ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jun; 21 (6): 516-520
http://www.chinaphar.com

Nociceptin, endomorphin-1 and -2 do not interact with invertebrate immune and neural μ_3 opiate receptor

Christos M RIALAS, Benjamins WEEKS[1], Patrick CADET, Yannick GOUMON, George B STEFANO[2]
(*Neuroscience Research Institute, State University of New York at Old Westbury, Old Westbury, NY 11568-0210, USA*; [1]*Biology Department, Adelphi University, Garden City, New York 11530, USA*)

KEY WORDS opioid peptides; morphine; nitric oxide; *Mytilus edulis*; immunocytes; mu opioid receptors

ABSTRACT

AIM: To determine if endomorphin-1,-2 and nociceptin (orphanin FQ) bind to the μ_3 opiate receptor subtype or release nitric oxide as μ_3 selective ligands do. **METHODS**: These opioid peptides were examined for their ability to displace [^{3}H]dihydromorphine (DHM) binding from the invertebrate (immunocytes and pedal ganglia) μ_3 opiate receptor in membrane homogenates. The ligands were also tested for their ability to release nitric oxide from the same intact tissues utilizing an amperometric probe that measures nitric oxide in real-time. **RESULTS**: Endomorphin-1,-2 and nociceptin do not displace [^{3}H]DHM binding from immunocyte or pedal ganglia membrane homogenates nor do they release nitric oxide from these tissues. **CONCLUSION**: Since these newly discovered opioid peptides do not interact with the μ_3 opiate receptor subtype, endogenous morphine's significance is enhanced because it appears to be the only naturally occurring opiate ligand for the receptor. Furthermore, since this study involves invertebrate tissues, this signal system had to evolve early during evolution.

INTRODUCTION

The newly discovered opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and -2 (Tyr-Pro-Phe-Phe-NH_2) exhibit a high affinity for the mammalian mu opiate receptor[1] and they produce short acting antinociception[2]. Nociceptin (orphanin FQ), another novel opioid peptide, has also been demonstrated in mammalian tissues[3,4]. Recently, we have demonstrated that within the invertebrate prodynorphin precursor a peptide sequence is found exhibiting 50 % sequence identity to nociceptin[5]. Therefore, it was of interest to determine if these novel opioid peptides have the ability to interact with the opioid peptide insensitive and opiate alkaloid selective receptor, designated μ_3, found on human monocytes, granulocytes, human vascular tissues and invertebrate immunocytes[6-9].

In the present study we sought to determine if the invertebrate opiate μ_3 receptor exhibited binding properties found in mammals. In human vascular tissues endomorphin-1 and -2 do not displace opiate alkaloids from this opiate receptor subtype[10]. This phenomenon has been verified by Makman in other tissues[11], further demonstrating the opiate alkaloid selectivity of this receptor. The finding of μ opiate receptor transcripts in *Mytilus* ganglia[12] prompted us to determine its binding properties in regard to these newly discovered opioid peptides. In this regard, the invertebrate μ receptor behaves in a similar manner noted for mammals, demonstrating that this receptor has been conserved for over 500 million years of evolution.

MATERIALS AND METHODS

Mytilus edulis were obtained from the shores of Long Island Sound at Montauk, NY. They were maintained in the laboratory for 3 wk prior to their use in these experiments, as described elsewhere in great detail[13]. *Mytilus edulis* immunocytes were obtained by aspiration from the heart cavity of animals maintained at 9 ℃. The hemolymph (100 mL) from 100 animals (10^9 cells/L) was centrifuged at 800 × *g* for 10 min to pellet the cells which were then processed for receptor binding. Pedal ganglia (300) were dissected on ice and prepared for

[2]Correspondence to Prof George B STEFANO PhD, *Neuroscience Research Institute, State University of New York at Old Westbury, Old Westbury, NY* 11568-0210, *USA*.
Phn 1-516-876-2732. Fax 1-516-876-2727.
E-mail: gbs11@banet.net
Received 2000-01-05 Accepted 2000-03-28

binding analysis as described elsewhere[14]. For nitric oxide experiments 10 pedal ganglia per chamber were used.

Binding analysis The immunocytes or pedal ganglia were prepared separately and as previously noted in detail elsewhere[6-8,15]. The identity and characteristics of the μ_3 receptor become quite apparent in a displacement analysis, using a tritiated opiate alkaloid ligand[6-8,15] and thus represent an important method for μ_3 recognition. [^{3}H]dihydromorphine ([^{3}H]DHM; 1.665 GBq per mmol/L) binding was performed in Tris 50 mmol/L, pH 7.4 at 35 ℃ without enzyme inhibitors for 90 min[6]. The membranes were then washed twice with ice-cold Tris buffer, resuspended to half the original volume in Tris-HCl 50 mmol/L (pH 7.4) and stored at −70 ℃ for binding assays. For IC_{50} determination (defined as the concentration of drug which elicits half-maximal inhibition of specific [^{3}H]DHM binding), aliquots of immunocyte or ganglia membrane suspensions were incubated with nonradioactive opioid compounds at 6 different concentrations for 10 min at 22 ℃ and then with [^{3}H]DHM for 60 min at 4 ℃ as previously noted in detail[6,7,14]. One hundred per cent binding is defined as bound [^{3}H]DHM in the presence of 10 mmol/L dextrorphan minus bound [^{3}H]DHM in the presence of 10 mmol/L levorphanol. The $\bar{x} \pm s$ for the experiments is calculated for each compound tested. Each point represents the mean of the experiments made with four different membrane preparations

Morphine was obtained from Winthrop Pharmaceuticals, naloxone from Endo Laboratories, and DHM from New England Nuclear Inc. Endomorphin -1 and -2 were obtained from Peptide International (Louisville, KY) and nociceptin and other peptides from Sigma (St. Louis MO).

Monitoring of nitric oxide (NO) release CNS (10 pedal ganglia per treatment replicated 4 times) or immunocytes (10^{10}/L centrifuged 800 × g per treatment and replicated 4 times) of *M edulis* were bathed in a saline incubation medium[14]. NO release was monitored with an NO-selective microprobe manufactured by World Precision Instruments (Sarasota, FL). The redox current was detected by a current-voltage converter circuit and continuously recorded. The NO release was monitored through the probe (200 μm) of a micromanipulator (Zeiss-Eppendorff) attached to the stage of an inverted microscope (Nikon Diaphot) with the sensor positioned 20 μm above the respective tissue surface. The system was calibrated daily by adding potassium nitrite to a solution of potassium iodide, resulting in the liberation of a known quantity of NO (World Precision Instruments). The probe was allowed to equilibrate for 12 h in the incubation medium free of tissue before being transferred to vials containing the ganglia or immunocytes for another 30 min. Manipulations/handling of the ganglia was only performed with glass instruments. The NO mean values were graphed every two min to represent the actual NO release ($\bar{x} \pm s$). Each experiment was performed simultaneously with a control (vehicle minus drug) from the same animal. Thus, the experiment was performed with 2 probes measuring the different experimental preparations (control, drug exposed). This strategy eliminated the probability of artifactual electrode drifts.

The data so obtained was then evaluated by t-test. Data acquisition was by the computer-interfaced software and for measuring NO release DUO-18 software (WOI, S, FL) was used. The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, CA) for graphic representation and evaluation. Data gatherer was unaware of the experimental conditions.

RESULTS

Endomorphin-1, -2, and nociceptin do not stimulate NO release at the same concentrations as morphine in all the tissues examined. Even at higher concentrations (10 μmol/L) NO release was not found in response to either endomorphin or nociceptin exposure. However, morphine at 0.1 and 0.01 μmol/L released maximum NO which peaked at 11.6 ± 2.7 and 5.3 ± 1.6 nmol/L ($P < 0.05$; t-test compared to control value of 1.1 ± 0.3 nmol/L) (Fig 1, Fig 2, Tab 1).

In examining the displacement analysis of the endomorphins and nociceptin for ^{3}H-DHM binding to the μ_3 opiate receptor subtype, the endomorphins and nociceptin exhibited a lack of affinity compared to the opiate alkaloids (Tab 2). In this regard, the lack of affinity of kappa and delta-type ligands is as expected[6-9,16,17].

DISCUSSION

In the past we have demonstrated that opiate alkaloids, not opioid peptides, exhibit a strong and selective affinity for the μ_3 opiate receptor found on human monocytes, granulocytes and human vascular endothelial cells, including *M edulis* immunocytes and ganglia[6-9,16,17]. More recent studies by our laboratory have demonstrated

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jun; 21 (6)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

that the μ_3 receptor is coupled to NO release in these tissues[7-9,14,17]. Furthermore, in *Mytilus* immunocytes, the μ_3 receptor coupling to NO release occurs only after intracellular calcium transients are stimulated[18].

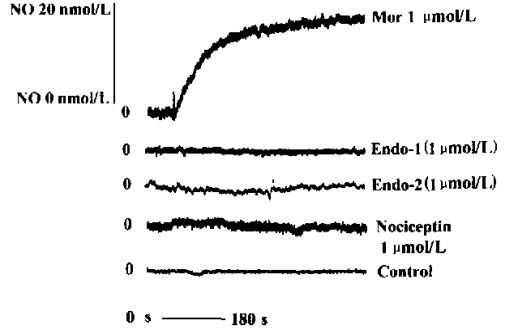

Fig 1. Real-time representation of opioid exposure to *Mytilus edulis* immunocytes and the subsequent release of nitric oxide. Morphine (Mor 1 μmol/L), the opioid peptides endomorphin-1 and -2 (Endo, 1 μmol/L), nociceptin (1 μmol/L), and control (vehicle minus drug). Each experiment was replicated 4 times.

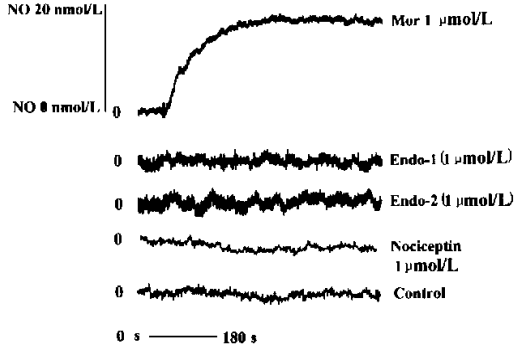

Fig 2. Real-time representation of opioid exposure to *Mytilus edulis* pedal ganglia and the subsequent release of nitric oxide. Morphine (Mor 1 μmol/L), the opioid peptides endomorphin-1 and -2 (Endo, 1 μmol/L), nociceptin (1 μmol/L) and control (vehicle minus drug). Each experiment was replicated 4 times.

Recently, Zadina and colleagues[1] have demonstrated the presence of novel and naturally occurring opioid peptides, endomorphin -1 and -2, that exhibit potent μ-associated activities. One of the activities noted is that of initiating hypotension in rat and rabbit[19,20], suggesting that these compounds may release NO. Given this, and based on the results of our studies documenting the presence of the μ_3 opiate receptor on vascular tissue causing vasodilation via NO, we examined the interactions of these novel peptides with the alkaloid selective receptor in vascular tissues[10]. In this regard, the data strongly demonstrates that endomorphin-1 and -2 exhibit no affinity for the μ_3 receptor and do not release NO under our experimental conditions. This finding is supported by the results of the present study that demonstrate a lack of NO release in invertebrate tissues containing the opiate μ_3 receptor subtype.

Tab 1. Nitric oxide release following the exposure of Mytilus edulis pedal ganglia to opioid compounds. Nitric oxide determinations were monitored every 5 min. Each experiment was replicated 4 times. $\bar{x} \pm s$. All drugs were given at a concentration of 1 μmol/L. Endo-1 = Endomorphin-1, Endo-2 = Endomorphin-2, Noc = Nociceptin, and Mor = Morphine. $^cP < 0.01$ *vs* time 0.

Time/min	*Nitric oxide/nmol·L^{-1}*			
	Noc	Endo-1	Endo-2	Mor
0	0.9±0.03	1.1±0.3	0.8±0.2	0.9±0.3
5	1.0±0.2	1.0±0.2	0.9±0.3	19.3±4.1^c
10	1.3±0.3	1.1±0.3	0.7±0.2	11.1±3.0^c
15	1.4±0.4	1.0±0.3	1.7±0.5	5.6±2.2
20	1.7±0.6	1.4±0.5	1.5±0.4	1.6±0.4

Tab 2. Displacement of [^{3}H] dihydromorphine binding (IC_{50}, nmol/L) by opioid ligands in *Mytilus* immunocytes and pedal ganglia membrane suspensions. DPDPE = (*D*-Pen^2, *D*-Pen^5)-enkephalin; DAMGO = [Tyr-*D*-Ala^2, Gly-*N*-Me-Phe^4, Gly(ol)^5-enkephalin].

Ligand	Immunocytes	Ganglia
Agonists		
δ-agonist		
DPDPE	> 1000	> 1000
μ-agonist		
Endomorphin-1	> 1000	> 1000
Endomorphin-2	> 1000	> 1000
Nociceptin	> 1000	> 1000
DAMGO	> 1000	> 1000
Dihydromorphine	48 ± 6	45 ± 7
Morphine	49 ± 7	47 ± 8
κ-agonist		
Dynorphin 1 – 17	> 1000	> 1000
Antagonists		
Naltrexone	34 ± 5	37 ± 4
Naloxone	68 ± 7	72 ± 8

Regarding nociceptin (orphanin FQ), we have char acterized a prodynorphin molecule in hemocytes of *Mytilus edulis*[5]. *Mytilus* prodynorphin contains, α-neo-endorphin, dynorphin-A and dynorphin-B at the C-terminus, exhibiting 100 %, 70.5 %, and 85 % sequence identity with the rat material. The number of leucine-enkephalins in this precursor is identical to that found in vertebrates. *Mytilus* prodynorphin is distinguishable from that found in leeches in that its N-terminus is longer[21]. Additionally, by sequence comparison, the presence of an orphanin FQ-like peptide, exhibiting 50 % sequence homology with that found in mammals, was demonstrated. It then is quite important to determine if nociceptin could bind to the μ_3 receptor since the dynorphins exhibited little affinity[6~8,16]. In this regard, we demonstrate that nociceptin does not exhibit an affinity for this opiate receptor, maintaining its opiate alkaloid selectivity, a result also supported by its inability to release NO from these tissues.

In conclusion, both endomorphins and nociceptin do not bind to the selective opiate alkaloid receptor in mammals and in *Mytilus* neural tissues. In a recent report using macrophages, astrocytes, and HL-60 human promyelocytic leukemia cells Makman and colleagues[11] also found that these novel peptides did not bind to the μ_3 receptor, further supporting the present results. Thus, it would appear that naturally occurring morphine found in mammals and invertebrates is the ligand for this unique receptor[8,22]. This conclusion is enhanced by the recent finding of mu opiate receptor transcripts in *Mytilus* pedal ganglia[12]. Clearly, the presence of this receptor and morphine in animals 500 million years divergent in evolution highlights the significance of this particular signaling mechanism.

ACKNOWLEDGMENTS This work was in part supported by the following grants: NIMH COR 17138, NIDA 09010 and the Research Foundation and Central Administration of the State University of New York and NIH Fogarty INT 00045. This work was performed in part for the MS degree at Adelphi University for CMR.

REFERENCES

1 Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the mu opiate receptor. Nature 1997; 386: 499 – 501.

2 Stone LS, Fairbanks CA, Laughlin TM, Nguyen HO, Bushy TM, Wessendorf MW, *et al*. Spinal analgesic actions of the new endogenous opioid peptides endomorphin -1 and -2. Neuroreport 1997; 8: 3131 – 5.

3 Civelli O, Douglass J, Goldstein A, Herbert E. Sequence and expression of the rat prodynorphin gene. Proc Natl Acad Sci USA 1985; 82: 4291 – 5.

4 Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, *et al*. Orphanin FQ: A neuropeptide that activates an opioid like G protein-coupled reaction. Science 1995; 270: 792 – 4.

5 Stefano GB, Salzet-Raveillon B, Salzet M. Mytilus edulis hemolymph contain prodynorphin. Immunol Lett 1998; 63: 33 – 9.

6 Stefano GB, Digenis A, Spector S, Leung MK, Bilfinger TV, Makman MH, *et al*. Opiatelike substances in an invertebrate, a novel opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression. Proc Natl Acad Sci USA 1993; 90: 11099 – 103.

7 Stefano GB, Hartman A, Bilfinger TV, Magazine HI, Liu Y, Casares F, *et al*. Presence of the mu3 opiate receptor in endothelial cells: coupling to nitric oxide production and vasodilation. J Biol Chem 1995; 270: 30290 – 3.

8 Stefano GB, Scharrer B, Smith EM, Hughes TK, Magazine HI, Bilfinger TV, *et al*. Opioid and opiate immunoregulatory processes. Crit Rev Immunol 1996; 16: 109 – 44.

9 Bilfinger TV, Hartman A, Liu Y, Magazine HI, Stefano GB. Cryopreserved veins used for myocardial revascularization: a 5 year experience and a possible mechanism for their increased failure. Ann Thorac Surg 1997; 63: 1063 – 9.

10 Rialas C, Bilfinger TV, Salzet M, Stefano GB. Endomorphin 1 and 2 do not interact with the m3 opiate receptor subtype. Acta Pharmacol Sin 1998; 19: 403 – 7.

11 Makman MH, Dobrenis K, Surratt CK. Properties of m3 opiate alkaloid receptors in macrophages, astrocytes, and HL-60 human promyelocytic leukemia cells. Adv Exp Med Biol 1998; 437: 137 – 48.

12 Cadet P, Stefano GB. *Mytilus edulis* pedal ganglia express μ opiate receptor transcripts exhibiting high sequence identity with human neuronal μ1. Brain Res Mol Brain Res 1999; 74: 242 – 6.

13 Stefano GB, Teoh M, Grant A, Reid C, Teoh H, Hughes TK. *In vitro* effects of electromagnetic fields on immunocytes. Electro-Magnetobiology 1994; 13: 123 – 36.

14 Stefano GB, Scharrer B. The presence of the m3 opiate receptor in invertebrate neural tissues. Comp Biochem Physiol 1996; 113C: 369 – 73.

15 Stefano GB, Melchiorri P, Negri L, Hughes TK, Scharrer B. (*D*-Ala2)-Deltorphin I binding and pharmacological evidence for a special subtype of delta opioid receptor on human and invertebrate immune cells. Proc Natl Acad Sci USA 1992; 89: 9316 – 20.

16 Makman MH, Bilfinger TV, Stefano GB. Human granulocytes contain an opiate receptor mediating inhibition of cytokine-induced activation and chemotaxis. J Immunol 1995; 154: 1323 – 30.

17 Magazine HI, Liu Y, Bilfinger TV, Fricchione GL, Stefano GB. Morphine-induced conformational changes in human monocytes, granulocytes, and endothelial cells and in inverte-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jun; 21 (6)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

brate immunocytes and microglia are mediated by nitric oxide. J Immunol 1996; 156: 4845 – 50.

18 Nieto-Fernandez FE, Mattocks DW, Cavani F, Salzet M, Stefano GB. Morphine coupling to invertebrate immunocyte nitric oxide release is dependent on intracellular calcium transients. Comp Biochem Physiol 1999; 123: 295 – 9.

19 Champion HC, Zadina JE, Kastin AJ, Hackler L, Ge LJ, Kadowitz PJ. Endomorphin 1 and 2, endogenous ligands for the mu-opioid receptor, decrease cardiac output, and total peripheral resistance in the rat. Peptides 1997; 18: 1393 – 7.

20 Champion HC, Zadina JE, Kastin AJ, Hackler L, Ge LJ, Kadowitz PJ. The endogenous mu-opioid receptor agonists endomorphins 1 and 2 have novel hypotensive activity in the rabbit. Biochem Biophys Res Commun 1997; 235: 567 – 70.

21 Salzet M, Stefano GB. Prodynorphin in invertebrates. Mol Brain Res 1997; 52: 46 – 52.

22 Stefano GB. The μ3 opiate receptor subtype. Pain Forum 1999; 8: 206 – 9.

孤啡肽、内吗啡肽-1 和-2 与无脊椎动物免疫和神经系统的 μ_3 阿片受体无相互作用

Christos M RIALAS, Benjamin WEEKS[1], Patrick CADET, Yannick GOUMON, George B STEFANO[2]

(*Neuroscience Research Institute, State University of New York at Old Westbury, Old Westbury, NY 11568 – 0210, USA*; [1]*Biology Department, Adelphi University, Garden City, New York 11530, USA*)

关键词 阿片样肽类; 吗啡; 一氧化氮; 贻贝; 免疫细胞; mu 阿片受体

(责任编辑 朱倩蓉)