Effects of β-Casomorphins on Neuronal Survival in Culture of Embryonic Chick Dorsal Root Ganglion Neurons

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ABSTRACT—We studied the effects of β-casomorphins (β-CMs, μ-acting opioid peptides from milk protein β-casein) on survival of primary-cultured chick dorsal root ganglion neurons in the presence of nerve growth factor. β-CM-5 and β-CM-7 had potent neuronal survival-promoting activities. β-CM-4 amide (morphiceptin) and des-Tyr1-β-CM-7 also exhibited similar promoting effects, although their effects were very weak. The promoting effect of β-CM-5 was prevented by co-administration of naloxone, or pretreatment with pertussis toxin. These results suggest that the neuronal survival-promoting effects of β-CMs might be mediated through opioid receptors coupled to G proteins.

Keywords: β-Casomorphin, Neuronal survival, Chick dorsal root ganglion neuron

β-Casomorphins (β-CMs) belong to the family of endogenous opioid peptides originally isolated from an enzymatic digests (peptone) of the bovine milk protein, β-casein (1). They all contain the common N-terminal amino acid sequence Tyr-Pro-Phe-Pro, differing from that of the other endogenous opioid peptides (e.g., enkephalins, β-endorphins, dynorphins and endomorphins) and possess preferential μ-opioid receptor agonist activity (2). However, their physiological meanings are poorly understood.

Endogenous opioid peptides and opiate drugs are known to affect development of the nervous system (3, 4), typically by inhibiting proliferation, survival and differentiation of neuronal cells. Apart from the possible role as an inhibitory factor, endogenous opioids also increase neuronal survival during naturally occurring neuronal death period in some developing nervous system (5). Recently we reported the promoting effects of [Leu5]enkephalin, [Met5]enkephalin and selective μ-agonist, [D-Ala2,N-Me-Phe4,Gly5-ol]enkephalin (DAMGO) on neuronal survival in a culture of embryonic chick dorsal root ganglion (DRG) neurons in the presence of nerve growth factor (NGF) (6). In the present study, we report the effects of β-CMs on survival of cultured embryonic DRG neurons.

DRG neurons were isolated as previously described (6). DRGs were removed from 8-day chick embryos. After dissociation with 0.2% trypsin at 37°C for 30 min, the cells were preplated in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) for 4 h on tissue culture flasks (Iwaki Scitech, Funabashi) to remove the non-neuronal cells that adhered to the plastic surface during this period. The rest of the cells were plated in 24-well plates (Iwaki Scitech) coated with polyethyleneimine (Sigma Chemical Co., St. Louis, MO, USA) at a density of 1 × 104 cells in 1.0 ml of DMEM and Ham’s F-12 medium (1:1) (Gibco BRL) supplemented with 10% FBS and kanamycin (50 µg/ml) (Gibco BRL) in each well. Test compound (one of the opioids examined), mouse 2.5S NGF (10 ng/ml) (Takara Shuzo, Otsu) or both were added immediately to the wells and then the plates were incubated at 37°C in 5% CO2. After 2 days, cells were fixed with 1% glutaraldehyde in phosphate-buffered saline and the numbers of surviving neurons were determined by counting under a phase-contrast microscope at ×100 in ten fields in each well. Survived neurons were defined as round, phase-bright cells bearing processes, and were easily distinguished from non-neuronal cells that had a flat, phase dark appearance. The cultured cells, which were judged as viable neurons from their morphological character, were labeled by antibodies to neurofilament, and were stained by the MTT method. In our culture system, the percentages of neuronal cells in culture were more than 95%. All measurements were done on 2-day-old cultures. Neuronal survival was expressed as the percentage of neurite-bearing cells relative to the control cultures that were untreated with opioid(s). All data are given as the mean ± S.E.M. percentages. Statistical comparisons were made by ANOVA fol-
lowed by Tukey’s test.

The following drugs were used: \(\beta\)-CM-5 (Tyr-Pro-Phe-Pro-Gly), \(\beta\)-CM-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) (Peptide Institute, Minoh); des-Tyr\(^1\)\(\beta\)-CM-7, morphiceptin, naloxone (Sigma Chemical Co.); pertussis toxin (List Biological Laboratories, Inc., Campbell, CA, USA).

Here we tested the effects of various \(\beta\)-CMs on survival of DRG neurons of 8-day chick embryos in the presence of NGF. A comparison of the effects of various peptides on neuronal survival is shown in Fig. 1. We found that all of \(\beta\)-CMs used in this study promoted neuronal survival. The survival-promoting effects of \(\beta\)-CM-5 and \(\beta\)-CM-7 were especially potent and dose-dependent with maximum effects (144.0 \(\pm\) 2.5\%, \(P < 0.01\) and 150.9 \(\pm\) 2.4\%, \(P < 0.01\), respectively) at \(10^{-5}\) M. The activity of des-Tyr\(^1\)\(\beta\)-CM-7 was weak; and at \(10^{-5}\) M, conversely, the neuronal survival was significantly reduced, indicating that the N-terminal tyrosine residue was important in survival-promoting activity. Morphiceptin, i.e., \(\beta\)-CM-4 amide having more potent opioid activity than \(\beta\)-CM-5 and \(\beta\)-CM-7 (7), produced a significant increase (113.6 \(\pm\) 4.5\%, \(P < 0.05\)) in the number of neurons at \(10^{-8}\) M, and yet at concentrations greater than \(10^{-6}\) M, this effect was attenuated. On the other hand, there were no viable neurons after culturing in the presence of \(\beta\)-CMs without NGF (data not shown), indicating that \(\beta\)-CMs by themselves had no survival effects on DRG neurons.

The survival-promoting effect of \(\beta\)-CM-5 (\(10^{-6}\) M) was blocked by the concomitant presence of naloxone, an opioid receptor antagonist at concentration over \(10^{-7}\) M, (Fig. 2). To determine whether the G protein system was involved in the effect of \(\beta\)-CM-5, we pretreated DRG neurons with pertussis toxin (10 ng/ml, PTX) for 6 h. Under this condition, \(\beta\)-CM-5 (\(10^{-6}\) M) failed to exert its promoting effect (Fig. 3). These results suggested the possibility of involvement of the PTX-sensitive opioid receptor in the survival-promoting effect of \(\beta\)-CM-5.

The present study demonstrated the potent neuronal survival-promoting effects of \(\beta\)-CM-5 and \(\beta\)-CM-7 in the presence of NGF in dissociated chick DRG neuronal culture. The effect of \(\beta\)-CM-5 was dose-dependent and prevented by co-administration of naloxone or by pretreatment with PTX. The results indicated that the promoting effects were mediated through opioid receptors coupled to G proteins. The neuronal survival-promoting effects of [Leu\(^5\)]enkephalin and [Met\(^5\)]enkephalin in the same culture
Cells were cultured for 2 days with NGF (10 ng/ml). Effects of PTX on the survival-promoting effect of β-CM-5 and β-CM-7 might be mediated via a μ-opioid receptor. An interesting observation in the present study is that, at high concentrations (micromolar range), the survival-promoting activities of β-CM-5 and β-CM-7 were more potent than that of morphiceptin, while the binding affinities of β-CM-5 and β-CM-7 to μ-receptor were 25 times and 100 times less potent than that of morphiceptin, respectively (7). It is possible that the lack of response at high concentrations of morphiceptin might have been due to the desensitization of μ-receptor in DRG neurons. It was reported that the efficacy of the opioids was related to desensitization, and partial agonists failed to down regulate the opioid receptors in neuroblastoma (8, 9) and in vivo (10). Thus, β-CM-5 and β-CM-7 might exhibit appropriate opioid efficacy for neuronal survival-promoting effect.

β-CMs have various effects on the central nervous, endocrine, cardiovascular and gastrointestinal system (11). Furthermore, these peptides seem to cross different barriers in the body, including the brush-border (12) and blood-brain barrier (13). The results of the present study, indicatin neuronal survival-promoting effects of β-CMs suggest a possible physiological role in development of the neuronal system.

REFERENCES

1. Brantl V, Teschemacher H, Henschens A and Lottspeich F: Novel opioid peptides derived from casein (β-casomorphins). I. Isolation from bovine casein pepton. Hoppe Seylers Z Physiol Chem 360, 1211 – 1216 (1979)
2. Brantl V, Teschemacher H, Blasig J, Henschens A and Lottspeich F: Opioid activities of β-casomorphins. Life Sci 28, 1903 – 1909 (1981)
3. Hammer RP and Hauser KF: Consequences of early exposure to opioids on cell proliferation and neuronal morphogenesis. In Development of the Central Nervous System: Effects of Alcohol and Opiates, Edited by Miller MW, pp 319 – 339, Wiley-Liss, New York (1992)
4. Hauser KF and Stiene-Martin A: Opiates and the regulation of nervous system development: evidence from in vitro studies. In Neurobiology of Opiates, Edited by Hammer RP, pp 23 – 61, CRC Press, Boca Raton FL (1993)
5. Meriney SD, Ford MJ, Oliva D and Pilar G: Endogenous opioids modulate neuronal survival in the developing avian ciliary ganglion. J Neurosci 11, 3705 – 3717 (1991)
6. Sakaguchi M, Fujimori T, Satoh T, Satoh M, Takeuchi M and Matsumura E: Effects of opioids on neuronal survival in culture of embryonic chick dorsal root ganglion neurons. Neurosci Lett 262, 17 – 20 (1999)
7. Chang K-J, Killian A, Hazum E and Cuatrecasas P: Morphiceptin (NH$_2$-Tyr-Pro-Phe-Pro-CONH$_2$): A potent and specific agonist for morphine (μ) receptors. Science 212, 75 – 77 (1981)
8. Law P-Y, Hom DS and Loh HH: Opiate receptor down-regulation and desensitization in neuroblastoma-glioma NG 108-15 hybrid cells are two separate cellular adaptation processes. Mol Pharmacol 424, 413 – 424 (1983)
9. Zadina JE, Chang SL, Ge L-J and Kastin AJ: Mu opiate receptor down-regulation by naloxone and opioid agonists in maternal rat brain. J Pharmacol Exp Ther 265, 254 – 262 (1993)
10. Sternini C, Spann M, Anton B, Keith DE Jr, Bunnett NW, Zastrow MV, Evans C and Breche NC: Agonist-selective endocytosis of μ-opioid receptor by neurons in vivo. Proc Natl Acad Sci USA 93, 9241 – 9246 (1996)
11. Ramabadrak K and Bansinath M: Pharmacology of β-casomorphins, opioid peptides derived from milk protein. Asia Pacific J Pharmacol 4, 45 – 58 (1989)
12. Mahe S, Tome D, Dumontier AM and Desjeux JF: Absorption of intact β-casomorphins (β-CM) in rabbit ileum in vitro. Reprod Nutr Dev 29, 725 – 733 (1989)
13. Nyberg F, Lieberman H, Lindström LH, Lyren æs S, Koch G and Terenius L: Immunoreactive β-casomorphin-8 in cerebrospinal fluid from pregnant and lactating women: correlation with plasma levels. J Clin Endocrinol Metab 68, 283 – 289 (1989)