Agouti-related protein (83–132) is a competitive antagonist at the human melanocortin-4 receptor: no evidence for differential interactions with pro-opiomelanocortin-derived ligands

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Abstract

Interactions between pro-opiomelanocortin (POMC)-derived peptides, agouti-related protein (AGRP) and the melanocortin-4 receptor (MC4-R) are central to energy homeostasis. In this study we have undertaken comprehensive pharmacological analysis of these interactions using a CHOK1 cell line stably transfected with human MC4-R. Our main objectives were (1) to compare the relative affinities and potencies of POMC-derived peptides endogenously secreted within the hypothalamus, (2) to investigate the potency of AGRP(83–132) antagonism with respect to each POMC-derived peptide and (3) to determine whether AGRP(83–132) and POMC-derived peptides act allosterically or orthosterically. We have found that beta melanocyte-stimulating hormone (β MSH), desacetyl alpha MSH (da- α MSH) and adrenocorticotrophic hormone all have very similar affinities and potencies at the MC4-R compared with the presumed

natural ligand, α MSH. Moreover, even MSH precursors, such as beta lipotrophic hormone, showed significant binding and functional activity. Therefore, many POMC-derived peptides could have important roles in appetite regulation and it seems unlikely that α MSH is the sole physiological ligand. We have shown that AGRP(83–132) acts as a competitive antagonist. There was no significant difference in the potency of inhibition by AGRP(83–132) or agouti(87–132) at the MC4-R, regardless of which POMC peptide was used as an agonist. Furthermore, we have found that AGRP(83–132) has no effect on the dissociation kinetics of radiolabelled Nle⁴,D-Phe⁷ MSH from the MC4-R, indicating an absence of allosteric effects. This provides strong pharmacological evidence that AGRP(83–132) acts orthosterically at the MC4-R to inhibit Gs-coupled accumulation of intracellular cAMP.

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Introduction

In the hypothalamus, interactions between pro-opiomelanocortin (POMC)-derived peptides and agouti-related peptide (AGRP) at the melanocortin-4 receptor (MC4-R) play a key role in energy homeostasis. The central melanocortin axis is responsive to a wide range of peripheral signals including leptin, insulin, glucose and ghrelin (Cone *et al.* 2001 and references therein), and mediates the appetite reducing effect of serotonin reuptake inhibitors (Heisler *et al.* 2002). It is well known that anorexigenic signals upregulate POMC gene expression and downregulate the endogenous MC4-R antagonist, AGRP (Cone 1999 and references therein). Conversely, orexigenic signals downregulate POMC and upregulate AGRP. This ying–yang relationship tightly coordinates perceived energy requirement with appetite and food

intake. A precise understanding of the pharmacological interactions between endogenous agonists and antagonists at the MC4-R is of critical importance in order to design potential anti-obesity therapies that modulate central melanocortin signalling.

It is widely thought that alpha melanocyte-stimulating hormone (α MSH) is the natural ligand for the MC4-R, but a range of other POMC-derived peptides are secreted in the hypothalamus, including desacetyl alpha MSH (da- α MSH), adrenocorticotrophin (ACTH), beta lipotrophic hormone (β LPH) and the unprocessed prohormone, POMC (Nilaver *et al.* 1979, O'Donohue *et al.* 1981, Pritchard *et al.* 2003). All of these peptides include the core Phe-Arg-Trp residues characteristic of melanocortin agonists and could therefore play important physiological roles in the central melanocortin signalling pathway. *In vitro* experiments have shown that some

POMC-derived peptides, in addition to α MSH, can bind and activate the MC4-R (Gantz *et al.* 1993, Mountjoy *et al.* 1994, Abbott *et al.* 2000). Furthermore, *in vivo* experiments have indicated that several POMC-derived peptides can inhibit food intake with varying efficacies when injected intracerebroventrically (Abbott *et al.* 2000, Kask *et al.* 2000, Al-Barazani *et al.* 2001, Millington *et al.* 2001). This suggests that several POMC-derived peptides are physiologically relevant in the melanocortin system. It is important, therefore, that the relative pharmacological properties of these peptides at the MC4-R are studied in detail, particularly their functional relationship with AGRP.

It is not known whether AGRP antagonises the effect of all POMC-derived peptides at the MC4-R to an equal extent. Mountjoy *et al.* (1999) have suggested that agouti protein, an AGRP homologue, is more potent at antagonising da- α MSH than α MSH coupling to the mouse MC4-R *in vitro*. Moreover, agouti appears to competitively antagonise α MSH and non-competitively antagonise da- α MSH. The implication of these findings is uncertain, as agouti is not normally expressed in the hypothalamus. However, by extrapolation, these observations suggest that there may be subtle differences in the way AGRP interacts with different POMC-derived peptides at the MC4-R and this may be a physiologically relevant means of regulating melanocortin signalling in the hypothalamus.

It is not totally clear whether AGRP and POMC-derived peptides act via physically distinct binding pockets (allosteric interaction) or whether they act via the same binding pocket (orthosteric interaction). This is a critical question to address with respect to future drug design. Most studies indicate that AGRP acts as a classic competitive antagonist (i.e. orthosterically) (Rosenfeld *et al.* 1998, Yang *et al.* 1999b) rather than as a non-competitive antagonist (i.e. allosterically) (Ollmann *et al.* 1997). However, this seems difficult to reconcile with the fact that the structures of α MSH and AGRP are substantially different and the two peptides have different receptor subtype specificities (Yang *et al.* 2000). Mutational analyses of the MC4-R (Yang *et al.* 1999a, 2000, 2003, Haskell-Luevano *et al.* 2001), and three dimensional structure analysis of AGRP (Tota *et al.* 1999, McNulty *et al.* 2001), indicate that melanocortin peptides and AGRP share binding determinants to the MC4-R, but that AGRP has additional points of contact. Unfortunately, it is impossible to discern whether the pharmacological consequences of receptor mutagenesis are due to direct or indirect disruption of ligand receptor interactions. Consequently, none of these studies definitively answer the question of whether POMC-derived peptides and AGRP interact allosterically or orthosterically at the MC4-R. The best approach to address this point is to analyse the dissociation kinetics of radioligands from the receptor.

In order to investigate these issues in detail, we have undertaken comprehensive binding and functional cAMP

coupling analysis of a range of POMC-derived peptides at the human (h) MC4-R, utilising a stably transfected CHOK1 cell line. We have compared relative binding affinities, potencies and the effect of the C-terminal domain of AGRP, AGRP(83–132), on each peptide. Moreover, we have analysed the effect of AGRP(83–132) on the dissociation kinetics of radiolabelled Nle⁴,D-Phe⁷ MSH (NDP-MSH), an α MSH analogue, to determine whether AGRP(83–132) acts as an allosteric or orthosteric antagonist.

Materials and Methods

Peptides

α MSH, da- α MSH and ACTH were purchased from Sigma (Poole, Dorset, UK). β LPH was supplied by Dr A Parlow of the Pituitary Hormone and Antisera Centre (Harbor-UCLA Medical Centre, CA, USA). AGRP(83–132) and agouti peptide(87–132) were purchased from Phoenix Peptides Inc. (Belmont, CA, USA).

Peptide binding assays

CHOK1 cells were stably transfected with full-length hMC4-R and a cAMP reporter construct consisting of a cAMP response element and three vasoactive intestinal peptide enhancer elements upstream of a lac Z reporter gene (kindly provided by Drs M Needham and D Scanlan, AstraZeneca, Cheshire, UK). Cells were grown to complete confluence in Dulbecco's modified Eagle's medium (DMEM) (Sigma), 10% fetal calf serum, 1% sodium hypoxanthine and thymidine supplement (Gibco), 1% non-essential amino acids (Gibco), 200 μ g/ml G418 (Gibco) and 500 μ g/ml hygromycin B (Roche). Cells were washed with 500 ml ice-cold buffer A (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, pH 7.4), harvested and homogenised. Homogenates were centrifuged at 1700 g for 5 min at 4 °C. The supernatant was then centrifuged at 48 000 g for 1 h at 4 °C and the resultant pellet resuspended in ice-cold buffer A to approximately 5–10 mg/ml and aliquots stored at –80 °C. Protein concentration was determined by the method of Lowry *et al.* (1951).

The affinities of a range of POMC peptides were determined by inhibition of equilibrium [¹²⁵I]NDP-MSH binding. A range of concentrations of the competing ligand diluted in HBSS buffer (140 mM NaCl, 5.3 mM KCl, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 0.5 mM MgCl₂, 0.04 mM MgSO₄, 5.6 mM (+)D-glucose, 0.34 mM Na₂HPO₄, 1.3 mM CaCl₂, 20 mM HEPES, 0.1% BSA, pH 7.4) were incubated with 10–30 μ g membrane protein and 50 pM [¹²⁵I]NDP-MSH (Amersham) in a total volume of 0.1 ml at room temperature for 3 h. Experiments were terminated by rapid filtration through Whatman GF/C glass fibre filters,

presoaked in 0.5% polyethylenimine using a Brandel cell harvester, followed with four washes of 1 ml ice-cold buffer B (25 mM HEPES, 1.5 mM CaCl_2 , 1 mM MgSO_4 , 100 mM NaCl). Filter plates were air dried before adding 50 μl MicroScint 40 scintillation fluid (Packard) and radioactivity was determined by scintillation spectroscopy.

cAMP reporter assays

Ligand stocks ($2 \times$) were prepared in indicator-free DMEM and 50 μl aliquots were added, in quadruplicate, into poly-lysine-coated 96-well plates. CHOK1 cells expressing the human MC4-R were added to each well at a density of 50 000 cells/well and the plate was incubated for 5 h at 37 °C/5% CO_2 . Cyclic AMP was detected by addition of 1 mM chlorophenol red- β -D-galactopyranoside (CPRG) (Roche) in buffer containing a final concentration of 40 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 7 mM KCl and 0.7 mM MgSO_4 . β -Galactosidase converts CPRG to give a red colour. Results were quantified by reading absorbance at 590 nm on a Spectrafluor (Tecan) plate reader. Each experiment was performed a minimum of three times with quadruplicate wells. Dose-response data were fitted to a sigmoid curve using nonlinear squares regression (Origin 6.0, Microcal Software, Inc, Northampton, MA, USA). Mean relative potency ($\text{pEC}_{50} \pm \text{S.E.M.}$) values are reported. Data from dose-response curves were transformed according to the method of Arunlakshana and Schild (1959) and pKb values were determined.

Dissociation kinetics

NDP-MSH (50 pM) was incubated with 10–30 μg membrane protein in HBSS buffer for 3 h at room temperature in a total volume of 100 μl . Non-specific binding was determined by inclusion of 10 μM NDP-MSH in one triplicate at the outset. Dissociation was initiated by adding 100 μl NDP-MSH to a final concentration of 10 μM in the presence or absence of 0.1 μM AGRP(83–132). Addition times were staggered so that dissociation could be followed over a period of 10 h and all wells could be harvested simultaneously while ensuring that the association period was 3 h in each well. Data from each individual experiment were fitted to a monophasic exponential decay equation and mean dissociation rate constants reported.

Results

Binding affinities of POMC-derived peptides at cloned hMC4-R

Figure 1a shows binding curves generated for POMC-derived peptides at the human MC4-R. Desacetyl α MSH

Figure 1 (a) MC4-R binding. Competition of peptides for [125]NDP-MSH binding to membranes prepared from CHOK1 cells expressing the hMC4-R. Radioligand (50 pM) was incubated for 3 h in the presence of a range of concentrations of NDP-MSH (■), α MSH (▲), β MSH (▼), da- α MSH (●), AGRP(83–132) (□), ACTH (◆), or β LPH (◇). Data were expressed as a proportion of the maximum specific binding of [125]NDP-MSH in each experiment and the mean and S.E.M. of three independent experiments are shown for each data point. Four parameter logistic equations were fitted independently to data from each individual experiment and IC_{50} values determined. Mean affinity values are reported in the text and curves shown are defined by these mean values. (b) MC4-R activation. CHOK1 cells stably expressing hMC4-R and a β -galactosidase reporter construct were treated with increasing concentrations of β MSH (□), α MSH (■), da- α MSH (○), ACTH (●), β LPH (△) and γ MSH (▲). Datapoints represent means of quadruplicate measurements. The curves are representative of five independent experiments.

($\text{pKi} \pm \text{S.E.M.} = 6.89 \pm 0.2$ (127 nM)), β MSH (6.85 ± 0.06 (140 nM)), α MSH (6.82 ± 0.08 (150 nM)) and ACTH (6.75 ± 0.1 (176 nM)) all had approximately equivalent affinities at the receptor. The high molecular weight MSH precursor, β LPH, also showed significant binding (6.43 ± 0.11 (371 nM)). It is noteworthy that AGRP had a considerably higher affinity for the MC4-R (8.65 ± 0.17 (2 nM)) than any of the POMC-derived peptides.

Figure 2 Schild analysis demonstrating that AGRP(83–132) acts as a competitive antagonist at the hMC4-R. CHOK1 cells stably expressing hMC4-R and a β -galactosidase reporter construct were treated with increasing concentrations of α MSH, and coincubated with 0 nM (■), 1 nM (●), 5 nM (▲), 10 nM (▼), 50 nM (◆) and 100 nM (+) AGRP(83–132). Datapoints represent means of quadruplicate measurements. Data shown are one representative of three independent experiments. Inset shows a graph representing a Schild analysis linear regression plot.

Potencies of POMC-derived peptides at cloned hMC4-R

The mean relative potencies ($pEC_{50} \pm S.E.M.$) of the POMC-derived peptides were: β MSH (8.47 ± 0.08 (3.4 nM)) $\geq \alpha$ MSH (8.4 ± 0.18 (4 nM)) $\geq$ da- α MSH (8.39 ± 0.08 (4.1 nM)) $\geq$ ACTH (8.1 ± 0.15 (7.9 nM)) $> \beta$ LPH (7.12 ± 0.11 (76 nM)) (Fig. 1b). Many POMC-derived peptides, therefore, can activate the MC4-R with equivalent potencies leading to an accumulation of intracellular cAMP. Gamma MSH, as expected, did not elicit a significant cellular response (Gantz *et al.* 1993, Mountjoy *et al.* 1994).

AGRP(83–132) antagonism of POMC-derived peptides at cloned hMC4-R

We have investigated the functional relationship between AGRP(83–132) and a range of different POMC-derived peptides at cloned hMC4-R. Figure 2 shows dose–response curves for α MSH stimulation of cAMP production in the presence of increasing concentrations of AGRP(83–132), as determined by the β -galactosidase reporter assay. Clearly AGRP(83–132) causes a rightward shift in the agonist dose–response curve without affecting maximal response. Data were transformed according to the method of Arunlakshana and Schild (1959), plotting log concentration of AGRP(83–132) versus log dose ratio minus one (DR–1). The slope of the Schild plot was 0.99. The fact that the slope approaches unity is consistent with the hypothesis of competitive interaction between AGRP(83–132) and α MSH at the MC4-R. Similar data were obtained when cAMP accumulation was assessed by direct cAMP measurement (data not shown).

Table 1 lists mean pKb values for AGRP(83–132) when coupled with each POMC-derived peptide. The data

demonstrate that there is no significant difference in the potency of AGRP(83–132) antagonism with respect to any of the peptides tested.

Effect of AGRP(83–132) on the dissociation kinetics of NDP-MSH at the MC4-R

To define the nature of AGRP antagonism, we investigated the effect of AGRP(83–132) on the dissociation kinetics of radiolabelled NDP-MSH, using membrane preparations from our MC4-R transfected CHOK1 cells. If AGRP(83–132) acted allosterically with POMC-derived peptides, it would be expected to accelerate or retard the rate of dissociation of radiolabelled NDP-MSH. A competitive ligand, on the other hand, would have no effect on the dissociation rate (Vauquelin *et al.* 2002). Figure 3 demonstrates that AGRP(83–132) does not have any effect on dissociation rate, which confirms that it competitively antagonises NDP-MSH binding at the MC4-R. Similar data were obtained when dissociation

Table 1 Mean pKb values for AGRP(83–132), coupled to a range of POMC-derived peptides. Schild plots were generated from three independent experiments and mean pKb values and Schild gradients are indicated

	Schild gradient	pKb
α MSH	0.94 ± 0.05	8.6 ± 0.14
β MSH	1.04 ± 0.02	8.67 ± 0.15
da- α MSH	0.9 ± 0.07	8.78 ± 0.09
ACTH	1.08 ± 0.17	8.44 ± 0.22
β LPH	1.07 ± 0.21	8.53 ± 0.16

Figure 3 Effect of AGRP on the dissociation kinetics of specific [125 I]NDP-MSH binding at the MC4-R. Membranes expressing the recombinant human MC4-R were incubated to equilibrium with [125 I]NDP-MSH (50 pM); dissociation was initiated by the addition of an excess of unlabelled NDP-MSH (10 μ M) either in the absence ($\blacksquare$) or presence of AGRP (0.1 μ M) ($\bullet$). Inset shows the predicted extrapolation of these dissociation curves assuming first order kinetics. Data are the means $\pm$ S.E.M. from three experiments carried out in triplicate.

kinetics were analysed using cell membranes prepared from HEK293 cells stably transfected with MC4-R (data not shown).

Agouti(87–132) antagonism of POMC-derived peptides at cloned hMC4-R

It has been suggested that agouti antagonises α MSH and da- α MSH coupling to human MC4-R by different mechanisms (Mountjoy *et al.* 1999). We have tested this hypothesis in our MC4-R cAMP reporter gene assay. Figure 4 shows representative dose-response curves and Schild analyses of α MSH and da- α MSH in the presence of increasing concentrations of agouti(87–132). Both α MSH (1.01 ± 0.02) and da- α MSH (0.98 ± 0.11) yield Schild plots with gradients approaching unity. Moreover, there was no significant difference in mean pK_B for agouti(87–132) when either α MSH (8.09 ± 0.05 (8.1 nM)) or da- α MSH (8.04 ± 0.06 (9.1 nM)) was used as an antagonist. Similar results were obtained when cAMP was measured directly (data not shown). In our hands, therefore, the interactions of agouti(87–132) with α MSH and da- α MSH at the MC4-R are consistent with competitive antagonism and there is no significant difference in the potency of antagonism of agouti peptide regardless of whether α MSH or da- α MSH is used as an agonist.

Discussion

In this study we have undertaken comprehensive pharmacological analysis of interactions between POMC-derived peptides and AGRP(83–132) at the human MC4-R, using a CHOK1 cell line stably transfected with human MC4-R and a cAMP reporter construct. Our main objectives were (1) to compare the relative affinities and potencies of POMC-derived peptides endogenously secreted within the hypothalamus at the MC4-R, (2) to investigate the potency of AGRP(83–132) antagonism with respect to each POMC-derived peptide, based on the previous observation that agouti antagonises α MSH and da- α MSH differently (Mountjoy *et al.* 1999) and (3) to determine whether AGRP(83–132) and POMC-derived peptides interact allosterically or orthosterically.

Although α MSH is regarded as the natural ligand for the MC4-R, we and others have shown that other POMC-derived peptides are produced and secreted in the hypothalamus, including da- α MSH, β MSH, ACTH, β LPH and POMC (Nilaver *et al.* 1979, O'Donohue *et al.* 1981, Bertagna *et al.* 1988, Pritchard *et al.* 2003). We have found that da- α MSH, β MSH and ACTH all bind MC4-R with similar affinity to α MSH and were essentially equipotent in a functional assay, which is consistent with previous studies (Gantz *et al.* 1993, Mountjoy *et al.* 1994). We have also demonstrated that high molecular

a) α MSH b) Desacetyl α MSH

Figure 4 Effect of agouti(87–132) on (a) α MSH and (b) da- α MSH dose responses. The ability of α MSH or da- α MSH to induce a cellular response was determined by β -galactosidase reporter gene assay in the presence of a range of concentrations 0 nM (■), 10 nM (●), 50 nM (▲) and 100 nM (▼) of agouti(87–132) peptide. Data shown are means and S.E.M. of triplicate determinations in one representative of three independent experiments. For each independent experiment data were subject to Schild analysis and a pKb value determined (inset). Mean values for gradient and pKb are reported in the text.

weight MSH precursors produced in the hypothalamus, such as β LPH, also bind and activate the receptor. These observations cast doubt on the assertion that α MSH is the sole physiological MC4-R ligand. Interestingly, intracerebroventricular injection of ACTH, β MSH and da- α MSH, significantly reduce food intake in rodents with varying potencies (Abbott *et al.* 2000, Kask *et al.* 2000, Al-Barazangi *et al.* 2001). These peptides, therefore, are likely to be important endogenous ligands in the central melanocortin system. An important functional role of ACTH is particularly likely, given our previous observations that ACTH is more predominant than α MSH in rat cerebrospinal fluid and its levels are altered depending on energy requirement (Pritchard *et al.* 2003). Recent data also suggest that β MSH is important in melanocortin signalling (Harrold *et al.* 2003). Whilst it is not clear

whether β MSH is produced in rodents, human studies implicate β MSH in the pathogenesis of obesity (Challis *et al.* 2002, Krude *et al.* 2002). Also, the precise role of da- α MSH in the regulation of food intake is difficult to delineate experimentally given its relative instability compared with other POMC-derived peptides (O'Donohue *et al.* 1981, Abbott *et al.* 2000). Our data further support the concept that many POMC-derived peptides are important in the central melanocortin system. However, it will require more investigation to define precisely the role of each peptide. The generation of a series of recombinant mice, where production of individual peptides is disrupted will be extremely useful. We believe it is likely that the relative levels of the many POMC-derived peptides secreted in the hypothalamus could be important in regulating flux through the melanocortin system with

respect to energy requirement. This is supported by observations in humans where subtle changes in POMC processing lead to gross obesity (Challis *et al.* 2002).

The main objective of this study was to define precisely the mechanism of interaction of POMC-derived peptides and AGRP(83–132) at the MC4-R. Our data clearly support a competitive antagonistic role of AGRP(83–132) at the MC4-R. In cAMP accumulation assays, we found that increasing concentrations of AGRP(83–132) cause a characteristic rightward shift in agonist dose–response curves, but had no effect on the maximal response to the agonist. This observation was apparent regardless of whether intracellular cAMP levels were measured directly, or via a reporter gene system. Schild plots generated from dose–response data yielded gradients of close to unity for all POMC-derived peptides tested. This is consistent with AGRP(83–132) acting as a competitive antagonist. It is possible that different POMC-derived peptides secreted in the hypothalamus have very different functional relationships with the endogenous antagonist, AGRP, despite having very similar potencies at the MC4-R, i.e. AGRP may be significantly more potent as an antagonist for certain POMC peptides compared with others. For example, MSH precursor molecules, such as the 89-amino acid β LPH could impede AGRP binding due to their relative size and bulk compared with smaller peptides, such as the 14-amino acid α MSH. Therefore, having confirmed that AGRP(83–132) acts as a competitive antagonist, we then calculated pK_B values for AGRP(83–132) with respect to each POMC-derived peptide. This analysis demonstrated that there is no difference in AGRP(83–132) potency regardless of which POMC peptide engages with the MC4-R.

Clearly the data from the MC4-R functional assays suggest that AGRP(83–132) acts as a competitive antagonist. However, it is not possible, based on these data alone, to ascertain whether POMC-derived peptides and AGRP(83–132) act via physically distinct or overlapping domains on the MC4-R. Comprehensive mutational analysis of MC4-R (Yang *et al.* 2000, Haskell-Luevano *et al.* 2001) and analysis of MC1-R/MC4-R chimeric molecules (Yang *et al.* 1999a, 2003) suggest that AGRP possesses both overlapping and unique receptor interactions as compared with POMC-derived agonists. We have, for the first time, undertaken receptor dissociation experiments to further explore these interactions. Our data demonstrate that the presence of a melanocortin ligand (NDP-MSH) at the receptor sterically prevents the binding of AGRP(83–132). This is consistent with classic competitive antagonism and explains why we observed Schild gradients approaching unity when the interactions of POMC-derived peptides/AGRP(83–132) were assessed by cAMP reporter assay. Although this observation does not necessarily rule out additional points of contact for AGRP(83–132) elsewhere within the MC4-R molecule, it demonstrates, in pharmacological terms, that

melanocortins and AGRP(83–132) act orthosterically, rather than allosterically, at the MC4-R.

It seems that AGRP has additional physiological effects that are independent of its role as a competitive antagonist of POMC-derived peptide/MC4-R interactions. For example, intracerebroventricular injection of AGRP into rats has a far more prolonged effect on food intake as compared with POMC-derived peptides (Hagan *et al.* 2000, Kim *et al.* 2002). Also, recent data indicate that AGRP may act as an inverse agonist at the MC4-R (Haskell-Luevano & Monck 2001, Nijenhuis *et al.* 2001), and may also inhibit internalisation of the receptor following agonist exposure (Shinyama *et al.* 2003). It seems likely, therefore, that AGRP may influence intracellular pathways in addition to the adenylyl cyclase/protein kinase A pathway. This would not be surprising as cellular responses mediated by G-protein-coupled receptors often involve the functional integration of more than one intracellular signalling pathway (Marinissen & Gutkind 2001). Indeed, there is evidence that this is the case for the MC4-R, which has been shown to mobilise intracellular free calcium as well as cAMP (Mountjoy *et al.* 2001). Understanding the role of AGRP is further complicated by the existence of syndecan-3, a heparan sulphate proteoglycan that seems to act as a co-receptor for AGRP by interacting with its N-terminal fragment (Reizes *et al.* 2001). Therefore, it may be pertinent to carefully examine pharmacological interactions of full-length AGRP at the MC4-R and its interaction with POMC-derived ligands. We have used AGRP(83–132), a C-terminal truncated fragment, in our analysis. This peptide does not interact with syndecan-3 (Reizes *et al.* 2001). It is probably more physiologically relevant to study the pharmacology of AGRP(83–132), rather than full-length AGRP, as there is evidence that AGRP is post-translationally cleaved into C-terminal AGRP and one or more N-terminal fragments (Li *et al.* 2000). Moreover, the AGRP C-terminal domain is alone capable of exerting potent effects on energy balance *in vivo* (Rossi *et al.* 1998).

Our results demonstrate that there is no difference in the potency of AGRP(83–132) antagonism at the MC4-R regardless of which POMC-derived peptide acts as the agonist. Interestingly, Mountjoy *et al.* (1999) have previously shown that agouti antagonism of the MC4-R is significantly greater when da- α MSH, rather than α MSH, is used as an agonist. Also, in their study, agouti seems to act as a non-competitive antagonist. It would appear, therefore, that the mechanism of action of AGRP and agouti may differ. As this phenomenon could have implications for the complexity of intracellular melanocortin signalling, we investigated the antagonistic characteristics of agouti(87–132) in our systems. Our data are consistent with competitive antagonism and did not show any significant difference in the effect of agouti(87–132) on α MSH and da- α MSH coupling to the MC4-R. These findings contrast with a previous study (Mountjoy *et al.*

1999), although the conflicting sets of data could be due to the fact that different cell lines were used, or to the variation in experimental parameters. Further pharmacological studies in cell lines that endogenously express MC4-R, such as GT1-1 (Khong *et al.* 2001), could shed further light on these issues.

In summary, we have undertaken pharmacological studies to gain further insight into agonist/antagonist interactions that occur at the MC4-R. We conclude that other POMC-derived peptides in addition to α MSH, particularly ACTH, are likely to act as physiological ligands for the receptor. We also demonstrate that MSH precursors, such as β LPH, can bind and activate the MC4-R. Both our cAMP assays and dissociation kinetic experiments support the view that AGRP(83–132) acts as a competitive antagonist. Moreover, dissociation kinetics experiments demonstrate that AGRP's role in downregulating cAMP accumulation is probably via the same binding site as that of the melanocortin agonists. This is an important finding to be considered during the quest for anti-obesity strategies that target the central melanocortin pathway.

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