Life Sciences 90 (2012) 306–312

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

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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ARTICLE INFO

Article history:
Received 4 July 2011
Accepted 5 December 2011

Keywords:
Nociceptin
Orphanin FQ
NOP receptors
Periaqueductal gray
Potassium channels
[Tyr^{10}]N/OFQ(1-11)
(+)-5a Compound
Ro 64-6198
Mu-opioid receptors
cAMP

ABSTRACT

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 ^{125}I-[Tyr^{14}]N/OFQ in rodent brains, can be a tool for revealing the NOP receptor heterogeneity. We have previously founded 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.
Main 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 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.

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 (Mollereau 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 antinocicpetive. 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.*) (Kotlinska 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 ^{125}I-[Tyr^{10}]N/OFQ(1-11) and ^{125}I-[Tyr^{14}]N/OFQ as radioligands showed that the binding density of ^{125}I-[Tyr^{10}]N/OFQ(1-11) was less than that of ^{125}I-[Tyr^{14}]N/OFQ in rodent brains (Letchworth et al., 2000; Mathis et al., 1999). Two (high and low affinity) binding sites for ^{125}I-[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 ^{125}I-[Tyr^{10}]N/OFQ(1-11) was proposed to be the high affinity site for ^{125}I-[Tyr^{14}]N/OFQ (Mathis et al., 1999).

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doi:10.1016/j.lfs.2011.12.004

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 homomeric receptors, displayed similar affinity for N/OFQ but had higher affinity for some opioid receptor ligands, such as NalBzOH, fentanyl and dynorphin. Interestingly, those ligands also displayed high affinity at the binding site of ^{125}I-[Tyr^{10}]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 (vlPAG) 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 vlPAG neurons (Chiou et al., 2004; Liao et al., 2011b).

In the PAG, the binding density of ^{125}I-[Tyr^{10}]N/OFQ(1-11) is one-sixth of that of ^{125}I-[Tyr^{14}]N/OFQ (Letchworth et al., 2000). We, therefore, hypothesize that the binding site for ^{125}I-[Tyr^{10}]N/OFQ(1-11) is the NOP receptor sensitive to Ro 64-6198/(+)-5a Compound in the vlPAG and the NOP–MOP receptor. To verify these hypotheses, we synthesized [Tyr^{10}]N/OFQ(1-11), (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Tyr-Ala), which was more selective to the binding site of ^{125}I-[Tyr^{10}]N/OFQ(1-11) than to the site of ^{125}I-[Tyr^{14}]N/OFQ, as compared with N/OFQ(1-11) (Letchworth et al., 2000; Mathis et al., 1999), and examined if [Tyr^{10}]N/OFQ(1-11) can reveal the NOP receptor heterogeneity. We have characterized pharmacological properties of [Tyr^{10}]N/OFQ(1-11) quantitatively and examined its interactions with (+)-5a Compound and N/OFQ in vlPAG neurons, and investigated whether the pharmacological profiles of [Tyr^{10}]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 vibratome (Microslicer DTK-100, Dosaka). Slices were immediately transferred to a submerged chamber and equilibrated at room temperature in oxygenated (95% O_2/5% CO_2) artificial cerebral spinal fluid (aCSF), which consisted of (in mM) 117 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ 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 $CaCl_2$, 5 BAPTA, 10 HEPES, 5 MgATP and 0.33 GTPtris (pH 7.3). To study if [Tyr^{10}]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 −60 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 [Tyr^{10}]N/OFQ(1-11), the percent increment of I_{-140} in each neuron was normalized to the maximal effect (E_{max}) produced by 1 μM N/OFQ, which was 39.4% ± 4% increment (n = 26) (Chiou et al., 2002). The EC_{50} of [Tyr^{10}]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 [Tyr^{10}]N/OFQ(1-11) in the same neuron, the antagonist was applied after the response to [Tyr^{10}]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 [Tyr^{10}]N/OFQ(1-11), [Tyr^{10}]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% CO_2 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 EC_{50s} of [Tyr^{10}]N/OFQ(1-11) and N/OFQ were determined using logistic fitting equations.

Chemicals

[Tyr^{10}]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). [Tyr^{10}]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).

Statistic 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

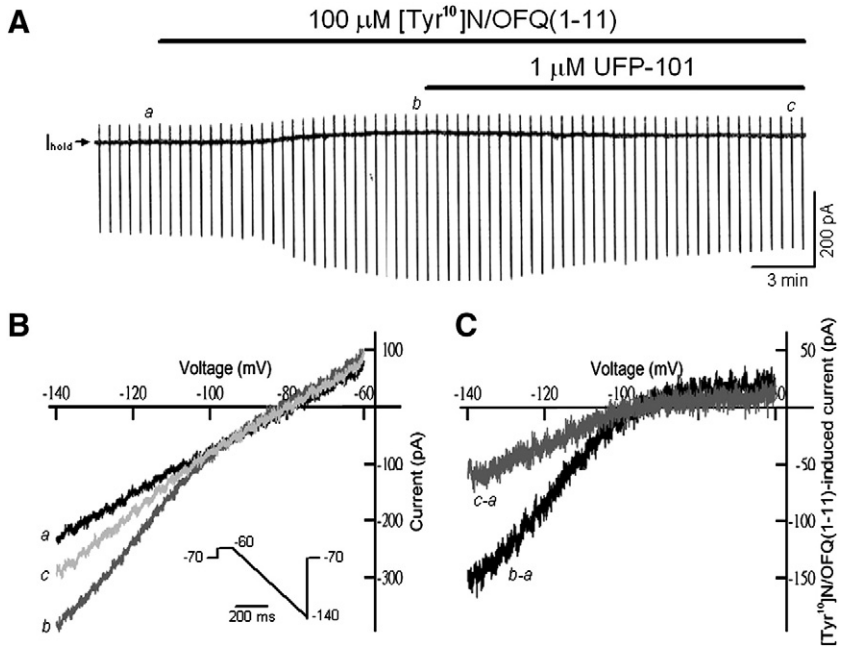

Fig. 1. [Tyr[10]]N/OFQ(1-11)-induced G-protein-coupled inwardly rectifying K^+ currents in rat vlPAG neurons in a manner antagonized by UFP-101, an NOP receptor antagonist. Membrane currents were evoked by hyperpolarization ramps from −60 to −140 mV at 0.2 mV/ms every 30 s from a holding potential of −70 mV (inset). (A) The chart recording of the membrane currents of a vlPAG neuron treated with 100 μM [Tyr[10]]N/OFQ(1-11) and further with 1 μM UFP-101. The holding current (I_{hold}) is the baseline of the recording traces. (B) Current–voltage (I–V) curves of the membrane current in the control (*a*) or the presence of [Tyr[10]]N/OFQ(1-11) (*b*) or [Tyr[10]]N/OFQ(1-11) plus UFP-101 (*c*). (C) The I–V curves of the [Tyr[10]]N/OFQ(1-11)-induced currents that were obtained by subtracting the current in the control from that during exposure to [Tyr[10]]N/OFQ(1-11) in the absence (*b*–*a*) or presence (*c*–*a*) of UFP-101.

value (0%). Differences were considered to be significant if a *p* value <0.05.

Results

[Tyr[10]]N/OFQ(1-11) activated GIRK channels in vlPAG neurons

[Tyr[10]]N/OFQ(1-11) (100 μM) shifted the holding current (I_{hold} 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 [Tyr[10]]N/OFQ(1-11)-induced current, which was obtained by subtracting the currents in the control from that in the presence of [Tyr[10]]N/OFQ(1-11), is characterized with inward rectification (Fig. 1C). The reversal potential of [Tyr[10]]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, [Tyr[10]]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).

[Tyr[10]]N/OFQ(1-11) was as efficacious as, but less potent than, N/OFQ

The effect of [Tyr[10]]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 [Tyr[10]]N/OFQ(1-11) was quantified from the increment of I_{-140} as described in Materials and methods. The maximal increment was induced by 100 μM [Tyr[10]]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 [Tyr[10]]N/OFQ(1-11) with that of N/OFQ, Ro 64-6198 and (+)-5a Compound, the increment of [Tyr[10]]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 EC_{50} value of [Tyr[10]]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. [Tyr[10]]N/OFQ(1-11) is also less potent than Ro 64-6198 or (+)-5a Compound (Fig. 2A).

The effect of [Tyr[10]]N/OFQ(1-11) was antagonized by UFP-101, but not naloxone

To verify if the effect of [Tyr[10]]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 [Tyr[10]]N/OFQ(1-11) had reached the steady state. UFP-101 decreased the current induced by [Tyr[10]]N/OFQ(1-11) but did not change its reversal potential (Fig. 1B). The I_{-140} induced by [Tyr[10]]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 [Tyr[10]]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 [Tyr[10]]N/OFQ(1-11) was unaffected by naloxone, a non-selective opioid receptor antagonist. The I_{-140} increments after treatment with 100 μM [Tyr[10]]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).

[Tyr[10]]N/OFQ(1-11) further increased GIRK currents in (+)-5a Compound-sensitive neurons

[Tyr[10]]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

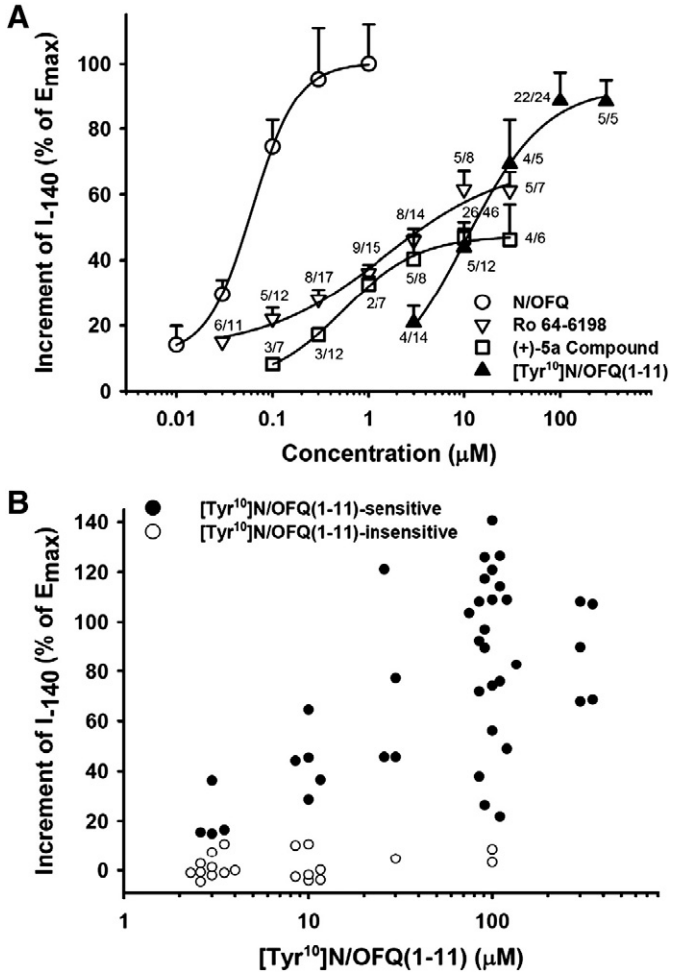

Fig. 2. Concentration-dependent activation of GIRK currents induced by [Tyr^{10}]N/OFQ(1-11), (+)-5a Compound, Ro 64-6198 and N/OFQ in vlPAG neurons. The ordinate is the increment of the membrane current at −140 mV (I_{-140}) induced by various NOP receptor agonists and is expressed as the percentage of the maximal increment (E_{max}) produced by N/OFQ which was 39.4%±4% (n=26) and was obtained with 1 μM N/OFQ (Chiou et al., 2002). (A) The concentration–response curves of N/OFQ (open circles), Ro 64-6198 (open inverted triangles), (+)-5a Compound (open squares) and [Tyr^{10}]N/OFQ(1-11) (filled triangles). The curves of N/OFQ, Ro 64-6198 and (+)-5a Compound were taken from our previous studies (Chiou et al., 2004; Chiou et al., 2002; Liao et al., 2011b). The EC_{50} for [Tyr^{10}]N/OFQ(1-11) is 8.98±0.85 μM. The numbers next to each point of the curve of Ro 64-6198, (+)-5a Compound or [Tyr^{10}]N/OFQ(1-11) are the numerical ratios of Ro 64-6198-sensitive, (+)-5a Compound-sensitive or [Tyr^{10}]N/OFQ(1-11)-sensitive neurons to the tested neurons. Data are mean±S.E.M. (B) A scatter plot for the effect of [Tyr^{10}]N/OFQ(1-11) (3–300 μM) in each recorded neuron. Note that there is no significant cut-off in the responses between [Tyr^{10}]N/OFQ(1-11)-sensitive (filled circles) and -insensitive neurons (open circles).

receptors in only a portion, but not all, of vlPAG neurons. However, the higher the concentration of [Tyr^{10}]N/OFQ(1-11) tested, the fewer the insensitive neurons (Fig. 2B). This might be due to the low potency of [Tyr^{10}]N/OFQ(1-11). When its concentration was too low, the GIRK current was too small to be distinguished from the baseline. This is unlike the case of (+)-5a Compound or Ro 64-6198. They were ineffective in a portion of tested neurons even at the highest tested concentrations (Chiou et al., 2004; Liao et al., 2011b). There is a distinct cut-off in the response histogram in neurons treated with (+)-5a Compound (Liao et al., 2011b), but not with [Tyr^{10}]N/OFQ(1-11) (Fig. 2B).

To examine if the population of [Tyr^{10}]N/OFQ(1-11)-sensitive vlPAG neurons is the same subset as those affected by (+)-5a Compound, we applied (+)-5a Compound first, followed by [Tyr^{10}]N/OFQ(1-11) in the same vlPAG neurons. (+)-5a Compound (10 μM) activated GIRK channels in 12 out of 22 recorded neurons and had no effect in the remaining 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, [Tyr^{10}]N/OFQ(1-11) (100 μM) further increased GIRK currents (Fig. 3A), increasing I_{-140} from 123.6%±4.2% to 135.6%±6.3% (n=12, $p<0.01$, one sample *t*-test), a level that was produced by [Tyr^{10}]N/OFQ(1-11) alone (134.9%±5.5%, n=22). The effect of [Tyr^{10}]N/OFQ(1-11) in these neurons was mediated by NOP receptors, confirmed by the blockade with 1 μM UFP-101 (Fig. 3B).

[Tyr^{10}]N/OFQ(1-11) induced GIRK currents in (+)-5a Compound-insensitive neurons

In those (+)-5a Compound-insensitive neurons, [Tyr^{10}]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 [Tyr^{10}]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 [Tyr^{10}]N/OFQ(1-11) alone (134.9%±5.5%, n=22).

[Tyr^{10}]N/OFQ(1-11) occluded the effect of N/OFQ

The interaction of [Tyr^{10}]N/OFQ(1-11) with N/OFQ was further investigated. In neurons treated with [Tyr^{10}]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 [Tyr^{10}]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 [Tyr^{10}]N/OFQ(1-11) occludes the effect (GIRK channel activation) of N/OFQ in vlPAG neurons.

[Tyr^{10}]N/OFQ(1-11) decreased cAMP accumulation with similar concentration–response curves in NOP cells and NOP–MOP cells

Effects of N/OFQ, [Tyr^{10}]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 IC_{50} 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. [Tyr^{10}]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 IC_{50} 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 IC_{50} 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 [Tyr^{10}]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 [Tyr^{10}]N/OFQ(1-11) was comparable to that of N/OFQ at either NOP cells or NOP–MOP cells (Fig. 5A). The inhibitory effects of [Tyr^{10}]N/OFQ(1-11) (15 μM) on forskolin-induced cAMP formation in NOP cells and NOP–MOP cells,

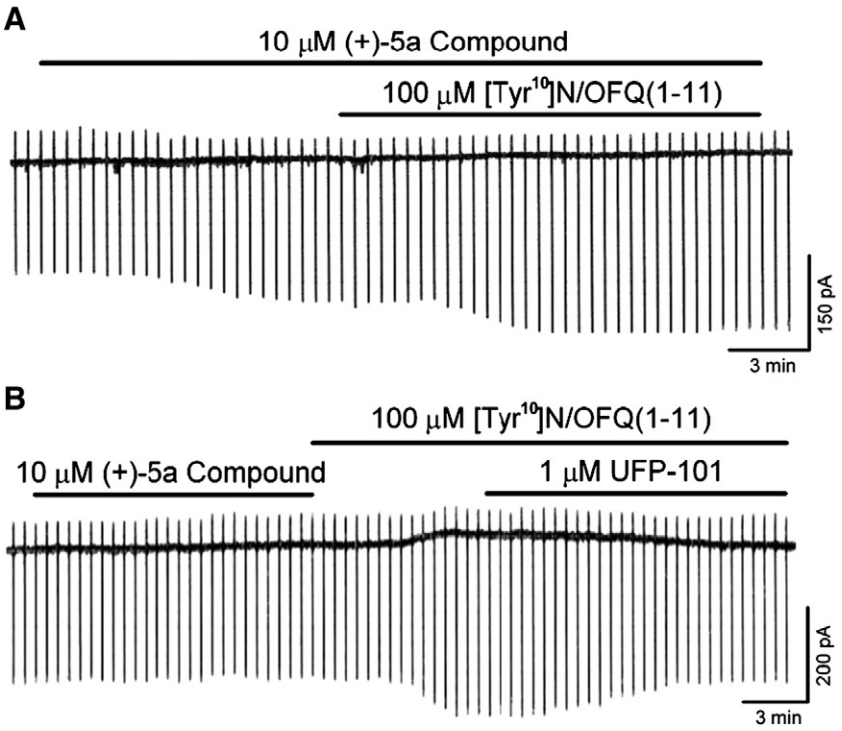

Fig. 3. Effect of [Tyr^{10}]N/OFQ(1-11) in (+)-5a Compound-sensitive and -insensitive neurons. (A) [Tyr^{10}]N/OFQ(1-11) did further increase GIRK currents in neurons which were responsive to a pretreatment with (+)-5a Compound at the maximal effective concentration (10 μM). (B) The chart recording of membrane currents in a vlPAG neuron which was insensitive to 10 μM (+)-5a Compound, but responsive to 100 μM [Tyr^{10}]N/OFQ(1-11). The effect of [Tyr^{10}]N/OFQ(1-11) was reversed by 1 μM UFP-101. The results were reproduced in another 11 and 7 neurons, respectively.

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 μM) (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

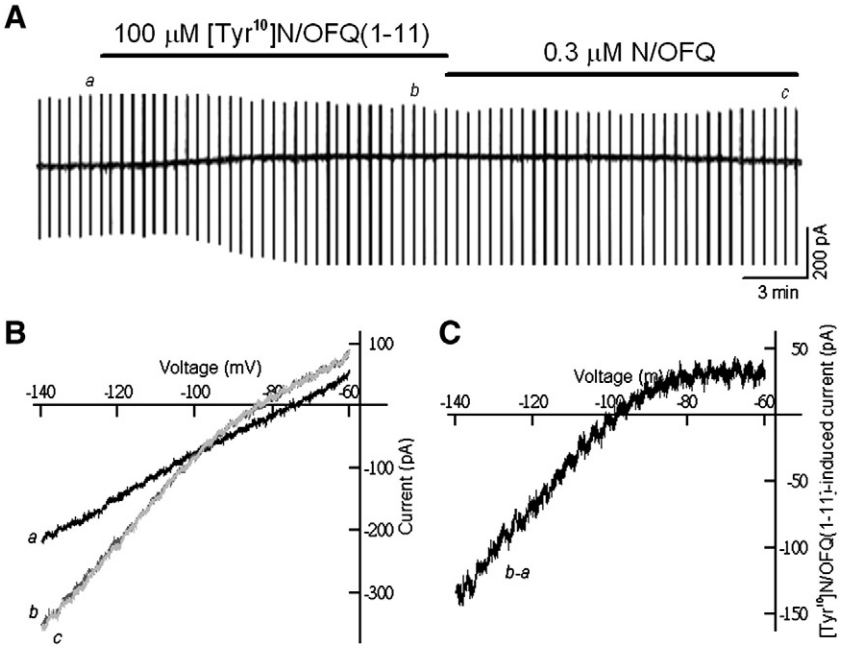

Fig. 4. [Tyr^{10}]N/OFQ(1-11) precludes the effect of N/OFQ. N/OFQ did not further increase GIRK currents in [Tyr^{10}]N/OFQ(1-11)-sensitive neurons. Membrane currents were elicited and recorded as described in Fig. 1. (A) The chart recording of the membrane current of a neurons treated with 100 μM [Tyr^{10}]N/OFQ(1-11) and further with 0.3 μM N/OFQ. (B) I–V curves of the membrane current in the control (*a*) or the presence of [Tyr^{10}]N/OFQ(1-11) (*b*) or N/OFQ (*c*). (C) The I–V curve of [Tyr^{10}]N/OFQ(1-11)-induced current that was obtained by subtracting the current in the control from that during exposure to [Tyr^{10}]N/OFQ(1-11) (*b*–*a*).

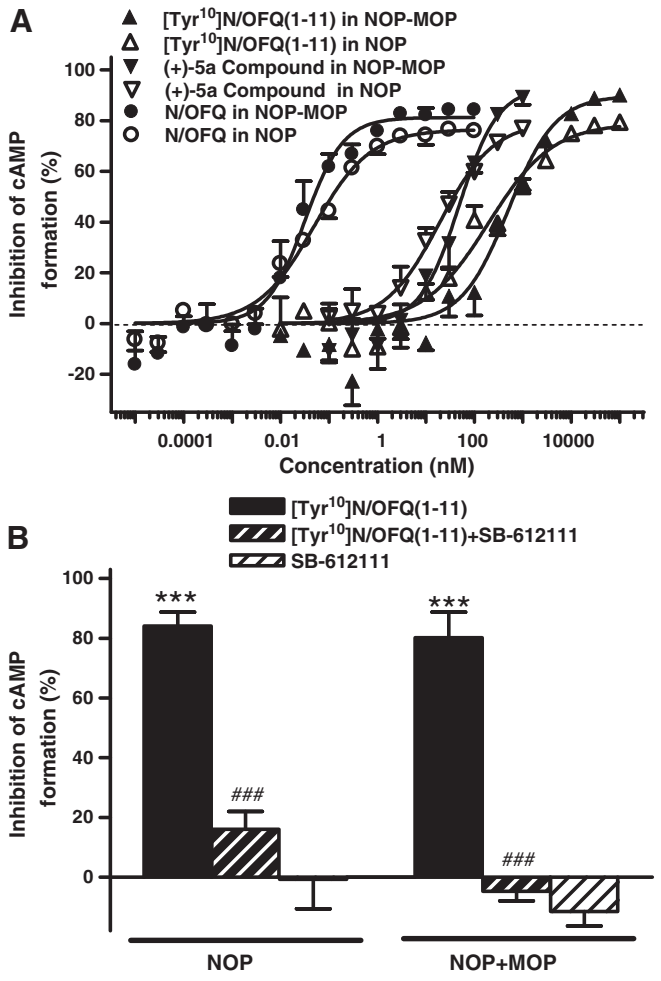

Fig. 5. Inhibitory effects of N/OFQ, [Tyr^{10}]N/OFQ(1-11) and (+)-5a Compound on forskolin-stimulated cAMP formation in HEK293 cells expressing NOP receptors. (A) Concentration–inhibition curves of N/OFQ, [Tyr^{10}]N/OFQ(1-11) and (+)-5a Compound. The ordinate is the percent inhibition of forskolin (10 μM)-stimulated cAMP formation produced after treatment with N/OFQ, [Tyr^{10}]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 [Tyr^{10}]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). $^{\#\#\#}p<0.005$ vs. [Tyr^{10}]N/OFQ(1-11) alone (Student's *t*-test). Data are mean ± S.E.M. $n \geq 3$.

effect of N/OFQ in vlPAG neurons, suggesting that [Tyr^{10}]N/OFQ(1-11) affects the NOP receptors in vlPAG neurons sensitive to N/OFQ, which weigh up to almost all (96%) neurons (Chiou et al., 2002). This pharmacological profile of [Tyr^{10}]N/OFQ(1-11) is different from that of (+)-5a Compound or Ro 64-6198, which activated NOP receptors in only a subset of vlPAG neurons. Furthermore, [Tyr^{10}]N/OFQ(1-11) was effective in both (+)-5a Compound-sensitive and -insensitive neurons. Therefore, the functional heterogeneity of NOP receptors in vlPAG neurons cannot be revealed by [Tyr^{10}]N/OFQ(1-11). In addition, [Tyr^{10}]N/OFQ(1-11) displayed similar concentration–response curves in inhibiting forskolin-induced cAMP formation in HEK293 cells expressing NOP receptors and in those co-expressing MOP and NOP receptors. Nevertheless, [Tyr^{10}]N/OFQ(1-11) was 3–4 order of magnitude less potent than N/OFQ in both cell lines.

[Tyr^{10}]N/OFQ(1-11) activates GIRK channels via NOP, but not MOP, receptors

The current induced by [Tyr^{10}]N/OFQ(1-11) had a reversal potential resembling the equilibrium potential of K^+ ions and was characterized with inward rectification. Therefore, [Tyr^{10}]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 [Tyr^{10}]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 [Tyr^{10}]N/OFQ(1-11).

[Tyr^{10}]N/OFQ(1-11) is a full agonist of NOP receptors and less potent than N/OFQ

The maximal increment of I_{-140} induced by [Tyr^{10}]N/OFQ(1-11) is comparable to that produced by N/OFQ, suggesting that [Tyr^{10}]N/OFQ(1-11) is a full agonist of NOP receptors in vlPAG neurons. However, [Tyr^{10}]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.

[Tyr^{10}]N/OFQ(1-11) fails to differentiate NOP receptor subsets in vlPAG neurons

High and low affinity binding sites of ^{125}I-[Tyr^{14}]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 ^{125}I-[Tyr^{10}]N/OFQ(1-11) (Mathis et al., 1999). In the rat PAG, the binding density of ^{125}I-[Tyr^{10}]N/OFQ(1-11) is one sixth of that of ^{125}I-[Tyr^{14}]N/OFQ (Letchworth et al., 2000). We, therefore, suggested that [Tyr^{10}]N/OFQ(1-11) might affect a portion of N/OFQ-sensitive NOP receptors in vlPAG 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, [Tyr^{10}]N/OFQ(1-11) affected both (+)-5a Compound-sensitive and -insensitive neurons. Second, [Tyr^{10}]N/OFQ(1-11) precluded the effect of N/OFQ in the same neuron, suggesting that [Tyr^{10}]N/OFQ(1-11) affects all the N/OFQ-sensitive NOP receptors.

[Tyr^{10}]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 ^{125}I-[Tyr^{10}]N/OFQ(1-11), as compared to that of ^{125}I-[Tyr^{14}]N/OFQ in mouse brains (Mathis et al., 1999). We, therefore, hypothesize that the binding site of ^{125}I-[Tyr^{10}]N/OFQ(1-11) might be heteromeric NOP–MOP receptors. However, the results in this study nullify this hypothesis since [Tyr^{10}]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 vlPAG 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 [Tyr^{10}]N/OFQ(1-11) and Ro 64-6198 nor (+)-5a Compound can differentiate NOP–MOP hetermeric receptors from NOP homomerized receptors.

Conclusion

[Tyr^{10}]N/OFQ(1-11) acted as a full agonist of N/OFQ-sensitive NOP receptors and is less potent than N/OFQ in vlPAG 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 heteromeric 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 ^{125}I-[Tyr^{10}]N/OFQ(1-11) in the brains of NOP receptor knock-out mice, as compared with the wild types (Majumdar et al., 2009). Therefore, the functional role of the binding site of ^{125}I-[Tyr^{10}]N/OFQ(1-11) in the brain remains to be further clarified.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgment

This study was supported by the grants from the National Health Research Institutes, Taiwan (NHRI-EX99-9506NI), National Science Council, Taiwan (NSC 98-2320-B-002-011-MY3 and 98-2323-B-002-012) and National Taiwan University, Taipei, Taiwan (Excellence Research Program 98R0066-51). We appreciate the generous gifts of (+)-5a Compound and Ro 64-6198 from F. Hoffmann-La Roche Ltd., Basle, Switzerland, UFP-101 from Dr Calo' and Dr Guerrini, University of Ferrara, Ferrara, Italy, and SB-612111 from Dr. Toll, SRI International, Menlo Park, California, USA.

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