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Co-operative regulation of ligand binding to melanocortin receptor subtypes: Evidence for interacting binding sites

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Abstract

This study evaluates the binding the melanocyte stimulating hormone peptide analogue [^{125}I]NDP-MSH to melanocortin receptors MC_1, MC_3, MC_4 and MC_5 in insect cell membranes produced by baculovirus expression systems. The presence of Ca^{2+} was found to be mandatory to achieve specific [^{125}I]NDP-MSH binding to the melanocortin receptors. Although association kinetics of [^{125}I]NDP-MSH followed the regularities of simple bimolecular reactions, the dissociation of [^{125}I]NDP-MSH from the melanocortin receptors was heterogeneous. Eleven linear and cyclic MSH peptides studied displaced the [^{125}I]NDP-MSH binding to the studied melanocortin receptors, with the shapes of their competition curves varying from biphasic or shallow to super-steep (Hill coefficients ranging from 0.4 to 1.5). Notably the same peptide often gave highly different patterns on different melanocortin receptor subtypes; e.g. the MC_4 receptor selective antagonist HS131 gave a Hill coefficient of 1.5 on the MC_1 receptor but 0.5–0.7 on the MC_{3-5} receptors. Adding a mask of one of the peptides to block its high affinity binding did not prevent other competing peptides to yield biphasic competition curves. The data indicate that the binding of MSH peptides to melanocortin receptors are governed by a complex dynamic homotropic co-operative regulations.

Keywords: Melanocortin receptor; Radioligand binding; Cooperativity; Melanocyte stimulating hormone; Sf9 cells; Calcium

1. Introduction

The melanocortin receptors belong to the G-protein coupled receptors, and contain five known subtypes, MC_{1-5}. These subtypes of melanocortin receptors are specific for melanocyte stimulating hormones (MSH) and the MC_2 receptors for adrenocorticotropic hormone (ACTH). The melanocortin receptors show distinct distributions to various parts of the body and evidence suggest that the MC_1 receptors could serve as a novel target for anti-inflammatory therapies, the MC_3 and MC_4 receptors for treatment of sexual dysfunctions, the MC_4 receptors for treatment of eating disorders and the MC_5 receptors for treatment of dysfunctions of exocrine glands (Wikberg et al., 2000). Accordingly, the melanocortin receptors have become important targets for drug developments (Andersson et al., 2001; Wikberg, 2001).

The melanocortin receptors were cloned almost a decade ago and several types of assays were developed for evaluation of their interactions with drugs. Early studies utilised radioligand binding in cells expressing the recombinant receptors. For the MC_1 and MC_{3-5} receptors the stable high affinity MSH peptide analogue [^{125}I][Nle^4, D-Phe^7]α-MSH ([^{125}I]NDP-MSH) was used as radioligand (Schiöth et al., 1995; 1996b). For the MC_2 receptors [^{125}I]-adrenocorticotropic hormone ([^{125}I]ACTH) was used (Schiöth et al., 1996a). The melanocortin receptors couple in a positive fashion to adenylate cyclase and assays of cAMP have been used for their characterization in cells expressing the recombinant melanocortin receptors (Chen et al., 1995).

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In the course of our studies aiming to develop subtype selective organic agonists and antagonists for melanocortin receptors (Mutulis et al., 2002a,b) we were interested in improving the accuracy of our radioligand binding assays. We reasoned that using a simpler system, where the receptors were confined in a membrane fraction rather than in cells, should be beneficial as less confounding effects should become introduced by ligand diffusion into the cells, cell metabolism and alike. During these studies we found that the melanocortin receptors show complex ligand binding patterns, indicating the presence of both positive and negative co-operative interactions. We here report our new findings, which indicate that the melanocortin receptors are subject to much more complex molecular interactions in the cell membrane than was previously thought.

2. Materials and methods

2.1. Materials

[^{125}I][Nle^4, D-Phe^7]α-MSH ([^{125}I]NDP-MSH) was prepared radiochemically pure (2190 Ci/mmol) by iodination using chloramine T followed by High Performance Liquid Chromatography (HPLC). [Nle^4, D-Phe^7]α-MSH (NDP-MSH), α-MSH, β-MSH, γ^1-MSH, γ^2-MSH, Lys-γ^1-MSH, [Nle^4]-γ^2-MSH, Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (MS05) (Szardenings et al., 2000), cyclic [Ac-Cys^{11}, D-Nal^{14}, Cys^{18}, Asp-NH_2^{22}]-β-MSH(11–22) (HS014) (Schiöth et al., 1998), cyclic [Ac-Cys^3, Nle^4, Arg^5, D-Nal^7, Cys-NH_2^{11}]-α-MSH(3–11) (HS024) (Kask et al., 1998), cyclic [Ac-Cys-Gly-D-Nal-Arg-Trp-Cys-NH_2] (HS131) (Wikberg, 2001) were synthesised on solid phase, purified by HPLC and checked by Liquid Chromatography/Mass Spectrometry (LC/MS). PFASTBac1, DM5, DHlO-bac, restriction enzymes, synthetic Sf900 II medium and cell culturing reagents were from Invitrogen LifeTechnologies. Sf9 cells (Chlorocebus Aethiopus 1999-06-07) were from ATCC (Manassas, VA, USA). Guanosine-5′-*O*-3-thiotriphosphate (GTPγS) and all other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Construction of baculovirus

PFastBac1 was *Hin*DIII linearized, S1 digested and religated to remove the original *Hin*DIII site. The resulting vector (pFdH1) was linearized with *Xba*I, a *Spe*I/*Xba*I fragment from pcDNA3.1 (Zeo)(+)-hMC2R (Mountjoy et al., 1992) was cloned into this site and the vector pFdHP-hMC2R, with the fragment in correct orientation was isolated. Human melanocortin receptor genes MC_3 (Gantz et al., 1993a), MC_4 (Gantz et al., 1993b) and MC_5 (Chhajlani et al., 1993) were then *Hin*DIII/*Xba*I cloned into this vector. The additional sequence for all receptors, besides the coding region at the 3′-end, was *Hin*DIII-AAGCTTCACATATG and TAGTTCTAGA-*Xba*I at the 5′-end as described (Schiöth et al., 1997a).

Generation of the baculovirus clones followed the recommendations from LifeTechnologies (i.e. modified from Luckow et al., 1993). Vector inserts were sequenced and the correct plasmids used for the transformation of DH10bac for transposition into baculovirus DNA. Positive colonies of transformed DH10bac were regrown on agar plates with kanamycin (40 μg/ml), gentamycin (6 μg/ml) and BluoGal (100 μg/ml) alone to check for clones with reverse transposition events. Recombinant baculovirus DNA was prepared from ampicillin sensitive colonies, checked by PCR with a pair of primers binding outside of the multiple cloning site of pFdH2: 5′-CTGTTTTCGTAA-CAGTTTTG-3′ (PFB-UP) and 5′-CATTTTATGTTT-CAGGTTCA-3′ (PFB-DOWN). These were also used for sequencing. Baculovirus DNA was transfected into Sf9 cells (Smith et al., 1985) using cellfectin. The viruses were amplified about 2–3 times and stocks were prepared. Baculovirus DNA was then re-transfected into Sf9 cells (Smith et al., 1985) performing a serial dilution of the virus stock in 96 hole plates (Dee and Shuler, 1997). About 1×10^4 Sf9 cells were added per well to a total volume of 100 μl. After 1 week viruses were isolated from wells where cells did not grow confluent, amplified and checked for receptor expression. The clonal baculoviruses yielding the highest receptor expression [clones vVPH4 (MC_3), v119-7 (MC_4) and v113-9 (MC_5)] were selected. Cloning of the human MC_1 receptor gene (Chhajlani and Wikberg, 1992) into the baculovirus was described earlier (Lindblom et al., 2001). We here used the v506-1 clone encoding Flag-MC_1-His receptor (Lindblom et al., 2001). The MC_2 receptor was not used for receptor expression herein.

2.3. Cell cultures

Sf9 cells were grown in 50–100 ml Sf-900 II medium at 27 °C in small spinner bottles (250 ml) as described (O'Reilly et al., 1992). Recombinant viruses were added to the cell culture ($2–3 \times 10^6$ cells/ml) and the incubation continued for additional 72 h before harvest. B16 melanoma cells were grown as described (Schiöth et al., 1997b). Data reported herein were obtained on melanocortin receptors in Sf9 cell membranes, unless otherwise stated.

2.4. Membrane preparations

Cells were collected by centrifugation at $800 \times g$ for 5 min and Dounce homogenized (5 times by 10 stokes with 30 s intervals) in ice-cold homogenization buffer containing 20 mM Na-HEPES, 0.1 mM phenylmethanesulfonyl fluoride, 0.25 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, pH 7.4 at concentration $5 \cdot 10^8$ cells/50 ml. The homogenate was centrifuged at $700 \times g$ for 5 min at 4 °C, the pellet was then

rehomogenized and centrifuged again. The combined supernatants were collected, sedimented at 70,000 × *g* (60 min at 4 °C) and washed once in new buffer and re-centrifuged. The final pellet was resuspended in the homogenization buffer at a protein concentration 1–3 mg of protein/ml and aliquots were stored at −80 °C. Protein was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as standard. All studies reported herein were, for each respective receptor, performed on the same batch of membranes.

2.5. Radioligand binding assays

Assays were (unless otherwise stated) performed by incubating membranes (10–20 μg protein/100 μl) in the incubation buffer (20 mM K-HEPES, 5 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mg/ml BSA, pH 7.4) with appropriate concentrations of [^{125}I]NDP-MSH and non-labelled ligands. Saturation studies included 1 pM to 4.7 nM [^{125}I]NDP-MSH. Displacement studies included 0.21–0.27 nM [^{125}I]NDP-MSH and appropriate concentrations of non-labelled compounds. Incubations were for 3 h at 25 °C (unless otherwise stated) and were terminated by rapid filtration through 0.3% polyethyleneimine and 1 mg/ml BSA pre-treated GF/B glass-fibre filters (Whatman Int. Ltd., Madistone, UK) using a Brandell cell harvester and three washes of 5 ml of ice-cold 12 mM NaK-phospate buffer (pH 7.4) containing 100 mM NaCl). Non-specific binding was measured using 3 μM NDP-MSH.

Kinetic association experiments were started by addition of [^{125}I]NDP-MSH (final concentration 0.23 nM) to a membrane suspension in the incubation buffer. At timed intervals aliquots (100 μl) were filtered on GF/B. Parallel incubations with 0.23 nM [^{125}I]NDP-MSH and 3 μM non-labelled NDP-MSH were used to estimate non-specific binding.

Dissociation experiments were performed after preincubating the membrane suspension with [^{125}I]NDP-MSH (0.23 nM) for 3 h at 25 °C. Dissociation was then initiated by adding non-labelled NDP-MSH (3 μM final concentration). Non-specific binding was determined in the same fashion except that the 3 μM non-labelled NDP-MSH was added already from the start of the experiment.

2.6. Data analysis

Steady state binding data were analysed by computer modelling fitting it to appropriate formulas using nonlinear least-squares regression and assuming that ligands bound to one or two independent binding sites.

Kinetic association data were fitted into Eq. (1), which assumes that association occurs in exponential fashion to *n* sites:

$$B(t) = B_{\text{nonsp}} + \sum^{n} B_n\left(1 - e^{-k_n t}\right), \quad (1)$$

where $B(t)$ is the binding at time t, B_{nonsp} the non-specific binding, B_n the equilibrium binding and k_n the observed association rate constant for site n.

Kinetic dissociation data were fitted into Eq. (2), which assumes that dissociation occurs in exponential fashion from *n* sites:

$$B(t) = B_{\text{nonsp}} + \sum^{n} B_n e^{-k_n^{\text{d}} t}, \quad (2)$$

where $B(t)$ is the binding at time t, B_{nonsp} the non-specific binding, B_n the binding at $t=0$ and k_n^{d} the observed dissociation rate constant for site n.

Ca^{2+} concentration effect data were analysed by fitting it to a generalisation of the logistic function:

$$R = \sum_{i}^{m} \frac{\alpha_i C^n}{C^n + C_{50_i}^n} + N \quad (3)$$

where R is the "response", C the concentration of the added agent, C_{50_i} the concentration giving 50% response of the "i-th component", α_i the maximum response of the i-th component, n the "pseudo" Hill-coefficient ("slope"), and $N=0$, $m=1$ or $m=2$ and $n=1$. Competition binding data were also analysed by fitting it into Eq. (3).

Data are presented as mean ± S.D. of 2–4 independent determinations. Statistical comparison of non-linear regression models were performed by an F-test using the extra sums of squares principle, as described (Draper and Hunter, 1967). Non-linear nonlinear least-squares regression was done using programs GraphPad PRISM™ 4.02 (GraphPad Software, San Diego, CA, USA) and BindAid (Wan System, Umeå, Sweden).

3. Results

3.1. Effects of Ca^{2+}

Some quite early work had suggested that binding of [^{125}I]NDP-MSH to native melanocortin receptors in melanoma cells (i.e. the MC_1 receptors) requires the presence of Ca^{2+} (Gerst et al., 1987; Salomon, 1990). In the present study it was shown that Ca^{2+} is mandatory for specific binding of [^{125}I]NDP-MSH to all the four studied melanocortin receptors, when these are confined in membranes prepared from insect cells expressing the recombinant human melanocortin receptor variants. Varying the concentration of Ca^{2+} from 10^{-8} to 10^{-2} M showed that already at 3–10 μM of Ca^{2+} some specific binding of [^{125}I]NDP-MSH could be achieved. Then the binding increased with increase of Ca^{2+}, achieving a maximum at 1 mM (Fig. 1). Fitting the data to Eq. (3) with $m=1$ showed that the C_{50} values of Ca^{2+} were 6 ± 3 μM, 25 ± 8 μM, 14 ± 4 μM and 158 ± 39 μM for the MC_1, MC_3, MC_4 and MC_5 receptors, respectively. For the MC_5 receptors the Ca^{2+} dose–response curve was considerably more shallow than for the other receptors. Computer modelling assuming a two binding site model

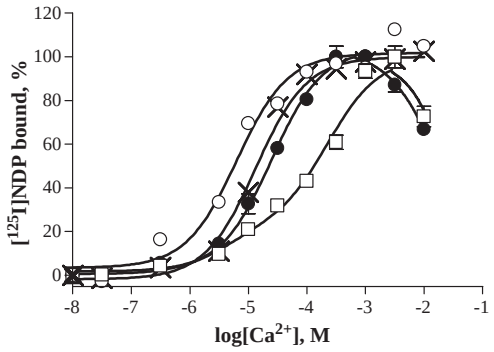

Fig. 1. Effect of the concentration of Ca^{2+} on the binding of [^{125}I]NDP-MSH to membranes of Sf9 cells infected with baculoviruses expressing different melanocortin receptor subtypes. The specific binding of 0.23 nM [^{125}I]NDP-MSH was measured after 3 h incubation with membranes of the MC_1 (○), MC_3 (●), MC_4 (×) and MC_5 (□) receptors in the presence of different concentrations of Ca^{2+}. Data are normalized to the maximal specific binding of [^{125}I]NDP-MSH (100%) for the respective subtype and presented as the mean of 3 independent measurements carried out in duplicates.

(e.g. Eq. (3); $m=2$; $n=1$) gave a significantly better fit than an one site model (Eq. (3) with $m=1$; $n=1$) ($P<0.001$). Accordingly the Ca^{2+} effect could be described with a $C_{50_1}=3.8\pm2.9$ μM and a $C_{50_2}=233\pm112$ μM, with the fraction of the first component $\alpha_1=0.23\pm0.07$. At high concentrations Ca^{2+} inhibited the specific [^{125}I]NDP-MSH binding. For the MC_3 and MC_5 receptors inhibition was seen at 3 mM (Fig. 1) and for the other subtypes studied at 50 mM Ca^{2+}. Mg^{2+} also supported the specific binding of [^{125}I]NDP-MSH, but only up to 20% of the maximal level achieved with Ca^{2+} (data not shown). All following experiments were carried out in the presence of 1 mM Ca^{2+}.

3.2. Kinetic studies

Association of [^{125}I]NDP-MSH (0.23 nM) to melanocortin receptors were adequately fitted to Eq. (1) assuming a pseudo first-order reaction to homogenous binding sites, which was preferred over two-phase exponential models ($P \ll 0.05$) for all receptor subtypes studied (Fig. 2). The half-times of the reactions were estimated to be 14 ± 1, 10 ± 1, 19 ± 1 and 2.9 ± 0.4 min for the MC_1, MC_{3-5} receptors, respectively ($n=2$–14).

Dissociations of [^{125}I]NDP-MSH from the melanocortin receptor subtypes were initiated by the non-labelled NDP-MSH after 180 min preincubation of the radioligand with the receptor. Only in the case of the MC_5 receptors the dissociation was essentially complete, while for the other melanocortin receptors the dissociation reached a plateau, which was considerably higher than the non-specific binding (Fig. 3). Fitting the data to Eq. (2) indicated that the dissociation occurred in a mono-exponential fashion, while the plateau of the MC_1, MC_3 and MC_4 receptors could be considered as an additional receptor bound fraction with a very slow (or even negligible) rate of dissociation. $52\pm8\%$ of the specifically bound [^{125}I]NDP-MSH dissociated from the MC_1 receptors with $\tau_{1/2}=40\pm14$ min. For the MC_3 receptors the corresponding numbers were $80\pm3\%$ and

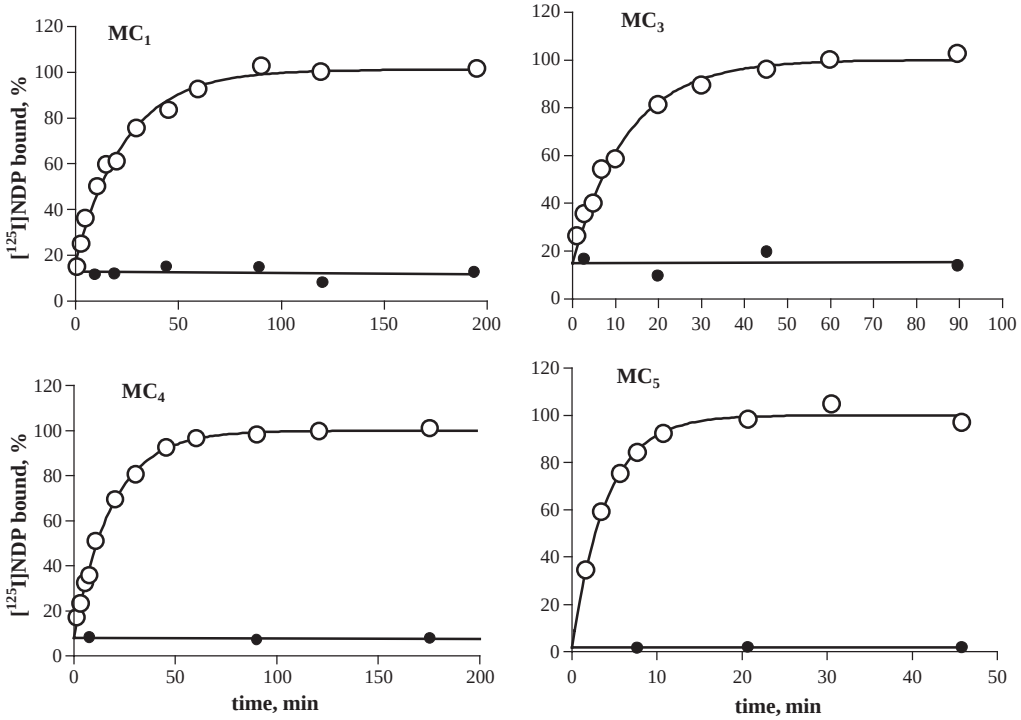

Fig. 2. Time course of total (○) and non-specific (●) binding of [^{125}I]NDP-MSH (0.23 nM) to membranes of Sf9 cells expressing different melanocortin receptors. Data of representative experiments are normalized to the maximal specific binding of [^{125}I]NDP-MSH (100%) for the respective melanocortin receptor subtype. The curved lines represent the best fit of the data to Eq. (1).

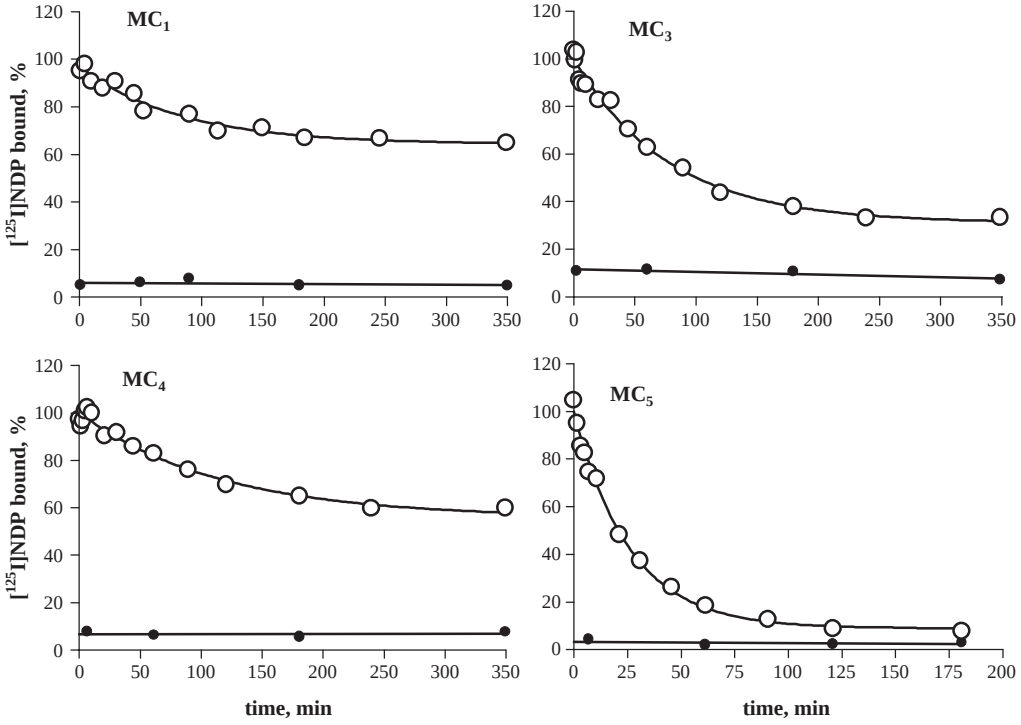

Fig. 3. Time course for the dissociation of [^{125}I]NDP-MSH from its complexes with different melanocortin receptors in membranes of Sf9 cells. The complexes were formed by incubation of membranes with 0.23 nM [^{125}I]-NDP-MSH for 3 h at 25 °C, and dissociation was initiated addition of 3 μM NDP-MSH (final concentration) (○). For the determination of non-specific binding (●), the preincubation with [^{125}I]-NDP-MSH was carried out in the presence of 3 μM non-labelled NDP-MSH. Data of representative experiments are normalized to the maximal binding of [^{125}I]NDP-MSH (100%) for the respective melanocortin receptor subtype. The curved lines represent the best fit of the data to Eq. (2).

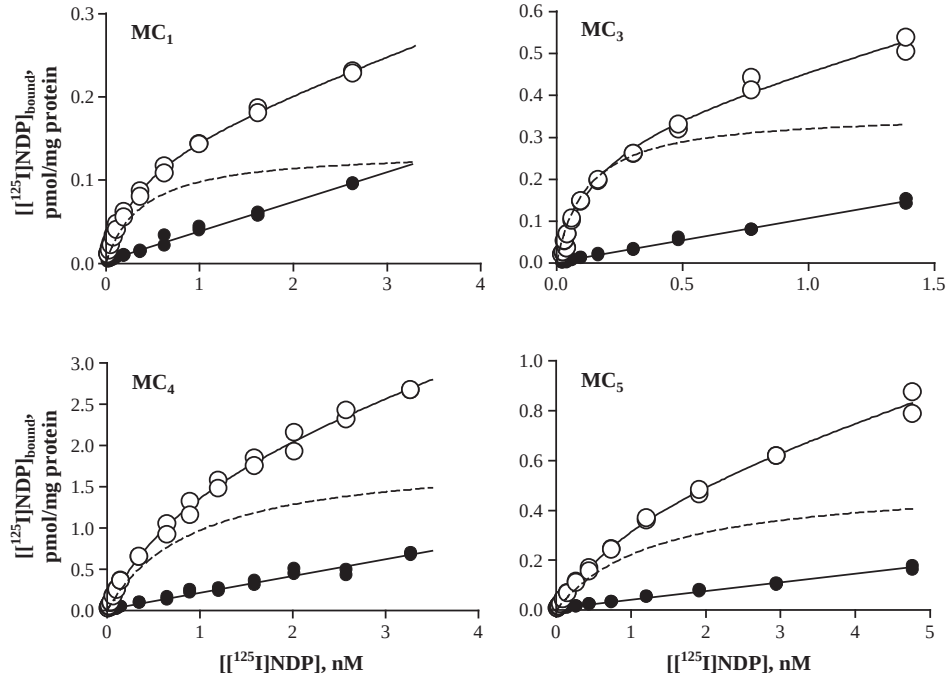

Fig. 4. Saturation curves for the binding of [^{125}I]NDP-MSH to different melanocortin receptors confined in membranes of Sf9 cells. The indicated preparations of melanocortin receptors were incubated with different concentrations of [^{125}I]NDP-MSH in the absence (○, total binding) and presence (●, non-specific binding) of 3 μM NDP-MSH for 3 h at 25 °C, and bound radioactivity was determined as described in Materials and methods. The specific binding defined as the difference between total and non-specific bindings are presented with dotted lines.

54 ± 6 min, and for the MC_4 50 ± 4% and 80 ± 15 min. For the MC_5 receptors the $\tau_{1/2}$ was 18 ± 2 min and the non-displaceable portion negligible.

3.3. Steady state saturation binding

The binding of [^{125}I]NDP-MSH to the MC_1, MC_{3-5} receptors in the Sf9 cell membranes was saturable with high affinity (Fig. 4). Fitting of the data to a simple binding isotherm assuming one binding site estimated the K_d of [^{125}I]NDP-MSH to 0.33 ± 0.04, 0.20 ± 0.02, 1.2 ± 0.2 and 2.8 ± 0.2 nM for the MC_1, MC_{3-5} receptors, respectively. The expression levels of the respective melanocortin receptor subtype was 0.14 ± 0.01, 0.36 ± 0.05, 1.9 ± 0.3 and 0.5 ± 0.1 pmol/mg protein (average of at least 3 independent experiments). In order to describe the binding in more detail the same data (which included measurements at 24 different radioligand concentrations) were fitted to a model that assumed two independent binding sites. A two site model was then clearly statistically preferred ($P<0.05$) over one site model, for all melanocortin receptors studied. The analysis showed that the high affinity site of the MC_1 receptors had $K_d^H = 0.12 \pm 0.03$ nM and $\alpha_H = 0.31 \pm 0.06$ (i.e. fraction of binding sites), while the low affinity site had a $K_d^L = 1.1 \pm 0.4$ nM. For the MC_3 receptors the corresponding values were: $K_d^H = 0.15 \pm 0.05$ nM, $\alpha_H = 0.55 \pm 0.12$, $K_d^L = 1.5 \pm 0.4$ nM; for the MC_4 receptors they were: $K_d^H = 0.14 \pm 0.06$ nM, $\alpha_H = 0.52 \pm 0.07$, $K_d^L = 2.2 \pm 0.8$ nM. For the MC_5 receptors the $K_d^H = 1.3 \pm 0.5$ nM, corresponded to 30 ± 4% of the binding calculated according to the one-site binding model, but the affinity of the low affinity site showed so low affinity ($K_d^L \gg 5$ nM) that it could not be properly computed.

Addition of 10 μM GTPγS had no significant influence on the [^{125}I]NDP-MSH binding to the MC_1 receptors, thus suggesting that G-proteins do not cause the observed heterogeneity (data not shown). When we used membranes prepared from native Sf9 insect cells, which had not been infected with melanocortin receptor containing baculovirus no specific binding of [^{125}I]NDP-MSH could be detected.

3.4. Steady state competition binding

Eleven linear and cyclic MSH related peptides were studied in competition with [^{125}I]NDP-MSH, and they all displaced the specific binding of the radioligand from the $MC_{1,3-5}$ receptors (Fig. 5, Table 1). The protein concentrations were taken so that measurements were under pseudo-monomolecular conditions, where the concentration of the free radioligand exceeded the bound radioligand concentration at least 10 times. However, the shapes of the competition curves ranged from strongly biphasic to super-steep. Notably the very same peptide behaved grossly different on the different melanocortin receptor subtypes. For example, the competition curve of the MC_4 receptor

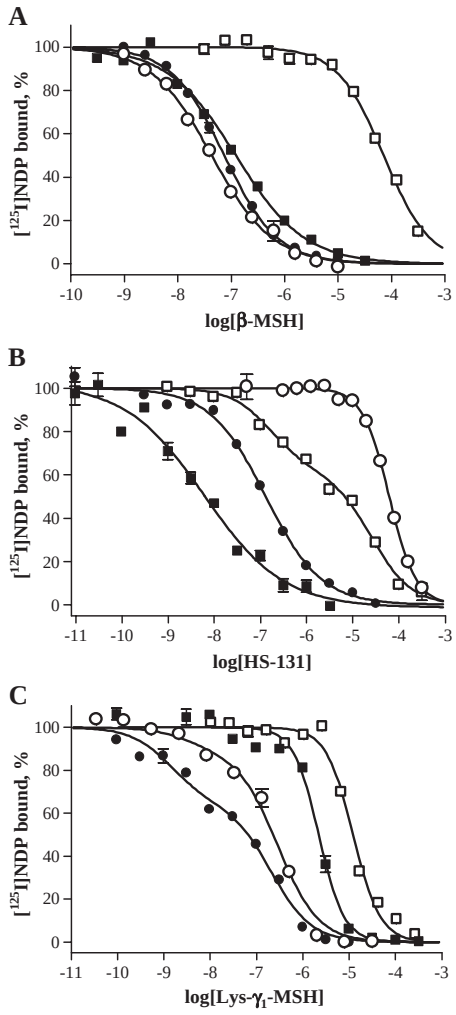

Fig. 5. Displacement of [^{125}I]NDP-MSH binding to MC_1 (○), MC_3 (●), MC_4 (■) and MC_5 (□) receptors by β-MSH (A), HS131 (B) and Lys-γ^1-MSH (C). The membranes of Sf9 cells of corresponding melanocortin receptors were incubated with 0.23 nM [^{125}I]NDP-MSH and different concentrations of competitive ligand for 3 h at 25 °C and the bound radioligand was determined as described in Materials and methods and presented as the percentage of the specific binding in the absence of competitive peptide.

selective antagonist HS131 (Wikberg, 2001) was clearly biphasic on the MC_5 receptors with $n_H = 0.51 \pm 0.05$, while on the MC_1 receptors the curve was super-steep with a Hill-coefficient 1.5 ± 0.1 (Fig. 5B). For the other melanocortin receptors the Hill coefficients resided between these extremes (Table 1). Another example is the MC_3 receptor selective peptide Lys-γ^1-MSH, which gave a clearly biphasic competition curve on the MC_3 receptors ($n_H = 0.53 \pm 0.05$), while on the MC_4 and MC_5 receptors the curves were super-steep with Hill-coefficients 1.5 ± 0.1 (Fig. 5C; Table 1). This contrasted to e.g. the displacement curves of β-MSH which were only shallow (MC_1 and MC_4 receptors) or uniphasic (MC_3 and MC_5 receptors) (Fig. 5A; Table 1). Addition of 10 μM GTPγS had no significant influence on the shape of the biphasic competition curve of NDP-MSH on the MC_5 receptors,

Table 1
pIC_{50}-values, Hill-slopes and fraction of apparent high affinity sites (α_H) for the binding of various MSH-peptides to MCR subtypes determined in competition with [^{125}I]NDP-MSH

	MC_1			MC_3			MC_4			MC_5		
	pIC_{50}[a] (Hill slope)	pIC_{50}^{high}[b], pIC_{50}^{low}	α_H	pIC_{50}[a] (Hill slope)	pIC_{50}^{high}[b], pIC_{50}^{low}	α_H	pIC_{50}[a] (Hill slope)	pIC_{50}^{high}[b], pIC_{50}^{low}	α_H	pIC_{50}[a] (Hill slope)	pIC_{50}^{high}[b], pIC_{50}^{low}	α_H
NDP-MSH	8.70 ± 0.09 (0.49 ± 0.04)	9.82 ± 0.25 8.06 ± 0.12	0.34 ± 0.03	8.95 ± 0.09 (0.55 ± 0.05)	10.09 ± 0.21 8.71 ± 0.017	0.38 ± 0.07	8.62 ± 0.11 (0.43 ± 0.06)	9.94 ± 0.13 7.79 ± 0.14	0.42 ± 0.07	8.32 ± 0.04 (0.32 ± 0.06)	9.84 ± 0.25 7.76 ± 0.11	0.33 ± 0.04
α-MSH	8.20 ± 0.07 (0.49 ± 0.04)	9.66 ± 0.14 7.77 ± 0.03	0.29 ± 0.02	7.24 ± 0.03 (0.73 ± 0.03)	7.98 ± 0.012 6.81 ± 0.05	0.45 ± 0.07	6.52 ± 0.05 (0.50 ± 0.02)	7.50 ± 0.04 5.73 ± 0.03	0.52 ± 0.04	4.28 ± 0.03 (1.27 ± 0.07)	–	ND[c]
β-MSH	7.15 ± 0.04 (0.68 ± 0.03)	7.70 ± 0.14 6.80 ± 0.13	0.49 ± 0.13	7.09 ± 0.02 (0.90 ± 0.07)	–	ND[c]	6.89 ± 0.07 (0.61 ± 0.04)	7.52 ± 0.05 6.04 ± 0.12	0.58 ± 0.05	4.18 ± 0.07 (0.95 ± 0.08)	–	ND[c]
γ^1-MSH	6.73 ± 0.10 (0.52 ± 0.06)	8.45 ± 0.21 5.98 ± 0.23	0.28 ± 0.03	6.40 ± 0.05 (0.99 ± 0.04)	–	ND[c]	5.33 ± 0.10 (1.39 ± 0.12)	–	ND[c]	4.62 ± 0.10 (1.24 ± 0.07)	–	ND[c]
γ^2-MSH	6.58 ± 0.19 (0.50 ± 0.04)	7.96 ± 0.21 6.28 ± 0.09	0.31 ± 0.04	6.03 ± 0.18 (0.90 ± 0.08)	–	ND[c]	4.67 ± 0.11 (1.33 ± 0.17)	–	ND[c]	3.94 ± 0.25 (1.32 ± 0.12)	–	ND[c]
Lys-γ^1-MSH	6.75 ± 0.07 (0.69 ± 0.08)	8.81 ± 0.27 6.63 ± 0.10	0.27 ± 0.05	7.12 ± 0.13 (0.53 ± 0.05)	8.34 ± 0.10 6.53 ± 0.08	0.33 ± 0.02	5.54 ± 0.21 (1.49 ± 0.13)	–	ND[c]	5.18 ± 0.16 (1.53 ± 0.14)	–	ND[c]
Nle-γ^2-MSH	7.39 ± 0.06 (0.40 ± 0.04)	9.79 ± 0.23 6.67 ± 0.17	0.40 ± 0.03	6.08 ± 0.09 (0.95 ± 0.06)	–	ND[c]	4.88 ± 0.09 (1.28 ± 0.08)	–	ND[c]	4.22 ± 0.23 (1.61 ± 0.25)	–	ND[c]
HS014	5.83 ± 0.03 (0.63 ± 0.05)	6.42 ± 0.07 5.14 ± 0.05	0.60 ± 0.04	7.21 ± 0.04 (0.70 ± 0.02)	8.25 ± 0.021 6.85 ± 0.11	0.34 ± 0.05	8.18 ± 0.10 (0.78 ± 0.09)	8.96 ± 0.16 7.49 ± 0.16	0.48 ± 0.08	5.34 ± 0.11 (0.45 ± 0.09)	7.00 ± 0.19 4.65 ± 0.04	0.38 ± 0.04
HS024	6.74 ± 0.04 (0.67 ± 0.19)	8.87 ± 0.36 6.55 ± 0.08	0.21 ± 0.05	7.30 ± 0.15 (0.89 ± 0.12)	–	ND[c]	8.63 ± 0.30 (0.67 ± 0.14)	10.02 ± 0.25 7.58 ± 0.15	0.45 ± 0.18	7.24 ± 0.12 (1.05 ± 0.15)	–	ND[c]
HS131	4.45 ± 0.05 (1.47 ± 0.07)	–	ND[c]	6.79 ± 0.03 (0.70 ± 0.03)	8.08 ± 0.14 6.11 ± 0.20	0.45 ± 0.15	8.23 ± 0.17 (0.50 ± 0.15)	8.85 ± 0.13 6.89 ± 0.24	0.62 ± 0.10	5.44 ± 0.07 (0.51 ± 0.05)	6.64 ± 0.13 4.79 ± 0.09	0.40 ± 0.04
MS05	8.44 ± 0.25 (0.42 ± 0.05)	9.93 ± 0.15 7.83 ± 0.19	0.37 ± 0.05	6.10 ± 0.06 (0.90 ± 0.10)	–	ND[c]	6.27 ± 0.22 (0.44 ± 0.06)	7.44 ± 0.14 5.37 ± 0.05	0.41 ± 0.03	3.94 ± 0.09 (1.18 ± 0.15)	–	ND[c]

Data are the mean ± S.D. of 3 independent experiments carried out in duplicates using 12–24 different concentrations of the ligand.

[a] Log of the concentration of the ligand causing inhibition of 50% of 0.23 nM [^{125}I]NDP-MSH binding.

[b] Log of the IC_{50} values of ligands obtained by fitting of displacement data of 0.23 nM [^{125}I]NDP-MSH binding to two-binding site model.

[c] ND—not detected: the one-site model was preferred over two-site model by *F*-test ($P > 0.05$).

supporting the notion that G-proteins do not cause directly the observed heterogeneity.

Fitting the data to equations assuming two binding sites revealed IC_{50} values of low and high affinity sites for the biphasic and shallow curves (results summarized in Table 1). For the γ^1-MSH, Lys-γ^1-MSH and Nle-γ^2-MSH binding to MC_1 receptors, and for the HS024 binding to MC_4 receptors, the IC_{50} values of the low and high affinity differed more than two orders of magnitude (Table 1). For the other cases the differences in affinities were smaller, or could not be delineated at all. In the latter cases the data was fitted only to a model assuming one site (Table 1).

For the cases when significant two-site fits were obtained the proportions of apparent high and low affinity sites differed substantially for the different peptide and receptor combinations (Table 1). For example, for the MC_1 receptors the fraction of high affinity sites ranged from 0.27 (Lys γ^1-MSH) to 0.60 (HS014), while for the MC_4 receptors it ranged from 0.41 (MS05) to 0.62 (HS131) (Table 1). However, the order of potencies of the evaluated peptides was essentially in agreement with previous reports on their selectivities for particular subtypes of the melanocortin receptors. Thus, all three γ-MSH peptides showed clear preference for the MC_3 over the MC_4 receptors, the antagonists HS014 (Schiöth et al., 1998), and HS131 (Wikberg, 2001) showed preference for the MC_4, and the peptide MS05 (Szardenings et al., 2000) had highest affinity for the MC_1 receptors.

3.5. Steady state competition binding using masks

The differences in the proportion of low- and high-affinity binding sites for different peptides led us to check if the fractions were statically pre-formed, or if they might form dynamically during the binding of the ligands. In the pre-formed case the high-affinity site could be blocked with appropriate concentration of a peptide giving strongly biphasic competition curves, while keeping the other site essentially free. During these conditions the binding of other peptides, having also heterogeneity, should give a uniphasic competition curve since only one site–the unmasked one–should remain for the binding.

NDP-MSH was selected as a mask for characterizing the binding of γ^1-MSH to the MC_1 receptors (Fig. 6A). NDP-MSH showed a clearly biphasic binding curve for the MC_1 receptors with $IC_{50}^H = 0.2$ nM and $IC_{50}^L = 9$ nM; the fraction of high affinity binding sites being 0.34 ± 0.03. From these data it could be estimated that 1.8 nM of NDP-MSH should block more than 99% of the high-affinity binding sites of the MC_1 receptors.

The competition curve of γ^1-MSH alone was also clearly biphasic (Fig. 6A open symbols) and was characterized with $IC_{50}^H = 5.5$ nM, $IC_{50}^L = 1.5$ μM and $\alpha_H = 0.28 \pm 0.03$. Addition of the NDP-MSH mask (1.8 nM) gave the expected reduction of [^{125}I]NDP-MSH binding (55%), but the shape of the competition curve of γ^1-MSH remained practically

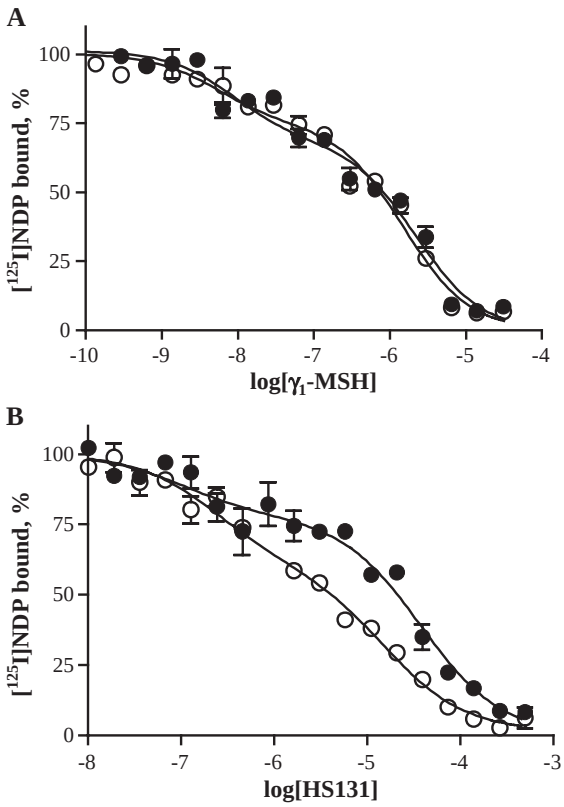

Fig. 6. Displacement curves of [^{125}I]NDP-MSH by γ^1-MSH (A) and HS131(B) to masked (○) and unmasked (●) MC_1 receptors (A) and MC_5 receptors (B). The high affinity binding of peptides were masked by the pre-treatment of membranes with 1.8 nM (MC_1) or 10 nM (MC_5) of NDP-MSH. Then different concentrations of competitive ligands were incubated with 0.23 nM [^{125}I]NDP-MSH for 3 h at 25 °C and the bound radioligand was determined as described in Materials and methods. The data of representative experiment are presented as the percentage of the specific binding of the radioligand in the absence of competitive peptide.

unchanged, being as biphasic as before (Fig. 6A filled symbols), and was characterized with $IC_{50}^H = 9$ nM, $IC_{50}^L = 2.2$ μM and $\alpha_H = 0.32 \pm 0.04$.

Similar experiments were carried out also to characterize the binding of the antagonist HS131 to the MC_5 receptors. The competition curve of HS131 alone was also clearly biphasic (Fig. 6B open symbols) and characterized with $IC_{50}^H = 230$ nM, $IC_{50}^L = 16$ μM and $\alpha_H = 0.40 \pm 0.04$. Similar experiments and considerations as above indicated that 10 nM NDP-MSH should to block more than 99% of the high affinity binding sites of the MC_5 receptors, but in the presence of this concentration of NDP-MSH the HS131 competition curves remained biphasic with binding parameters: $IC_{50}^H = 110$ nM, $IC_{50}^L = 38$ μM and $\alpha_H = 0.23 \pm 0.04$ (Fig. 6B filled symbols).

3.6. Steady state competition binding on melanoma cell membranes

In order to assess if the ligand binding heterogeneity is a general property of melanocortin receptors, NDP-MSH/

[^{125}I]NDP-MSH competition experiments were carried out also using membranes prepared from mouse B16 melanoma cells, which express MC_1 receptors naturally (Wikberg et al., 2000). The displacement curve of NDP-MSH in this system was also very shallow (Hill coefficient $n_H = 0.45 \pm 0.08$), and a model of two binding sites was preferred over a model with one binding site ($P < 0.0001$). The parameters from the two site model were $IC_{50}^{H} = 0.1$ nM, $IC_{50}^{L} = 14$ nM and $\alpha_H = 0.67 \pm 0.07$, which are in good agreement with the corresponding values for the recombinant human MC_1 receptors in membranes of Sf9 cells, although the fraction of high-affinity binding sites in melanoma cell membranes was higher.

4. Discussion

[^{125}I]NDP-MSH has been widely used for the characterization of melanocortin receptors (Schiöth et al., 1995, 1996b), but little attention has been given on its mechanisms of binding. The data of our present study show that binding of peptides to the different melanocortin receptors are governed by complex regulations, which cannot be described by simple reversible bimolecular reactions. The heterogeneity of binding was revealed already in a detailed analysis on the binding of [^{125}I]NDP-MSH, where the kinetic studies indicated that only a part of the specifically bound radioligand could be released from the MC_1, MC_3 and MC_4 receptors at a measurable rate, while another part remains firmly bound over long periods of time. Different MSH peptides also give grossly different shapes of their competition curves. Some peptides yielded shallow curves that resolved into apparent two-site fits, but other peptides yielded super-steep curves with Hill coefficients up to 1.6. Notably, for some peptides the shape depended on the melanocortin receptor subtype used, but also different peptides at one subtype showed different shapes. For example, HS131 gave a Hill-slope of 1.5 on the MC_1 receptor, while the slopes on the other melanocortin receptors ranged from 0.5 to 0.7. At the same time, the slopes of Lys-γ^1-MSH binding to MC_4 and MC_5 receptors were close to 1.5, while on the MC_3 and MC_1 receptors they were only 0.5–0.7 (Table 1). These observations clearly link the effects to the melanocortin receptor subtypes expressed in these cells, and exclude the possibility that the complex shapes of curves were due to experimental artefacts (e.g. dilution series error, adsorption to plastic walls or metabolic transformations of the ligands; see Wikberg et al., 1998). Moreover, the observation that only the dissociation kinetics of the MC_5 receptors were fast and lacked the slow component precludes the possibility that the observed heterogeneous steady-state shapes arose solely due to states of non-equilibrium. Similar complex competition curves we also found also using melanoma cell membranes that express MC_1 receptors constitutively, indicating that the effect is not due to some peculiar property of melanocortin receptors when they are confined in insect cell membranes.

Shallow and biphasic competition curves are usually connected with the presence of two or more independent binding sites for the ligand. However in the present case this can be ruled out, as the proportion of high- and low-affinity binding sites varied for different peptides. The fraction of high affinity sites ranged 0.21–0.60 for the MC_1, 0.33–0.45 for the MC_3, 0.41–0.62 for the MC_4 and 0.33–0.40 for the MC_5 receptors, depending on the ligand used. In these experiments the same batch of membranes was used for respective receptor, eliminating the possibility that the differences were caused by differences in membrane preparations.

Another distinct possibility is that the binding sites are dynamically formed and regulated. This possibility was strongly supported by the competition experiments using masks. Thus, when one competing ligand was applied in a concentration blocking the high affinity sites while leaving the low affinity sites open, other ligands still generated shallow competition curves that were resolved into two-site fits. This behaviour can be explained only by assuming that sites were dynamically formed during the ligand binding process.

The most straightforward explanation for our present findings is that the melanocortin receptors are subjected to homotropic allosteric regulation. By virtue of a positive co-operative interaction the binding of a ligand to one site may induce an effect on adjacent site(s) increasing their affinity for the ligand. This would lead to ligand binding curves with Hill-coefficients larger than unity. Through negative co-operative interactions the binding of a ligand to one site would reduce the affinity of the ligand for adjacent sites. This would lead to ligand binding curves with Hill-coefficients smaller than unity (Colquhoun, 1973). Very similar behaviour as in the present study (i.e. competition curves ranging super-steep to shallow and varied proportion of apparent high and low affinity sites depending on the competing ligand used) was seen for ligand binding to imidazoline I_2 receptors, which are (receptors) also subject to positive and negative co-operative regulations (Wikberg et al., 1992).

Co-operative regulation may be formalised into more or less complex models, such as the independent subunit models, Monod–Wyman–Changeux model and lattice model (Colquhoun, 1973). Unfortunately, as the shapes of binding isoterms show only marginal differences between such models, the present data are not sufficient to distinguish between these possibilities.

Here it should also be mentioned that a model giving theoretical basis for shallow competition curves is the ternary receptor model. It assumes that a conformational state of a binding unit is stabilised by another protein subunit. If a ligand binds to the binding unit and induces this conformational state the stabilising effect will induce a

distortion of binding isotherm so that Hill-coefficients become less than unity. The ternary receptor model has been used to explain the shallow competition curves by agonists competing with antagonist ligands for G-protein coupled receptors (de Lean et al., 1980; Wreggett and de Lean, 1984). However, G-protein-receptor interactions do not seem to be prominent in the present case as a stable GTP analogue did not affect the ligand binding. As the ternary model cannot also explain super-steep competition curves (de Lean et al., 1980), it is not sufficient to explain the present data.

One possibility to explain the heterogenous binding pattern of ligands is the formation of dimers/oligomers for the melanocortin receptors. There are already known many types of G-protein coupled receptors which form dimers/oligomers. Radioligand binding, radiation inactivation, fluorescence resonance energy transfer (FRET), bioluminiscence resonance energy transfer (BRET) and various other molecular biological and biochemical approaches have been used to provide evidence for the formation of conglomerates of G-protein coupled receptors (Rios et al., 2001). Although most of these methods are not able to delineate how many receptors are present in each aggregate the phenomenon is often referred to as dimerisation, but oligomer formation is still a distinct possibility. Ample evidence exist for the formation of homodimers of, e.g. bradykinin, agiotensin, opiod, muscarinic, dopamine D_1, D_2 and D_3, histaminergic H_2, and α_2- and β_2-adrenergic receptors (Rios et al., 2001). Formation of heterodimers have been suggested for β_2 and α_2-adrenoceptors, μ and δ opioid receptors, and several other G-protein coupled receptors (Rios et al., 2001), including melanocortin receptors (Dolby et al., 2004; Mandrika et al., 2005).

Reviewing the literature for radioligand binding data for G-protein coupled receptors similar to the present data reveals that the behaviour of the melanocortin receptors is quite unique. Maggi et al. (1980) showed that the β-adrenergic agonist isoproterenol causes an increase in α_2-adrenergic receptor binding in membranes from the rat brain, findings which have been taken as evidence for heteromeric interactions between β- and α_2-adrenoceptors (Rios et al., 2001). Vasoactive intestinal peptide (VIP) decreased the apparent affinity and increased the numbers of specific high affinity binding sites for [^{3}H]5-hydroxytryptamine (presumed serotonin receptors) in the dorsal hippocampus (Rostene et al., 1983), findings which may indicate the presence of heteromeric interactions between VIP and serotonin receptors (Rios et al., 2001). Similar cross regulation of affinities of different receptors has been used as evidence of formation receptor heteromers also for several other G-protein coupled receptors (Agnati et al., 2003).

However, the data of radioligand binding experiments accumulated from the above studies give quite weak evidence for heteromeric receptor interactions. G-protein interactions or other intracellular signalling events might have given rise to the observed receptor-cross-talk effects. We have actually found only one other example besides the present, where binding curves for a G-protein coupled receptor was modulated in a fashion distinctly indicating the existence of homotropic positive co-operative regulation. This was for [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding to muscarinic receptors in dog heart sarcolemmal membranes (Mattera et al., 1985). In the presence of the stable guanine nucleotide guanylylimidodiphosphate (Gpp(NH)p) and Mg^{2+} saturation binding of the muscarinic antagonist [^{3}H]QNB yielded a super-steep binding curve (Hill-coefficient 1.44), whereas in the absence of guanine nucleotide (and presence of Mg^{2+}) the Hill-coefficient was close to unity. However, the curves of the agonist ligands carbachol and oxotremorine obtained in competition with [^{3}H]QNB were shallow and right shifted by Gpp(NH)p. These data may indicate that the muscarinic receptor interact co-operatively concomitantly with its regulation by G-proteins (Mattera et al., 1985).

A very interesting observation of the present study is that the positive and negative co-operative regulations of the melanocortin receptors do not bear any relation to agonistic and antagonistic properties of ligands. For example, both NDP-MSH and α-MSH are agonists on the MC_5 receptors. However, whereas the former gave a Hill coefficient of 0.32 the latter gave a Hill coefficient of 1.27 on the MC_5 receptors (Table 1). Another example is HS131 (an MC_4 receptor antagonist; Lindblom et al., 2001), which gave a Hill-coefficient of 0.50 on the MC_4 receptors, while HS024 (also an MC_4 receptor antagonist; Kask et al., 1998) gave a value 0.7. Moreover, the MC_4 receptor agonistic peptide α-MSH gave here a Hill-coefficient of 0.50 (Table 1). These observations give a theoretical possibility of differential multiple signalling by different ligands via differential G-protein activation and melanocortin receptor homomeric (or even heteromeric) receptor cross-talks. Although it can yet only be speculated if such differential signalling are present or not for the melanocortin receptors, our present data have wide implications for the ongoing strives to develop subtypes selective agonists and antagonists for melanocortin receptor subtypes for treatment of various conditions such as obesity, anorexia and impotence.

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