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# Neurite Outgrowth-stimulating Activities of β-Casomorphins in Neuro-2a Mouse Neuroblastoma Cells

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**Endogenous opioid peptides and opiate drugs are known to affect the development of the nervous system. β-Casomorphins (β-CMs) belong to a family of exogenous opioid peptides derived from the milk protein β-casein by proteolytic fragmentation. We investigated the effects of various fragments and analogues of β-CM on neurite outgrowth in Neuro-2a mouse neuroblastoma cells. The fragments β-CM-5 to -9 and β-CM-5 amide stimulated neurite outgrowth. Fragments shorter than β-CM-5 (β-CM-3, -4, and β-CM-4 amide) and longer than β-CM-9 (β-CM-13 and -21) had no effects. The activity of β-CMs to promote neurite outgrowth does not correlate with their opioid activity in guinea-pig ileum. The effect of the most potent fragment, β-CM-5, was prevented by the μ-opioid receptor-selective antagonist D-Phe-Cys$^2$-Tyr$^3$-D-Trp-Orn$^5$-Thr$^6$-Pen$^7$-Thr$^8$-NH$_2$ (CTOP), or by pretreatment with pertussis toxin. These results suggest that the stimulatory effects of β-CMs on neurite outgrowth were mediated through G protein-coupled μ-opioid receptors.**

**Key words:** β-casomorphin; neuroblastoma; neurite outgrowth; μ-opioid receptor; G-protein

β-casomorphins (β-CMs) are opioid peptides, which are released from the milk protein, β-casein by proteolytic fragmentation. β-CM-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) was the first β-CM isolated, from peptone of bovine casein.[1)] [Pro$^8$]-β-CM-8 and β-CM-4 amide (morphiceptin) were also isolated from an enzymatic digest of casein.[2)] They all contain the common N-terminal amino acid sequence Tyr-Pro-Phe-Pro, corresponding to the peptidic sequence 60–63 of bovine β-casein and have preferential μ-opioid receptor agonist activity.[3)] Afterward, the amino acid sequences of β-CM were found in analogous positions of β-casein in many species of mammals.[4)] β-CMs have various effects as neurotransmitters and neuromodulators on the central nervous, endocrine, cardiovascular, and gastrointestinal systems.[5)] Evidence for the liberation of β-CMs from casein into the intestinal lumen of mammals after the intake of milk or a casein-containing diet under *in vivo* conditions, has already been obtained.[6,7)] Recently, Jinsmaa and Yoshikawa[8)] reported the conditions for the release of β-CM-7 from bovine β-casein by gastrointestinal proteases *in vitro*. In their study, long β-CMs (β-CM-9, -13, and -21) were also released. As to the fragments longer than β-CM-7, there are two types ([His$^8$]-β-CMs and [Pro$^8$]-β-CMs) derived from different genetic variants of β-casein containing His or Pro at the 67th position.

Endogenous opioid peptides and opiate drugs are known to affect the development of the nervous system, including neuroblast replication, survival, and differentiation of neuronal cells.[9,10)] While the inhibitory activity of opioids against the growth and differentiation of neurons is well recognized, the stimulatory effects are less well established. Recently we reported the stimulatory effects of β-CM-5 on neurite outgrowth in Neuro-2a mouse neuroblastoma cells.[11)] Because milk is the only source of nutrition for newborn mammals, β-CMs may influence neural development during the suckling period. Further, β-CMs may provide medical or health benefits, including the prevention and treatment of neurodegenerative disease and neuronal injury.

In this study, to build on our previous report we tested the effects of various β-CM fragments and some analogues on neurite outgrowth in Neuro-2a cells, and the participation of the opioid receptor in the stimulatory effects of β-CMs.

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*Abbreviations*: β-CM, β-Casomorphin; CTOP, D-Phe-Cys$^2$-Tyr$^3$-D-Trp-Orn$^5$-Thr$^6$-Pen$^7$-Thr$^8$-NH$_2$; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PTX, pertussis toxin; GPI, Guinea-pig ileum; nor-BNI, nor-binaltorphimine

## Materials and Methods

*Materials.* β-CM-5 and -7 were obtained from Peptide Institute, Inc. (Osaka, Japan) and β-CM-3, -4, -6, morphiceptin, [D-Ala$^2$]-β-CM-5, [D-Pro$^2$]-β-CM-5, and β-CM-5 amide from Bachem (Bubendorf, Switzerland). Des-Tyr-β-CM-7, naloxone, and naltrindole were from Sigma (St. Louis, MO, USA). D-Phe-Cys$^2$-Tyr$^3$-D-Trp-Orn$^5$-Thr$^6$-Pen$^7$-Thr$^8$-$NH_2$ (CTOP) and nor-binaltorphimine (nor-BNI) were from Tocris Cookson Inc. (Ballwin, MO, USA). Pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA, USA). Other β-CM fragments were synthesized using SAM-2 (Biosearch, San Rafael, CA, USA) and PS3 (Rainin, Woburn, MA, USA) peptide synthesizers by the Fmoc method. Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, kanamycin, fetal bovine serum (FBS) and N2 supplement were from Gibco Life Sciences (Grand Island, NY, USA). The cAMP RIA kit was from Yamasa Shouyu (Chiba, Japan). 3-isobutyl-methyl-xanthine (IBMX) and forskolin were from Wako Pure Chemical Industries (Osaka, Japan). Plastic tissue culture dishes and plates were from Iwaki (Chiba, Japan).

*Cell culture.* The Neuro-2a cell line (IFO 50081) was obtained from the Institute for Fermentation, Osaka (Osaka, Japan). Cells were grown on plastic dishes in DMEM supplemented with 10% FBS and kanamycin (50 μg/ml) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

*Neurite outgrowth assay.* The neurite outgrowth assay was done as described previously.[11] Briefly, Neuro-2a cells in exponential growth phase were plated in 24-well plates at a density of $1 \times 10^4$ cells per ml in each well, and grown for 24 h in DMEM with 1% FBS and kanamycin (50 μg/ml). After removal of the medium, 1 ml of serum-free chemically defined N2 medium[12] (50/50 mixture of Ham's F-12 and DMEM containing N2 supplement) with or without test compound(s) was added. After culturing for 3 h, each culture was fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS). In this experiment, to avoid the effects of serum, which may contain endogenous opioids, we used serum-free medium. Neurite formation in this cell line is induced, reportedly, on serum removal.[13] Under our experimental conditions, neuritogenesis in Neuro-2a cells was evident after serum removal for 1 h, and then the percentage of cells bearing neurites increased gradually. The neurite outgrowth assay was done after switching from serum-containing to serum-free medium with or without test compound(s) for 3 h. The outgrowth of neurites from cells was monitored under a phase-contrast microscope. Cells bearing neurites of at least one cell body in length were scored as positive. At least 200 cells were scored in each well to find the percentage of cells with neurites. Cultures were coded and counted without knowledge of their treatment. Data are given as the mean ± SEM values from two or three separate experiments with quadruplicate measurements. Statistical comparisons were made using one-way ANOVA followed by Scheffe's test or Student's *t*-test. The significance level was set at $P < 0.05$.

*Guinea-pig ileum (GPI) assay.* The opioid activity of various β-CM fragments and analogues was measured by the GPI assay as described previously.[8] $IC_{50}$ means the concentration of peptide inhibiting maximum electrically-induced contraction by 50%.

*Measurement of cAMP accumulation.* Neuro-2a cells in an exponential growth phase were plated in 24-well plates at a density of $4 \times 10^5$ cells per ml in each well, and grown in DMEM with 1% FBS and kanamycin (50 μg/ml) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. After 24 h, the medium was removed from the individual wells, and replaced with 0.5 ml of DMEM supplemented with 0.5 mM 3-isobutyl-methyl-xanthine (IBMX) at 37°C. After 10 min, the reaction was started with the addition of 0.5 ml of DMEM containing β-CM-5 with or without 20 μM forskolin. After another 30 min, the medium was removed and the reaction was stopped by the addition of 1 ml of cold 6% trichloroacetic acid (TCA). The cells were disrupted by repeated freezing and thawing three times, and each cell extract was centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatant was freed from TCA by ether extractions, and the pellet saved for protein assay. The amount of cAMP recovered was then assayed by RIA according to the instructions of the manufacturer. The initial protein pellet from the TCA-treatment was dissolved in 1 N NaOH and a portion of the solution was assayed for protein.

## Results

*Effects of β-CM fragments on neurite outgrowth*

We tested the effects of various β-CM fragments on neurite outgrowth. In addition to naturally occurring β-CMs (see Introduction), several short fragments were also examined. [Val$^0$, Pro$^8$]-β-CM-9 was present in the digest of β-casein treated with gastrointestinal proteases.[14] Table 1 summarizes the effects of β-CM fragments on neurite outgrowth. β-CM-5, β-CM-6, and [Pro$^8$]-β-CM-9 produced significant increases in the percentage of cells with neurites at concentrations over $10^{-9}$ M. β-CM-7 also had a significant effect at concentrations over $10^{-8}$ M. However, β-CM-5 was the most potent and efficacious with a maximum effect (52.1 ± 1.5%,

**Table 1.** Effects of β-CM Fragments on Neurite Outgrowth in Neuro-2a Cells

| Fragment | Name | % Cells with neurites (mean ± SEM) | | | | |
|---|---|---|---|---|---|---|
| | | Peptide (log M) | | | | |
| | | -9 | -8 | -7 | -6 | -5 |
| Control | | 38.8 ± 1.2 | | | | |
| YPF | β-CM-3 | 42.3 ± 2.7 | 42.9 ± 2.1 | 42.1 ± 2.6 | 42.8 ± 2.6 | 42.6 ± 2.8 |
| YPFP | β-CM-4 | 43.2 ± 2.8 | 43.9 ± 1.9 | 45.1 ± 2.3 | 44.6 ± 2.5 | 42.9 ± 2.8 |
| YPFPG | β-CM-5 | 48.5 ± 2.0** | 50.3 ± 2.0** | 50.5 ± 1.4** | 52.1 ± 1.5** | 51.2 ± 1.6** |
| YPFPGP | β-CM-6 | 47.4 ± 1.9* | 48.6 ± 2.5* | 48.1 ± 2.1* | 49.1 ± 2.3** | 49.0 ± 2.3** |
| YPFPGPI | β-CM-7 | 47.3 ± 3.1 | 48.7 ± 2.3* | 48.0 ± 1.8* | 47.0 ± 1.9* | 47.0 ± 2.1* |
| PFPGPI | des-Tyr-β-CM-7 | 40.6 ± 3.3 | 39.5 ± 3.8 | 44.8 ± 2.8 | 42.0 ± 3.4 | 39.5 ± 3.3 |
| YPFPGPIH | [$His^8$]-β-CM-8 | 43.8 ± 1.6 | 45.0 ± 1.8 | 46.2 ± 2.4* | 45.1 ± 1.5 | 44.9 ± 2.2 |
| YPFPGPIHN | [$His^8$]-β-CM-9 | 43.5 ± 2.2 | 42.2 ± 1.0 | 43.7 ± 1.8 | 44.0 ± 1.6 | 45.2 ± 1.3* |
| YPFPGPIHNSLPQ | [$His^8$]-β-CM-13 | 40.9 ± 1.4 | 39.0 ± 1.2 | 38.4 ± 1.4 | 39.9 ± 1.6 | 40.5 ± 1.3 |
| YPFPGPIHNSLPQNIPPLTQT | [$His^8$]-β-CM-21 | 40.2 ± 2.2 | 38.9 ± 0.9 | 40.1 ± 1.8 | 39.7 ± 2.6 | 40.9 ± 1.3 |
| YPFPGPIP | [$Pro^8$]-β-CM-8 | 42.8 ± 1.6 | 43.5 ± 1.7 | 43.3 ± 1.3 | 41.5 ± 1.8 | 40.8 ± 1.3 |
| YPFPGPIPN | [$Pro^8$]-β-CM-9 | 47.2 ± 1.0** | 45.7 ± 1.8** | 45.9 ± 1.1** | 48.7 ± 1.6** | 48.4 ± 1.3** |
| YPFPGPIPNSLPQ | [$Pro^8$]-β-CM-13 | 37.5 ± 1.5 | 38.4 ± 0.8 | 38.3 ± 1.1 | 38.3 ± 2.0 | 39.0 ± 0.9 |
| YPFPGPIPNSLPQNIPPLTQT | [$Pro^8$]-β-CM-21 | 40.1 ± 0.9 | 41.0 ± 2.7 | 41.6 ± 1.5 | 39.7 ± 1.8 | 41.6 ± 1.9 |
| VYPFPGPIPN | [$Val^0$, $Pro^8$]-β-CM-9 | 35.7 ± 2.7 | 35.8 ± 1.6 | 37.5 ± 1.9 | 41.1 ± 1.2 | 36.7 ± 2.1 |

Cells were treated with various peptides for 3 h. The numbers of neurite-bearing cells were counted as described in Materials and Methods. Data are represented as the means ± SEM from six to eight observations. *$P<0.05$; **$P<0.01$ compared with control by Scheffe's test.

**Table 2.** Effects of β-CM Analogues on Neurite Outgrowth in Neuro-2a Cells

| Analogue | Name | % Cells with neurites (mean ± SEM) | | | | |
|---|---|---|---|---|---|---|
| | | Peptide (log M) | | | | |
| | | -9 | -8 | -7 | -6 | -5 |
| Control | | 38.8 ± 2.0 | | | | |
| YPFP-$NH_2$ | Morphiceptin | 41.1 ± 1.7 | 40.6 ± 1.4 | 43.4 ± 1.3 | 41.7 ± 1.7 | 39.2 ± 1.8 |
| YPFPG-$NH_2$ | β-CM-5 amide | 46.0 ± 1.6 | 46.3 ± 2.0* | 48.3 ± 2.1** | 48.2 ± 3.2** | 47.8 ± 2.9** |
| Y-D-AFPG | [D-$Ala^2$]-β-CM-5 | 41.9 ± 2.5 | 44.7 ± 2.0 | 43.1 ± 2.2 | 42.5 ± 2.6 | 43.4 ± 2.7 |
| Y-D-PFPG | [D-$Pro^2$]-β-CM-5 | 42.0 ± 2.3 | 41.9 ± 1.5 | 44.2 ± 1.6 | 43.0 ± 1.9 | 45.3 ± 2.6 |

Cells were treated with various peptides for 3 h. The numbers of neurite-bearing cells were counted as described in Materials and Methods. Data are represented as the means ± SEM from six to eight observations. *$P<0.05$; **$P<0.01$ compared with control by Scheffe's test.

$P<0.01$ versus control value of 38.8 ± 1.2%) at $10^{-6}$ M. Significant stimulatory effects of [$His^8$]-β-CM-8 and [$His^8$]-β-CM-9 were observed only at $10^{-7}$ M and $10^{-5}$ M, respectively. Fragments shorter than β-CM-5 or longer than β-CM-9 produced no significant change. [$Val^0$, $Pro^8$]-β-CM-9 and des-Tyr-β-CM-7 were also ineffective, indicating that the N-terminal tyrosine residue was essential for the activity stimulating neurite outgrowth.

*Effects of β-CM analogues on neurite outgrowth*

We next examined the effects of β-CM analogues on neurite outgrowth. The modification by amidation of the carboxy-terminal or by the substitution of D-Ala for L-$Pro^2$ of β-CMs increases the affinity for the opioid receptor[15,16] and an L-configuration at the $Pro^2$ residue is required for opioid activity.[17,18] A comparison of the effects of β-CM analogues is shown in Table 2. The β-CM-5 amide produced a significant change at concentrations over $10^{-8}$ M though the β-CM-4 amide (morphiceptin) was ineffective. The β-CM-5 analogues in which L-$Pro^2$ was replaced by D-Ala or D-Pro also did not have a significant stimulatory effect.

*Opioid activities of β-CMs in the GPI assay*

In order to find whether the activity of β-CMs to promote neurite outgrowth was correlated with their opioid activities, we measured the latter by inhibiting electrically-induced contractions of a guinea-pig ileum muscle preparation (GPI assay). The $IC_{50}$ values are summarized in Table 3. β-CM-5 and the β-CM-5 amide, which had a potent and efficacious stimulatory effect on neurite outgrowth, showed strong opioid activities. However, morphiceptin and [D-$Ala^2$]-β-CM-5, which had no stimulatory activity, showed stronger opioid activities than β-CM-5. Further, all fragments longer than β-CM-5 had similar $IC_{50}$ values in the GPI assay, regardless of whether or not they had activity to promote neurite outgrowth. These results indicate that the stimulatory activities of β-CMs for neurite outgrowth do not

**Table 3.** Opioid Activities of β-CMs in GPI Assay

| Neurite outgrowth activity Peptide | GPI assay $IC_{50}$ value (μM) |
|---|---|
| Active peptides | |
| β-CM-5 | 0.48 |
| β-CM-5 amide | 0.31 |
| β-CM-6 | 3.3 |
| β-CM-7 | 3.2 |
| [$Pro^8$]-β-CM-9 | 4.4 |
| [$His^8$]-β-CM-8 | 5.2 |
| [$His^8$]-β-CM-9 | 3.4 |
| Inactive peptides | |
| Morphiceptin | 0.10 |
| [D-$Ala^2$]-β-CM-5 | 0.22 |
| [D-$Pro^2$]-β-CM-5 | No activity |
| [$Pro^8$]-β-CM-8 | 8.2 |
| [$Pro^8$]-β-CM-13 | 4.2 |
| [$Pro^8$]-β-CM-21 | 8.5 |
| [$His^8$]-β-CM-13 | 2.9 |
| [$His^8$]-β-CM-21 | 4.4 |

The opioid activity of various β-CM fragments and analogs was measured by GPI assay as described in Materials and Methods. $IC_{50}$ means the concentration of peptide inhibiting maximum electrically-induced contraction by 50%.

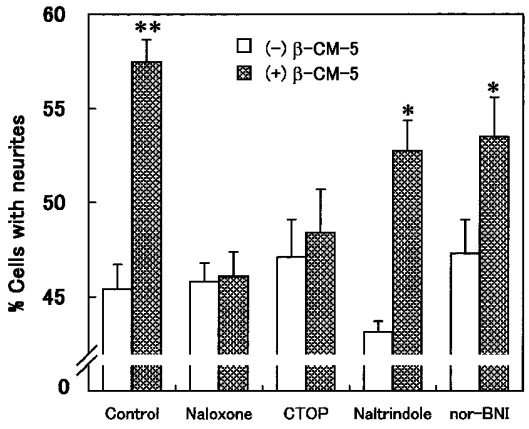


**Fig. 1.** Effects of Antagonists on the Neurite Outgrowth-stimulatory Effect of β-CM-5.

Cells were treated with β-CM-5 ($10^{-6}$ M) in the presence or absence of an antagonist ($10^{-6}$ M) for 3 h. The number of neurite-bearing cells were counted as described in Materials and Methods. Data are represented as the means ± SEM from eight observations. $^*P<0.05$, $^{**}P<0.01$ compared with the data of (−) β-CM-5 in respective antagonist treatment by Student's *t*-test.

correlate with their opioid potencies in the GPI assay.

*Effects of opioid antagonists and pertussis toxin (PTX) on the neurite outgrowth-stimulating activity of β-CM-5*

The effects of selective opioid antagonists on the stimulatory effect of β-CM-5 are shown in Fig. 1. The effect of β-CM-5 ($10^{-6}$ M) was completely inhibited by $10^{-6}$ M of naloxone and the μ selective antagonist, CTOP. On the other hand, neither a δ-selective antagonist, naltrindole, nor a κ-selective antagonist, nor-BNI, blocked the stimulatory effect of β-CM-5. To find whether the G protein system was involved in the effect of β-CM-5, we pretreated cells with PTX (100 ng/ml) for 4 h. Under these conditions, β-CM-5 ($10^{-6}$ M) failed to have an effect (Fig. 2).

**Table 4.** Effects of β-CM-5 on cAMP Levels in Neuro-2a Cells

| Treatment | cAMP Concentration (pmol/mg protein) | |
|---|---|---|
| | basal level | forskolin stimulated |
| Control | 1.8 ± 0.1 | 11.1 ± 1.3 |
| β-CM-5 ($10^{-6}$ M) | 2.1 ± 0.2 | 8.7 ± 0.3* |

The intracellular cAMP concentration was measured by radioimmunoassay as described in Materials and Methods. Data are represented as the means ± SEM from four observations. $^*P<0.05$ compared with forskolin stimulated control by Student's *t*-test.

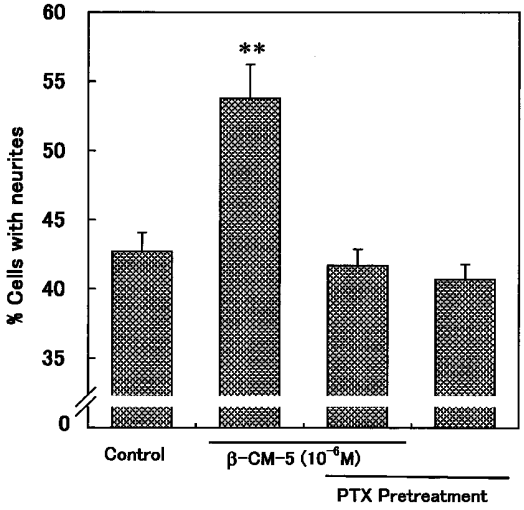


**Fig. 2.** Effect of Pretreatment with PTX on the Neurite Outgrowth-stimulatory Effect of β-CM-5.

Cells were treated with PTX (100 ng/ml) for 4 h, and then treated with β-CM-5 ($10^{-6}$ M) for 3 h. The number of neurite-bearing cells were counted as described in Materials and Methods. Data are represented as the means ± SEM from eight observations. $^{**}P<0.01$ compared with control untreated with β-CM-5 and PTX by Scheffe's test.

*Effects of β-CM-5 on cAMP level in Neuro-2a cells*

Opioid receptors are known to act *via* G proteins to inhibit adenylyl cyclase,[19] whereas opioids also have stimulatory effects on cAMP production.[20] Since reagents that elevate intracellular cAMP levels promote neurite outgrowth in this cell line,[21] we tested the effects of β-CM-5 on cAMP levels. Table 4 shows that $10^{-6}$ M of β-CM-5 reduced the level of forskolin-stimulated cAMP production by 22%, and it had no effect on the basal cAMP level. Thus, the β-CMs-stimulated neurite outgrowth did not involve the elevation of intracellular cAMP level.

## Discussion

We previously reported that the stimulatory action of β-CM-5 was blocked by a non-selective antagonist, naloxone, and that DAMGO had a similar effect, which was very weak.[11] Therefore, the effect of β-CM-5 on neurite outgrowth is expected to be regulated *via* an opioid receptor, which has μ-like characteristics. From the structure-activity relationships of β-CM fragments and analogues in this study, affinity for the opioid receptors was concluded to be essential for the activity to stimulate neurite outgrowth. [$Val^0$, $Pro^8$]-β-CM-9 and des-Tyr-β-CM-7 were ineffective, indicating that the N-terminal tyrosine residue was essential for the activity to stimulate neurite outgrowth. It is known that the presence of a tyrosine residue at the N-terminal end is an important structural motif that fit into the binding site of the opioid receptors.[17] β-CM-3, β-CM-4, and [D-$Pro^2$]-β-CM-5 which reportedly show little affinity for opioid receptors,[17,18,22] were also ineffective in Neuro-2a cells. On the other hands, all peptides that stimulated neurite outgrowth had the opioid activities in the GPI assay, without exception. The effect of β-CM-5 was prevented by co-administration of CTOP or by pretreatment with PTX. These results suggest that the G protein-coupled μ-opioid receptor is involved in the stimulatory effects of β-CMs on neurite outgrowth in Neuro-2a cells. However, the activity to stimulate neurite outgrowth was only shown by fragments of a certain length (β-CM-5 to -9).

An interesting observation in this study is that the stimulatory activities of β-CMs did not correlate with their opioid activities in the GPI assay. Notably, morphiceptin and [D-$Ala^2$]-β-CM-5 did not stimulate neurite outgrowth despite having potent opioid activity in the GPI assay (Table 2, 3). A similar result was obtained by us in terms of neuronal survival-promoting effects of β-CM-5, β-CM-7 and morphiceptin.[23] The efficacy of morphiceptin on neuronal survival was much less than that of β-CM-5 or -7 at high concentrations (micromolar range). The inconsistency may be explained by the experimental differences of treatment periods with opioids. The opioid activity measured in the GPI assay reflected the acute action of the peptide, as compared with the neurite outgrowth activity induced by prolonged exposure to opioids. Thus, the lack or weakness of the activity to promote neurite outgrowth by morphiceptin and [D-$Ala^2$]-β-CM-5 might be due to the desensitization of the μ-receptor. Receptor internalization and down-regulation in response to chronic agonist treatment contribute to receptor desensitization, which affects cellular responsiveness to ligands. The potency and efficacy of signal transduction of opioids are related to desensitization, and partial agonists caused less internalization of opioid receptors than full agonists in neuroblastoma[24] and *in vivo*.[25] Thus, stimulation of μ-receptors with β-CM-5, -6, and -7 might result in appropriate efficacies for signaling subsequent to neurite outgrowth-stimulation without the loss of μ-receptors from the cell surface. On the other hand, the reason the longer β-CM fragments had less neurite outgrowth-stimulatory activity, did not appear to be desensitization, considering their low levels of opioid activity in the GPI assay. The μ-receptors have been subdivided into $\mu_1$ and $\mu_2$ types.[26] The $\mu_1$-receptors display a high affinity for both opioid peptides and opiate alkaloids. In contrast, the $\mu_2$-receptor has a higher affinity for opiate alkaloids than for opioid peptides. It is known that the GPI preparation mainly contains $\mu_2$ receptors.[26] The failure of long β-CMs to stimulate neurite outgrowth might be due to the ligand selectivities of opioid receptors in the Neuro-2a cells. Further study is required to characterize the μ-receptors in Neuro-2a cells.

Opioids have been shown to influence neuritic differentiation *in vivo* and *in vitro*. Addition of low concentrations of various enkephalin and endorphin peptides increased the neurite outgrowth of superior cervical ganglia explants from rodents.[27] Morphine was reported to show biphasic dose-dependent inhibitory and stimulatory effects on neuritogenesis in PC12 pheochromocytoma in the presence of nerve growth factor (NGF); the effect might be mediated *via* a receptor having δ-like characteristics.[28] On the other hand, perinatal administration of morphine induced a reduction in dendritic growth in the cerebral cortex of 5-day-old rats.[29] Morphine decreased neurite arborization in neuron-enriched cultures derived from chick embryonic brain,[30] and reduced the length of Purkinje cell dendrites in organotypic explants of cerebellum from 1-day-old mice.[31] Further, Met-enkephalin and [D-$Ala^2$]-deltorphin II reduced the differentiation of cerebellar external granular layer neurons from postnatal mice through $\delta_2$ receptor subtypes.[32] Together, these findings suggest that the effects of opioids on neuronal differentiation are variable and cell-type specific. Our study suggests μ-opioid receptor agonists to be stimulators of neurite outgrowth.

At present, the intracellular signaling mechanisms for neurite outgrowth after the stimulation of G protein-coupled μ-opioid receptors by β-CMs are not clear. In neuronal cells, reagents that elevate intracellular cAMP levels evoke differentiation signals.[33] In the present study, the β-CMs-stimulated neurite outgrowth did not involve the regulation of intracellular cAMP. It was recently reported that opioids can activate ras/mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) *in vitro* and *in vivo*.[34,35] ERK1/2 activity is known to play a role in the neuritogenesis of

Neuro-2a cells.[36] Thus, $\beta$-CMs may trigger a G protein-mediated activation of a PI3K/MAPK signaling pathway, subsequently to elongation of neurite.

In summary, these findings suggest that $\mu$-opioid receptor activation stimulates neuronal differentiation *via* a G protein transduction system. Interestingly, some $\beta$-CM fragments ($\beta$-CM-5 to -9) had this stimulatory effect. Apart from their possible role as neurotransmitters and neuromodulators, $\beta$-CMs may also influence neural development or regeneration.

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