FEBS Letters 439 (1998) 152–156 FEBS 21116

Endomorphins fully activate a cloned human mu opioid receptor

Jianhua Gong[a], Judith A. Strong[a], Shengwen Zhang[a], Xia Yue[a], Robert N. DeHaven[c], Jeffrey D. Daubert[c], Joel A. Cassel[c], Guangling Yu[c], Erik Mansson[c], Lei Yu[a,b,*]

[a] *Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0521, USA*
[b] *Department of Psychiatry, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521, USA*
[c] *Adolor Corporation, Malvern, PA 19355, USA*

Received 17 August 1998; received in revised form 6 October 1998

Abstract Endomorphins were recently identified as endogenous ligands with high selectivity for mu opioid receptors. We have characterized the ability of endomorphins to bind to and functionally activate the cloned human mu opioid receptor. Both endomorphin-1 and endomorphin-2 exhibited binding selectivity for the mu opioid receptor over the delta and kappa opioid receptors. Both agonists inhibited forskolin-stimulated increase of cAMP in a dose-dependent fashion. When the mu opioid receptor was coexpressed in *Xenopus* oocytes with G protein-activated K^+ channels, application of either endomorphin activated an inward K^+ current. This activation was dose-dependent and blocked by naloxone. Both endomorphins acted as full agonists with efficacy similar to that of [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO). These data indicate that endomorphins act as full agonists at the human mu opioid receptor, capable of stimulating the receptor to inhibit the cAMP/adenylyl cyclase pathway and activate G-protein-activated inwardly rectifying potassium (GIRK) channels.

Key words: Endomorphin; Human mu opioid receptor

1. Introduction

Opioids, including both endogenous peptides and exogenous alkaloids, exert their physiological effects by interacting with membrane-bound opioid receptors. These effects include regulation of pain perception, reinforcement and reward, neurotransmitter release, hormone secretion, gastrointestinal motility, and respiratory activity [1]. Pharmacological studies have identified three major types of opioid receptors in the brain and spinal cord: mu, delta, and kappa, of which the mu opioid receptor plays an important role in opioid analgesia and development of opioid dependence [2,3]. Extensive studies have also led to the identification of three major classes of endogenous ligands in the mammalian brain: endorphins, enkephalins, and dynorphins [4]. Endorphins and enkephalins can activate both the mu and delta opioid receptors, while dynorphins are considered to be relatively selective for the kappa opioid receptor.

Zadina et al. [5] isolated two endogenous opioid peptides from the mammalian brain, named endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), that display high affinity and selectivity for the mu opioid receptor. Endomorphin-1 was also demonstrated to have higher binding affinity than the synthetic mu-selective analogue [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO) in vitro, and to produce potent and prolonged analgesia in mice [5]. However, these studies were conducted in brain tissues and in vivo, in which multiple types of opioid receptors are present.

The major cellular effects of activation of all three types of opioid receptors by their agonists are inhibition of adenylyl cyclase and modulation of membrane Ca^{2+} and K^+ conductances [6,7]. Opioid-mediated increases in K^+ conductance and decrease in Ca^{2+} conductance both serve to reduce membrane excitability and contribute to the analgesic properties of the opioids. Because cAMP is an important second messenger in cell signalling, opioid inhibition of adenylyl cyclase implies a more complex pathway for opioid regulation of cellular mechanisms. These two major cellular effects of mu opioid receptor activation have been studied by using cloned mu opioid receptors since expression of the cloned receptor provides a cellular model with a single defined receptor type [8–10]. In this study, we evaluated the functional effects, including both efficacy and potency, of endomorphins on the cloned human mu opioid receptor.

2. Materials and methods

2.1. Receptor expression and binding assay

Chinese hamster ovary (CHO) cells transfected with receptor cDNAs in pcDNA3 (Invitrogen) using the calcium phosphate method [11] and expressing the human mu (hMOR), delta (hDOR), or kappa (hKOR) receptor [12] were used in this study. When 90% confluence was reached, the cells were scraped from the plates and were harvested by centrifugation at $1000\times g$ for 10 min at 4°C. The cell pellets were then washed once and resuspended in ice-cold binding assay buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM EGTA, 5 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 200 μg/ml bacitracin, and 0.5 μg/ml aprotinin, and homogenized using a Polytron homogenizer (Brinkmann). The homogenates were centrifuged at $48\,000\times g$ for 20 min at 4°C to recover the membrane fractions. For the binding assay, a membrane sample (50–100 μg protein) was mixed with a radio-labelled ligand ([^{3}H]DAMGO or [^{3}H]diprenorphine, Amersham Life Science) and a test compound in a final volume of 500 μl of the assay buffer in 96-well deep-well polystyrene titer plates (Beckman). The final concentration of [^{3}H]DAMGO or [^{3}H]diprenorphine after mixing was 1 nM. Following incubation at 22°C for 60 min, binding was terminated by vacuum filtration with a Brandel MPXR-96T Harvester through GF/B filters that had been pretreated with a solution of 0.5% (w/v) polyethyleneimine and 0.1% (w/v) bovine serum albumin. The filter-bottom plates were washed four times with 1 ml each of ice-cold 50 mM Tris-HCl (pH 7.8). Radioactivity on the filters was then

*Corresponding author. Fax: (1) (513) 558-3367.
E-mail: lei.yu@uc.edu

Abbreviations: DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; GIRK, G-protein-activated inwardly rectifying potassium channel; hMOR, human mu opioid receptor; hDOR, human delta opioid receptor; hKOR, human kappa opioid receptor; CHO cells, Chinese hamster ovary cells; HK, high potassium; EDM, endomorphin

PII: S0014-5793(98)01362-3

Table 1
Binding affinity values of endomorphins to human mu, delta, and kappa opioid receptors

	K_i (nM)		
	Mu	Delta	Kappa
Endomorphin-1	5.5 ± 0.4	824.3 ± 362.9	> 10 000
Endomorphin-2	8.1 ± 1.7	> 10 000	> 10 000

Summary of the data from three or more separate experiments. The results are presented as mean ± standard error. The K_i values of endomorphins for the mu opioid receptor were determined by displacing [^{3}H]DAMGO bound to the receptor, while the K_i values for delta and kappa receptors were estimated by displacement of bound [^{3}H]diprenorphine.

determined by scintillation spectrometry in a Packard TopCount in Microscint-20 (Packard Instruments, Meriden, CT, USA). Binding data were analyzed by non-linear regression using the GraphPad Prism software program. The K_i values were calculated from IC_{50} by the Cheng-Prusoff equation [13] using the K_d values of [^{3}H]DAMGO and [^{3}H]diprenorphine (4.1 nM for DAMGO binding to the mu opioid receptor and 0.33 nM for diprenorphine binding to the delta receptor).

Preliminary experiments were performed to show that no specific binding was lost during the wash of the filters, that binding was at equilibrium by 60 min and remained at equilibrium for at least an additional 60 min, and that the binding was within the linear range with regard to the protein amounts used for the assay. Non-specific binding, determined in the presence of 10 μM unlabeled naloxone, was approximately 10% of total binding.

2.2. cAMP assay

CHO cells stably transfected with hMOR were seeded in 24-well plates at the density of 5×10^4 cells/well. After incubation at 37°C to reach 90% confluence in a F-12 Nutrient Mixture (HAM, Gibco-BRL) supplemented with 1 mM glutamine, 10% (v/v) fetal calf serum, 100 μg/ml each of penicillin and streptomycin, and 500 μg/ml geneticin, the cells were incubated in 0.5 ml of F-12 containing 500 μM of isobutylmethylxanthine (IBMX) at 37°C for 15 min prior to the treatment with 500 μM IBMX, 25 μM forskolin, and agonists at appropriate concentrations in 0.5 ml of F-12 at 37°C for 20 min. The reactions were terminated by adding 0.1 ml of luciferase cell culture lysis reagent (Promega) in 0.1 M HCl after removal of F-12. Aliquots were removed and the concentrations of cAMP were determined using the radioimmunoassay kit (NEN-DuPont) according to the manufacturer's instructions. The data were analyzed by non-linear regression using the GraphPad Prism software program.

2.3. Oocyte expression and electrophysiology

Xenopus oocytes were isolated using standard methods [14]. Synthetic mRNAs for the human mu opioid receptor [9] and the G-protein-activated inwardly rectifying K^+ channels, GIRK1 [15,16] and GIRK2 [17,18], were transcribed in vitro, as described [8]. Thirty-two nl of the mRNA solution (1 ng of the mu receptor mRNA and 0.5 ng each of GIRK1 and GIRK2 mRNAs) was injected into each mature (stage V–VI) oocyte using a Drummond automatic microinjector. The injected oocytes were incubated in 50% (v/v) L-15 medium supplemented with 0.8 mM glutamine and 10 μg/ml gentamycin at 20°C. Two to three days after injection, oocytes were voltage-clamped at −80 mV using a two-microelectrode voltage clamp, the Axoclamp-2A (Axon Instruments) under the control of pCLAMP software (Axon Instruments). The electrodes were filled with 3 M KCl and had tip resistance of 0.5–3.0 MΩ. During recording, oocytes were superfused with either ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5), a high K^+ solution (HK: 96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5), or HK containing agonists. The data were analyzed with the Microcal Origin software program.

3. Results

3.1. Binding of endomorphins to human opioid receptors

Zadina et al. [5] showed that in rodent brain homogenates, endomorphins bind selectively to mu opioid receptors. To determine the binding selectivity of endomorphins for the

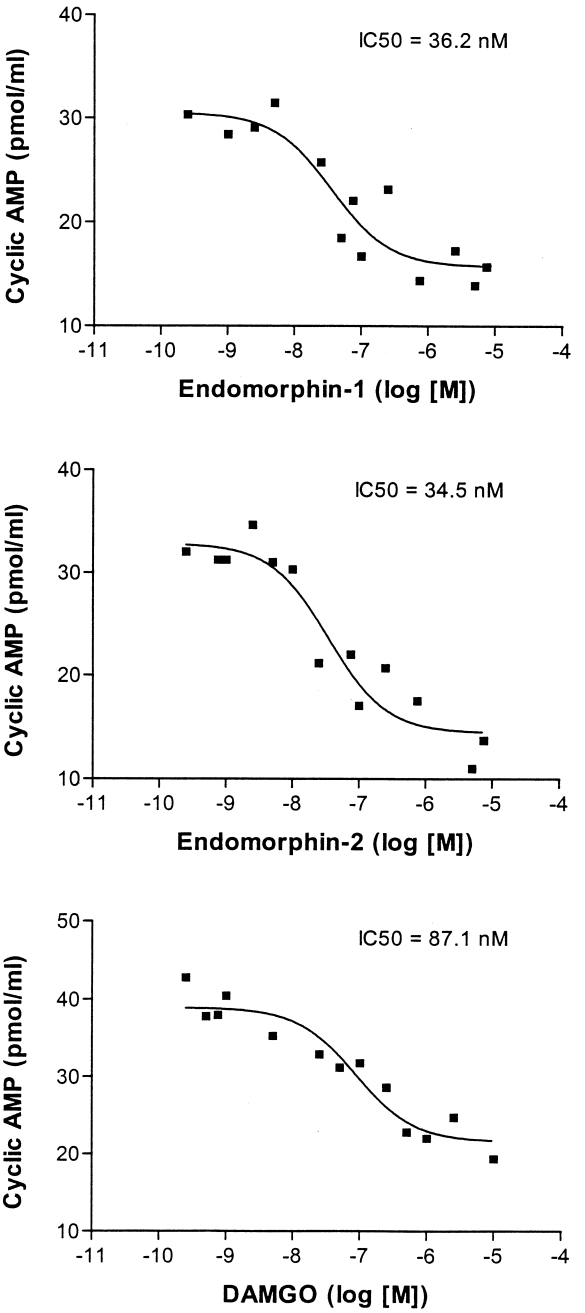

Fig. 1. Inhibition of forskolin-stimulated cyclic AMP increase by endomorphins and DAMGO. CHO cells stably transfected with the human mu opioid receptor were treated with 25 μM forskolin in the presence or absence of endomorphins or DAMGO at various concentrations from 0.25 nM to 10 μM. The data for each point were from three determinations of a representative experiment.

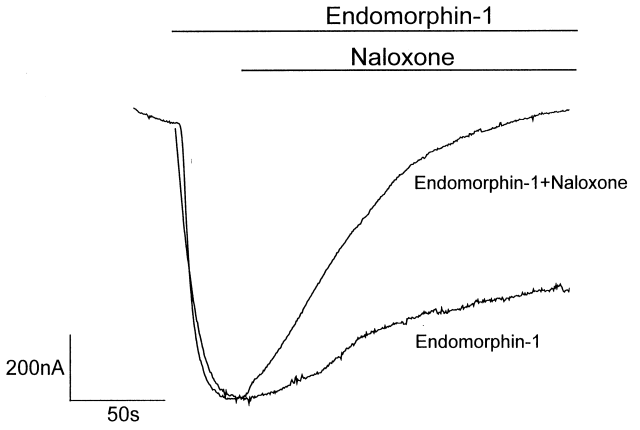

Fig. 2. Example of endomorphin-evoked K^+ current mediated by the human mu opioid receptor. Two representative endomorphin-evoked current traces at a holding potential at −80 mV are shown. Following superfusion with HK, the oocytes were perfused with HK containing endomorphin-1 (100 nM) alone or followed by endomorphin-1 (100 nM) plus naloxone (10 μM), as indicated by the bars above the current traces.

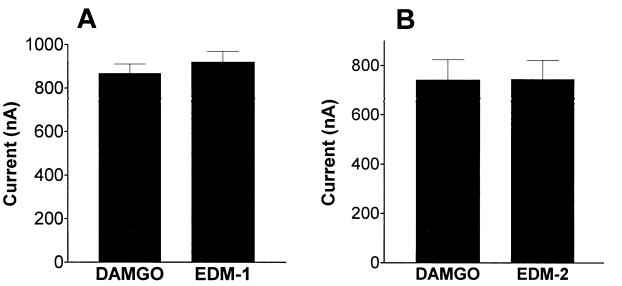

Fig. 3. Efficacy of endomorphins and DAMGO in activation of an inwardly rectifying K^+ current mediated by the human mu opioid receptor. Data in panels A and B were from two different experiments, presented as mean ± S.E. The concentration of endomorphin-1 and DAMGO was 50 or 100 nM in panel A, and 50 nM in panel B. A: The K^+ current evoked by endomorphin-1 or DAMGO. Endomorphin-1: 921 ± 49 nA, $n = 9$; DAMGO: 868 ± 43 nA, $n = 9$. B: The K^+ current evoked by endomorphin-2 or DAMGO. Endomorphin-2: 745 ± 77 nA, $n = 5$; DAMGO: 742 ± 82 nA, $n = 5$.

cloned human opioid receptors, we used CHO cells transfected with the human mu, delta, and kappa opioid receptor clones for binding studies. Binding affinity values of endomorphins for the human mu, delta, and kappa opioid receptors are shown in Table 1. Both endomorphins exhibited high affinity and selectivity for the human mu opioid receptor, with little or no binding for the delta or kappa opioid receptors. The estimated K_i value of endomorphin-1 for the human mu opioid receptor is 5.5 nM, an affinity 150-fold higher than that for the delta opioid receptor and over 1500-fold higher than that for the kappa opioid receptor, indicating its selectivity for the mu opioid receptor. Similarly, endomorphin-2 has a K_i value of 8.1 nM for the mu opioid receptor with very little binding to the delta or kappa opioid receptors.

3.2. Inhibition of forskolin-stimulated cyclic AMP increase

Opioid receptors, including the mu opioid receptor, have been shown to inhibit adenylyl cyclase activity and thus reduce the intracellular cAMP content [6]. To examine the functional effect of endomorphins on the cloned human mu opioid receptor, the potency with which endomorphins inhibit the increase of cAMP stimulated by forskolin was determined. As shown in Fig. 1, endomorphins 1 and 2 have IC_{50} values of 36.2 and 34.5 nM, respectively, and DAMGO has an IC_{50} value of 87.1 nM. These data indicate that both endomorphins, like DAMGO, can exert an inhibitory effect on the cAMP/adenylyl cyclase pathway via the cloned human mu opioid receptor. The maximum inhibition of the cAMP increase achieved in our experiments was 58% for endomorphin-1 (95% confidence level 49–66%), 65% for endomorphin-2 (95% confidence level 56–73%), and 55% for DAMGO (95% confidence level 49–61%). There were no significant differences between these values. Thus, comparable levels of maximum cAMP inhibition between endomorphins and DAMGO indicate that endomorphins are of similar efficacy as DAMGO.

3.3. Activation of the mu opioid receptor coupled to G-protein-activated K^+ channels

The mu opioid receptor has been shown to activate cloned G-protein-activated K^+ channels in *Xenopus* oocytes upon activation by its agonists, such as DAMGO [8,9,18]. To examine whether endomorphins can activate the mu opioid receptor in this functional assay, hMOR, GIRK1 and GIRK2 were coexpressed in oocytes, and the functional coupling of the receptor to the K^+ channels was assessed by two-electrode voltage clamp. When the receptor was activated by superfusing the oocytes with either of the endomorphins, an inward current was observed (Fig. 2). This K^+ current was induced by activation of the expressed receptor, since it was completely blocked by the opioid receptor antagonist naloxone (Fig. 2). Both endomorphins induced maximum K^+ currents comparable to those induced by the full agonist DAMGO (Fig. 3), indicating that both endomorphins are full agonists at the mu opioid receptor in this functional assay.

To examine the potency of endomorphins in activating the mu opioid receptor, the dose effects of endomorphins were determined. Because of the variability of individual oocytes in expressing exogenous proteins, we standardized the receptor-mediated response by taking the ratio of the test agonist-evoked current (I_{test}) at a particular dose to the maximum current induced by DAMGO at a saturating concentration (100 nM), as indicated in Fig. 4A. The activation of the mu opioid receptor by endomorphins is dose dependent within the range from 0.1 nM to 50 nM (Fig. 4B). The EC_{50} values calculated from the dose response curves are 4.6 nM for endomorphin-1, 9.7 nM for endomorphin-2, and 14.0 nM for DAMGO. These data demonstrate that both endomorphins can fully induce the functional coupling between the mu opioid receptor and G-protein-activated K^+ channels with similar efficacy and potency to DAMGO.

4. Discussion

Activation of the mu opioid receptor by its agonists has two major cellular effects. One is the inhibitory effect on the cAMP/adenylyl cyclase pathway, resulting in reduced levels of intracellular cAMP [6,19,20], an important second messenger in cell signalling. The other is the modulation of ion channels, such as increasing K^+ conductance or decreasing Ca^{2+} conductance, to reduce cellular excitability [8,9,21–24]. In this study, we were interested in whether endomorphin-1 and endomorphin-2, two recently discovered endogenous pep-

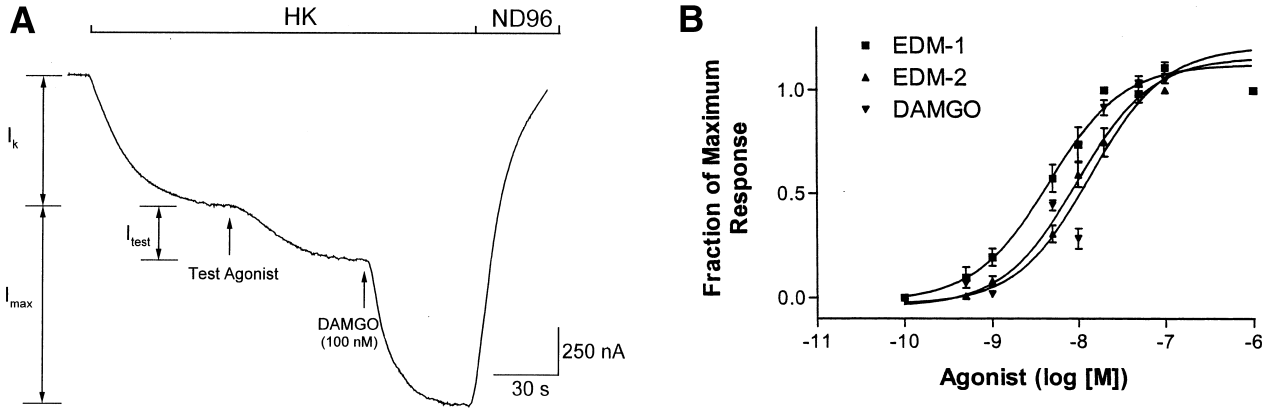

Fig. 4. Dose response curves for human mu opioid receptor-mediated activation of K^+ currents by endomorphins and DAMGO. A: An example of a current trace showing the experimental protocol and calculation method for the agonist-induced response. Oocytes were clamped at a holding potential of −80 mV and superfused with different solutions as indicated. Application of agonists in HK started at the points indicated by the arrows. I_k: the basal inward K^+ currents in HK. I_{max}: maximum K^+ currents evoked by DAMGO at a saturating concentration (100 nM). I_{test}: K^+ currents evoked by the test dose of endomorphins or DAMGO. B: Dose response curves of receptor activation. The tested concentrations of agonists ranged from 0.1 nM to 1 μM. The response to a test dose is expressed as the fraction of the maximum activation (I_{test}/I_{max}). Data are presented as mean ± S.E. ($n = 4–5$). All oocytes were used only once to avoid desensitization. The EC_{50} values calculated from the curves are 4.6 nM for endomorphin-1, 9.7 nM for endomorphin-2, and 14.0 nM for DAMGO.

tides with high affinity and selectivity for the mu opioid receptor [5], can activate the human mu opioid receptor to exert these two major cellular effects. Our binding data demonstrated that both endomorphins bound to the mu opioid receptor with affinity values comparable to that with the high affinity agonist DAMGO, and both endomorphins were highly selective for the human mu opioid receptor over the delta and kappa opioid receptors (Table 1). These observations are consistent with those reported by Zadina et al. [5] in rodent brain tissues which contain several types of opioid receptors.

The effect of agonists on activation of opioid receptors and their potency and efficacy in the activation can be determined in functional assays, as activation of mu opioid receptors by agonists results in reduction in intracellular levels of cAMP and in activation of co-expressed K^+ channels in *Xenopus* oocytes to produce an inward current [25]. In this study, both endomorphins were shown to reduce the intracellular level of cAMP and to evoke an inward K^+ current with high potency by activation of the cloned human mu opioid receptor (Figs. 1, 3 and 4B), supporting the initial observation in functional assays by Zadina et al. [5] using the guinea-pig ileum system.

During the preparation of this report, two separate studies published in which endomorphins were compared with DAMGO using mu opioid receptor-stimulated [^{35}S]GTPγS binding as the assay [26,27]. Based on this assay, the authors reported that endomorphins are partial agonists at the mu opioid receptor. The mu opioid receptor similar to other G protein-coupled receptors, achieves its cellular activity by first activating G proteins. Thus, receptor-stimulated [^{35}S]GTPγS binding serves as a valuable marker to indicate the ability of an agonist to activate the receptor qualitatively. Whether the extent of [^{35}S]GTPγS binding parallels the efficacy of an agonist quantitatively remains to be established. In this context, it is interesting to speculate on the difference between the outcome of these two studies and that in this report. We examined the major cellular functions of the mu opioid receptor: inhibition of cAMP synthesis and activation of K^+ channels. In both functional assays, the endomorphins showed full agonist activity as compared with DAMGO, a well-recognized full agonist at the mu opioid receptor. One explanation for the different conclusions from our study and those by Sim et al. [26] and Hosohata et al. [27] is that, while endomorphins may not stimulate [^{35}S]GTPγS binding to the same extent as that by DAMGO, the amount of activated G protein is sufficient for a full-scale cellular effect.

In summary, this study showed that endomorphins are selective full agonists for the cloned human mu opioid receptor, capable of conferring high affinity binding and efficacious activation of the human mu opioid receptor. Thus, the discovery of endomorphins by Zadina et al. [5] opens the door to further exploration of the physiological roles of these endogenous peptides.

Acknowledgements: This work was supported by National Institutes of Health Grants DA09444 and DA11891.

References

[1] Pasternak, G.W. (1993) Clin. Neuropharmacol. 16, 1–18.
[2] Pasternak, G.W., Childers, S.R. and Snyder, S.H. (1980) Science 208, 514–516.
[3] Chaillet, P., Coulaud, A., Zajac, J.M., Fournie-Zaluski, M.C., Costentin, J. and Roques, B.P. (1984) Eur. J. Pharmacol. 101, 83–90.
[4] Akil, H., Watson, S.J., Young, E., Lewis, M.E., Khachaturian, H. and Walker, J.M. (1984) Annu. Rev. Neurosci. 7, 223–255.
[5] Zadina, J.E., Hackler, L., Ge, L.J. and Kastin, A.J. (1997) Nature 386, 499–502.
[6] Childers, S.R. (1991) Life Sci. 48, 1991–2003.
[7] North, R.A. (1993) in: Handbook of Experimental Pharmacology, Vol. 104, Opioids I (Herz, A., Ed.) pp. 773–797, Springer, Berlin.
[8] Chen, Y. and Yu, L. (1994) J. Biol. Chem. 269, 7839–7842.
[9] Mestek, A., Hurley, J.H., Bye, L.S., Campbell, A.D., Chen, Y., Tian, M., Schulman, H. and Yu, L. (1995) J. Neurosci. 15, 2396–2406.
[10] Kovoor, A., Henry, D.J. and Chavkin, C. (1995) J. Biol. Chem. 270, 589–595.
[11] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752.

[12] Zhang, S., Tong, Y., Tian, M., DeHaven, R.N., CortesBurgos, L., Mansson, E., Simonin, F., Kieffer, B.L. and Yu, L. (1998) J. Pharmacol. Exp. Ther. 286, 136–141.
[13] Cheng, Y.-C. and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099–3108.
[14] Gurdon, J.B. and Wickens, M.P. (1983) Methods Enzymol. 101, 370–386.
[15] Dascal, N., Lim, N.F., Schreibmayer, W., Wang, W., Davidson, N. and Lester, H.A. (1993) Proc. Natl. Acad. Sci. USA 90, 6596–6600.
[16] Kubo, Y., Reuveny, E., Slesinger, P.A., Jan, Y.N. and Jan, L.Y. (1993) Nature 364, 802–806.
[17] Patil, N., Cox, D.R., Bhat, D., Faham, M., Myers, R.M. and Peterson, A.S. (1995) Nat. Genet. 11, 126–129.
[18] Tong, Y., Wei, J., Zhang, S., Strong, J.A., Dlouhy, S.R., Hodes, M.E., Ghetti, B. and Yu, L. (1996) FEBS Lett. 390, 63–68.
[19] Chen, Y., Mestek, A., Liu, J., Hurley, J.A. and Yu, L. (1993) Mol. Pharmacol. 44, 8–12.
[20] Chen, Y., Liu, J. and Yu, L. (1996) Addict. Biol. 1, 49–59.
[21] Williams, J.T., Egan, T.M. and North, R.A. (1982) Nature 299, 74–77.
[22] North, R.A., Williams, J.T., Surprenant, A. and Christie, M.J. (1987) Proc. Natl. Acad. Sci. USA 84, 5487–5491.
[23] Wimpey, T.L. and Chavkin, C. (1991) Neuron 6, 281–289.
[24] Twitchell, W.A. and Rane, S.G. (1994) Mol. Pharmacol. 46, 793–798.
[25] Yu, L. (1996) Addict. Biol. 1, 19–30.
[26] Sim, L.J., Liu, Q., Childers, S.R. and Selley, D.E. (1998) J. Neurochem. 70, 1567–1576.
[27] Hosohata, K., Burkey, T.H., Alfaro-Lopez, J., Varga, E., Hruby, V.J., Roesk, W.R. and Yamamura, H.I. (1998) Eur. J. Pharmacol. 346, 111–114.