Dynorphin Peptides: Antagonists of Melanocortin Receptors

J. Mark Quillan[1,2] and Wolfgang Sadée[1,2]

Received January 30, 1997; accepted March 21, 1997

Purpose. To identify possible targets that mediate the non-opioid effects of dynorphin A (DynA), effects that include inflammation and aggravation of traumatic nerve injury.

Method. We examined dynorphin peptides for functional interaction with the closely related melanocortin (MC) system.

Results. DynA-(1-13)NH_2 and other related opioid dynorphin peptides antagonize the human MC1, MC3 and MC4 receptors, and an amphibian MC receptor, with dissociation constants (K_d's) of 40 to 150 nM. The affinity of dynorphin's interaction with MC receptors is therefore greater than with other previously proposed non-opioid targets of dynorphin, which require micromolar concentrations. Dynorphin also antagonizes the adrenocorticotropic hormone (ACTH; MC2) receptor and an MC-like receptor endogenous to COS-7 cells, but with lower efficacy. In contrast DynA had no effect on seven control receptors and was only weakly effective at two others. Metabolites of dynorphin derived from cleavage of the amino terminal Tyr residue, such as DynA(2-17), lack opioid activity yet still produce a number of well established non-opioid effects. These des-Tyr derivatives also antagonized each of the five MC receptors examined.

Conclusions. DynA peptides were found to antagonize MC receptors *in vitro* with potencies that parallel those reported for pharmacological non-opioid effects of dynorphins *in vivo*. The combination of DynA and its active metabolites may reach levels sufficient to inhibit MC receptors physiologically. Dynorphin inhibition of MC receptors could prove to be an example of crosstalk between two distinct yet phylogenetically related neurotransmitter systems.

KEY WORDS: opioid peptides; [des-Tyr]dynorphin; non-opioid dynorphin effects; melanocortin receptors; receptor cross-reactivity; melanocortin peptides; α-melanocyte-stimulating hormone (α-MSH); adrenocorticotropic hormone (ACTH); tolerance; dependence; shock; trauma.

INTRODUCTION

Dynorphin peptides derived from prodynorphin constitute a distinct branch of the opioid peptide family tree and produce effects strikingly different from those of their opioid cousins, the enkephalins and endorphins (1–4). Many effects of dynorphin can be accounted for by activation of dynorphin's preferred receptor, the κ-opioid receptor, but a number of prominent effects cannot, such as aggravation of spinal cord injury and inflammation (1,5). These activities are "non-opioid" in nature; they are not blocked by opioid antagonists such as naloxone, and are mimicked by [des-Tyr]dynorphin metabolites that do not bind opioid receptors (6).

Non-opioid binding sites of intermediate affinity (K_d's 100 to 1000 nM) for dynorphin and [des-Tyr]dynorphin peptides have been demonstrated in a number of tissues (6,7), but the identity of these sites has yet to be determined. These sites differ substantially from dynorphin's high affinity interaction with opioid receptors (8), and from its "opioid-like" interaction ($K_d \approx 40$ nM) with the ORL_1 (nociceptin/orphanin FQ) receptor (9). Studies on the neuropathology of spinal cord injury has led to speculation that dynorphin may produce its non-opioid effects by direct interaction with N-methyl-D-aspartate (NMDA) receptors (6). However, because affinity of dynorphin for NMDA receptors is low (K_d's in the μM range), it has been suggested that this interaction may involve indirect mechanisms (6). More recently dynorphin has been shown to bind the neuropeptide Y (NPY) receptor (10), but as a possible site of dynorphin's non-opioid action the NPY receptor is a poor candidate; binding affinity is weak and functional antagonism has yet to be demonstrated.

In this report we examine melanocortin (MC) receptors as possible targets for dynorphin's non-opioid action. Since melanocortin and opioid peptides are involved in a number of similar physiological processes such as analgesia, memory, arousal, thermoregulation, and cardiovascular regulation (3,11–13), demonstration of a direct interaction between these two systems could be of great interest. Dynorphin peptides also share with melanopeptides the ability to modulate analgesia and opioid tolerance and dependence (11,14–18), and specific non-opioid effects of dynorphin—including hind-limb paralysis, neurotoxicity, aggravation of spinal cord injury, brain ischemia, and inflammation (1,5)—contrast markedly with the protective role that melanocortins such as α-MSH and ACTH play under similar conditions (19).

Opioid and melanocortin peptides not only produce contrasting physiological effects, they are related signaling systems. Neurons and receptors from both systems are often colocalized (13,20), and endorphins share with melanopeptides—α-MSH, β-MSH, γ-MSH, and ACTH—a common precursor, pro-opiomelanocortin (13). Although the idea of an "opio-melanocortin homeostatic regulatory system" has been proposed previously (4,21), direct interactions between the two systems have not been demonstrated conclusively. For example, reported interactions between melanopeptides and μ-opioid receptors, and between β-endorphin and MC receptors, are of low affinity and of doubtful physiological relevance (11,17). However, recent evidence of an interaction of nanomolar affinity between the μ-opioid receptor selective peptide CTAP with MC receptors (22) led us to examine the possibility that non-proopioimelanocortin opioid peptides may interact with MC receptors. We therefore tested dynorphin and other opioid peptides for interaction with several melanocortin receptor subtypes including the human MC1, MC3, and MC4 receptors, the human MC2 receptor (ACTH receptor), and a frog melanophore MC receptor. In this report we demonstrate that both dynorphin and non-opioid [des-Tyr]dynorphin peptides antagonize each of six MC receptors tested, and have potencies consistent with non-opioid actions of dynorphin reported elsewhere.

MATERIALS AND METHODS

Cell Cultures

Human embryonic kidney (HEK-293) cells were maintained in modified Eagle's medium (DMEM/H-16/F-12) with

[1] Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry, University of California Medical Center, San Francisco, California 94143-0446.

[2] To whom correspondence should be addressed.

0724-8741/97/0600-0713$12.50/0

10% fetal calf serum (FBS), 100 U/mL penicillin "G" and 100 μg/mL streptomycin (P/S; Gemini). The African green monkey kidney COS-7 cells were maintained in DME H-12 with 4.5g/L-glucose, 0.584 g/L glutamine, and 2.5 g/L $NaHCO_2$, 10% FBS, and P/S. Murine adrenocortical Y1 cells were maintained in Ham's F-10 medium (Gibco), 15% horse serum (Hyclone), 2.5% FBS, and P/S.

Melanophores have been used extensively to study the effects of melanocortins in vitro, and recently pure cultures of melanophores derived from *Xenopus laevis* embryos have been used by Michael Lerner and coworkers to study a variety of G-protein-coupled receptors (23–25). Cultured melanophore cells retain their ability to aggregate (lightened state) or disperse (darkened state) cytoplasmic pigment vesicles in response to hormonal stimulation; melatonin causes aggregation whereas α-MSH, VIP/PACAP, vasotocin, histamine, noradrenaline, and serotonin cause dispersion (22). *Xenopus* fibroblast and melanophore cell cultures were maintained in 5 parts Leibovitz's L-15 medium (Gibco), 2 parts FBS, 3 parts deionized-distilled H_2O, and P/S as described elsewhere (22,23).

Transformations and Stable Cell Lines

Transfections were performed using cDNA containing the human MC1 receptor (pcDNAI/NEO · hMC1), MC3 (pcCMV · hMC3), or MC4 (pcCMV · hMC4) receptors. Transient transfections of *Xenopus* fibroblast cells were performed by electroporation [$\approx 8 \times 10^6$ cells per 600 αL in 70% Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (0.14 g/L KH_2PO_4, 1.51 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.14 g/L KCl, 5.6 g/L NaCl, pH 7.2), plus 20 μg of test cDNA] in 0.4 cm cuvettes using a Gene Pulser transfection apparatus (450 V, 960 μF; BioRad, Hercules CA). After transfection, cells were transferred to culture medium and plated to confluency in 12-well tissue culture plates (Falcon), washed with fresh medium 3 hrs later, and allowed to incubate an additional 48 hr before use.

HEK-293 cells were stably transformed with the human MC1, MC3, and MC4 receptors by electroporating 20 μg of plasmid cDNA [$\approx 8 \times 10^6$ cells per 800 μL in 100% Ca^{2+}/Mg^{2+}-free phosphate-buffered saline in 0.4 cm cuvettes at 400V, and 960 μF]. After six days stable MC clonal cell lines were selected by addition of 400 μg/mL G-418 (GENETICIN; GIBCO/BRL) to the medium.

Cyclic-AMP and Concentration-Response Measurements

Concentration-response measurements were made in *Xenopus* fibroblasts, COS-7, HEK-293, and Y1 cells by quantifying changes in cAMP levels an [8-^{3}H]-cAMP kit from Amersham essentially as described elsewhere (22). Briefly, *Xenopus* fibroblasts transiently transfected with MC receptors, plated to confluency in 12-well tissue culture plates, were rinsed for 1 hr with 70% (vol/vol) L-15 medium containing 0.5% bovine serum albumin, and again for 5 min with added 0.5 mM 3-isobutyl-1-methlylxanthine (IBMX). After a 30 min exposure to test ligands, in presence of IBMX, cells were rinsed twice with ice-cold 70% phosphate-buffered saline (0.07 g/L $CaCl_2$, 0.07 g/L $MgCl_2 \cdot 6H_2O$, 0.14 g/L KH_2PO_4, 1.51 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.14 g/L KCl, 5.6 g/L NaCl, pH 7.4), and intracellular cAMP extracted with 1 mL of 60% (vol/vol) ethanol. Cellular debris was separated from extracted samples by centrifugation, 400 μL aliquots of the supernatant lyophilized, and total cAMP content quantified. cAMP measurements in COS-7, HEK-293 and Y1 cells were conducted in similar manner from 96-well plates after a 10 min exposure to test compounds (no IBMX was used), and the reaction was then stopped with 100% ethanol, bringing the final concentration to 60:4O (vol/vol) ethanol/medium.

Concentration-response curves were measured in melanophores by quantifying the change in absorbance at 630 nm through a monolayer of cells using a BT2000 Microplate Reader (FisherBiotech, Pittsburgh PA) after treatment with test compounds, and curves were fit using a logistic equation as described elsewhere (22,24,25). Briefly, initial absorbance (A_i) was measured upon addition of test compounds after 90 min pretreatment with carrier alone [7 parts L-15 medium in 3 parts distilled-deionized H_2O containing 0.5% (w/w) bovine serum albumin], or carrier plus 1 nM melatonin, to initiate tests from either the dispersed state (high absorbance) or an aggregated state (low absorbance), respectively. Test compounds were allowed to react with melanophores for 90 min, or as otherwise specified, and the absorbance measured again. This second absorbance reading (A_f) was used to calculate the percent of initial absorbance (%A_i) using the equation $\%A_i = 100(A_f/A_i)$. Test drugs included: endothelin-3, [Arg^8]-vasotocin, histamine, isoproterenol, PACAP-27, DynA-(1-6), Leu-enkephalin, somatostatin, Leu-enkephalin amide; α-MSH, and ACTH(1-24) from Sigma; neuropeptide Y from Peninsula Laboratories (Belmont, CA); opioid receptor-like (ORL_1) peptide and DynA-(6-17) from Phoenix Pharmaceuticals (Mountain View, CA); DynA fragments (1-13)-amide, (1-13), (1-11), (1-9), (1-8), (2-17), (2-13), (2-11), and (2-8), DADLE (Tyr-D-Ala-Gly-Phe-D-Leu), DAMGO (Tyr-D-Ala-Gly-Phe(N-Me)-Glyol), DPDPE (Tyr-cyclic[D-Pen-Gly-Phe-D-Pen]), DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr), ICI-174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu), Met-enkephalin amide, and β-endorphin from Multiple Peptide Systems (San Diego, CA).

RESULTS

Dynorphin, [des-Try]dynorphin, and other opioid peptides were tested for their ability to stimulate or inhibit activation of recombinant human MC1, MC3, and MC4 receptors stably expressed in HEK-293 cells, and transiently expressed in *Xenopus* fibroblast cells. MC receptors endogenous to *Xenopus* melanophores, COS-7, and Y1 adrenocortical cells were also tested. Dynorphin peptides had no direct stimulatory effect on any of the receptors tested, and so were reexamined for inhibitory effects when added in combination with melanocortin peptides.

Dynorphin Peptides Block α-MSH Activation of the Human MC1, MC3, and MC4 Receptors

Dynorphin peptides were found to inhibit α-MSH-mediated activation of three recombinant human MC receptors stably expressed in HEK cells. Dynorphin inhibition of α-MSH mediated stimulation of the MC1 receptor is shown in Fig. 1. The opioid congener DynA-(1-13)NH_2 (30 μM) produced a 300- to 1500-fold shift in concentration-response curves to α-MSH at each of the MC1, MC3, and MC4 receptor subtypes tested. The EC_{50} for α-MSH alone was 1.4 ± 0.6, 4.2 ± 2.1, and

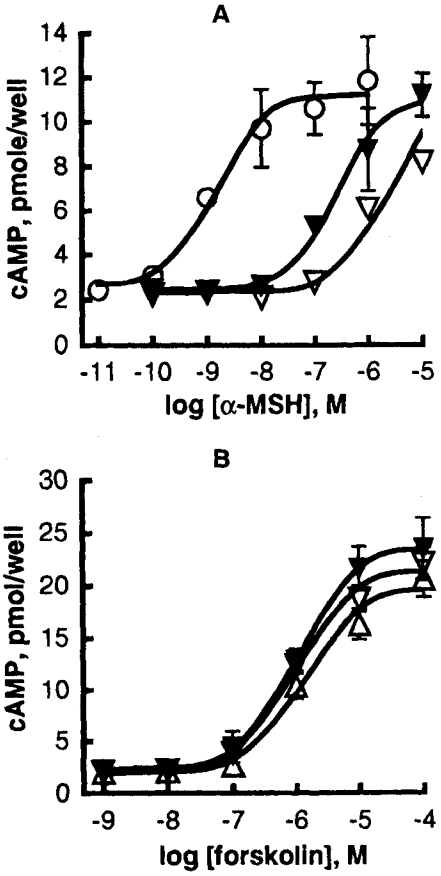

Fig. 1. Dynorphins were tested for their ability to block responses to (A) α-MSH and (B) forskolin in HEK cells stably expressing a human MC1 receptor. The EC_{50} of α-MSH alone (○), was shifted 1000- and 100-fold to the right in the presence of 30 μM DynA-(1-13)NH_2 (▽) and DynA-(2-13) (▼), respectively. The EC_{50} of forskolin (▽), however, was not significantly altered by the same concentrations of dynorphin. Each point represents the mean and SD of n = 4 for α-MSH and forskolin alone, and n = 3 in the presence of dynorphin.

3.0 ± 1.7 nM, for the MC1, MC3, and MC4 receptors respectively, and increased to 2,100 ± 900, 1,600 ± 1,100, and 2,000 ± 1,200 nM, respectively, in the presence of 30 μM DynA-(1-13)NH_2. Shifts in concentration-response curves to α-MSH were not blocked by naloxone (10 μM), indicating that opioid receptors are not involved. Dynorphin induced shifts in α-MSH response curves expressed as dose ratios were used to compare relative dynorphin effects at a number of MC and other receptor types (Fig. 2). Dose ratios for the MC1, MC3, and MC4, receptors ranged from approximately 500 to 1000.

Since the non-opioid effects of dynorphin include those produced by [des-Tyr]dynorphin metabolites, compounds which do not bind opioid receptors, we also tested DynA-(2-13) interactions with MC receptors. At each receptor subtype, DynA-(2-13) produced significant shifts in concentration-response curves that were 3 to 5 times smaller than DynA-(1-13)NH_2 (shown for the MC1 receptor in Fig. 1). Dose ratios calculated from α-MSH dose-response curves measured in the presence and absence of 30 μM DynA-(2-13) indicated shifts of 250 ± 31, 128 ± 44, and 147 ± 13 for the MC1, MC3, and MC4 receptors, respectively. Therefore DynA-(2-13) was an effective antagonist of α-MSH, but was of slightly lower potency than DynA-(1-13)NH_2. Wild-type (non-transformed) HEK cells, which lack endogenous MC receptors, were unresponsive to α-MSH at concentrations up to and beyond 10 μM, and in the stably transformed cells, neither DynA-(1-13)NH_2 nor DynA-(2-13) caused any change in cAMP levels when α-MSH was not present.

DynA-(1-13)NH_2 had no direct effect on the β-adrenergic receptor endogenous to HEK cells, nor did it block isoproterenol mediated receptor stimulation. The EC_{50} of isoproterenol alone (492 ± 103 nM; mean ± SD, n = 3), was not significantly different from isoproterenol in the presence of 30 μM of DynA-(2-13) (469 ± 283 nM) or 30 μM DynA-(1-13)NH_2, (584 ± 199 nM). Therefore, dynorphin does not impede receptor coupling to adenylyl cyclase. To test further the possibility of receptor-independent effects of dynorphin, DynA-(1-13)NH_2 and DynA-(2-13) were assayed for their ability to inhibit cAMP production stimulated directly with forskolin. Neither dynorphin analog was able to significantly change the EC_{50} of forskolin when used in place of α-MSH in wild-type or transformed HEK cells (Fig 1B). The EC_{50} of forskolin alone was 1.3 ± 0.5 μM, and was 1.0 ± 0.5 μM and 0.9 ± 0.3 μM in the presence of 30 μM DynA-(2-13) and 30 μM DynA-(1-13)NH_2, respectively. Thus, dynorphin appears to directly antagonize MC receptors.

Although shifts in α-MSH dose-response curves provide a *relative* measure of antagonist potency, estimates of the absolute potency were calculated from dose-response curves measured in the presence of several concentrations of antagonist. Schild regression analysis was used to estimate equilibrium dissociation K_d values for DynA-(1-13)NH_2 at the human MC1, MC2, and MC4 receptors and the MC receptor endogenous to melano-

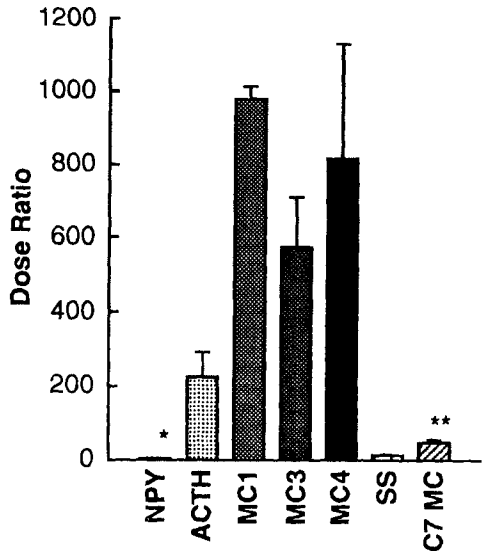

Fig. 2. Relative inhibitory potency of DynA-(1-13)NH_2 (30 μM) at the NPY receptor, various MC receptor subtypes including the adrenocortical Y1 MC2 (ACTH) receptor, and the somatostatin (SS) receptor. DynA-(1-13)NH_2 produced a marginal 4-fold shift in EC_{50} values at the NPY receptor, significantly less than at each of the other receptors tested (single asterisk; $P < 0.001$, t-Test), and produced a 15-fold shift in the EC_{50} to somatostatin, significantly less than at each MC receptor subtype tested except the COS-7 (C7) receptor (double asterisk; $P < 0.05$, t-Test). Each bar represents the mean and range of n = 3 for NPY and n = 2 for all others.

Table 1. Equilibrium K_d's Determined by Schild Regression Analysis

(A) K_d values for DynA-(1-13)NH_2 interaction with human MC receptor subtypes	
Receptor subtype	K_d (nM)
hMC1	87 ± 42
hMC3	43 ± 23
hMC4	115 ± 60
(B) K_d's for DynA-(1-13)NH_2 and DynA-(2-13) using *Xenopus* melanophore cells	
Substrate	K_d (nM)
DynA-(1-13)NH_2	71 ± 48
DynA-(2-13)	79 ± 12

phore cells (Table 1). For these K_d measurements, recombinant human MC1, MC3, and MC4 receptors were transiently expressed in *Xenopus* fibroblast cells, with results similar to those observed in stably transformed HEK-293 cells. Thus dynorphin's effects are independent of cell type. Fibroblast cells transfected with vector alone were unresponsive to α-MSH (24), but in cells transfected with the same vector containing MC receptor inserts, α-MSH concentration-response curves were clearly evident. These curves were shifted to the right upon addition of DynA-(1-13)NH_2. Calculated K_d's for DynA-(1-13)NH_2 ranged from 43 to 115 nM for the MC1, MC3, and MC4 receptors (Table 1).

Schild plots were also used to estimate K_d's for both DynA-(1-13)NH_2 and DynA-(2-13) at the MC receptor endogenous to melanophore cells. This analysis yielded K_d values in the same range, 70 to 80 nM, similar to those observed for human receptors (Table 1). An example Schild regression plot is presented in Fig. 3. The regression slope, which is close to unity, suggests a mechanism of competitive inhibition.

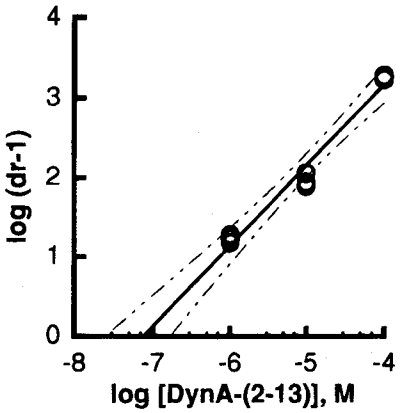

Fig. 3. Schild regression analysis was used to calculate K_d values for the inhibition of α-MSH by dynorphin peptides. In the example shown, the negative logarithm of the equilibrium K_d for DynA-(2-13) in *Xenopus* melanophores is 79 ± 12 nM, and the slope of the regression is 1.02 ± 0.02. Broken lines indicate the 99% confidence level. Each point (○) represents a dose ratio (dr) of EC_{50} values for α-MSH measured in the absence and presence of 1, 10, and 100 μM DynA-(2-13).

Dynorphin Antagonizes the ACTH and Cos-7 MC Receptors

DynA-(1-13)NH_2 and DynA-(2-13) were also tested for their ability to inhibit the ACTH (MC2) receptor endogenous to murine Y1 adrenocortical cells (26). At 30 μM, DynA-(2-13) and DynA-(1-13)NH_2 shifted the concentration-response curve of ACTH-(1-24) approximately 15 and 200-fold to the right, respectively (Fig. 4). The EC_{50} of ACTH-(1-24) alone was 1.8 ± 1.2 nM, and 28 ± 13 and 364 ± 112 nM in the presence 30 μM DynA-(2-13) and DynA-(1-13)NH_2, respectively. The DynA-(1-13)NH_2 induced shift in the concentration-response curve to ACTH-(1-24), expressed as a dose ratio, is included in Fig. 2. This result indicates that dynorphin's interaction with the MC2 receptor is significant but less potent than observed with the MC1, MC3, and MC4 receptors. The shift in the ACTH concentration-response curve suggests an estimated K_d for DynA-(1-13)NH_2 in the submicromolar range. α-MSH by itself, up to concentrations of 10 μM, was not effective at stimulating cAMP accumulation in Y1 cells, and DynA-(1-13)-NH_2 did not significantly affect basal, or inhibit forskolin (3 μM) stimulated, cAMP production.

In Cos-7 cells, cells that contain an atypical MC receptor (27), α-MSH elevated cAMP production, but did so with relatively low potency (EC_{50} ~80 nM). This EC_{50} value was shifted ~50-fold by 30 μM DynA-(1-13)NH_2 (Fig. 2), demonstrating that DynA-(1-13)NH_2 also antagonizes the MC receptor subtype found in Cos-7 cells, but with somewhat lower potency (K_d ~1 μM).

Dynorphin Fails to Antagonize Non-MC Receptors

The ability of DynA-(1-13)NH_2 to inhibit responses at a variety of different receptor types was tested for comparison to its activity at MC receptors. Examples of these experiments are shown in Fig. 5. The failure of DynA-(1-13)NH_2 to affect directly the β-adrenergic receptor endogenous to HEK cells and its inability to inhibit stimulation of cAMP production by

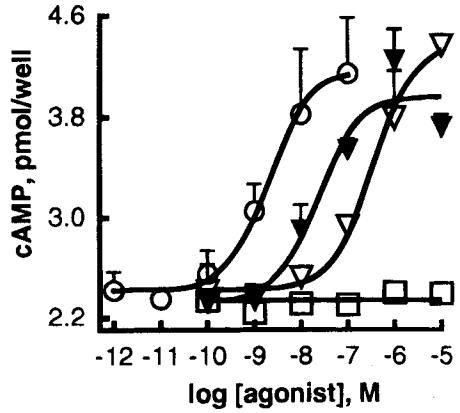

Fig. 4. Inhibition of an ACTH (MC2) receptor endogenous to Y1 adrenocortical cells by opioid and non-opioid analogs of dynorphin. The EC_{50} of ACTH-(1-24) in the presence of 30 μM DynA-(1-13)NH_2 (▽) and DynA-(2-13) (▼), was shifted 200- and 15-fold to the right as compared to ACTH-(1-24) alone (○). Neither α-MSH alone (□), nor DynA-(1-13)NH_2 in the presence or absence of 3 μM forskolin (data not shown) significantly altered cAMP levels. Each point represents the mean and SD of n = 3 measurements.

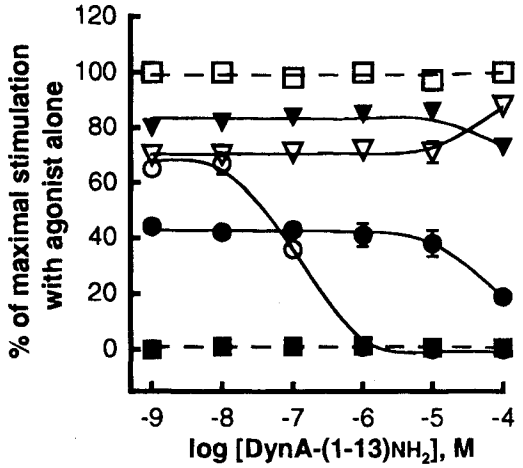

Fig. 5. Inhibition of other receptor types by DynA-(1-13)NH_2. Increasing concentrations of DynA-(1-13)NH_2 inhibited melanophore pigment response to 600 pM α-MSH (○; EC_{50} ~600 pM), but did not inhibit response to 1 nM [Arg^8]-vasotocin (●; EC_{50} ~1 nM), 100 nM endothelin-3 (▽; EC_{50} ~60 nM), or 100 nM histamine (▼; EC_{50} ~30 nM). Each point is expressed as % of response to supramaximal ligand concentration in the absence of dynorphin (solid lines) in the presence of 1 nM melatonin. Minimum values were defined as the response measured in the presence of 1 nM melatonin alone. DynA-(1-13)NH_2 produced no response in the absence of other drugs (□), and did not affect the reponse to 1 nM melatonin alone (■) (broken lines). Each point represents the mean and SD of n = 4 measurements.

isoproterenol was described above. DynA-(1-13)NH_2 also failed to inhibit responses evoked by near EC_{50} concentrations of [Arg^8]-vasotocin, histamine, endothelin-3, serotonin, and PACAP-(1-27) in melanophore cells (Fig. 5). Endogenous receptors for each of these ligands are present in melanophores, and all cause pigment dispersion when stimulated after a 90 min pretreatment with 1 nM melatonin. Included in Fig. 5 for comparison was the inhibition of α-MSH by DynA-(1-13)NH_2 shown as open circles. Fig. 5 also shows that DynA-(1-13)NH_2 had no effect if no other drugs were present (open squares), nor did it reverse responses evoked by stimulation of the melatonin receptor (solid squares). These results indicate that dynA selectively antagonizes α-MSH stimulation of the MC receptor in melanophore cells, but not agonist-induced stimulation of other receptors.

DynA-(1-13)NH_2 did appear to have some inhibitory effect at the NPY and somatostatin receptors as shown in Fig. 2. The NPY receptor endogenous to Y1 cells (26) appeared to be only marginally inhibited as DynA-(1-13)NH_2 (30 μM) produced less than a 5-fold shift in the concentration-response curve. The somatostatin receptor endogenous to HEK-293 cells, on the other hand, was significantly more susceptible to block by the same concentration of DynA-(1-13)NH_2. The resulting 15-fold shift in the concentration-response to somatostatin was still considerably less, however, than that for any of the MC receptors tested (Fig. 2).

Relative Potencies of Dynorphin Fragments Are Compared

To assess which structural elements of dynorphin are required for antagonism of the MC receptor, the *relative* potency of various DynA fragments were compared by taking IC_{50} measurements against a fixed concentration of α-MSH. Melanophores were used since dynorphins were of comparable potency at both frog and human MC receptors. DynA-(1-13)NH_2 was found to be the most potent of a series of DynA-(1-x) analogs (where x is the number of the carboxy terminal amino acid) tested for their ability to inhibit pigment dispersion in melanophore cells (Fig. 6A and Table 2). [Opioid peptides DynA-(1-17) and (1-32) were not tested.] DynA-(1-13)NH_2 had an IC_{50} of 110 nM against 600 pM α-MSH compared to IC_{50} values of 250 to 600 nM for the non-amidated DynA-(1-13) and DynA-(1-11). Further shortening of the carboxy terminal end caused an additional reduction in antagonist potency, with DynA-(1-9) being 40-fold less potent than DynA-(1-13)NH_2. Removal of the Arg from position 9 to yield DynA-(1-8) caused an

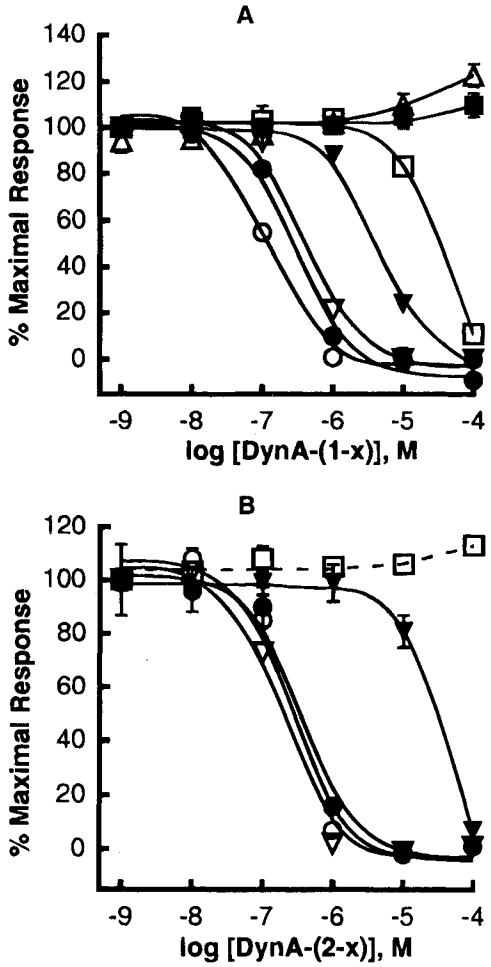

Fig. 6. Inhibition of α-MSH by dynA peptides in melanophore cells (see Tab 2). (A) DynA fragments-(1-x), where x represents the carboxy-terminal amino acid, were tested for their ability to antagonize 600 pM α-MSH. Fragments 1-13-NH_2 (○), 1-13 (●), 1-11 (▽), 1-9 (▼), and 1-8 (□) each blocked responses to α-MSH, whereas fragments 1-6 (●) and 1-5 (△; Leu-enkephalin) were ineffective. (B) Non-opioid [des-Tyr]DynA fragments-(2-x) (solid lines), also inhibited responses to 600 pM α-MSH; fragments 2-17 (○), 2-13 (●), 2–11 (▽), and 2–8 (▼) were each effective, but dynA-(6-17) (□, broken line) was not. Values expressed as % of maximal response to 600 pM α-MSH alone. Each point represents the mean and SD of n = 4 measurements taken in the presence of 1 nM melatonin.

Table 2. Relative Inhibitory Potencies of DynA Peptides as Measured in Melanophores[a]

	IC_{50} (nM)
(A) DynA fragment	
(1-13)-NH_2	110 ± 20
(1-13)	305 ± 78
(1-11)	421 ± 157
(1-9)	4,700 ± 600
(1-8)	>10,000
(1-6)	>10,000
(1-5) {Leu-Enkephalin}	>10,000
(B) [des-Try]DynA fragment	
(2-17)	259 ± 121
(2-13)	362 ± 152
(2-11)	188 ± 73
(2-8)	>10,000
(C) Control peptides[b]	
DynA-(6-17)	>10,000
Dynorphin B	2,100 ± 1,000
ORL_1	~10,000
β-Endorphin	~10,000
Leu-Enkephalin-NH_2	>10,000
Met-Enkephalin	>10,000

[a] Measurements were taken in the presence of 600 pM α-MSH and 1 nM melatonin.
[b] Other compounds with IC_{50} values > 10 μM include: PLO17; DTLET; ICI-174,864; naloxone; morphine; dermorphin; DAMGO; DADLE; DPDPE; and DSLET.

additional 10-fold decrease in potency. DynA-(1-6) and DynA-(1-5), which corresponds to Leu-enkephalin, were devoid of antagonist activity. Since melanophores lack endogenous opioid receptors, pigment translocation was not directly affected by any of opioid drugs tested. This is in contrast to the clearly visible responses seen after introduction of a recombinant opioid receptor to melanophores (22). Inhibition of α-MSH by DynA-(1-13)NH_2 was also unaffected by naloxone at concentrations up to 10 μM (data not shown), another indication that the effects of dynorphin are non-opioid in nature and are produced by direct action at the MC receptor.

Analogs of [des-Tyr]dynA were also tested for their ability to inhibit the melanophore MC receptor (Fig. 6B). DynA-(2-17), DynA-(2-13), and DynA-(2-11) each blocked the MC receptor with IC_{50} values in the range of 190 to 360 nM against 600 pM α-MSH. As seen with opioid analogs of dynorphin, shortening the C-terminus of the non-opioid des-Tyr analog to position-8 decreased its inhibitory potency approximately 100-fold (Fig 6B). And, like their opioid relatives, [des-Tyr]DynA also caused no response when applied to melanophores in the absence of α-MSH (data not shown).

A number of other opioid peptides, with the exception of dynorphin B, failed to significantly inhibit α-MSH (Table 2). Neither Leu- nor Met-enkephalin displayed antagonist activity, whereas ORL_1 nociceptin/orphanin-FQ peptide and β-endorphin had IC_{50} values of similar to 10 μM. Dynorphin B had an IC_{50} of 2 μM. Other opioid peptide and non-peptide drugs tested, DTLET, PLO17, DAMGO, DADLE, DPDPE, DSLET, naloxone, morphine, dermorphin, and ICI-174,864 and others, did not affect response to α-MSH in melanophores at concentrations in excess of 10 μM.

DISCUSSION

The present study demonstrates that dynorphin peptides can directly antagonize melanocortin receptors. Specifically, dynorphins block the agonist effects of α-MSH at the human MC1, MC3, and MC4 receptors, and the MC receptor in *Xenopus* melanophores, with K_d's of the more potent DynA analogs ranging form 40 to 200 nM. Schild analyses of α-MSH dose-response curves in the presence of increasing concentrations of dynorphin peptides were consistent with a mechanism of competitive antagonism. Dynorphin peptides also blocked the MC2 receptor (selective for ACTH) and, less potently, a recently identified MC-like receptor in COS-7 cells (27). They were considerably less effective, however, at blocking the somatostatin receptor and the NPY receptor, a recently proposed target for dynorphin (10). Dynorphin failed to antagonize seven other receptors. These results indicate that dynorphin's action at the MC receptor is relatively selective and considerably more potent than its reported interaction with NMDA and NPY receptors (10,28). The affinity of dynorphin for MC receptors is consistent with non-opioid binding sites that have been reported for ^{3}H-dynorphin in several tissues including rat heart (7), sites that could represent MC receptor subtypes.

One characteristic of dynorphin that distinguishes its opioid and non-opioid effects *in vivo* is that removal of the amino-terminal Tyr residue, yielding [des-Tyr]dynorphin peptides, abolishes opioid activity but fails to alter significantly their non-opioid effects. Accordingly, we see here that *in vitro* [des-Tyr]DynA peptides are nearly as potent as the corresponding full length dynorphin analogs in antagonizing MC receptors. If some of the non-opioid effects of dynorphin observed *in vivo* are indeed due to the action of dynorphin on MC receptors, the slight differences in potency between [des-Tyr]dynorphin observed here may simply reflect differences in metabolic stability. While we cannot exclude the possibility of some protease activity in the tissue cultures used in this report, rapid degradation is unlikely and therefore should not affect conclusions derived from these *in vitro* experiments.

To establish more detailed structure-activity relationships, we tested twelve dynorphin and [des-Tyr]dynorphin analogs, and a number of other opioid peptides, for antagonist activity using frog melanophores. Successive removal of residues from the C-terminus of dynorphin failed to affect antagonist activity strongly until residue 11, regardless of whether the amino-terminal Tyr was present. DynA-(1-8) and DynA-(2-8) still retain weak activity, whereas DynA-(6-17) was inactive. Therefore, residues between positions 2-11 appear crucial to dynorphin's activity at the MC receptor. It remains to be seen, however, whether these results will also hold for human MC receptors.

These structure-activity data may aid in future evaluation of dynorphin/MC receptor interactions *in vivo,* and may in combination with other information be used to assess possible sites of action. For example, Dumont and Lemaire (7) demonstrated the presence of non-opioid binding sites with a K_d of 385 nM for DynA-(1-13) in cardiac tissue, tissue were dynorphin and melanocortins are both known to have effects. While

DynA-(2-13) and DynA-(1-13) had approximately equal affinity, non-opioid binding affinity for DynA-(1-8) was considerably lower, similar to the relative potencies reported here for dynorphin peptides and MC receptors. Therefore, cardiac tissue might be used to examine possible interactions between opioid peptides and MC receptors.

Other systems may also warrant attention. The modulatory effects of dynorphins on pain perception, opioid analgesia, tolerance and dependence share striking similarities to effects that melanocortins produce *in vivo*. Both melanocortin and dynorphin, for example, can modulate analgesia and can affect tolerance and dependence (1,15,29,30). Further experiments may help clarify whether antagonism of MC receptors by dynorphin peptides can account for their non-opioid effects *in vivo*. The present study provides structure-activity data that can help address this question specifically. This study also demonstrates that significant cross-reactions can occur between different neuropeptide systems and their respective receptors. Such interactions may provide an added dimension of regulatory control within the neuroendocrine system.

ACKNOWLEDGMENTS

This work was supported by grants NIDA-DAO4166 and NIH-GM43102 from the National Institutes of Health. We thank Dr. Roger Cone of the Vollum Institute, Portland OR, and Dr. Ira Gantz of the University of Michigan, for plasmids containing human MC1, MC3 and MC4 receptors, and Drs. Katharine Winans and Neil Burford for valuable comments on this manuscript.

REFERENCES

1. L. Stanfa, and A. Dickenson. *Inflamm. Res.* **44**:231–41 (1995).
2. J. M. Walker, H. C. Moises, D. H. Coy, G. Baldrighi, and H. Akil. *Science* **218**:1136–8 (1982).
3. H. Akil, S. J. Watson, E. Young, M. E. Lewis, H. Khachaturian, and J. M. Walker. *Ann. Rev. Neurosci.* **7**:223–55 (1984).
4. R. Poggioli, A. V. Vergoni, and A. Bertolini. *Peptides* **7**:843–8 (1986).
5. A. I. Faden. *J. Neurosci.* **12**:425–9 (1992).
6. V. K. Shukla, and S. Lemaire. *Trends Pharmacol. Sci.* **15**:420–4 (1994).
7. M. Dumont, and S. Lemaire *J. Mol. Cell Cardiol.* **25**:983–91 (1993).
8. C. Chavkin, I. F. James, and A. Goldstein. *Science* **215**:413–5 (1982).
9. S. Zhang, and L. Yu. *J. Biol. Chem.* **270**:22772–6 (1995).
10. M. Miura, A. Inui, K. Sano, N. Ueno, A. Teranishi, Y. Hirosue, M. Nakajima, M. Okita, J. Togami, K. Koshiya, S. Baba, and M. Kasuga. *Am. J. Physiol.* **267**:E702–9 (1994).
11. W. H. Gispen, J. Buitelaar, V. M. Wiegant, L. Terenius, and D. De Wied. *Eur. J. Pharmacol.* **39**:393–7 (1976).
12. M. E. Hadley. *The Melanotropic Peptides,* Vols 1–3, CRC Press Inc., Boca Raton, Florida, 1988.
13. A. N. Eberle. *The Melanotropins: Chemistry, Physiology and Mechanism of Action,* Karger, Basel, Switzerland, 1988.
14. J. M. Walker, H. C. Moises, D. H. Coy, E. A. Young, S. J. Watson, and H. Akil. *Life Sci.* **31**:1821–4 (1982).
15. A. E. Takemori, H. H. Loh, and N. M. Lee. *J. Pharmacol. Exp. Ther.* **266**:121–4 (1993).
16. L. P. Hooke, L. He, and N. M. Lee. *J. Pharmacol. Exp. Ther.* **273**:292–7 (1995).
17. P. C. Contreras and A. E. Takemori. *J. Pharmacol. Exp. Ther.* **229**:21–6 (1984).
18. J. I. Szekely, E. Miglecz, Z. Dunai-Kovacs, I. Tarnawa, A. Z. Ronai, L. Graf, and S. Bajusz. *Life Sci.* **24**:1931–8 (1979).
19. A. Bertolini, S. Guarini, E. Rompianesi, and W. Ferrari. *Eur. J. Pharmacol.* **130**:19–26 (1986).
20. J. D. Alvaro, J. B. Tatro, J. M. Quillan, M. Fogliano, M. Eisenhard, M. R. Lerner, E. J. Nestler, and R. S. Duman. *Mol. Pharm.* **50**:583–91 (1996).
21. A. Bertolini, S. Guarini, W. Ferrari, and E. Rompianesi. *Life Sci.* **39**:1271–80 (1986).
22. J. M. Quillan and W. Sadée. *Pharm. Res.* **13**:1622–28 (1996).
23. A. Daniolos, A. B. Lerner, and M. R. Lerner. *Pigment Cell Res.* **3**:38–43 (1990).
24. J. M. Quillan, C. K. Jayawickreme, and M. R. Lerner. *Proc. Natl. Acad. Sci. (USA)* **92**:2894–98 (1995).
25. J. M. Quillan. *Development of a Combinatorial Diffusion Assay and Discovery of MSH Receptor Antagonists,* UMI, Ann Arbour, Michigan, 1995.
26. G. Weng, F. Yee, P. Michl, D. Reis, and C. Wahlestedt. *Mol. Pharmacol.* **48**:9–14 (1995).
27. W. E. Polgar, C. M. Bitler, and L. Toll. *Life Sci.* **58**:1223–9 (1996).
28. L. Chen, Y. Gu, and L. Y. Huang. *J. Neurosci.* **15**:4602–11 (1995).
29. J. M. Walker, G. G. Berntson, C. A. Sandman, A. J. Kastin, and H. Akil. *Eur. J. Pharmacol.* **69**:71–9 (1981).
30. P. C. Datta, C. A. Sandman, and F. K. Hoehler. *Peptides* **3**:433–7 (1982).