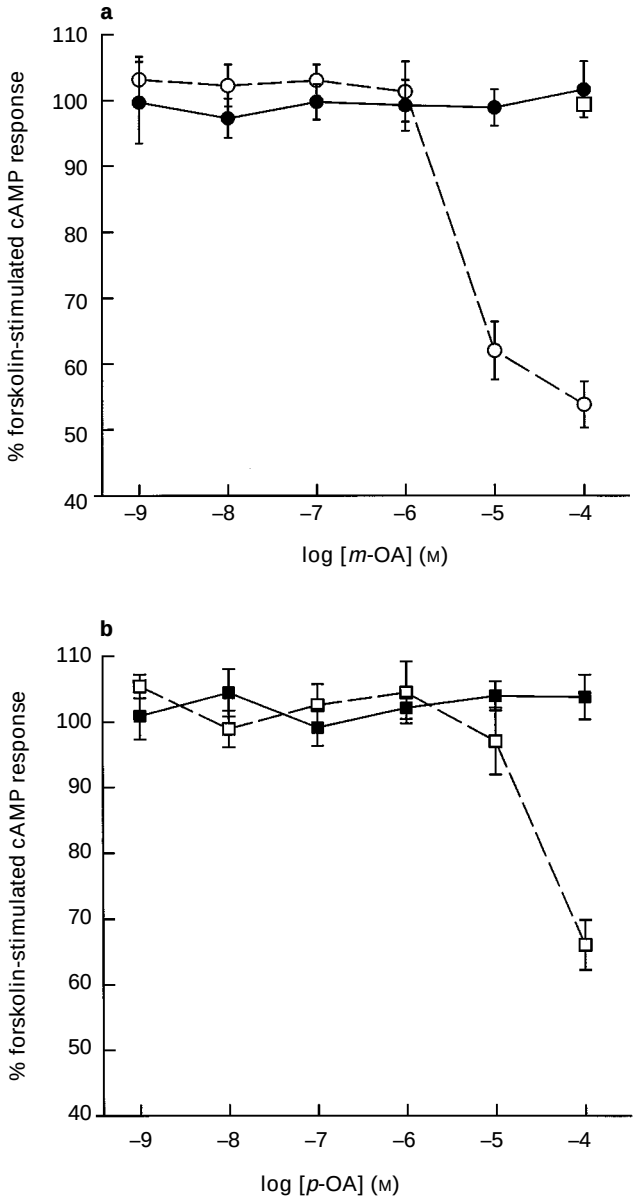

Figure 2 The *meta*-isomer (a) and the *para*-isomer (b) of (±)-octopamine (OA) both caused a dose-dependent inhibition of cyclic AMP production in the absence of PTX pretreatment (open symbols); pretreatment with PTX (solid symbols) abolished inhibition of cyclic AMP production, but failed to reveal catecholamine-style stimulation of cyclic AMP production. The *meta*-isomer of (±)-octopamine was more potent in inhibiting cyclic AMP production than the *para*-isomer. In (a), co-application of the α_2-adrenoceptor antagonist, rauwolscine (□), also abolished octopamine-sensitive inhibition of cyclic AMP production in the absence of PTX pretreatment. Data represent means and vertical lines show s.e.mean, $n \geqslant 6$.

octopamine-sensitive inhibition of cyclic AmP production, confirming that the effects of octopamine were mediated by the α_{2A}-adrenoceptor stably expressed in this cell line (Figure 2a). Non-transfected CHO-K1 cells showed no response to treatment with either octopamine or the catecholamines (data not shown).

The *para* configuration of (±)-octopamine was less potent than the *meta* positional isomer (Figure 2b), causing significant inhibition in the rate of cyclic AMP production only at a concentration of 100 μM ($F=9.98$; d.f. = 5,60; $P<0.001$). The maximum inhibition of cyclic AMP production by (±)-*p*-octopamine was less than that brought about by (±)-*m*-octopamine in the concentration range tested. Pre-incubation with pertussis toxin also abolished the inhibition of cyclic AMP production resulting from activation of the α_{2A}-adrenoceptor by (±)-*p*-octopamine (Figure 2b).

Stereospecificity of adenylyl cyclase regulation

Inhibition of cyclic AMP production by the enantiomers of *m*-octopamine was stereospecific (Figure 3a); significant inhibition of the rate of cycic AMP production occurred at a concentration of 10 μM for (−)-*m*-octopamine ($F=24.56$; d.f. = 5,30; $P<0.001$) whilst (+)-*m*-octopamine significantly inhibited cyclic AMP production only when administered at 100 μM ($F=19.34$; d.f. = 5,30; $P<0.001$). The magnitude of maximum inhibition of cyclic AMP production at agonist concentrations of 100 μM was very similar for the two enantiomers, although the (−) form was slightly more effective than either the (+) form or (±)-*m*-octopamine.

The response to *p*-octopamine showed similar stereospecificity (Figure 3b), with (−)-*p*-octopamine causing significant inhibition in the rate of cyclic AMP accumulation when administered at 100 μM ($F=4.67$; d.f. = 5,30; $P<0.01$) whilst (+)-*p*-octopamine had no significant effect on adenylyl cyclase activity ($P<0.05$).

Neither of the optical isomers of *ortho*-octopamine significantly altered the rate of cyclic AMP accumulation at any concentration over the range from 1 nM to 100 μM (data not shown) and *o*-octopamine has not been detected in any organ of the rat, even after treatment with monoamine oxidase (MAO) inhibitors (Williams *et al*., 1984; 1987).

It has been demonstrated that the (−)-isomer of *m*-synephrine (phenylephrine) elicits physiological changes via α-adrenoceptors (Brown *et al*., 1988) and the enantiomers of *m*-synephrine, the N-methylated analogue of *m*-octopamine, showed similar stereospecificity to the corresponding antipodes of *m*-octopamine in their inhibition of cyclic AMP production (Figure 4). As with the octopamines, (−)-*m*-synephrine was the more potent stereoisomer. However, both the (−) and (+) forms of synephrine were more potent than the corresponding enantiomers of *m*-octopamine by approximately one order of magnitude. Also, in common with the octopamines, but not the catecholamines, (±)-*m*-synephrine did not show a stimulation of cyclic AMP production after pertussis toxin pretreatment, even at a concentration of 1 mM (110.8 ± 8.2% of control; $n=6$).

Interactions of octopamine and noradrenaline

The interactive effects of (±)-*m*-octopamine and (−)-noradrenaline on the rate of cyclic AMP production were investigated by exposing plates of CHO cells expressing the α_{2A}-adrenoceptor to mixtures of the two agonists at various ratios. In the absence of pertussis toxin, inhibition of cyclic AMP production was greatest when the concentration of (±)-*m*-octopamine was 100 μM and (−)-noradrenaline was present at a concentration of 1 μM (Figure 5a). The rate of cyclic AMP accumulation was significantly lower for cells exposed to a mixture of 1 nM (−)-noradrenaline and 100 μM (±)-*m*-octopamine than for cells exposed only to the catecholamine at the same concentration ($F=5.37$; d.f. = 10,78; $P<0.001$). This trend, of octopamine-enhanced inhibition of cyclic AMP production, was maintained over the range of (−)-noradrenaline concentrations tested. A sub-threshold concentration of octopamine (1 μM) added to solutions of (−)-noradrenaline did not significantly alter the rate of cyclic AMP production ($P>0.05$) in comparison with the responses to treatment with (−)-noradrenaline alone (Figure 5a).

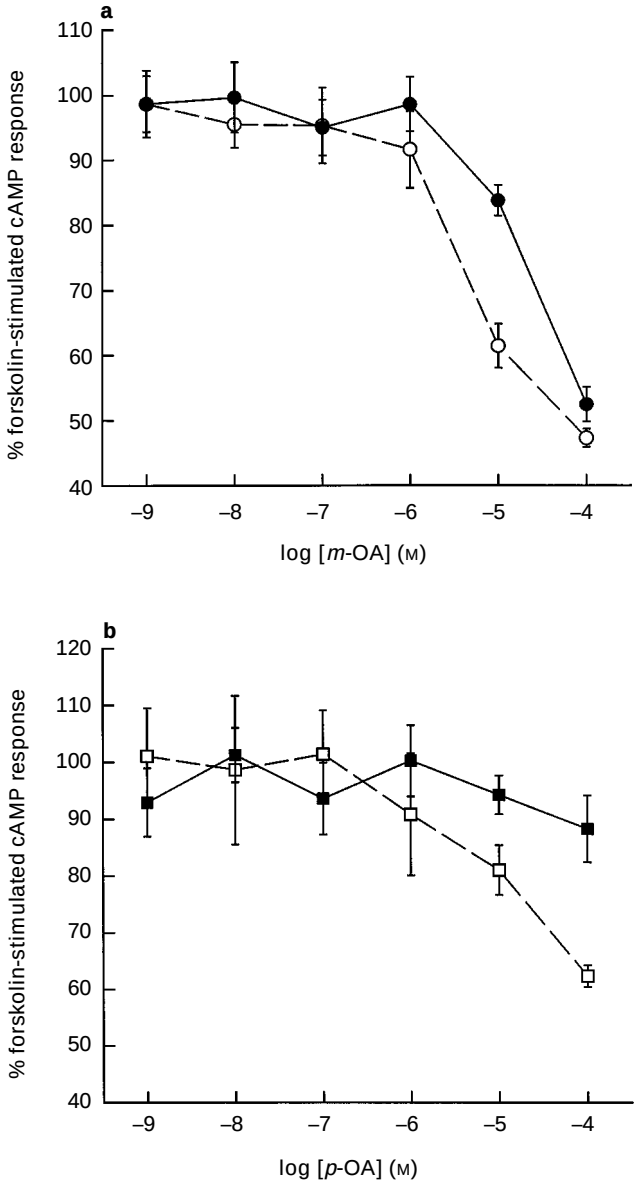

Figure 3 Stereospecificity of octopamine (OA) in the inhibition of cyclic AMP production by the human cloned α_{2A}-adrenoceptor. (a) (−)-*m*-Octopamine (○) was more potent than the (+)-stereoisomer (●) ($n=6$). (b) Similarly, (−)-*p*-octopamine (□) was more effective than (+)-*p*-octopamine (■) in inhibiting cyclic AMP production ($n\geqslant 5$), although neither enantiomer was as potent as the corresponding *meta* forms. Data represent means and vertical lines show s.e.mean.

After pre-incubation of the cultured cells with pertussis toxin, to abolish the agonist-mediated inhibition of cyclic AMP production, the interactive effects of (±)-*m*-octopamine and (−)-noradrenaline were most obvious at a sub-maximal (but effective) concentration of the catecholamine (Figure 5b). At a concentration of 100 μM, octopamine significantly depressed the noradrenaline-induced stimulation of cyclic AMP production ($F=4.63$; d.f. = 2,18; $P<0.05$). The same concentration of octopamine was unable to moderate the maximal stimulation of cyclic AMP production produced by noradrenaline at its most effective concentrations (10 μM).

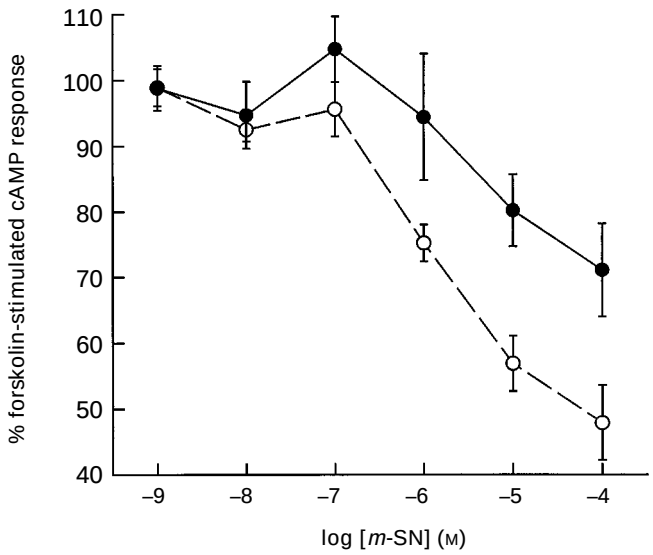

Figure 4 Stereospecificity of inhibition of cyclic AMP production by *m*-synephrine acting via the human cloned α_{2A}-adrenoceptor expressed in CHO cells. As with the octopamines, the (−)-*meta* isomer of synephrine (○) was more potent than the (+)-*meta* isomer (●) in its ability to inhibit the production of cyclic AMP. Maximal inhibition by synephrine was slightly greater than that produced by the corresponding octopamine (see Figure 3), and the threshold for the affects of synephrine was about one order of magnitude lower than that for octopamine. Data represent means and vertical lines show s.e.mean, $n=5$.

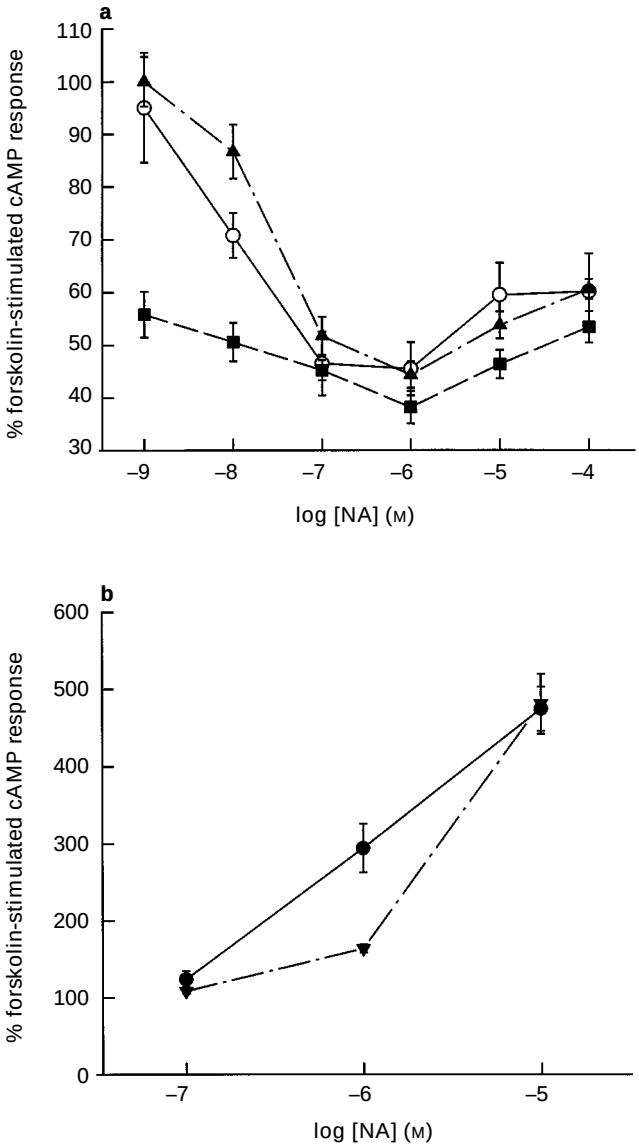

Figure 5 The interactions of octopamine- and noradrenaline-mediated modulation of cyclic AMP production by the human cloned α_{2A}-adrenoceptor expressed in CHO cells. (a) The combined effects of (−)-noradrenaline (NA) and (±)-*m*-octopamine (OA) on the inhibition of cyclic AMP production. The NA-induced inhibition of cyclic AMP production (○) was not augmented in the presence of subthreshold concentrations of OA (1 μM) (▲) whilst OA at 100 μM enhanced the inhibition of cyclic AMP production (■) over the NA concentration range tested ($n=4$). (b) Pretreatment of the CHO cells with pertussis toxin, to abolish coupling via inhibitory G-proteins, showed different interactive effects of NA and OA. However, although (±)-*m*-octopamine by itself has no effect in the presence of PTX, due to uncoupling of the inhibitory pathways, the NA-mediated stimulation of cyclic AMP production (●) was attenuated in the presence of 100 μM (±)-*m*-octopamine (▼), at submaximal doses of NA ($n=4$). Presumably, this effect is due to inhibition of NA binding to the receptor active site by OA, despite the lack of activity of the latter in this situation. Data represent means and vertical lines show s.e.mean.

Relative time courses of agonist-induced responses

In order to investigate the potential physiological role for two co-released agonists acting via the same receptor, the relative time courses of agonist-induced inhibition and stimulaton of cyclic AMP production were compared (Figure 6). Inhibition of cyclic AMP production by both (±)-*m*-octopamine (100 μM) and (−)-noradrenaline (100 nM) began to occur within 30 s after the start of incubation with the agonist solutions, as did the stimulation of cyclic AMP production by (−)-noradrenaline in the presence of pertussis toxin. The time course of the initial peaks of stimulation and inhibition did not differ, suggesting that coupling to both excitatory and inhibitory intracellular pathways occurs with equal efficacy. The stimulation observed in response to noradrenaline in the presence of pertussis toxin lasted much longer than the inhibition associated with either octopamine or noradrenaline.

Discussion

The results of the present study suggest that the cloned human α_{2A}-adrenoceptor expressed in CHO cells can be differentially coupled to the inhibition or stimulation of cyclic AMP production by naturally occurring agonists. The *m*- and *p*-positional isomers of octopamine occur naturally in sympathetic neurones and the corresponding isomers of synephrine, the N-methylated analogue of octopamine, occur in the adrenal glands (Ibrahim *et al*., 1985; Williams *et al*., 1987). In addition, the positional isomers of octopamine are co-released with the catecholamines from sympathetic nerve endings (Molinoff & Axelrod, 1969; Reimann, 1984). Previous studies have examined the effects of the enantiomers of the *m*- and *p*-structural isomers of both octopamine and synephrine on α-adrenoceptor classes in intact tissue preparations (Brown *et al*., 1988). In the case of the α_1-adrenoceptors from rat aorta and the α_2-adrenoceptors from rabbit saphenous vein, noradrenaline was between 6 and 150 fold more potent than the (−)-isomers of *meta*-octopamine and *meta*-synephrine, whereas on the α_1-adrenoceptors of the rat anococcygeus muscle noradrenaline was equipotent with (−)-*m*-synephrine. In all cases the *m*-isomers of synephrine and octopamine were more potent than the *p*-isomers, and the (−)enantiomers were more potent than the (+)enantiomers. In these studies the predominant receptor subtype expressed in the different tissue types was assumed and a sub-classification into different α_1 and α_2 receptor subtypes was not attempted. In addition, it is

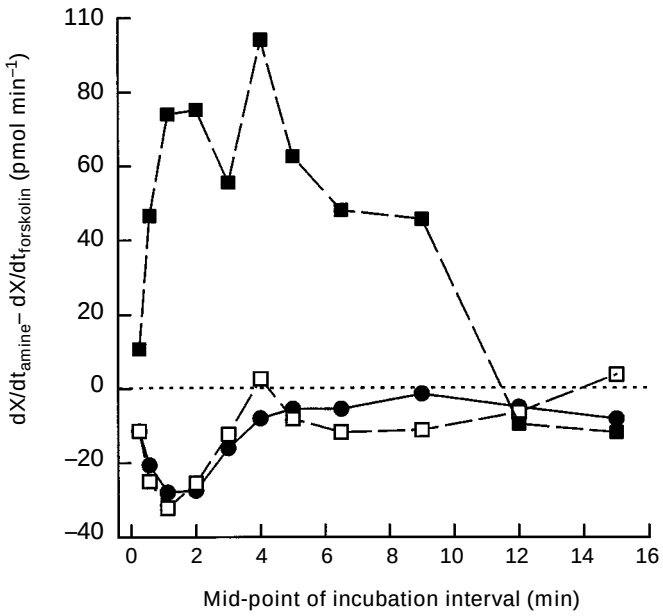

Figure 6 Time courses of noradrenaline- and octopamine-mediated modulation of cyclic AMP production by the human cloned α_{2A}-adrenoceptor expressed in CHO cells. The time course of the stimulation of cyclic AMP production by 10 μM noradrenaline in the presence of PTX (■) and inhibition by 100 nM (−)-noradrenaline (□) are shown as the difference in rate of cyclic AMP production in cells treated with either forskolin alone (dashed line) or solutions of agonist + forskolin. The time course of the inhibition of cyclic AMP production by 100 μM(±)-*m*-octopamine (●) is also shown. Both stimulant and inhibitory effects reached a peak about one minute after application of agonists, but the stimulation of cyclic AMP production was of longer duration, remaining elevated above the basal (forskolin-stimulated rate) for 9 min or longer of the 15 min incubation period shown. The results shown represent the mean of six determinations at each time point.

not clear if any of the tissues express mixed populations of receptor subtypes.

In the present investigation, our studies on a clonal human α_{2A}-adrenoceptor subtype have enabled us to characterize definitively the pharmacological profile of this receptor subtype in detail. In terms of the inhibition of cyclic AMP production we can confirm the above pharmacological profile found in studies on intact tissues, with the catecholamines being 2 to 3 orders of magnitude more potent than the (−)-*m*-isomers of synephrine and octopamine. In addition, the *m*-positional isomers of both phenolamines were more potent than the corresponding *p*-isomers and the (−)-enantiomers were more potent than the corresponding (+)-enantiomers. Further, the synephrine isomers were almost an order of magnitude more potent than the corresponding octopamine isomers. In contrast, the results obtained with the stimulation of cyclic AMP production after pertussis toxin pretreatment (to abolish the coupling of the receptor to inhibition of cyclic AMP production), show a marked difference to the above pharmacological profile. The catecholamines were 1 to 2 orders of magnitude less potent at stimulating than inhibiting cyclic AMP production, in agreement with previous studies (Fraser *et al*., 1989; Eason *et al*., 1992; 1994). In contrast, the phenolamines, octopamine and synephrine, did not exhibit any stimulation of cyclic AMP production at any of the concentrations tested in the present study up to a concentration of 1 mM. We conclude, therefore, that (−)-*m*-synephrine (the most potent of the enantiomers of the structural isomers of the phenolamines tested at inhibiting cyclic AMP production through this receptor), is a minimum of at least 4 orders of magnitude less potent at stimulating cyclic AMP production.

The ability of the human cloned α_{2A}-adrenoceptor, utilized in the present investigation, to couple primarily to the inhibition of cyclic AMP production when activated by octopamine and synephrine, but to couple to both the inhibition and stimulation of cyclic AMP production when activated by the catecholamines, could be explained by a number of mechanisms including those dependent on the strength of the signal and those involving agonist-specific coupling (agonist trafficking) (Kenakin, 1995). In the former case only a single active state of the receptor would be required and agonists of high efficacy (e.g. noradrenaline and adrenaline) would activate multiple G-proteins, whereas agonists of low efficacy (e.g. octopamine and synephrine) would couple the receptor only to the most efficiently coupled G-protein. A similar explanation has been suggested (Kenakin, 1995) for the coupling of the α_{2A}-adrenoceptor to only G_i by oxymetazoline and to both G_i and G_s by adrenaline (Eason *et al*., 1994). However, evidence is mounting to suggest that a simple two-state model is an operational simplification of the interactions of a ligand with a G-protein coupled receptor (e.g. see Scheer *et al*., 1996) and that a more general model of allosteric transition implies that a receptor will undergo intramolecular transitions among an endless number of substates, all of which exist, but with different proportions in the unbound and bound receptor forms (Scheer *et al*., 1996). Thus, it appears possible that agonist-specific coupling of the α_{2A}-adrenoceptor to intracellular signal transduction pathways may underlie the results observed in the present investigation. Because of the close structural homologies between the catecholamines and the octopamines (including the absolute configurations of the (−)-isomers; Midgley *et al*., 1989), it can be suggested that the failure of the octopamines to stimulate cyclic AMP production results from the absence of a second hydroxyl group on the aromatic ring of this ligand. Thus, the specific receptor-mediated responses to agonist binding appear to be due to different conformations of the activated receptor-agonist complex (see Robb *et al*., 1994; Evans *et al*., 1995a,b; Kenakin, 1995; 1996), resulting from the availability of a second hydroxyl group to interact with the amino acid residues in the binding region of the receptor. Support for the contention that the crucial region responsible for agonist-specific effects is the aromatic ring is provided by the finding that adrenaline and noradrenaline can couple to both stimulation and inhibition of cyclic AMP production, despite the difference in the structure of the side-chain. However, the positional isomers of octopamine share a common aromatic structure with the corresponding synephrines and the octopamines and synephrines have identical side-chains (including absolute configuration) to those of noradrenaline and adrenaline, respectively, but they are capable primarily of coupling only to the inhibition of cyclic AMP production.

In vitro mutagenesis studies on adrenoceptors have also emphasized the importance of serine residues in transmembrane region five (TMV) in binding catecholamine ligands and in activation of the receptor. For example, in the β_2-adrenoceptor, it is thought that Ser^{204} and Ser^{207} interact via hydrogen bonds with the *meta*-hydroxyl and *para*-hydroxyl groups, respectively, on the catecholamine ring and that both interactions are required for full agonist activity (Strader *et al*., 1989). In contrast, the situation with α-adrenoceptors appears to be more complex. Wang *et al*. (1991) suggested that Ser^{204} of the α_{2A}-adrenoceptor (which corresponds to Ser^{207} of the β_2-adrenoceptor) interacts with the *para*-hydroxyl group; whereas Ser^{200} (which corresponds to Ser^{204} of the β_2-adrenoceptor) does not participate directly in receptor-agonist interactions. Further, Hwa and Perez (1996) suggest that hydrogen bond interaction between Ser^{188} on the rat α_{1A}-adrenoceptor (which corresponds to Ser^{204} of the β_2-adrenoceptor) and the *meta*-hydroxyl group and not that between the *para*-hydroxyl group and Ser^{192} (which corresponds to Ser^{207} of the β_2-adrenoceptor) allows receptor activation. The present study on the human α_{2A}-adrenoceptor emphasizes the importance of interactions with the *meta*-hydroxyl group of the phenolamines in inducing a receptor-agonist conformation that is optimal for coupling to

G_i, leading to the inhibition of cyclic AMP production. It also suggests that interactions with both the *para*- and the *meta*-hydroxyl groups on the catecholamine ring are essential to produce an agonist-receptor conformation that is capable of activating a G_s mediated activation of cyclic AMP production.

The concept that adrenoceptors can assume different conformations that can selectively and differentially couple them to specific second messenger pathways also receives support from additional mutagenesis studies. Conversion of Asp^{79} to asparagine in the α_{2A}-adrenoceptor results in a selective uncouping of the receptor from K^+ currents but the retention of its ability to inhibit cyclic AMP production and to open voltage-sensitive Ca^{2+} channels (Lakhlani *et al*., 1996). Similarly, conversion of Cys^{128} to Phe in TMIII of the α_{1B}-adrenoceptor induces constitutive activity of the receptor, resulting in G-protein coupling in the absence of agonist and activation of phospholipase C but not phospholipase A_2 activity (Perez *et al*., 1996).

Activation of the human α_{2A}-adrenoceptor by octopamine is likely to be particularly important under conditions such as hepatic and renal encephalopathy, where the circulating levels of octopamine are substantially increased (Kinniburgh & Boyd, 1979). Normal human plasma octopamine concentrations are found in the range 0.23 to 1 ng ml^{-1} (1.5 to 6.5 nM (Kinniburgh & Boyd, 1979; Bozzi *et al*., 1981) but concentrations up to 59.5 ng ml^{-1} (0.4 μM) have been found in patients with hepatic coma (Hörtnagl *et al*., 1981). The latter value is close to the threshold concentration for the effectiveness of (−)-*m*-octopamine demonstrated in the present study for the inhibition of cyclic AMP production. It is also in excess of the threshold concentrations for the effectiveness of octopamine stereoisomers on α-adrenoceptors in a number of whole tissue preparations (Brown *et al*., 1988). Based on evidence presented here, it is possible to predict that interactions between circulating catecholamines and octopamines, both at the receptor-agonist binding and intracellular transduction levels, may determine the final cellular response to α_{2A}-adrenoceptor activation *in vivo*.

The results of the present study demonstrate agonist-specific coupling for naturally occurring ligands of the human α_{2A}-adrenoceptor. Furthermore, the ability of the phenolamines, octopamines and synephrines, to couple the receptor selectively to the inhibition of cyclic AMP production, suggests a possible modulatory role for these compounds through the activation of α-adrenoceptors. This mode of action may explain why specific octopamine receptors have not been identified to date, despite the presence and capacity for neurosecretion of the octopamines and synephrines, in vertebrate nervous systems.

We thank Dr C.M. Fraser, The Institute for Genomic Research, Rockville, Maryland, U.S.A. for making the transfected CHO cell line expressing the cloned human α_{2A}-adrenoceptor available to us. This work was funded by the BBSRC through The Babraham Institute and by NSERC and AHFMR Fellowships to C.N.A. J.E.R. was supported by a BBSRC Postgraduate Studentship.

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(Received February 11, 1997
Revised May 19, 1997
Accepted June 4, 1997)