Quantitative study of [Tyr^{10}]nociceptin/orphanin FQ (1-11) at NOP receptors in rat periaqueductal gray and expressed NOP receptors in HEK293 cells

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Aim: The nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor was reported to be functionally heterogeneous. We investigated if [Tyr^{10}]N/OFQ(1-11), a peptide ligand reported to selectively bind to the high affinity site of 125I-[Tyr^{14}]N/OFQ in rodent brains, can be a tool for revealing the NOP receptor heterogeneity. We have previously found an NOP receptor subset insensitive to Ro 64-6198 and (+)-5a Compound, two non-peptide NOP agonists, in rat ventrolateral periaqueductal gray (vlPAG) neurons. Here, we examined if [Tyr^{10}]N/OFQ(1-11) differentiated (+)-5a Compound-sensitive and -insensitive vlPAG neurons. Certain mu-opioid (MOP) receptor ligands highly competing with [Tyr^{10}]N/OFQ(1-11) in binding studies also showed high affinity at expressed heteromeric NOP–MOP receptors. We also examined if [Tyr^{10}]N/OFQ(1-11) distinguished heteromeric NOP–MOP receptors from homomeric NOP receptors.

Methods: The NOP receptor activity was evaluated by G-protein coupled inwardly rectifying potassium (GIRK) currents in rat vlPAG slices, and by inhibition of cAMP accumulation in HEK293 cells expressing NOP receptors or co-expressing NOP and MOP receptors.

Key findings: In vlPAG neurons, [Tyr^{10}]N/OFQ(1-11), like N/OFQ, induced GIRK currents through NOP receptors. It was less potent (EC_{50}: 8.98 μM) but equi-efficacious as N/OFQ. [Tyr^{10}]N/OFQ(1-11) displayed different pharmacological profiles as (+)-5a Compound, and was effective in both (+)-5a Compound-sensitive and -insensitive vlPAG neurons. In NOP-expressing HEK293 cells and NOP- and MOP-co-expressing cells, [Tyr^{10}]N/OFQ(1-11) displayed similar concentration–response curves in decreasing cAMP accumulation.

Significance: [Tyr^{10}]N/OFQ(1-11) is an NOP full agonist and less potent than N/OFQ. However, it can neither reveal the functional heterogeneity of NOP receptors in vlPAG neurons nor differentiate heteromeric NOP–MOP and homomeric NOP receptors.

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Introduction

Nociceptin (Meunier et al., 1995), also named orphanin FQ (Reinscheid et al., 1995) (N/OFQ), is a heptadecapeptide agonist of N/OFQ peptide (NOP) receptors, a branch of opioid receptor family with little affinity for traditional opioids (Mollerau et al., 1994). N/OFQ and NOP receptors are widely distributed in the brain and involved in many biological functions (Chiou et al., 2007; Lambert, 2008).

N/OFQ(1-11) is an active metabolite of N/OFQ (Rossi et al., 1997). Unlike N/OFQ, which can be pronociceptive and antinociceptive depending on injection sites, N/OFQ(1-11) usually is antinociceptive. Like N/OFQ, N/OFQ(1-11) was antinociceptive in the tail-flick test when administered intrathecally (King et al., 1997), reduced capsaicin-induced nociception when given by intraplantar injection (Sakurada et al., 2005), and attenuated morphine-withdrawal syndrome intracerebroventricularly (i.c.v.) (Kotilnska et al., 2004). However, unlike N/OFQ (i.c.v.) which is pronociceptive (Rossi et al., 1997), N/OFQ(1-11) (i.c.v.) was antinociceptive (Mathis et al., 1998; Rossi et al., 1997).

Binding studies using 125I-[Tyr^{10}]N/OFQ(1-11) and 125I-[Tyr^{14}]N/OFQ as radioligands showed that the binding density of 125I-[Tyr^{10}]N/OFQ(1-11) was less than that of 125I-[Tyr^{14}]N/OFQ in rodent brains (Letchworth et al., 2000; Mathis et al., 1999). Two (high and low affinity) binding sites for 125I-[Tyr^{14}]N/OFQ in rodent brains were suggested from a saturation binding study (Mathis et al., 1997). Based on the maximal binding density, the binding site of 125I-[Tyr^{14}]N/OFQ(1-11) was proposed to be the high affinity site for 125I-[Tyr^{14}]N/OFQ (Mathis et al., 1999).

N/OFQ(1-11) usually is antinociceptive.
Pan et al. (2002) demonstrated that, heterodimeric NOP and mu-opioid (MOP) (NOP–MOP) receptors formed in Chinese Hamster Ovary (CHO) cells, as compared with NOP homomorphic receptors, displayed similar affinity for N/OFQ but had higher affinity for some opioid receptor ligands, such as NaBzOH, fentanyl and dynorphin. Interestingly, those ligands also displayed high affinity at the binding site of 125I-[Tyr10]N/OFQ(1-11) in the mouse brain (Mathis et al., 1999).

Previously, we have demonstrated that NOP receptors are functionally heterogeneous in rat ventrolateral periaqueductal gray (vPAG) neurons using Ro 64-6198 and (+)-5a Compound, two non-peptide NOP agonists (Chiou et al., 2004; Liao et al., 2011b). Both compounds were ineffective in one subset of NOP receptors while N/OFQ affected NOP receptors in almost all of recorded vPAG neurons (Chiou et al., 2004; Liao et al., 2011b).

In the PAG, the binding density of 125I-[Tyr10]N/OFQ(1-11) is one-sixth of that of 125I-[Tyr14]N/OFQ (Letchworth et al., 2000). We, therefore, hypothesize that the binding site for 125I-[Tyr14]N/OFQ(1-11) is the NOP receptor sensitive to Ro 64-6198/(+-)-5a Compound in the vPAG and the NOP-MOP receptor. To verify these hypotheses, we synthesized [Tyr18]N/OFQ(1-11), (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Tyr-Ala), which was more selective to the binding site of 125I-[Tyr18]N/OFQ(1-11) than to the site of 125I-[Tyr14]N/OFQ, as compared with N/OFQ(1-11) (Letchworth et al., 2000; Mathis et al., 1999), and examined if [Tyr18]N/OFQ(1-11) can reveal the NOP receptor heterogeneity. We have characterized pharmacological properties of [Tyr18]N/OFQ(1-11) quantitatively and examined its interactions with (+)-5a Compound and N/OFQ in vPAG neurons, and investigated whether the pharmacological profiles of [Tyr18]N/OFQ(1-11) in decreasing cAMP accumulation are different between human-embryonic-kidney 293 (HEK293) cells expressing NOP receptors only and those co-expressing NOP and MOP receptors.

Materials and methods

Brain slice preparations

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University. All reasonable efforts were made to minimize the number of animals used. The preparation of midbrain periaqueductal gray slices, electrophysiological recordings and data analysis was similar to our previous study (Liao et al., 2011a, 2011b).

Wistar rats of 9–18 day-old were decapitated using a guillotine, the midbrain blocks containing the PAG were rapidly dissected and cut into 300 μm-thick coronal slices using a vibrotome (Microlisier DTK-100, Dosaka). Slices were immediately transferred to a submerged chamber and equilibrated at room temperature in oxygenated (95% O2/5% CO2) artificial cerebral spinal fluid (aCSF), which consisted of (in mM) 117 NaCl, 4.5 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 25 NaHCO3 and 11.4 dextrose (pH 7.4). After equilibration for at least 1 h, the slice was mounted on a submerged recording chamber and continuously perfused with aCSF at a rate of 2–3 ml/min.

Electrophysiology

Blind patch-clamp whole cell recording was performed with 4–8 MΩ glass microelectrodes filled with the internal solution of the following composition (in mM): 125 K+ gluconate, 5 KCl, 0.5 CaCl2, 5 BaCl2, 10 HEPES, 5 MgATP and 0.33 GTPtris (pH 7.3). To study if [Tyr18]N/OFQ(1-11) increased the GIRK current, a hyperpolarization voltage ramp protocol was applied every 30 s. The recorded neurons were held at −70 mV, stepped to −100 mV for 100 ms, ramped from −60 mV to −140 mV for 400 ms, and then stepped back to −70 mV (Fig. 1, inset). The membrane currents elicited by voltage ramps were acquired through an Axopatch 700A amplifier (Molecular Devices/ Axon Instruments, Union City, CA) with a pClamp 7 software (Molecular Devices/Axon Instruments, Union City, CA) and simultaneously recorded with a chart recorder (Gould RS3200) to monitor the time course of drug effects. Only those neurons with unchanged access resistance (10–15 MΩ) before and after drug treatments were accepted to ensure that the clamp efficiency was not deteriorated during the recording period.

Data analyses

The effect of an NOP receptor agonist was quantified by the percent increment of the membrane current at −140 mV (I−140), taking its own I−140 before treatment as 100%. An increment of more than 5% and the induced current having a reversal potential at around −90 mV (the equilibrium potential of K+ ions) was considered to be effective. For establishing the concentration–response curves of [Tyr18]N/OFQ(1-11), the percent increment of I−140 in each neuron was normalized to the maximal effect (Emax) produced by 1 μM N/OFQ, which was 39.4%±4% increment (n=26) (Chiou et al., 2002). The EC50 of [Tyr18]N/OFQ(1-11) was determined by the reflection point of its concentration–response curve produced by logistic fitting. To investigate the interaction of a receptor antagonist with [Tyr18]N/OFQ(1-11) in the same neuron, the antagonist was applied after the response to [Tyr18]N/OFQ(1-11) had reached a steady state, which usually took 15–20 min. To examine whether (+)-5a Compound can occlude the effect of [Tyr18]N/OFQ(1-11), [Tyr18]N/OFQ(1-11) was tested in the same neuron which had been treated with (+)-5a Compound for 15–20 min.

cAMP assay in HEK293 cells expressing NOP and MOP receptors

The homogeneous time-resolved fluorescence (HTRF) cAMP assay was performed in HEK293 cells stably transfected with human NOP and MOP receptors, alone or in combination, as described previously (Lee et al., 2011). Briefly, HEK293 cells were dispensed with compound buffer in 96 half-well plates (Costar, Corning, NY, USA) on the day of the experiment. After an incubation of 1 h at 37 °C in a humidified 5% CO2 incubator, 10 μM forskolin and desired concentrations of drugs were added to the cells, followed by 30-min incubation at room temperature. Subsequently, cells were lysed and cAMP concentrations were determined by the HTRF detection kit (cAMP HiRange: Cisbio, Bagnols/Cèze Cedex, France). The EC50s of [Tyr18]N/OFQ(1-11) and N/OFQ were determined using logistic fitting equations.

Chemicals

[Tyr18]N/OFQ(1-11) was synthesized by Kelowna International Scientific Inc. (Taipei, Taiwan). (+)-5a Compound and Ro 64-6198 were kindly provided by Hoffmann-La Roche, Basel, Switzerland, UFP-101 by Drs. Calo’ and Guerrini, University of Ferrara, Ferrara, Italy, and SB-612111 by Drs. Toll and Jiang, SRI International, Menlo Park, CA. N/OFQ was purchased from Tocris (Bristol, UK). Naloxone, baclofen, forskolin and isobutylmethylxanthine were purchased from Sigma (St. Louis, MO). [Tyr18]N/OFQ(1-11), (+)-5a Compound, Ro 64-6198, SB-612111 and isobutylmethylxanthine were dissolved in dimethylsulfoxide (DMSO) and other drugs were dissolved in de-ionized water. The final concentration of DMSO was kept below 0.1%, which did not affect the membrane currents elicited by voltage ramps (Chiou et al., 2004) or cAMP assays (Lee et al., 2011).

Statistical analyses

Data were presented as mean±SEM. The n number is the number of tested neurons or cells. The Student’s t-test was used for statistical analysis of differences between groups and paired t-test was used for the difference within the same group. One sample t-test was used to analyze the change in a treatment group, as compared with its control.
value (0%). Differences were considered to be significant if a p value <0.05.

Results

[Tyr10]N/OFQ(1-11) activated GIRK channels in vlPAG neurons

[Tyr10]N/OFQ(1-11) (100 μM) shifted the holding current (Ihold in Fig. 1A) outwardly and increased the membrane current elicited by hyperpolarization ramps from −60 to −140 mV voltage-dependently in vlPAG neurons (Fig. 1B). The currents increased at more negative potentials were greater than those at less negative potentials. Thus, the current–voltage (I–V) relationship of [Tyr10]N/OFQ(1-11)-induced current, which was obtained by subtracting the currents in the control from that in the presence of [Tyr10]N/OFQ(1-11), is characterized with inward rectification (Fig. 1C). The reversal potential of [Tyr10]N/OFQ(1-11)-induced current was −92.4±1.8 mV (n=40), which corresponds to the equilibrium potential of potassium ions (−91 mV) according to the Nernst equation. Therefore, in vlPAG neurons, [Tyr10]N/OFQ(1-11), like N/OFQ (Liao et al., 2011a, 2011b), activated IRK channels which are coupled to G-protein (Ikeda et al., 1997).

[Tyr10]N/OFQ(1-11) was as efficacious as, but less potent than, N/OFQ

The effect of [Tyr10]N/OFQ(1-11) (3–300 μM) on GIRK channels was concentration-dependent. To establish its concentration–response curve (triangle symbols, Fig. 2A), the magnitude of GIRK channel activation induced by [Tyr10]N/OFQ(1-11) was quantified from the increment of I−90−140 as described in Materials and methods. The maximal increment was induced by 100 μM [Tyr10]N/OFQ(1-11), which was 34.9±5.5% (n=22) and similar to the maximal effect induced by N/OFQ (1 μM), being 39.4±4% (n=26), in the same preparation (Chiou et al., 2002).

In order to compare the potency of [Tyr10]N/OFQ(1-11) with that of N/OFQ, Ro 64-6198 and (+)-5a Compound, the increment of [Tyr10]N/OFQ(1-11) was normalized to the maximal increment (39.4±4%), which was produced by 1 μM N/OFQ (Chiou et al., 2002), and expressed as the percentage of the maximal effect of N/OFQ in Fig. 2A. The estimated EC50 value of [Tyr10]N/OFQ(1-11) is 8.98±0.85 μM, which is about 173 times lower than that of N/OFQ, 52.0±6.8 nM (Chiou et al., 2002) obtained in the same preparations. [Tyr10]N/OFQ(1-11) is also less potent than Ro 64-6198 or (+)-5a Compound (Fig. 2A).

The effect of [Tyr10]N/OFQ(1-11) was antagonized by UFP-101, but not naloxone

To verify if the effect of [Tyr10]N/OFQ(1-11) is mediated through NOP receptors, UFP-101, which competitively antagonized the effect of N/OFQ in the same preparation (Chiou et al., 2005), was applied after the effect of [Tyr10]N/OFQ(1-11) had reached the steady state. UFP-101 reduced the current induced by [Tyr10]N/OFQ(1-11) but did not change its reversal potential (Fig. 1B). The I−90−140 induced by [Tyr10]N/OFQ(1-11) (100 μM) was significantly reduced by UFP-101 (1 μM) from 132.1%±5.9% to 118.6±4.7% (n=7, p<0.05, one sample t-test). The reversal potentials of [Tyr10]N/OFQ(1-11)-induced currents in the absence and presence of UFP-101 were −92.4±1.8 mV (n=40) and −90.1±2.7 mV (n=20), respectively. Conversely, the effect of [Tyr10]N/OFQ(1-11) was unaffected by naloxone, a non-selective opioid receptor antagonist. The I−90−140 increments after treatment with 100 μM [Tyr10]N/OFQ(1-11) in the absence or presence of 1 μM naloxone were not significantly different (134.4%±5.1% vs. 134.5%±5.4%, n=6, p=0.96, one sample t-test).

[Tyr10]N/OFQ(1-11) further increased GIRK currents in (+)-5a Compound-sensitive neurons

[Tyr10]N/OFQ(1-11) (3–300 μM) activated GIRK channels in 40/60 of the recorded neurons. This phenomenon appears to be similar to the results obtained with (+)-5a Compound (Liao et al., 2011b) and Ro 64-6198 (Chiou et al., 2004), which also affected the NOP
10 neurons. In (+)-5a Compound-sensitive neurons, (+)-5a Compound reproduced a mean increment of I−140 (123.6% ± 4.2%, n = 12) equivalent to that (118.5% ± 1.9%, n = 26) obtained before (Liao et al., 2011b). In these (+)-5a Compound-sensitive neurons, [Tyr10]N/OFQ(1-11) (100 μM) further increased GIRK currents (Fig. 3A), increasing I−140 from 123.6% ± 4.2% to 135.6% ± 0.3% (n = 12, p < 0.01, one sample t-test), a level that was produced by [Tyr10]N/OFQ(1-11) alone (134.9% ± 5.5%, n = 22). The effect of [Tyr10]N/OFQ(1-11) in these neurons was mediated by NOP receptors, confirmed by the blockade with 1 μM UFP-101 (Fig. 3B).

[Tyr10]N/OFQ(1-11) induced GIRK currents in (+)-5a Compound-sensitive neurons

In those (+)-5a Compound-insensitive neurons, [Tyr10]N/OFQ(1-11) was effective in 8 out of 10 tested neurons. Fig. 3B demonstrates one of these neurons, in which (+)-5a Compound was ineffective, but [Tyr10]N/OFQ(1-11) activated GIRK channels. The mean increment of I−140 was 131.3% ± 5.9% in 8 neurons, which is not different from that produced by 100 μM [Tyr10]N/OFQ(1-11) alone (134.9% ± 5.5%, n = 22).

[Tyr10]N/OFQ(1-11) occluded the effect of N/OFQ

The interaction of [Tyr10]N/OFQ(1-11) with N/OFQ was further investigated. In neurons treated with [Tyr10]N/OFQ(1-11) at the maximal effective concentration (100 μM), further addition of N/OFQ (0.3 μM) failed to cause any additional change in membrane currents (Fig. 4) in all of 7 tested neurons. The I−140 values after treatment with 100 μM [Tyr10]N/OFQ(1-11) were 137.7% ± 3.7% of controls (n = 7), and were 138.1% ± 4.1% (n = 7, p = 0.72, one sample t-test) after further treatment with 0.3 μM N/OFQ. This result suggests that [Tyr10]N/OFQ(1-11) occludes the effect (GIRK channel activation) of N/OFQ in vIPAG neurons.

[Tyr10]N/OFQ(1-11) decreased cAMP accumulation with similar concentration–response curves in NOP cells and NOP–MOP cells

Effects of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound on forskolin-stimulated cAMP formation were compared in HEK293 cells expressing human NOP receptors only (NOP cells) or co-expressing NOP and MOP receptors (NOP–MOP cells). N/OFQ inhibited cAMP formation induced by forskolin (10 μM) in NOP cells and NOP–MOP cells in a similar concentration-dependent manner (Fig. 5A). The mean IC50 value of N/OFQ obtained in NOP cells was 0.05 ± 0.01 nM (n = 3), which is not significantly different from that (0.03 ± 0.01 nM) (n = 3) obtained in NOP–MOP cells. [Tyr10]N/OFQ(1-11) also produced a similar concentration-dependent inhibition of forskolin-stimulated cAMP formation in NOP cells and NOP–MOP cells (Fig. 5A) with IC50 values of 192 ± 91 nM (n = 4) and 500 ± 151 nM (n = 4, p = 0.083, Student t-test), respectively. Interestingly, (+)-5a Compound also produced a similar concentration-dependent inhibition of forskolin-stimulated cAMP formation in NOP cells and NOP–MOP cells (Fig. 5A) with IC50 values of 19 ± 8 nM (n = 3) and 48 ± 15 nM (n = 3, p = 0.738, Student t-test), respectively. The potency of N/OFQ was 3–4 order of magnitude higher than that of [Tyr10]N/OFQ(1-11) in both NOP cells and NOP–MOP cells. This result is in line with the finding in the heterodimeric NOP–MOP receptors expressed on CHO cells (Pan et al., 2002). (+)-5a Compound was 380–1600 times less potent than N/OFQ. In the study of Kolczewski et al. (2003), (+)-5a Compound was 25 folds less potent than N/OFQ in reducing cAMP accumulation in human NOP receptors expressed in HEK293 cells. The efficacy of [Tyr10]N/OFQ(1-11) was comparable to that of N/OFQ at either NOP or NOP–MOP cells (Fig. 5A). The inhibitory effects of [Tyr10]N/OFQ(1-11) (15 μM) on forskolin-induced cAMP formation in NOP cells and NOP–MOP cells,
which were insignificantly different (84.1%±4.7% vs 80.2%±8.2% inhibition, \( p = 0.71 \)) (Fig. 5B); were both significantly antagonized by SB-612111 (1 \( \mu M \)) (Fig. 5B), an NOP receptor selective antagonist (Liao et al., 2011a; Zaratin et al., 2004) which has similar potency and pharmacological profiles as UFP-101 in PAG slices (Chiou et al., 2005; Liao et al., 2011a).

**Discussion**

In this study, we demonstrated that [Tyr\(^{10}\)]N/OFQ(1-11) activates GIRK channels through NOP receptor activation in rat vlPAG neurons. [Tyr\(^{10}\)]N/OFQ(1-11) acted as a full agonist of NOP receptors and was 173 folds less potent than N/OFQ. [Tyr\(^{10}\)]N/OFQ(1-11) occluded the
Reduced after treatment with N/OFQ, [Tyr10]N/OFQ(1-11) or (+)-5a Compound for 311
ordinate is the percent inhibition of forskolin (10 μM)-stimulated cAMP formation in HEK293 cells expressing NOP receptors. (A) Concentration-inhibition curves of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound for 311
min in HEK293 cells stably expressing NOP receptors only or co-expressing NOP and MOP receptors. (B) Effects of 15 μM [Tyr10]N/OFQ(1-11) on both cell lines were antagonized by 1 μM SB-612111. ***p < 0.005 vs. control (0%) (one-sample t-test). Data are mean ± S.E.M. n ≥ 3.

Fig. 5. Inhibitory effects of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound on forskolin-stimulated cAMP formation in HEK293 cells expressing NOP receptors. (A) Concentration-inhibition curves of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound. The ordinate is the percent inhibition of forskolin (10 μM)-stimulated cAMP formation produced after treatment with N/OFQ, [Tyr10]N/OFQ(1-11) or (+)-5a Compound for 30 min in HEK293 cells stably expressing NOP receptors only or co-expressing NOP and MOP receptors. (B) Effects of 15 μM [Tyr10]N/OFQ(1-11) on both cell lines were antagonized by 1 μM SB-612111. ***p < 0.005 vs. control (0%) (one-sample t-test). Data are mean ± S.E.M. n ≥ 3.

The maximal increment of I_{\text{t,i.c.v.}} induced by [Tyr10]N/OFQ(1-11) is comparable to that produced by N/OFQ, suggesting that [Tyr10]N/OFQ(1-11) is a full agonist of NOP receptors in vPAG neurons. However, [Tyr10]N/OFQ(1-11) is 173 folds less potent than N/OFQ. This is in agreement with the finding that N/OFQ(1-11) is 10–726 folds less potent than N/OFQ in cultured cells (Reinscheid et al., 1996; Rossi et al., 1997) and 100 folds less potent in reducing cAMP formation in mouse brain homogenates (Mathis et al., 1997). N/OFQ(1-11) (i.t.) was also less effective than N/OFQ in increasing the mouse tail-flick latency (King et al., 1997). Conversely, N/OFQ(1-11) was more potent than N/OFQ, when given by i.c.v. injection in reducing the mouse tail-flick response (Rossi et al., 1997) or given by intraplantar injection in attenuating capsaicin-induced nociception (Sakurada et al., 2005). The more potent antinociceptive effect of N/OFQ(1-11), as compared with N/OFQ, was suggested to be attributed to its antinociceptive effect mediated by N/OFQ(1-11) (Rossi et al., 1997), but not by the parent compound N/OFQ.

Inhibitory effects of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound on forskolin-stimulated cAMP formation in HEK293 cells expressing NOP receptors only and in those co-expressing NOP and MOP receptors. (A) Concentration-inhibition curves of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound for 30 min in HEK293 cells stably expressing NOP receptors only or co-expressing NOP and MOP receptors. (B) Effects of 15 μM [Tyr10]N/OFQ(1-11) on both cell lines were antagonized by 1 μM SB-612111. ***p < 0.005 vs. control (0%) (one-sample t-test). Data are mean ± S.E.M. n ≥ 3.

Fig. 5. Inhibitory effects of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound on forskolin-stimulated cAMP formation in HEK293 cells expressing NOP receptors. (A) Concentration-inhibition curves of N/OFQ, [Tyr10]N/OFQ(1-11) and (+)-5a Compound. The ordinate is the percent inhibition of forskolin (10 μM)-stimulated cAMP formation produced after treatment with N/OFQ, [Tyr10]N/OFQ(1-11) or (+)-5a Compound for 30 min in HEK293 cells stably expressing NOP receptors only or co-expressing NOP and MOP receptors. (B) Effects of 15 μM [Tyr10]N/OFQ(1-11) on both cell lines were antagonized by 1 μM SB-612111. ***p < 0.005 vs. control (0%) (one-sample t-test). Data are mean ± S.E.M. n ≥ 3.

Tyr10]N/OFQ(1-11) activates GIRK channels via NOP, but not MOP receptors 311

The current induced by [Tyr10]N/OFQ(1-11) had a reversal potential resembling the equilibrium potential of K+ ions and was characterized with inward rectification. Therefore, [Tyr10]N/OFQ(1-11) mimics the action of N/OFQ, the endogenous peptide agonist of NOP receptors, to GIRK channels (Liao et al., 2011a, 2011b; Vaughan et al., 1997). The effect of [Tyr10]N/OFQ(1-11) is mediated through NOP receptors since it was antagonized by UFP-101. The ineffectiveness of naloxone excludes the involvement of opioid receptors in the effect of [Tyr10]N/OFQ(1-11).

[Tyr10]N/OFQ(1-11) is a full agonist of NOP receptors and less potent than N/OFQ.

High and low affinity binding sites of 125I-[Tyr14]N/OFQ were reported in rodent brains (Letchworth et al., 2000; Mathis et al., 1999), and the high affinity site was suggested to be the binding site for 125I-[Tyr10]N/OFQ(1-11) (Mathis et al., 1999). In the rat PAG, the binding density of 125I-[Tyr14]N/OFQ was one sixth of that of 125I-[Tyr10]N/OFQ (Letchworth et al., 2000). We, therefore, suggested that [Tyr10]N/OFQ(1-11) might affect a portion of N/OFQ-sensitive NOP receptors in vPAG neurons, as did (+)-5a Compound or Ro 64-6198 (Chiou et al., 2004; Liao et al., 2011b). However, the present results nullify this hypothesis. First, [Tyr10]N/OFQ(1-11) affected both (+)-5a Compound-sensitive and -insensitive neurons. Second, [Tyr10]N/OFQ(1-11) precluded the effect of N/OFQ in the same neuron, suggesting that [Tyr10]N/OFQ(1-11) affects all the N/OFQ-sensitive NOP receptors.

[Tyr10]N/OFQ(1-11) fails to differentiate homomeric NOP from heteromeric NOP–MOP receptors

Heterodimeric NOP–MOP receptors can be formed by co-expressing both receptors of mice in CHO cells (Pan et al., 2002) and those of rats in HEK293 cells (Wang et al., 2005). A few opioids, such as naloxone benzoylhydrazone (NalBzOH), fentanyl and dynorphin (1-17), with high affinity at expressed heterodimeric NOP–MOP receptors (Pan et al., 2002) also displayed higher affinity at the binding site of 125I-[Tyr10]N/OFQ(1-11), as compared to that of 125I-[Tyr14]N/OFQ in mouse brains (Mathis et al., 1999). We, therefore, hypothesize that the binding site of 125I-[Tyr10]N/OFQ(1-11) might be heteromeric NOP–MOP receptors. However, the results in this study nullify this hypothesis since [Tyr10]N/OFQ(1-11) displayed the same concentration–response curves in decreasing cAMP formation in HEK293 cells expressing NOP receptors only and in those co-expressing NOP and MOP receptors. Interestingly, Ro 64-6198 (Lee et al., 2011) and (+)-5a Compound (the current study), which activated a subset of NOP receptors in vPAG neurons (Chiou et al., 2004; Liao et al., 2011b), also displayed similar potencies and efficacies in both cell lines. In HEK293 cells co-expressing NOP and MOP
receptors, we have demonstrated dense colocalization of these two receptors on cell membrane (Lee et al., 2011). The high colocalization rate suggests the formation of heteromerized NOP-MOP receptors. These results suggest that neither [Tyr10]N/OFQ(1-11) and Ro 64-6198 nor (+)-5a Compound can differentiate NOP–MOP hetermeric receptors from NOP homomerized receptors.

Conclusion

[Tyr10]N/OFQ(1-11) acted as a full agonist of N/OFQ-sensitive NOP receptors and is less potent than N/OFQ in vPAG neurons. It can neither distinguish the subset of NOP receptors sensitive or insensitive to (+)-5a Compound/Ro 64-6198 (Chiou et al., 2004; Liao et al., 2011b) nor differentiate homomeric NOP receptors and NOP–MOP hetermeric receptors, which are very likely formed in cells co-expressing NOP and MOP receptors (Lee et al., 2011). Recently, N/OFQ was found to have the same affinity at the binding site of [125I]Y10-OFQ/N(1-11) in the brains of NOP receptor knock-out mice, as compared with the wild type (Majumdar et al., 2009). Therefore, the functional role of the binding site of [125I]Y10-OFQ/N(1-11) in the brain remains to be further clarified.

Conflict of interest

All authors declare no conflict of interest.

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