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Dynorphin peptides differentially regulate the human κ opioid receptor

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Abstract

Dynorphins, endogenous peptides for the κ opioid receptor, play important roles in many physiological and pathological functions. Here, we examined how prolonged treatment with three major prodynorphin peptides, dynorphin A (1-17) (Dyn A), dynorphin B (1-13) (Dyn B) and α -neoendorphin (α -Neo), regulated the human kappa opioid receptor (hKOR) stably expressed in Chinese hamster ovary (CHO) cells. Results from receptor binding and [35 S]GTP γ S binding assays showed that these peptides were potent full agonists of the hKOR with comparable receptor reserve and intrinsic efficacy to stimulate G proteins. A 4-h incubation with α -Neo at a concentration of $\sim 600\times$ EC₅₀ value (from [35 S]GTP γ S binding) resulted in receptor down-regulation to a much lower extent than the incubation with Dyn A and Dyn B at comparable concentrations ($\sim 10\%$ vs. $\sim 65\%$). Extending incubation period and increasing concentrations did not significantly affect the difference. The plateau level of α -Neo-mediated receptor internalization (30 min) was significantly less than those of Dyn A and Dyn B. Omission of serum from the incubation medium or addition of peptidase inhibitors into the serum-containing medium enhanced α -Neo-, but not Dyn A- or Dyn B-, mediated receptor down-regulation and internalization; however, the degrees of α -Neo-induced adaptations were still significantly less than those of Dyn A and Dyn B. Thus, these endogenous peptides differentially regulate KOR after activating the receptor with similar receptor occupancy and intrinsic efficacy. Both stability in the presence of serum and intrinsic capacity to promote receptor adaptation play roles in the observed discrepancy among the dynorphin peptides.

Keywords

Endogenous opioid peptides; Ligand-dependent; GPCR; Down-regulation; Internalization

Introduction

The κ opioid receptor (KOR) is one of the three major types (μ , δ and κ) of opioid receptors that mediate physiological and pharmacological effects of opioids in vivo, mainly through pertussis toxin-sensitive heterotrimeric G_{i/o} proteins (Liu-Chen, 2004). The cDNA clones of KORs have been isolated and characterized from several species including the human, mouse, rat, guinea pig, zebra fish, frog and *C. elegans* (Waldhoer et al., 2004).

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Several kappa-preferring endogenous peptides were identified in the late 1970s and early 1980s, and their common precursor, prodynorphin (PDYN), was cloned in 1982 (Akil et al., 1998). Seven peptides are generated from PDYN (Figure 1) by sequential enzymatic cleavage mainly by proprotein convertase 2 at C-terminal region at single or paired basic residues followed by carboxypeptidase (Day et al., 1998). All of the peptides show higher affinity for the KOR over the MOR and DOR (Mansour et al., 1995). Therefore, they are recognized as endogenous ligands for the KOR and collectively known as dynorphins.

Immunohistochemistry and in situ hybridization studies have congruously demonstrated that distribution of the PDYN gene products is quite widespread in the mammalian nervous system and very similar to that of KOR (Mansour et al., 1988; Mansour et al., 1996; Merchenthaler et al., 1997). α -Neo, Dyn A and Dyn B have similar distribution in the central nervous system. Quantitatively, α -Neo and Dyn B are the major peptides in the hippocampus, striatum and cortex, whereas Dyn A is the most abundant one in the spinal cord (Chavkin et al., 1983; Healy and Meador-Woodruff, 1994; Ramsdell and Meador-Woodruff, 1993; Day and Akil, 1989). Dynorphin peptides have distinct properties in terms of relative binding affinity and selectivity (Mansour et al., 1995; Merg et al., 2006), relative tissue abundance (Chavkin et al., 1983; Ramsdell and Meador-Woodruff, 1993; Evans et al., 1985) and enzymatic sensitivity (Silberring et al., 1992; Silberring and Nyberg, 1989). In addition, it is well documented that these endogenous opioid peptides play different roles in mediating many physiological, pharmacological and pathological effects including antinociception (Kuzmin et al., 2006), morphine withdrawal and tolerance (Takemori et al., 1993), anxiolytic-like activity (Kuzmin et al., 2006) and cytotoxicity (Tan-No et al., 2001).

Non-peptide agonist-induced adaptative events of the KOR have been extensively investigated. Following activation by an agonist, the KOR is phosphorylated by G protein-coupled receptor kinases and then non-visual arrestin is recruited which reduces coupling between the receptor and G protein, causing receptor desensitization. Through clathrin- and dynamin-dependent pathway, phosphorylated KOR is endocytosed (internalization) followed by either dephosphorylation and EBP50/NHERF-1-involved recycling (resensitization) or down-regulation via both lysosome and proteasome systems (Liu-Chen, 2004). We have shown previously that non-peptide κ full agonists have different efficacies in regulating the hKOR. While U50,488H causes robust phosphorylation, internalization and down-regulation, etorphine does not (Chen et al., 2006b; Li et al., 2000; Li et al., 1999; Li et al., 2003). In the present study, using CHO cells stably expressing the hKOR as the model system, we found that three major dynorphins, Dyn A, Dyn B and α -Neo, induced differential regulation of the hKOR after receptor activation. We then investigated the contribution of their stability in serum and intrinsic efficacy to the observed discrepancy.

Materials and Methods

Materials

Dyn A, Dyn B and α -Neo were purchased from Phoenix Pharmaceuticals (Belmont, CA). U50,488H [2-(3,4-dichlorophenyl)-N-methyl-N-[(2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide] was provided by the National Institute on Drug Abuse (Bethesda, MD). [35 S] GTP γ S (~1250 Ci/mmol) and [3 H]diprenorphine (~56 Ci/mmol) were purchased from PerkinElmer (Boston, MA). Bestatin, captopril, phosphoramidon, M $_1$ mouse anti-FLAG monoclonal antibody and rabbit anti-FLAG polyclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO). Cell media and fetal bovine serum (FBS) were acquired from Invitrogen (Carlsbad, CA). The following materials were bought from the indicated companies: GF/B filters from Brandel (Gaithersburg, MD), Alexa Fluo 488-conjugated goat anti-mouse IgG from Molecular Probes (Eugene, OR), 2-guanidinoethylmercaptosuccinic Acid (GEMSA) from EMD Biosciences (San Diego, CA), HRP-linked goat anti-rabbit IgG from New England

Biolabs (Beverly, MA), Prism 3.0 program from GraphPad Software Inc. (San Diego, CA), FACScan from BD Biosciences (San Jose, CA) and Fuji LAS-1000 Plus Gel Documentation System from Fuji Film (Tokyo, Japan).

Cell Lines

Clonal CHO cell line stably expressing the Flag-tagged human kappa opioid receptor (FLAG-hKOR) was generated previously (Li et al., 2002). The $B_{\max}$ value of FLAG-hKOR was determined to be 1.89 pmol/mg protein (Zhang et al., 2002). Cells were cultured in 10-cm Petri dishes in DMEM/F-12 medium supplemented with 10% FBS, 0.2 mg/ml geneticin in a humidified atmosphere consisting of 5% CO₂ and 95% air at 37°C.

Competitive binding assay

CHO cell membranes were prepared and frozen as described by Zhu et al. (1997). Competitive binding experiments were carried out as described by Wang et al. (2005). Briefly after thawing of membranes, competition inhibition by U50,488H, Dyn A, Dyn B and α -Neo of [³H] diprenorphine (0.4 nM) binding to the KOR was performed with ~10 μ g membrane proteins in the absence or presence of various concentrations (10^{-12} ~ 10^{-6} M) of each drug. Binding was carried out in TE buffer (50 mM Tris-HCl and 1 mM EGTA, pH 7.4) or in [³⁵S]GTP γ S binding buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂ and 1 mM EDTA, pH 7.4, with 10 μ M GDP added immediately before use) containing 80 pM cold GTP γ S. Naloxone (10 μ M) was used to define nonspecific binding. Bound and free [³H]diprenorphine were separated by filtration under reduced pressure with GF/B filters pre-soaked with blocking buffer A (50 mM Tris, pH 7.4, 0.1 mg/ml bovine serum albumin, and 0.2% polyethyleneimine). Radioactivity on filters was determined using PerkinElmer liquid scintillation counter. Binding data were analyzed and K_i values of each drug were determined with the Prism 3.0 program.

[³⁵S]GTP γ S binding assay

[³⁵S]GTP γ S binding to G α proteins was determined using a modified procedure that was previously reported by Zhu et al. (1997). Briefly, cell membranes were thawed and diluted with the [³⁵S]GTP γ S binding buffer mentioned above. Cell membranes (10 μ g) were incubated in the binding solution containing 80 pM [³⁵S]GTP γ S, 10 μ M GDP and U50,488H, Dyn A, Dyn B or α -Neo at different concentration (10^{-12} ~ 10^{-6} M) for 60 min at 30°C. Non-specific and basal levels of GTP γ S binding were defined by using 10 μ M cold GTP γ S and binding buffer to replace the agonist, respectively. Bound and free [³⁵S]GTP γ S were separated by filtration and radioactivity on the filters was determined. Data were fitted to the sigmoidal dose-response equation and EC₅₀ and maximal response ($E_{\max}$) values were calculated with the Prism program.

Calculation of intrinsic efficacy

Intrinsic efficacy (e) values of the peptides were calculated according to the equation that was firstly described by Ehlert (1985) and subsequently modified by Selley et al. (1998), using the K_i , EC₅₀ and $E_{\max}$ values determined in [³⁵S]GTP γ S binding buffer as described above.

$$e = \frac{E_{\max}^{\text{peptide}}}{E_{\max}^{\text{U50}}} \times \left(\frac{K_i}{EC_{50}} + 1 \right) \times \frac{1}{2}$$

SDS-PAGE, Western blotting and ligand-induced regulation of FLAG-hKOR

Cells were treated with the peptide ligands or vehicle in the complete medium (DMEM/F-12/10% FBS) at 37°C for 4 h. When Opti-MEM I reduced-serum medium with or without

10% FBS was employed in indicated experiments, complete medium was replaced 1 h prior to the ligand treatment. For studies assessing the effects of peptidases on peptide stability, a peptidase inhibitor cocktail containing 10 μ M bestatin (for aminopeptidase), 10 μ M captopril (for angiotensin-converting enzyme), 10 μ M GEMSA (for carboxypeptidase) and 1 μ M phosphoramidon (for endopeptidase 24.11) was added into the medium 1 h prior to the ligand treatment. These peptidases have been reported to be involved in the degradation of dynorphins (Jordan et al., 2000; Muller and Hochhaus, 1995). Cells were solubilized in 2 $\times$ Laemmli sample buffer (4% SDS, 100 mM DTT, 10% glycerol, 62.5 mM pH6.8 Tris and 0.1% Bromophenol Blue) and 2 $\times$ 10⁵ cells per lane were subjected to Tricine-SDS-PAGE on 8% separating gel. After protein transfer, the Immobilon-P PVDF membranes were incubated with the blocking solution B [5% nonfat dry milk in TBS-T buffer (25 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20, pH 7.6)] for at least 1 h and then overnight with rabbit polyclonal anti-FLAG antibody (1:5000) at 4°C. The membranes were washed three times with the TBS-T buffer and incubated at room temperature for 2 h with HRP-linked goat anti-rabbit IgG (1:1000). After washing, the protein bands were revealed using SuperSignal West Pico Chemiluminescent substrate and then digitalized with Fuji LAS-1000 Plus Gel Documentation System. The densitometric analyses of FLAG-hKOR bands were performed by using ImageGauge 4.1 software (Fuji Film). Percent change of mature FLAG-hKOR = 100% $\times$ (optical density in ligand-treated group - optical density in vehicle-treated group) / (optical density in vehicle-treated group).

Fluorescence flow cytometry and peptide-induced internalization of cell surface FLAG-hKOR

A fluorescence flow cytometry assay was conducted according to a modified protocol that was previously described by Li et al. (2003). Cells were incubated with the peptide ligands or vehicle in the complete medium at 37°C for 30 min. When Opti-MEM I reduced-serum medium with or without 10% FBS was employed in indicated experiments, complete medium was replaced 1 h prior to the treatment. Use of peptidase inhibitor cocktail followed the same protocol as described above. After harvest, 1.5 $\times$ 10⁶ cells were used. Because Ca²⁺ is necessary for M₁ anti-FLAG antibody binding with the antigen, all solutions used in the subsequent steps contain 1 mM CaCl₂. Cells were incubated with M₁ antibody (1:2000) in 1 ml of Opti-MEM I for 1 h. After three washes with PBS buffer, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:2000) in 1 ml of Opti-MEM I for 1 h. Finally, cells were resuspended in 1 ml PBS buffer. Surface FLAG-hKOR immunofluorescence of 1 $\times$ 10⁴ cells was quantitated using FACScan and mean fluorescence intensity of single cell was calculated. The mean fluorescence intensity of cells stained only with the second antibody was also measured and subtracted from the mean intensity. Ligand-induced %internalization of cell surface FLAG-hKOR = 100% $\times$ (fluorescence intensity in vehicle group - fluorescence intensity in ligand group) / (fluorescence intensity in vehicle group).

Data analysis

When data were acquired from at least three independent experiments, they were presented as mean $\pm$ S.E.. One-way analysis of variance followed by Tukey's *post hoc* test was used for determining between-group differences among multiple sets of data. The difference was defined to be significant if the *p* value was less than 0.05. All statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Results

Dyn A, Dyn B and α -Neo had similar receptor occupation and intrinsic efficacy to stimulate GTP γ S binding

Compared to the selective KOR full agonist U50,488H, all three peptides inhibited [³H] diprenorphine binding with higher affinity (Table 1). Moreover, three peptide ligands

functioned as full agonists in stimulating [35 S]GTP γ S binding (Table 1). Based on the EC₅₀ values, Dyn A, Dyn B and α -Neo were more potent than U50,488H. In addition, receptor binding conducted in [35 S]GTP γ S buffer showed decreased binding affinity of the ligands by 4-15 fold compared to in TE buffer.

The K_i /EC₅₀ ratios (Table 1) of these agonists (> 1.0), which are indexes of receptor reserve (Ehlert, 1985), indicate that in CHO-FLAG-hKOR cells, these agonists occupy only a fraction of the receptor to induce maximal stimulation of [35 S]GTP γ S binding. Among the tested ligands, U50,488H required lower receptor occupancy to generate its E_{max} than the three peptides, which occupied similar levels of the receptor for inducing their maximal effects. Correspondingly, U50,488H had the highest intrinsic efficacy (e), and that Dyn A, Dyn B and α -Neo appeared to have comparable intrinsic efficacy to stimulate G proteins.

Dyn A, Dyn B and α -Neo differentially regulated FLAG-hKOR

By SDS-PAGE followed by immunoblotting with anti-FLAG antibody, we reported previously that FLAG-hKOR was resolved as a broad and diffuse 55 kDa band and a less diffuse 45 kDa band, indicating that the receptor is glycosylated. Endoglycosidase H treatment cleaved the 45 kDa band to about 38 kDa, but did not change the 55 kDa band. These results demonstrate that the 55-kDa form is fully glycosylated with complex-type polysaccharides locating in trans-Golgi and plasma membranes and the 45 kDa band is *N*-glycosylated with high-mannose type polysaccharides, residing in the endoplasmic reticulum (ER) / Golgi apparatus and is a precursor of the mature 55-kDa form (Chen et al., 2006a).

For G protein-coupled receptors (GPCRs), cell surface form is the one responding to agonists leading to signal transduction. We have shown previously by ligand binding that ~80% of the receptor is located on cell surface (Xu et al., 2005), thus most of the 55-kDa band is likely located on cell surface. To evaluate whether ligands differentially regulated the FLAG-hKOR, we focused on the 55-kDa band using immunoblotting assay. As it is very difficult to remove these peptides from membranes by washing and the residual peptides will affect binding, we decided not to employ radioligand binding to quantitate ligand-induced change of the receptor. Three peptide ligands were examined for their abilities to change level of the receptor after 4-h treatment (Fig. 2). We previously demonstrated that EC₅₀ values of U50,488H and dynorphin A(1-17) in promoting internalization were about 20-fold those for stimulating [35 S]GTP γ S binding (Li et al., 2003). The concentration of each peptide used was ~600-fold of its EC₅₀ in stimulating [35 S]GTP γ S binding, therefore ensuring that maximal effects would be achieved. Unexpectedly, both Dyn A (0.2 μ M) and Dyn B (0.5 μ M) promoted receptor down-regulation to significantly higher extents than α -Neo (0.7 μ M) (~65% vs. ~10%).

Time- and concentration-dependence of peptide-mediated receptor down-regulation

The observed differences may be due to variations among the peptides in the time course or concentration–effect relationship. As shown in Fig. 3A, both Dyn A (0.2 μ M) and Dyn B (0.5 μ M) reached the respective plateaus (~65%) following 4-h treatment, but α -Neo (0.7 μ M) did (~10%) as early as 2 h after treatment. In addition, although these effects reached plateau rapidly (2 or 4 h), the peptides down-regulated FLAG-hKOR even at 16 h after incubation without adding fresh peptides.

When cells were incubated for 4 h, all the peptides promoted decreases of mature FLAG-hKOR in a concentration-dependent manner (Fig. 3B), attaining maximal effects at 0.2 μ M, 0.5 μ M and 7 μ M for Dyn A, Dyn B and α -Neo, respectively, which are approximately 800, 600 and 6,000-fold their respective EC₅₀ values in stimulating [35 S]GTP γ S binding. However, the maximum down-regulation (~25%) induced by α -Neo was substantially lower than those (~65%) by Dyn A and Dyn B.

Difference in peptides-mediated receptor internalization

We previously have reported that internalization is required for agonist-mediated down-regulation of hKOR and that receptor adaptation following activation is ligand-dependent (Li et al., 2000; Li et al., 2003). Accordingly, we tested whether these peptides promoted receptor endocytosis to different extents. Concentration-response curves were generated for each peptide (Fig. 4). Dyn A, Dyn B and α -Neo caused maximal receptor internalization at concentration of 0.2 μ M, 0.5 μ M and 7 μ M, respectively. Furthermore, there were significant differences between the maximal level ($\sim$ 40%) of α -Neo-mediated receptor internalization and those ($\sim$ 55%) of Dyn A and Dyn B. Therefore, the lower level of α -Neo-induced KOR internalization contributes to its smaller degree of down-regulation.

Two possibilities may account for lower degrees of KOR regulation induced by α -Neo. α -Neo may have a lower intrinsic activity than other two peptides to cause receptor internalization and down-regulation although these three peptides show the similar level of intrinsic efficacy to stimulate GTP γ S binding. In addition, α -Neo may be more susceptible than Dyn A and Dyn B to many endogenous proteases / peptidases present in serum in the incubation medium and / or associated with membranes (Egleton and Davis, 2005).

Effects of serum and peptidase inhibitors on the peptide-mediated receptor down-regulation and internalization

We tested the possibility that peptidases / proteases in serum and associated with cell plasma membrane may impact on α -Neo-promoted KOR regulation. For the following experiments, 20 μ M, 50 μ M and 70 μ M for Dyn A, Dyn B and α -Neo were used, respectively. These concentrations induced the plateau levels of internalization and down-regulation. As shown in Fig. 5, substitution of cell growth medium, DMEM-F12 HAM plus 10% FBS, with Opti-MEM I reduced-serum medium (which contains no serum) plus 10% FBS did not have any significant influence on the levels of KOR down-regulation (compared to Fig. 3) and internalization (compared to Fig. 4). On the other hand, omission of 10% FBS from or addition of the peptidase inhibitor cocktail into Opti-MEM I / 10% FBS medium significantly ($P < 0.05$) enhance the extent of α -Neo-, but not Dyn A- and Dyn B-, induced KOR internalization and down-regulation (Fig. 5B and 5C). The cocktail contains inhibitors of endopeptidase 24.11, aminopeptidase, carboxypeptidase and angiotensin-converting enzyme, which are important for metabolism of dynorphins (Jordan et al., 2000; Muller and Hochhaus, 1995). These observations indicate that degradation of α -Neo occurs as fast as 30 min after incubation in medium containing 10% FBS and that peptidases in the FBS significantly affect the stability of α -Neo, but not Dyn A and Dyn B. In addition, the results also suggest that the observed discrepancies among these ligands are not due to differential binding affinities of the peptides to serum albumin. Compared to cells incubated with Opti-MEM I only, however, addition of the peptidase inhibitor cocktail did not enhance α -Neo-induced receptor down-regulation and internalization further (Fig. 5). Therefore, cell membrane-associated aforementioned peptidases did not influence the stability of these peptides significantly. There were some differences in the levels of down-regulation and internalization in Opti-MEM I / 10% FBS medium between the two figures in Fig. 5B and in Fig. 5C, which were attributed to different passages of cells used in the studies. However, the finding that α -Neo caused lower extents of internalization and down-regulation was consistent.

Discussion

Agonist-dependent receptor adaptation following activation

It is somewhat surprising that the three major dynorphin peptides have differential abilities to regulate the hKOR. All the three peptides caused internalization and down-regulation in a dose-dependent manner in a medium containing 10% FBS, but the maximal effects of α -Neo were

substantially lower than those of Dyn A or Dyn B. Exclusion of serum from culture medium or addition of peptidase inhibitors into the medium containing serum increased the maximal levels of α -Neo-, but not Dyn A- or Dyn B-, induced internalization and down-regulation. However, the maximal effects of α -Neo were still lower than those of Dyn A or Dyn B. In addition, the peptides have similar intrinsic efficacies in activating G proteins. Therefore, degradation of α -Neo was a contributing factor for causing a lower level of regulation. These results suggest that these peptides may change the receptor conformations to different states, which lead to activation of G proteins, but only a subset of active conformational states results in internalization and down-regulation. Thus, KOR adaptation following G protein activation by these peptides is apparently agonist-specific.

Agonist-specific receptor adaptation has been reported for κ , μ and δ opioid receptors previously. While U50,488H and Dyn A promoted robust internalization and down-regulation of the hKOR, etorphine and levorphanol did not, although all four were full agonists (Li et al., 1999; Li et al., 2003). Both in vitro and in vivo studies demonstrated that etorphine evoked robust endocytosis of the MOR, but morphine did not, despite that the two agonists inhibited adenylyl cyclase activity to comparable extents (Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996). Etorphine and peptide agonists promoted internalization of the DOR, while levorphanol did not (Bot et al., 1997). In addition, internalized receptor may be sorted differently depending on the agonist used. Marie et al. (2003) reported that compared to DPDPE and deltorphin I, etorphine resulted in less lysosome-mediated degradation, but more recycling of the human DOR. However, it is not known whether there were differences in receptor occupancy and/or intrinsic efficacy of the tested agonists in these studies, which may contribute to the observed discrepancy. Regulation of the rat KOR expressed in CHO cells by dynorphin peptides was reported previously. Jordan et al. (2000) demonstrated that incubation with 0.1 μ M Dyn A, Dyn B or α -Neo for 24 h resulted in 42%, 23% and 23% down-regulation of the rat KOR in serum-containing medium. However, in serum-free medium, Dyn A caused less down-regulation (19%) and the effects of α -Neo and Dyn B were abolished. This latter finding is different from our observation that α -Neo induced a higher level of down-regulation in both peptidase inhibitor-containing and serum-free medium. The reasons for the discrepancy are not clear, but may be attributed to differences in species (human vs. rat KOR), concentration (600 $\times$ EC₅₀ vs. 0.1 μ M) and incubation time (4-16 h vs. 24 h).

We have previously demonstrated that non-peptide agonists down-regulated cell surface FLAG-hKOR to less extents than peptide agonists (Chen et al., 2006b). In addition to causing agonist-induced receptor degradation, non-peptide agonists function intracellularly as pharmacological chaperones to enhance cell surface receptor expression. We found that α -Neo, similar to Dyn A and Dyn B, did not function as a pharmacological chaperone (data not shown).

We attempted to examine whether there were differences in sorting of internalized hKOR by different peptides. Monensin, which blocks acidification of intracellular vesicles, has been used to block recycling (Pippig et al., 1995). However, we found that treatment with monensin greatly increased the 45-kDa form and decreased the 55-kDa mature form of the receptor (data not shown), indicating that it also blocks the trafficking along the biosynthesis pathway. Thus, we were not able to draw a conclusion about whether there is differential recycling.

Stability of peptides in the presence of serum

Proteolytic enzymes in human serum are generally composed of endopeptidase, aminopeptidase, carboxypeptidase and angiotensin-converting enzyme (Checler, 1991; Van, V et al., 1999), which result in rapid clearance of peptides from circulation (Egleton and Davis, 2005). All of those enzymatic activities have been found in FBS (Glavas-Obrovac et al., 2005; Kozlowski et al., 1992).

Degradation of Dyn A in human blood or plasma has been studied. Chou et al. (1996) found that, in human blood, Dyn A had a half-life of 180 min. Endopeptidase, aminopeptidase and carboxypeptidase have been found to play important roles in its degradation (Yu et al., 1996; Chou et al., 1996). In addition, Dyn B and α -Neo are processed to Leu-Enk-Arg⁶ in human plasma (Silberring et al., 1998). Consequently, it is highly likely that three peptides can be metabolized in the medium containing 10% FBS. However, metabolism of these peptides in the Opti-MEM I reduced-serum medium, which is serum-free, is negligible since insulin and transferrin are the only proteins in the medium (Manufacturer's manual).

Our present results suggest that FBS indeed contains peptidases and that α -Neo is more sensitive to the enzymes and is degraded more rapidly than the other two peptides in the presence of FBS. This may be due to their different sizes because extra C-terminal amino acid present in Dyn A had been demonstrated to be protective (Chou et al., 1996).

Long-lasting effects of the peptides on the FLAG-hKOR

Our results (Fig. 3A) show that these endogenous ligands have prolonged influences (at least 16 h) on the level of the mature receptor. Young et al. (1987) found that co-existence of Dyn A (1-17) with metabolic products such as Dyn A (2-17) and Dyn A (7-17) decreased degradation of the parent peptide via inhibiting proteolytic enzymes. Therefore, it is possible that peptide fragments slow further metabolism of the parent peptides, and thus prolong their actions. In addition, direct down-regulating effects by the degradation products on the hKOR could not be ruled out because many dynorphin fragments such as the C-terminal extension peptides of Leu-Enk are demonstrated to be KOR agonists (Merg et al., 2006; Mansour et al., 1995).

Conclusion

We have demonstrated that among the three major endogenous peptides derived from prodynorphin, α -Neo induced lower levels of internalization and down-regulation of the hKOR than Dyn A and Dyn B. All the three peptides are potent full agonists with similar receptor occupancy and intrinsic efficacy for activating G protein. Thus, the observed differential regulation is attributed to the lower stability of α -Neo than Dyn A and Dyn B in the serum-containing culture medium and to the difference in their intrinsic activity for promoting these changes.

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Figure 1. Generation and amino acid sequences of naturally occurring dynorphins

(A). Schematic representation of human preprodynorphin shows that endogenous dynorphins are generated from the precursor through differential cleavages by proprotein convertases at paired and single basic amino acids followed by carboxypeptidases. (B). Amino acid sequences of prodynorphin gene products display that all peptides contain Leu⁵-Enk at the N-termini, but have different C-terminal extensions.

Figure 2. Dyn A, Dyn B and α -Neo differentially down-regulated the mature form of FLAG-hKOR (A). Immunoblotting of the mature FLAG-hKOR. Cells were incubated with each peptide at 37°C for 4 h at a concentration $\sim$ 1000-fold of its EC₅₀ in stimulating [³⁵S]GTP γ S binding. Subsequently 2×10^5 cells were solubilized and subjected to SDS-PAGE. FLAG-hKOR was detected by immunoblotting with polyclonal anti-FLAG antibody. (B). Quantitation and comparison of peptide-induced down-regulation of mature receptor. Each value represents mean $\pm$ S.E. of four independent experiments performed with similar results. *** $P < 0.001$ compared to Dyn A- or Dyn B-treated cell group using one-way ANOVA followed by Tukey's *post hoc* test.

Figure 3. Time- and concentration-dependence of peptide-mediated down-regulation of mature FLAG-hKOR

Cells were treated with (A) Dyn A (0.2 μM), Dyn B (0.5 μM) or α-Neo (0.7 μM) for indicated time periods or (B) different concentrations of the peptides for 4 h. FLAG-hKOR was detected by immunoblotting and quantitated (mean ± S.E., n=3) by densitometry.

*** $P < 0.001$ compared to Dyn A- or Dyn B-treated (16 h) cell group;

** $P < 0.01$ compared to Dyn A (20 μM)- and Dyn B (50 μM)-treated cell group using one-way ANOVA followed by Tukey's *post hoc* test.

Figure 4. Concentration-dependence of peptide-mediated internalization of surface FLAG-hKOR
Cells were treated with different concentrations of Dyn A, Dyn B and α -Neo for 30 min. Surface receptors were labeled by monoclonal M₁ anti-FLAG antibody and then Alexa Fluo 488-conjugated goat anti-mouse IgG antibody. Immunofluorescence intensity was determined using fluorescence activated cell sorter (FACS). Each value represents mean $\pm$ S.E. of three independent experiments.

* $P < 0.05$ compared to Dyn A (20 μ M)- and Dyn B (50 μ M)-treated cell group using one-way ANOVA followed by Tukey's *post hoc* test.

Figure 5. Effects of serum and peptidase inhibitors on the peptide-promoted down-regulation and internalization of FLAG-hKOR

(A). Immunoblotting of mature FLAG-hKOR. One hour prior to ligand treatment, complete medium was removed and Opti-MEM I reduced-serum medium with or without 10% FBS and/or peptidase inhibitor cocktail was added as indicated. Ligand treatment and immunoblotting assay were conducted as described in Fig. 2A. (B). Quantitation and comparison of peptide-induced down-regulation of mature receptor. (C). Quantitation and comparison of peptide-induced receptor internalization. Replacement of incubation medium and addition of 10% FBS and/or peptidase inhibitor cocktail followed the same protocol as described in (A), and the

internalization assay for surface FLAG-hKOR was conducted as described in Fig. 4. Each value represents mean $\pm$ S.E. of three independent experiments performed with similar results.

* $P < 0.05$ compared to corresponding Dyn A- or Dyn B-treated cell group;

$P < 0.05$ compared to α -Neo-treated cell group in the presence of 10% FBS by one-way ANOVA followed by Tukey's *post hoc* test.

Binding and functional parameters of Dyn A, Dyn B, α -Neo and U50,488H at FLAG-hKOR stably expressed in CHO cells
 K_i values were obtained from competitive inhibition assay using [3 H]diprenorphine as the radiolabelled ligand in the presence of either TE binding buffer or GTP- γ S binding buffer containing 80 pM cold GTP- γ S. EC₅₀ and E_{max} values were calculated from [35 S]GTP- γ S binding stimulated by the agonists. Each value represents mean $\pm$ S.E. of four independent experiments.

Table 1

Ligand	K_i (nM) (TE buffer)	K_i (nM) (GTP- γ S buffer)	EC ₅₀ (nM)	E _{max} *	K_i /EC ₅₀ #	Intrinsic Efficacy (e) #
U50,488H	1.32 $\pm$ 0.12	6.16 $\pm$ 0.6	2.02 $\pm$ 0.37	100	3.05	2.02
Dyn A	0.05 $\pm$ 0.02	0.39 $\pm$ 0.04	0.26 $\pm$ 0.04	90.6 $\pm$ 6.0	1.50	1.13
Dyn B	0.11 $\pm$ 0.03	1.51 $\pm$ 0.15	0.85 $\pm$ 0.10	91.7 $\pm$ 7.9	1.78	1.27
α -Neo	0.16 $\pm$ 0.06	2.23 $\pm$ 0.33	1.17 $\pm$ 0.21	86.5 $\pm$ 8.5	1.91	1.26

* Normalized to the E_{max} of U50,488H-induced stimulation on 35 S-GTP- γ S binding.
K_i value from competition binding assay using GTP- γ S buffer was employed to determine K_i /EC₅₀ and intrinsic efficacy.