Association of aminopeptidase N and endopeptidase 24.15 inhibitors potentiate behavioral effects mediated by nociceptin/orphanin FQ in mice

Florence Noble, Bernard P. Roques*

Département de Pharmacochimie Moléculaire et Structurale, INSERM U266-CNRS URA D 1500, Université René Descartes, UFR des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire, 75270 Paris Cedex 06, France

Received 12 December 1996

Abstract The behavioral effects induced by central administration in mice of the endogenous ORL_1 (opioid receptor-like$_1$) ligand, nociceptin/orphanin FQ, were investigated in the absence or presence of inhibitors of aminopeptidase N (bestatin) and endopeptidase 24.15 ($Z\text{-}_{(L,D)}Phe\Psi(PO_2CH_2)_{(L,D)}Ala\text{–}Arg\text{–}Phe$) recently shown to be involved in the metabolism of the heptadecapeptide in vitro. A severe reduction in motor activity induced by nociceptin/orphanin FQ was measured in two tests (spontaneous motor activity and open field). This pharmacological effect was shown to be potentiated by the association of bestatin and $Z\text{-}_{(L,D)}Phe\Psi(PO_2CH_2)_{(L,D)}Ala\text{–}Arg\text{–}Phe$, confirming in vivo the involvement of these peptidases in nociceptin/orphanin FQ inactivation. In our conditions, these inhibitors were devoid of intrinsic effects, suggesting a low tonic regulation by the heptadecapeptide of the measured behaviour.

Key words: Nociceptin/orphanin FQ; Metabolism; Selective inhibitor; Aminopeptidase N; Endopeptidase-24.15; Motor activity; Open field

1. Introduction

In addition to the three major types of opioid receptors, μ, δ and κ (review in [1]), a novel receptor (ORL_1, opioid receptor-like$_1$) was identified on the basis of its homology with the amino acid sequence of opioid receptors [2–8]. The ORL_1 receptor has a very low affinity for traditional opioid peptides (dynorphins, endorphins, and enkephalins). Two groups have recently isolated a heptadecapeptide, nociceptin/orphanin FQ, FGGFTGARKSARKLANQ [9,10], structurally resembling dynorphin A, and which behaves as a potent endogenous ligand of the ORL_1 receptor. As expected, nociceptin/orphanin FQ exhibits a nanomolar affinity for the ORL_1 receptor and a low affinity for the μ, δ, κ opioid receptors despite the presence in N-terminal position of the FGGF sequence instead of YGGF in endogenous opioid receptor ligands. Although the functional role of nociceptin/orphanin FQ remains unknown, the wide distribution of ORL_1 mRNA and nociceptin/orphanin FQ precursor in the central nervous system of rodents, particularly in the limbic system and in several areas known to be involved in the control of nociceptive stimuli, including the spinal cord dorsal horn, have suggested that this peptide could be involved in pain perception [2–5,11–13]. The peptide has been reported to heighten sensitivity to pain following i.c.v. injection in mice but to be without effect after spinal application [9,10]. Nevertheless, a recent study disagrees with these results, showing that nociceptin/orphanin FQ has an inhibitory action on spinal dorsal horn neurones in rat [14]. Furthermore nociceptin/orphanin FQ was reported to decrease motor activity and to reduce the muscular tone of mice [10].

As clearly shown with the enkephalins, the interruption of peptidergic signals is ensured by peptidase-induced inactivation of the endogenous peptide effector (review in [15]). We have recently shown by using brain slices that nociceptin/orphanin FQ is hydrolysed essentially at its Phe^1–Gly^2 and Ala^7–Arg^8 peptide bonds by aminopeptidase N (APN) and endopeptidase 3.4.24.15 [16]. The biological relevance of this metabolic pathways was confirmed in this study by measuring the behavioral effects induced by i.c.v. administration in mice of nociceptin/orphanin FQ, or the metabolite peptides Gly^2–Gln^{17} and Phe^1–Ala^7 in the absence or in the presence of aminopeptidase and endopeptidase 24.15 inhibitors. Thus, these compounds potentiated the severe impairment in motor activity induced by the heptadecapeptide.

2. Materials and methods

2.1. Chemicals

Nociceptin/orphanin FQ and metabolite peptides were synthesized using the stepwise solid-phase method of Merrifield [17] on a Applied Biosystems model 431A automated peptide synthesizer with Applied Biosystems small-scale Fmoc chemistry. Peptides were cleaved from the resin, deprotected, diethyl ether-precipitated, and washed in accordance with Applied Biosystems guidelines. All the peptides were purified by reverse-phase HPLC on a Vydac C_{18} column (250×10 mm) using acetonitrile gradients in 0.1% trifluoroacetic acid. Bestatin was purchased from Roger Bellon (France), and the selective endopeptidase 24.15 inhibitor [$Z\text{-}_{(L,D)}Phe\Psi(PO_2CH_2)_{(L,D)}Ala\text{–}Arg\text{–}Phe$] was kindly provided by Dr. V. Dive (Commisariat à l'Energie Atomique, Centre d'Etudes Nucléaires de Saclay, Saclay, France). Nociceptin/orphanin FQ, metabolite peptides, and peptidase inhibitors were dissolved in saline.

2.2. Animals

Animals used in this study were acquired, cared for and used in accordance with the guidelines published in the European Communities Council directives (86/609/EEC).

Male CD_1 mice (20–22 g) were obtained from Charles River France (Saint-Aubin-Lès-Elbeuf, France). Animals were supplied with food and water ad libitum and housed in groups ($n = 20$) in an environment controlled for both temperature (22 ± 1°C) and humidity (45–55%) at least 2 days before the experiments were started. All behavioral measures were recorded between 10:00 h and 20:00 h.

2.3. Intracerebroventricular and systemic injections

Nociceptin/orphanin FQ, metabolite peptides, and peptidase inhibitors were injected free hand in the left lateral ventricule (i.c.v.) 15 min before the open field. The antagonist naloxone was administered s.c. 5 min before i.c.v. injection.

2.4. Behavioral tests

The open field was a rectangular area (70 cm wide, 90 cm long and 60 cm high); 63 squares (10/10) were drawn with black lines on the white floor of the field which was brightly illuminated from the top (500 lux). Three events were recorded for 5 min: (a) the latency (s) to move out from the center of the open field where the animal was

*Corresponding author. Fax: (33) 1-43-26-69-18

PII S0014-5793(96)01476-7

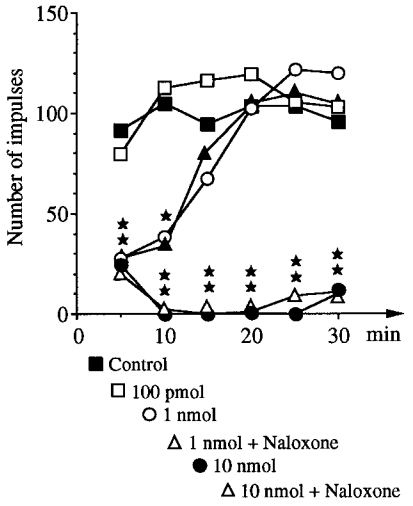

Fig. 1. Time course of the effects induced by i.c.v. administration of nociceptin/orphanin FQ in mice on the spontaneous motor activity in absence or presence of naloxone (0.1 mg/kg, s.c.). Results are expressed as means ± SEM ($n = 8$–10 mice for each group). Spontaneous motor activity was recorded in an automated activity monitor system containing photoelectric detectors. During the test, the mice cut the photoelectric beams resulting in an impulse. The number of impulses was recorded every 5 min for 30 min. $^{**}P < 0.01$ compared to control group (Newman-Keuls test).

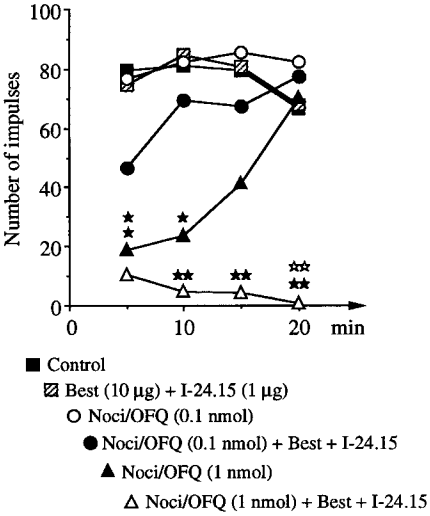

Fig. 2. Effects of bestatin (Best, 10 μg) and of the selective inhibitor of endopeptidase 24.15 (I-24.15, 1 μg) on the effects induced by i.c.v. administration of nociceptin/orphanin FQ (Noci/OFQ, 1 nmol) on spontaneous motor activity. Results are expressed as means ± SEM ($n = 8$–10 mice for each group). Spontaneous motor activity was recorded in an automated activity monitor system containing photoelectric detectors. During the test, the mice cut the photoelectric beams resulting in an impulse. The number of impulses was recorded every 5 min for 20 min. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control group; $^{☆☆}P < 0.01$ compared to nociceptin/orphanin FQ treated group (Newman-Keuls test).

placed and to cross two squares; (b) locomotion scored by the number of squares crossed; (c) the total number of rears. Spontaneous motor activity was recorded in an automated activity monitor system containing photoelectric detectors. During the test, the mice cut the photoelectric beams resulting in an impulse. The total number of impulses was recorded every 5 min for 90 min. The experiment was performed in a quiet and dimly illuminated room (50 lux).

2.5. Analysis of data

Statistical analysis was carried out by a 1-way analysis of variance (ANOVA) followed by Dunnett's t test for dose-response curves or Newman-Keuls test for multiple comparisons. The level of significance was set at $P < 0.05$.

3. Results and discussion

Intracerebroventricular administration of nociceptin/orphanin FQ induced a severe reduction in spontaneous motor activity observed in an automated activity monitor system. Indeed, i.c.v. administration of the heptadecapeptide (1 nmol) decreased the vertical and horizontal activity of mice for about 15 min, which was not modified in presence of naloxone (Fig. 1). Moreover, a long-lasting hypolocomotor effect was recorded when nociceptin/orphanin FQ was administered at a higher dose (10 nmol) (Fig. 1). Except for a complete loss of muscular tone observed following i.c.v. administration of the heptadecapeptide (10 nmol and 100 nmol) in all mice, no apparent toxic effect was observed. Animals recovered a motor activity similar to that of the control group ~80 min after i.c.v. administration (data not shown). These results have been confirmed in the open field, where a very strong increase of the latency time was observed, as a reduction of the number of squares crossed and number of rearings (Table 1).

In a previous study it has been shown that two metallopeptidases are involved in the nociceptin/orphanin FQ metabolism [16]. Both these enzymes, APN and endopeptidase 24.15 contain the typical sequence HExxH which has been found in numerous other zinc endopeptidases. The membrane-bound APN removes the N-terminal amino acid of protein and peptide substrates. This enzyme is mainly located in the small intestinal and kidney brush borders, but also found in lung, liver, primary cultures of fibroblasts and brain, where APN has been found to be involved in the degradation of neuropeptides (review in [15]). Several studies suggest that endopeptidase 24.15 is a cytosolic enzyme with a minor membrane-associated component (10–20% of total activity) [18], which

Table 1
Behavioral analysis of nociceptin/orphanin FQ injected i.c.v. 15 min before the open field

	Latency time(s)	Number of squares crossed	Number of rearing
Control	2.7 ± 0.5	375.0 ± 37.0	31.2 ± 2.9
1 pmol	2.8 ± 0.7	322.9 ± 45.3	26.8 ± 3.4
10 pmol	6.8 ± 2.3	347.5 ± 41.9	27.2 ± 3.5
100 pmol	10.7 ± 2.4	335.0 ± 38.8	25.1 ± 2.25
1 nmol	204.3 ± 42.1**	23.5 ± 10.2**	4.3 ± 3.1**

Events were recorded for 5 min.
Values are mean ± SEM ($n = 8$–10/group).
**$P < 0.01$ as compared to control group (Dunnett's t test).

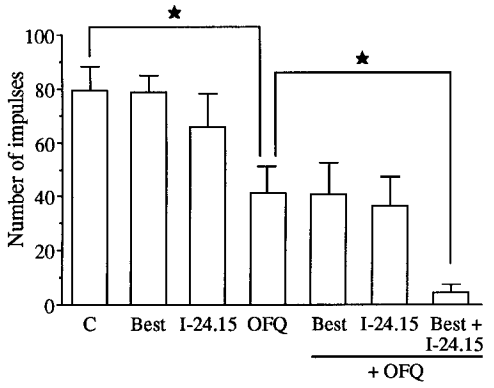

Fig. 3. Effects of bestatin (Best, 10 μg) and/or of the selective inhibitor of endopeptidase 24.15 (I-24.15, 1 μg) on the effects induced by i.c.v. administration of nociceptin/orphanin FQ (OFQ, 1 nmol) on spontaneous motor activity. Results are expressed as means ± SEM ($n = 8$–10 mice for each group). Spontaneous motor activity was recorded in an automated activity monitor system containing photoelectric detectors. During the test, the mice cut the photoelectric beams resulting in an impulse. The number of impulses was recorded for 5 min, 10 min after nociceptin/orphanin FQ administration. ★$P < 0.05$ (Newman-Keuls test).

could account for the involvement of this peptidase in the in vivo metabolism of several neuropeptides [19]. APN and endopeptidase 24.15 have been shown to hydrolyse nociceptin/orphanin FQ in vitro essentially at the Phe^1–Gly^2, and Ala^7–Arg^8 bonds, respectively, generating the metabolites Gly^2–Gln^{17} and Phe^1–Ala^7 [16]. At all doses tested, including concentrations 10 times higher (10 nmol) than those used for nociceptin/orphanin FQ, the metabolite peptides Gly^2–Gln^{17} and Phe^1–Ala^7 were found unable to modify the spontaneous motor activity (data not shown). These results are in agreement with structure–activity relationship studies, which have demonstrated that the des–Phe nociceptin/orphanin FQ, Gly^2–Gln^{17} has a 1000 times lower affinity and biological activity than its parent compound [20] and that the N-terminal part extending up to position 8 plays a critical role for recognition and activation of the ORL_1 receptor [20,21]. Thus, as for other neuropeptides, interruption of the responses induced by nociceptin/orphanin FQ is ensured by membrane-bound enzymes which cleave the peptide into inactive fragments. Several years ago, the characterization of APN and neutral endopeptidase 24.11 (NEP), followed by the design of selective inhibitors have been, and will continue to be, of great value in determining the role of endogenous enkephalins (review in [15]). Therefore to achieve a better understanding of the physiological role of nociceptin/orphanin FQ, selective inhibitors of the heptadecapeptide metabolism were co-administered. In contrast to NEP/APN inhibitors, which have an intrinsic opioidergic action [22], aminopeptidase and endopeptidase 24.15 inhibitors alone, or in association, did not induce pharmacological effects after central administration, indicating that there is little or no tonic participation of endogenous nociceptin/orphanin FQ in the behaviours measured (Figs. 2 and 3). However, a strong potentiation of the hypolocomotor effect induced by i.c.v. administration of the exogenous heptadecapeptide in the motor activity test was observed (Figs. 2 and 3) in the presence of the association of the aminopeptidase inhibitor, bestatin and the selective endopeptidase 24.15 inhibitor, $Z\text{-}_{(L,D)}Phe\Psi(PO_2CH_2)_{(L,D)}Ala$–Arg–Phe [23]. On the other hand, due to the complementary role of endopeptidase 24.15 and aminopeptidase in the nociceptin/orphanin FQ metabolism [16], selective inhibition of only one of these peptidases did not give a significant effect (Fig. 3). This has already been observed in the case of enkephalins which are metabolized by the concomitant action of neutral endopeptidase 24.11 and APN (review in [15]).

In conclusion, the involvement of aminopeptidase and endopeptidase 24.15 in nociceptin/orphanin FQ metabolism demonstrated on brain cortical slices [16] has been confirmed in vivo by the use of selective inhibitors. The use of peptidase inhibitors could permit a better understanding of the physiological role of nociceptin/orphanin FQ.

Acknowledgements: We wish to acknowledge gratefully C. Lenoir and Dr. F. Cornille for the synthesis of nociceptin/orphanin FQ and metabolite peptides and Dr. V. Dive for providing us the selective inhibitor of endopeptidase 24.15.

References

[1] Keiffer, B.L. (1995) Cell. Mol. Neurobiol. 15, 615–635.
[2] Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., Caput, D., Vassart, G. and Meunier, J.C. (1994) FEBS Lett. 341, 33–38.
[3] Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., Miyata, T., Houtani, T. and Sugimoto, T. (1994) FEBS Lett. 343, 42–46.
[4] Chen, Y., Fan, Y., Liu, J., Mestek, A., Tian, M., Kozak, C.A. and Yu, L. (1994) FEBS Lett. 347, 279–283.
[5] Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M. and Grandy, D.K.(1994) FEBS Lett. 347, 284–288.
[6] Wang, J.B., Johnson, P.S., Imai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M. and Uhl, G.R. (1994) FEBS Lett. 348, 75–79.
[7] Wick, M.J., Minnerath, S.R., Lin, X., Elde, R., Law, P.Y. and Loh, H.H. (1994) Mol. Brain Res. 27, 37–44.
[8] Lachowicz, J.E., Shen, Y., Monsma, F.G. Jr. and Sibley, D.R. (1995) J. Neurochem. 64, 34–40.
[9] Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M. and Costentin, J. (1995) Nature 377, 532–535.
[10] Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J. Jr. and Civelli, O. (1995) Science 270, 792–794.
[11] Houtani, T., Nishi, M., Takeshima, H., Nukuda, T. and Sugimoto, T. (1996) Biochem. Biophys. Res. Commun. 219, 714–719.
[12] Nothacker, H.P., Reinscheid, R.K., Mansour, A., Henningsen, R.A., Ardati, A., Monsma Jr., F.J., Watson, S.J. and Civelli, O. (1996) Proc. Natl. Acad. Sci. USA 93, 8677–8682.
[13] Mollereau, C., Simons, M.J., Soularue, P., Liners, F., Vassart, G., Meunier, J.C. and Parmentier, M. (1996) Proc. Natl. Acad. Sci. USA 93, 8666–8670.
[14] Stanfa, L.C., Chapman, V., Kerr, N. and Dickenson, A.H. (1996) Br. J. Pharmacol. 118, 1875–1877.
[15] Roques, B.P., Noble, F., Daugé, V., Fournié-Zaluski, M.C. and Beaumont, A. (1993) Pharmacol. Rev. 45, 87–146.
[16] Montiel, J.L., Cornille, F., Roques, B.P. and Noble, F. (1997) J. Neurochem. 68, in press.
[17] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[18] Acker, G.R., Molineaux, C. and Orlowski, M. (1987) J. Neurochem. 48, 284–292.
[19] Mentlein, R. and Dahms, P. (1994) J. Neurochem. 62, 27–36.
[20] Dooley, C.T. and Houghten, R.A. (1996) Life Sci. 59, PL23–PL29.
[21] Reinscheid, R.K., Ardati, A., Monsma, F.J. Jr. and Civelli, O. (1996) J. Biol. Chem. 271, 14163–14168.
[22] Fournié-Zaluski, M.C., Chaillet, P., Bouboutou, R., Coulaud, A., Chérot, P., Waksman, G., Costentin, J. and Roques, B.P. (1984) Eur. J. Pharmacol. 102, 525–528.
[23] Jiraceck, J., Yitakis, A., Vincent, B., Lecoq, A., Nicolaous, A., Checler, F. and Dive, V. (1995) J. Biol. Chem. 270, 21701–21706.