ELSEVIER

European Journal of Pharmacology 325 (1997) 29–34

ejp

Modulation of enkephalin release by nociceptin (orphanin FQ)

Alan R. Gintzler [a,*], Indira D. Adapa [b], Lawrence Toll [b], Victor M. Medina [a], Lin Wang [a]

[a] *Box 8, Department of Biochemistry, State University of NY, Health Sciences Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, USA*
[b] *Life Sciences Division, SRI International, Menlo Park, CA 94025, USA*

Received 26 August 1996; revised 27 January 1997; accepted 4 February 1997

Abstract

Nociceptin (orphanin FQ) is an endogenous peptide agonist for the newly discovered receptor (opioid receptor-like 1 receptor, ORL_1) that bears striking homology to opioid receptors. Initial reports claimed that this peptide had hypoalgesic effects following i.c.v. or i.t. administration. The present study demonstrates that, in the presence of opioid receptor blockade, nociceptin can substantially alter the magnitude of the stimulated release of methionine-enkephalin from the guinea pig myenteric plexus. This effect is concentration dependent. Low doses (1 or 10 nM) inhibit whereas higher concentrations (100 or 1000 nM) enhance evoked enkephalin release. In contrast, in the absence of opioid receptor blockade, a statistically significant inhibition of stimulated enkephalin release is observed in response to 1, 100 or 1000 nM nociceptin. However, the magnitude of this effect did not differ among these concentrations. Furthermore, at 10 nM nociceptin, either an inhibition or enhancement of stimulated enkephalin release is manifest. The ability of naloxone to alter the nociceptin modulation of enkephalin release suggests that a component of the nociceptin modulation of enkephalin release is mediated via opioid receptors. This is consistent with the observation that this peptide has modest affinity for opioid receptors ($\mu > \kappa > \delta$) which, under appropriate conditions, should be sufficient to permit interactions with multiple opioid receptor types. This complicates dose responsiveness for nociceptin since both the naloxone-resistant (ORL_1-mediated) and naloxone-sensitive (opioid receptor-mediated) component exhibit a concentration-dependent bimodality (albeit in opposite directions). Determination of i.c.v. or i.t. nociceptin dose responsiveness over several orders of magnitude is suggested before concluding the physiological effects of this peptide.

Keywords: Nociceptin; Pain threshold; Opioid receptor; Endorphin

1. Introduction

Opioid receptor-like 1 receptor (ORL_1) is a newly discovered G-protein-coupled receptor that has striking amino acid sequence homology to opioid receptors (Mollereau et al., 1994; Wang et al., 1994; Lachowicz et al., 1995). Recently, an endogenous agonist for this receptor (termed orphanin FQ or nociceptin) has been isolated and its sequence determined (Reinscheid et al., 1995; Meunier et al., 1995). This substrate for ORL_1 is a heptadecapeptide that resembles dynorphin A. The synthetic heptadecapeptide is a potent inhibitor of forskolin-stimulated accumulation of cAMP in the recombinant Chinese hamster ovary (CHO)/ORL_1 cell line. This effect is not altered by opioid agonists or antagonists, despite the high degree of homology between ORL_1 and opioid receptors.

The first demonstrated physiological action of nociceptin appears to be increased responsiveness to nociceptive stimuli. In the initial study, this function was inferred from the effect of intracerebroventricular (i.c.v.) application of either an antisense oligonucleotide to ORL_1 mRNA (Meunier et al., 1995) or i.c.v. application of the newly discovered peptide (Reinscheid et al., 1995; Meunier et al., 1995). In the former case, increased latencies to rearing and escape jumping were observed. In contrast, decreased jumping (Meunier et al., 1995) or tail flick (Reinscheid et al., 1995) latencies were observed following i.c.v. administration of the peptide itself. To date, however, the ability of nociceptin to alter the release of neurotransmitters known to modulate pain thresholds has not been demonstrated.

Accordingly, the present study examines the ability of nociceptin to modulate the stimulated release of methionine-enkephalin from the guinea pig myenteric plexus. The effect of nociceptin on the release of this opioid peptide was studied since the ability of enkephalin to mediate antinociception has been well documented (Heyman et al., 1988; Millan, 1986; Tung and Yaksh, 1982). The myenteric plexus was selected for study since it contains a significant intrinsic enkephalinergic innervation (Schultzberg et al., 1978) which is subject to opioid receptor-cou-

* Corresponding author. Tel.: (1-718) 270-2129; Fax: (1-718) 270-3316.

0014-2999/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved.
PII S0014-2999(97)00103-9

pled regulation (Xu et al., 1989; Gintzler and Xu, 1991; Glass et al., 1986; Gintzler et al., 1987). Moreover, the myenteric plexus contains the three predominant types of opioid receptor which are strikingly similar, if not identical, to those found centrally. Thus, any interaction between nociceptin and opioid receptors should be observable.

2. Materials and methods

2.1. Tissue preparation and experimental protocol

'Strips' of the longitudinal muscle with adherent myenteric plexus were prepared, mounted and superfused in a stimulating chamber, as described previously (Xu et al., 1989; Gintzler and Xu, 1991; Glass et al., 1986). The oxygenated (95% O_2/5% CO_2) Krebs' solution used to superfuse the tissue contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 11 mM glucose, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4. Additionally, captopril (10 mM), thiorphan (0.3 mM), bestatin (10 mM) and L-leucyl-L-leucine (2 mM) were added to protect against the action of proteases (McKnight et al., 1983). These protease inhibitors were present during the 30 min equilibration period before the start of each experiment and thereafter for its duration. Under the conditions of superfusion employed, 74% of standard ^{3}H-labeled enkephalin that was added to the Krebs' solution and then passed over the tissue during electrical stimulation was recovered in the form of either authentic enkephalin or its sulfoxide derivative (Glass et al., 1986).

The electrically induced increase in the rate of [Met^5]enkephalin release was measured first in the absence (cycle A), 3 min following pretreatment with varying concentrations of nociceptin while still in its presence (cycle B), and following a 15 min washout (cycle C). All 3 cycles were obtained using the same longitudinal muscle myenteric plexus preparation. In each cycle, tissue superfusate was collected before, during and after a 30 s period of electrical stimulation (40 Hz, 0.2 ms pulse duration). Fractions (0.5 ml) were collected on ice and oxidized immediately with hydrogen peroxide (0.3% at 4°C overnight) after which each sample was aliquoted in duplicate (100 μl each) and lyophilized to dryness. While in the presence of the above cocktail of protease inhibitors, nociceptin was not metabolized during its exposure to the longitudinal muscle myenteric plexus tissue via continuous superfusion. Reverse phase high pressure liquid chromatography fractionation (4–70% acetonitrile over 28 min) of [^{125}I]nociceptin that had been incubated with longitudinal muscle myenteric plexus tissue for 30 s (37°C; the time required to traverse the tissue chamber) indicated that $>$ 95% of the radiolabeled peptide eluted at a retention time corresponding to that of radiolabeled peptide not previously incubated with longitudinal muscle myenteric plexus tissue.

2.2. Determination of enkephalin content in longitudinal muscle myenteric plexus superfusate

Enkephalin-like immunoreactivity was determined using a radioimmunoassay (RIA) procedure that utilized an antibody generated against enkephalin sulfoxide as described previously (Kumar et al., 1990). A standard curve (1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 500 pg/assay tube) in which the percentage of inhibition of binding was plotted against the log of unlabeled oxidized enkephalin in the reaction tube was generated in each experiment. The concentration of salt that is contained in each unknown sample does not affect the appearance of the standard curve. The minimum detectable concentration is approximately 2 pg, at which approximately a 10% inhibition of binding is observed. Cross-reactivity with leucine enkephalin, α-endorphin, β-endorphin, γ-endorphin, was less than 0.1% (Kumar et al., 1990). High pressure liquid chromatography fraction in combination with RIA indicated that greater than 90% of enkephalin-like immunoreactivity is authentic [Met^5]enkephalin (Glass et al., 1986).

The percent rise above basal release in each cycle was calculated by subtracting the mean basal release (obtained prior to stimulation over 4 or 5 collection periods of 30 s each) from the peak release observed during electrical stimulation and dividing the difference by the mean basal release ((stimulated − basal)/basal). The percent rise (mean ± S.E.M.) above basal release observed in the presence of drug (cycle B) is expressed relative to that observed in its absence (cycle A). In order to determine the effect of opioid receptor blockade on nociceptin modulation of [Met^5]enkephalin release, all three cycles of release were obtained following a 3 min pretreatment with naloxone (1 μM) prior to electrical stimulation, after which it was immediately washed out. In cycle B, naloxone and nociceptin were added simultaneously. Thus, any effect of naloxone on release of enkephalin should not confound data interpretation.

2.3. Determination of nociceptin affinity at opiate receptors

Binding affinity to opioid receptors was determined using guinea pig brain membranes. Briefly, brains were removed from Hartley guinea pigs and homogenized in 50 mM Tris buffer, pH 7.5, using a polytron homogenizer. The homogenate was centrifuged at 40 000 × g for 15 min, re-homogenized and centrifuged once more. Incubations were for 1 h at 25°C and contained 6 mg original wet weight of tissue per ml. Samples were filtered over glass fiber filters using a cell harvester (Brandel). Radioligands used were [^{3}H][D-Ala^2,N-Me-Phe^4,Gly-ol]enkephalin (DAMGO; μ), [^{3}H][D-Pen^2,pClPhe^4,D-Pen^5]enkephalin (pCl-DPDPE; δ), [^{3}H][(5α,7α,8β)-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide] (U69,593; κ_1), [^{3}H]bremazocine (κ_2) and [^{3}H]naloxone

benzoylhydrate ([^{3}H]NalBzOH; κ_3). The incubation for κ_2 binding contained DAMGO, DPDPE and U69,593 (100 nM each) to prevent binding to μ, δ and κ_1 sites (Clark et al., 1989). The incubation for κ_3 binding contained 100 nM U69,593 and 5 mM EDTA to allow for selective binding to this site (Webster et al., 1993). K_i values were calculated according to the relationship $K_i = IC_{50}/(1 + [L]/K_d)$ where K_d is the dissociation constant of the radioligand and [L] represents the concentration of the radioligand (Cheng and Prusoff, 1973).

2.4. Data analyses

Significance of inhibitory or facilitatory actions of nociceptin within each group (with or without naloxone) was determined using Student's *t*-test. Differences among groups were determined by multiple analysis of variance (MANOVA). Planned comparisons between responses to different doses of nociceptin in naloxone-treated tissue were performed using the Duncan analysis.

2.5. Drugs

Nociceptin was synthesized by Research Genetics (Huntsville, AL, USA). Its purity was greater than 95% as assessed by high-pressure liquid chromatography analysis.

3. Results

3.1. Effects of nociceptin on stimulated and basal enkephalin release following opiate receptor blockade

As reported previously (Gintzler and Xu, 1991), electrical stimulation of untreated longitudinal muscle myenteric plexus tissue produced approximately a 2-fold increase in the rate of [Met^5]enkephalin release (3.3 nmol/min per two longitudinal muscle myenteric plexus strips). Following treatment with naloxone (1 μM for 3 min), electrically stimulated release of [Met^5]enkephalin was unaltered following pretreatment with 0.1 nM nociceptin. However, at higher concentrations (1, 10, 100, 1000 nM), significant effects were observed (Fig. 1A). The magnitude of this effect differed among the concentrations employed ($F(3,15) = 11.03$; $P < 0.001$). The nature of this modulation, however, is significantly different for high vs. low concentrations of nociceptin ($P < 0.001$ for 1 or 10 nM vs. 100 or 1000 nM). One or 10 nM nociceptin reduced the magnitude of evoked enkephalin release ($t(3) = 4.13$ and $t(4) = 3.19$, respectively; $P < 0.04$). A similar pretreatment with 100 or 1000 nM concentration also results in the modulation of the magnitude of enkephalin release. The direction of this modulation, however, is in the opposite direction, i.e., an enhancement of the magnitude of the increase in evoked enkephalin release is observed ($t(4) = -4.13$ and $t(4) = -3.14$; $P < 0.04$).

Experiments were also conducted to determine if the naloxone alteration of nociceptin modulation of enkephalin release was due to an effect on basal enkephalin release. In contrast to evoked enkephalin release, basal release remained unchanged following treatment with nociceptin while in the presence of naloxone (data not shown).

This study did not determine the effect of naloxone itself on the basal or stimulated release of enkephalin. However, it was present through out all three cycles of release. Thus, even if it were to have an effect on enkephalin release, it would not confound interpretation of results.

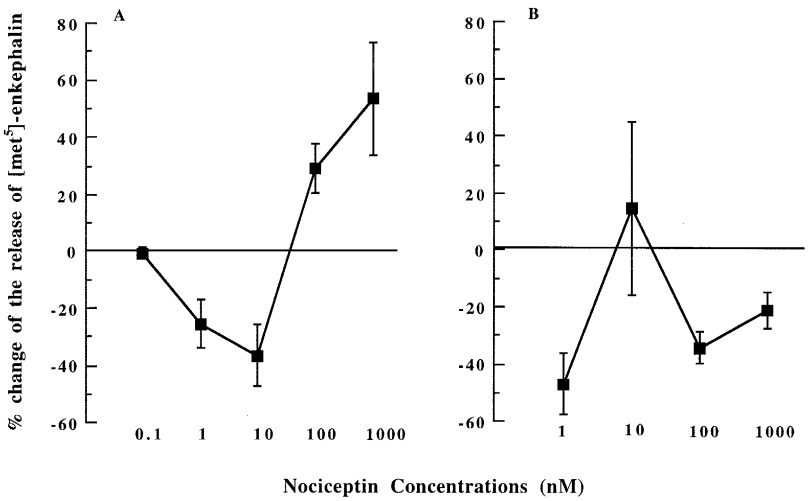

Fig. 1. Effect of nociceptin on stimulated enkephalin release in (A) the presence or (B) the absence of opioid receptor blockade. Treatment with naloxone (1 μM) was begun 3 min prior to electrical stimulation (concomitantly with nociceptin). Basal and stimulated release of [Met^5]enkephalin was quantitated before, during and after pretreatment (3 min) with the indicated concentration of nociceptin. The percent change in the magnitude of release induced by nociceptin is calculated as described in Section 2. $n = 3$, 4, 5, 5 and 5, for 0.1, 1, 10, 100 and 1000 nM nociceptin, respectively, in the presence of naloxone. $n = 5$, 8, 6, 5 for 1, 10, 100 and 1000 nM nociceptin, respectively, in the absence of naloxone.

Table 1
Affinity (K_i) of nociceptin and Tyr^{14}-nociceptin at opioid receptors

Opiate ligand	[^{3}H]DAMGO	[^{3}H]pCl-DPDPE	[^{3}H]U69,593	[^{3}H]Bremazocine	[^{3}H]NalBzOH
	μ	δ	κ_1	κ_2	κ_3
Nociceptin	133 ± 30	380 ± 21	267 ± 10	> 10 000	456 ± 150
Tyr^{14}-Noc [a]	163 ± 60	380 ± 13	321 ± 45	> 10 000	490 ± 75
DAMGO	1.1 ± 0.2	180 ± 16	1841 ± 22	> 10 000	26.9 ± 0.85
pCl-DPDPE	> 10 000	2.8 ± 0.4	> 10 000	> 10 000	> 1000
U69,593	692 ± 97	1358 ± 118	0.7 ± 0.05	6763 ± 417	1100 ± 2
Bremazocine	0.1 ± 0.01	0.3 ± 0.07	0.1 ± 0.03	2.2 ± 0.1	0.1 ± 0.02
NalBzOH	0.2 ± 0.01	1.4 ± 0.13	0.4 ± 0.1	66.5 ± 12	0.3 ± 0.07

Affinities at each of the sites were determined as described in Section 2. The K_i (nM) values shown are the means ± S.D. obtained from 3 experiments.
[a] Tyr^{14}-nociceptin.

3.2. Effects of nociceptin on the stimulated release of Met-enkephalin in the absence of opiate receptor blockade

In the absence of naloxone, the manifestation of nociceptin modulation of enkephalin release was substantially different from that observed in the presence of opioid receptor blockade (Fig. 1B). A two-way MANOVA indicated a significant interaction between groups (with or without naloxone) and dosage ($F(3,35) = 6.32,\ P < 0.01$). In the absence of opioid receptor blockade, the inhibition produced by 1 nM nociceptin was still manifest [$t(4) = 3.03,\ P < 0.04$]. Moreover, the magnitude of this inhibitory effect did not differ from that observed while in the presence of naloxone [$t(7) = 1.12,\ P > 0.2$]. However, at 10 nM concentration, the consistent inhibition of enkephalin release (observed in 5 out of 5 experiments while in the presence of naloxone) was no longer consistently manifest. Instead, in the absence of opioid receptor blockade, either an inhibition or enhancement of release was observed. In a total of 8 experiments, 50% of the responses were inhibitory (50 ± 4.5%) but the remainder manifested facilitatory responses (79 ± 39%). Moreover, instead of the facilitation of release observed in response to 100 or 1000 nM nociceptin while in the presence of naloxone, an inhibition of evoked release was now manifest [$t(5) = 6.85$ and $t(4) = 3.27;\ P < 0.04$]. The magnitude of inhibitory responses to nociceptin (1, 100 or 1000 nM) in the absence of opioid receptor blockade ranged from 47 ± 11% to 21 ± 6.4%. These differences, however, were not statistically significant [$F(2,15) = 2.80,\ P > 0.1$].

Experiments were conducted to determine if the nociceptin-induced negative modulation of stimulated enkephalin release, in the absence of naloxone, is due to alteration of basal activity or to the inhibition of release in response to electrical stimulation. In contrast to the stimulated release of [Met^5]enkephalin, basal release was not affected by the presence of nociceptin. Regardless of the concentration of nociceptin employed, basal release remained unchanged ($P > 0.09$; data not shown).

3.3. Affinity of nociceptin to opiate receptors

In order to better understand the apparent opioid receptor-mediated effect of nociceptin on enkephalin release, the affinity of nociceptin was determined at several types of opioid receptor. Moreover, the affinity of the Tyr^{14} analog of nociceptin at opioid receptors was also determined since it is commonly used as the radiolabelled ligand in binding experiments. As seen in Table 1, nociceptin and its Tyr^{14} analog have low to moderate affinity for several of the types of opioid receptor tested. Of the 5 binding sites tested, nociceptin has highest affinity for μ and second highest for κ. Although the affinity is low compared to its affinity for ORL_1 (Reinscheid et al., 1995; Meunier et al., 1995), it should be sufficient to permit interactions with multiple types of opioid receptors, depending on the local concentration of nociceptin.

4. Discussion

This study reports the first demonstration that nociceptin can modulate the stimulated release of [Met^5]enkephalin. As seen in Fig. 1A, following opioid receptor blockade, low concentrations of nociceptin clearly inhibit the electrically stimulated release of [Met^5]enkephalin whereas a facilitation of release occurs in response to higher concentrations (100 and 1000 nM) of this peptide. Since the endogenous synaptic concentration of nociceptin is currently unknown, it is difficult to determine the physiological relevant action(s) of this peptide. Antagonists selective for the ORL_1 receptor are currently not available. Thus, it is not possible to unequivocally establish that this receptor mediates the naloxone-insensitive bimodal effects of nociceptin on stimulated enkephalin release. However, the recent demonstrations of high levels of [^{3}H]nociceptin binding (A.T. McKnight, personal communication) and nociceptin immunoreactivity (S.J. Watson, personal communication) in guinea pig myenteric plexus underscore the likelihood of this possibility. The ability of an appropriate concentration of naloxone to significantly alter nociceptin responsiveness, long the standard for indicating opioid receptor activity, strongly infers that a component of the nociceptin modulation of enkephalin release is mediated via this receptor(s). In these

studies, a concentration of 1 μM naloxone was selected in order to guarantee that any effect of nociceptin remaining was not opioid mediated and to facilitate comparison with previously reported pharmacological analysis of nociceptin action in isolated rat vas deferentia (Dunnill et al., 1997). An interaction with a naloxone-sensitive receptor is not surprising in light of the moderate affinity of nociceptin at opioid receptors (see Table 1 for comparison with μ-, δ- and κ-selective opioids). Because nociceptin has comparable affinities for μ, δ and κ_1 sites, it could interact with any or all of the types of opioid receptor found in the myenteric plexus. It should be noted that although there are reports suggesting that ORL_1 and κ_3 are splice variants of a common gene (Pan et al., 1995), nociceptin has no higher affinity at this site than at μ, δ or κ_1. Thus, there is no reason to think that the opiate action of nociceptin is preferentially mediated via this site.

Although the affinity of nociceptin for opioid receptors is modest, greater than 50% of the μ-opioid receptors present would be occupied by this peptide at concentrations above 100 nM. With the known high opioid receptor reserve in the myenteric plexus (the spare receptor population for μ and κ types is about 90%; Chavkin and Goldstein, 1984), an opioid receptor-mediated effect could be manifest with a much lower percent occupancy. For example, 0.1 nM sufentanil (resulting in 10% receptor occupancy) is effective in facilitating the stimulated formation of myenteric cAMP (Wang and Gintzler, 1994). Thus, an opioid receptor-mediated effect by nociceptin at a concentration of 10 nM (also resulting in approximately 10% opioid receptor occupancy) is not surprising.

The dose-effect curve for nociceptin modulation of myenteric enkephalin release is complicated by the fact that this peptide exerts effects via an interaction with more than one population of myenteric receptor, i.e., naloxone-resistant receptor (presumably ORL_1) as well as naloxone-sensitive (opioid) types of receptor. Furthermore, the nociceptin effects on enkephalin release that are mediated via each receptor type exhibit bimodality. The bimodality of the former is readily apparent from the dose-effect curve obtained in the presence of opioid receptor blockade (Fig. 1A). The bimodality of the latter is inferred by the nature of the naloxone alteration of the nociceptin dose-effect curve. At 10 nM concentration of nociceptin, the facilitation of enkephalin release that is observed in 50% of the preparations is abolished by naloxone. Thus, in these tissues, the 10 nM nociceptin interaction with opioid receptors mediates enhancement of enkephalin release. In contrast, at 100 and 1000 nM concentrations, nociceptin mediates an inhibition of enkephalin release via opioid receptors. This is demonstrated by the fact that naloxone abolishes the inhibition of enkephalin release observed in the absence of opioid receptor blockade unmasking a facilitatory effect.

The finding that nociceptin can facilitate enkephalin release at low concentrations and inhibit the same at higher concentrations via opioid receptors is consistent with the observation that low doses of sufentanil (nM) enhance whereas higher concentrations (μM) inhibit evoked enkephalin release (Xu et al., 1989; Gintzler and Xu, 1991; Xu and Gintzler, 1992) and cAMP formation (Wang and Gintzler, 1994). It should be pointed out that data interpretation is not confounded by the ability of naloxone, itself, to alter release of enkephalin (Glass et al., 1986) since this antagonist was present throughout all three cycles of release.

It is interesting to note that, in the absence of naloxone, the magnitude of the inhibition of enkephalin release produced by 100 or 1000 nM nociceptin did not differ from that observed in response to 1 nM. This is presumably due to the concomitant interaction of nociceptin with a 'facilitatory' ORL_1 receptor and an inhibitory opioid receptor at the higher two concentrations. In this regard, it should be noted that the concentration dependence of the direction of nociceptin modulation of enkephalin release following opioid receptor blockade (inhibition at low and facilitation at high concentrations) is opposite that which has been reported for the opioid receptor modulation of enkephalin release and cAMP formation from the same preparation (Xu et al., 1989; Gintzler and Xu, 1991; Xu and Gintzler, 1992; Wang and Gintzler, 1994). It is, however, similar to the concentration dependence that has been reported for the bimodal α_2-adrenoceptor modulation of cAMP formation in CHO cells (Eason et al., 1992).

The variability of the response to 10 nM nociceptin in the absence of opioid receptor blockade is perplexing. It is possible that the relative abundance of 'excitatory' opioid receptors and the inhibitory ORL_1 receptor varies among ilea obtained from different guinea pigs. Blockade of the former by naloxone would negate the consequences of this variability and thus result in the consistently observed 10 nM nociceptin inhibition of enkephalin release.

Finally, it should be underscored that while nociceptin was able to modulate the evoked release of enkephalin, no effect was observed on basal release of this opioid. The striking parallels between the myenteric opioid system and those found centrally (Gyang et al., 1964) make it tempting to speculate that similar phenomena might also occur in the central nervous system. Were this to occur, the hyperalgesic actions of centrally administered nociceptin should be expected to be particularly robust when pain thresholds are elevated due, in part, to augmented enkephalin neurotransmission.

Acknowledgements

The authors wish to acknowledge the Scientific and Academic Computing Center, particularly Dr. Reuben Margolis, for statistical consultation and analyses.

References

Chavkin, C. and A. Goldstein, 1984, Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus, Proc. Natl. Acad. Sci. USA 81, 7253.

Cheng, Y.-C. and W.H. Prusoff, 1973, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Clark, J.A., L. Liu, M. Price, B. Hersh, M. Edelson and G.W. Pasternak, 1989, Evidence for two U50,488-sensitive kappa subtypes and a novel kappa$_3$ subtype, J. Pharmacol. Exp. Ther. 251, 461.

Dunnill, R.J., K. Kakizawa, A.T. McKnight and G. Henderson, 1997, Characterization of the actions of naloxone benzoylhydrazone at μ-opioid, κ-opioid, and ORL 1 receptors in isolated tissues from rat and guinea-pig, Br. J. Pharmacol. 119, 275P.

Eason, M.G., H. Kuroses, B.D. Holt, J.R. Raymond and S.B. Liggett, 1992, Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects, J. Biol. Chem. 267, 15795.

Gintzler, A.R. and H. Xu, 1991, Different G proteins mediate the opioid inhibition or enhancement of evoked [5-methionine]enkephalin release, Proc. Natl. Acad. Sci. USA 88, 4741.

Gintzler, A.R., W.C. Chan and J. Glass, 1987, Evoked release of methionine-enkephalin from tolerant/dependent enteric ganglia: paradoxical dependence on morphine, Proc. Natl. Acad. Sci. USA 84, 2537.

Glass, J., W. Chan and A.R. Gintzler, 1986, Direct analysis of the release of methionine-enkephalin from guinea pig myenteric plexus: modulation by endogenous opioids and exogenous morphine, J. Pharmacol. Exp. Ther. 239, 742.

Gyang, E.A., H.W. Kosterlitz and G.M. Lees, 1964, The inhibition of autonomic neuron effector transmission by morphine-like drugs and its use as a screening test for narcotic analgesic drugs, Naunyn-Schmiedeberg's Arch. Exp. Pathol. 248, 231.

Heyman, J.S., J.L. Vaught, R.B. Raffa and F. Porreca, 1988, Can supraspinal delta-opioid receptors mediate antinociception?, Trends Pharmacol. Sci. 9, 134.

Kumar, M.S.A., M. Haney, T. Becker, M.L. Thompson, R. Kream and K. Miczek, 1990, Effect of early exposure to delta-9-tetrahydrocannabinol on the levels of opioid peptides, gonadotropin, and substance P in the adult male rat brain, Brain Res. 525, 78.

Lachowicz, J.E., Y. Shen, F.J. Monsma Jr. and D.R. Sibley, 1995, Molecular cloning of a novel G protein-coupled receptor related to the opioid receptor family, J. Neurochem. 64, 34.

McKnight, A.T., A.D. Corbett and H.W. Kosterlitz, 1983, Increase in potency of opioid peptides after protease inhibition, Eur. J. Pharmacol. 86, 393.

Meunier, J.-C., C. Mollereau, L. Toll, C. Suaudeau, C. Moisand, P. Alvinerle, J.-L. Butour, J.-C. Guillemot, P. Ferrara, B. Monsarrat, H. Mazarguil, G. Vassart, M. Parmentier and J. Costentin, 1995, Isolation and structure of the endogenous agonist of opioid receptor-like ORL$_1$ receptor, Nature 377, 532.

Millan, M.J., 1986, Multiple opioid systems and pain, Pain 27, 303.

Mollereau, C., M. Parmentier, P. Mailleux, J.L. Butour, C. Moisand, P. Chalon, D. Caput, G. Vassart and J.C. Meunier, 1994, ORL$_1$, a novel member of the opioid receptor family. Cloning, functional expression and localization, FEBS Lett. 341, 33.

Pan, Y.-X., J. Cheng, J. Xu, G. Rossi, E. Jacobson, J. Ryan-Moro, A. Brooks, G.E. Dean, K.M. Standifer and G.W. Pasternak, 1995, Cloning and functional characterization through antisense mapping of a κ_3-related opioid receptor, J. Pharmacol. Exp. Ther. 47, 1180.

Reinscheid, R.K., H.-P. Nothacker, A. Bourson, A. Ardati, R.A. Henningsen, J.R. Bunzow, D.K. Grandy, H. Langen, F.J. Monsma Jr. and O. Civelli, 1995, Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor, Science 270, 792.

Schultzberg, M., C.F. Dreyfus, M.D. Gershon, T. Hokfelt, R. Elde, G. Nilsson, G. Said and M. Goldstein, 1978, Vip-, enkephalin-, substance P-, and somatostatin-like immunoreactivity in neurons intrinsic to the intestine: immunohistochemical evidence from organotypic tissue cultures, Brain Res. 155, 239.

Tung, A.S. and T.L. Yaksh, 1982, In vivo evidence for multiple opioid receptors mediating analgesia in the rat spinal cord, Brain Res. 247, 75.

Wang, L. and A.R. Gintzler, 1994, Bimodal opioid regulation of cAMP formation: implications for positive and negative coupling of opioid receptors to adenylyl cyclase, J. Neurochem. 63, 1726.

Wang, J.B., P.S. Johnson, Y. Imai, A.M. Persico, B.A. Ozenberger and C.M. Eppler, 1994, cDNA cloning of an orphan opioid receptor gene family member and its splice variant, FEBS Lett. 348, 75.

Webster, J.L., W.E. Polgar, S.R. Brandt and I.P. Berzetei-Gurske, 1993, Comparison of kappa 2-opioid receptors in guinea pig brain and guinea pig ileum membranes, Eur. J. Pharmacol. 231, 251.

Xu, H. and A.R. Gintzler, 1992, Opioid enhancement of evoked [Met^5]enkephalin release requires activation of cholinergic receptors: possible involvement of intracellular calcium, Proc. Natl. Acad. Sci. USA 89, 1978.

Xu, H., I. Smolens and A.R. Gintzler, 1989, Opioids can enhance and inhibit the electrically evoked release of methionine-enkephalin, Brain Res. 504, 36.