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European Journal of Pharmacology 515 (2005) 47 – 53

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[Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH$_2$ is a competitive antagonist of NOP receptors in the periaqueductal gray

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Received 20 December 2004; received in revised form 22 March 2005; accepted 31 March 2005
Available online 17 May 2005

Abstract

Nociceptin/orphanin FQ (N/OFQ) and N/OFQ peptide (NOP) receptors are implicated in many physiological functions including pain regulation. This study quantitatively investigated the interaction of a novel NOP receptor antagonist, UFP-101 ([Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH$_2$), with N/OFQ in the ventrolateral periaqueductal gray, a crucial midbrain area for pain regulation. N/OFQ concentration-dependently activated G-protein coupled inwardly rectifying K^+ (GIRK) channels in ventrolateral neurons of periaqueductal gray slices. UFP-101 antagonized N/OFQ-induced GIRK channel activation in a concentration-dependent manner and produced a parallel shift of the concentration–response curve of N/OFQ. The pA_2 value estimated from Schild plot is 6.92±0.06. At concentrations up to 1 μM, UFP-101 had no effect on membrane current per se and did not affect the GIRK current activated by [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkephalin, a μ-opioid receptor agonist. It is concluded that UFP-101 is a potent and competitive peptide antagonist of NOP receptors that mediate GIRK channel activation in ventrolateral periaqueductal gray neurons.

Keywords: [Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH$_2$; UFP-101; Nociceptin; Orphanin FQ; NOP receptor; Potassium channel; Periaqueductal gray

1. Introduction

A new member of opioid receptor family was identified and named initially as opioid like orphan receptor because it is highly homologous to classical opioid receptors but displays little affinity to opioids (Mollereau et al., 1994). This receptor was renamed after its endogenous ligand, nociceptin (Meunier et al., 1995) or orphanin FQ (Reinscheid et al., 1995) (N/OFQ), was identified. It was termed as N/OFQ peptide (NOP) receptor and classified as a non-opioid branch of opioid receptor family (NC-IUPHAR, 2004). N/OFQ has been implicated in many physiological or pathological functions, including pain regulation, stress response, feeding, learning and memory, pituitary functions, and immune and cardiovascular controls (Darland et al., 1998; Peluso et al., 1998; Calo' et al., 2000b; Mogil and Pasternak, 2001; Serhan et al., 2001; Fiset et al., 2003). Among these, effects of N/OFQ on pain regulation were actively pursued. Different from conventional opioids that usually produce analgesia, N/OFQ induces hyperalgesia and reverses morphine-induced analgesia at the supraspinal level. However, it has analgesic effect when administrated intrathecally (Darland et al., 1998; Calo' et al., 2000b; Mogil and Pasternak, 2001). Nevertheless, N/OFQ shares similar cellular actions with opioids, such as inhibition of c-AMP formation, activation of K^+ channels and inhibition of Ca^{2+} channels (Darland et al., 1998). Heterogeneity of NOP receptors has been suggested (Mathis et al., 1997) and splicing variants of NOP receptor transcripts have been reported (Peluso et al., 1998). Hence, the development and characterization of NOP receptor ligands would be of value in revealing the physiological roles of N/OFQ and clarifying the possible diversity of NOP receptors.

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doi:10.1016/j.ejphar.2005.03.036

UFP-101 ([Nphe^1,Arg^{14},Lys^{15}]N/OFQ-NH_2), is a heptadecapeptide derived by a combination of two N/OFQ-modified peptides, [Arg^{14}, Lys^{15}]N/OFQ and [Nphe^1]N/OFQ(1–13)-NH_2. The former is a full agonist with higher potency and longer duration of action than N/OFQ (Okada et al., 2000; Rizzi et al., 2002) and the later is a pure antagonist of NOP receptors (Calo' et al., 2000a). Quantitative studies indicated that UFP-101 is a competitive antagonist of NOP receptors expressed in CHO cells and peripheral preparations, and is more potent than [Nphe^1]N/OFQ-(1–13)-NH_2 (Calo' et al., 2002). UFP-101 also antagonized several in vivo effects of N/OFQ, including (i) supraspinal pronociceptive effects in the tail withdrawal assay in mice (Calo' et al., 2002), (ii) inhibitory effects on spontaneous locomotor activity in mice (Calo' et al., 2002; Kuzmin et al., 2004), (iii) bradycardiac and hypotensive action after iv administration in guinea pigs (Hashiba et al., 2003), and (iv) inhibition of alcohol induced gastric lesions in rats (Morini et al., 2005). The antagonistic nature of UFP-101 was also demonstrated in vitro in NOP receptor-mediated inhibition of [^{3}H]norepinephrine and [^{3}H]serotonin release in synaptosomes of the cerebral cortex (Marti et al., 2003) and K^+ channel activation in locus ceruleus and raphe slices (Gavioli et al., 2004). However, a quantitative assessment of the pharmacological action of UFP-101 has not been conducted at native NOP receptors of brain tissues. Therefore, the present study was designed to quantitatively investigate the interaction of UFP-101 and N/OFQ at native NOP receptors of rat brain slices containing the midbrain periaqueductal gray. This area has very dense distribution of NOP receptors (Anton et al., 1996) and the precursor of N/OFQ, preproN/OFQ (Nothacker et al., 1996).

Experiments were conducted in the ventrolateral region of the periaqueductal gray, which is a crucial site for morphine-induced supraspinal analgesia (Yaksh et al., 1976) as well as the site of action that N/OFQ reverses morphine-induced analgesia (Morgan et al., 1997). N/OFQ has been found to activate inwardly rectifying K^+ channels, which are coupled to G-protein (Ikeda et al., 1997), in most of the neurons tested in the ventrolateral periaqueductal gray (Vaughan and Christie, 1997; Chiou et al., 2004). Therefore, the effect of N/OFQ was quantified by the increment of this G-protein coupled inwardly rectifying K^+ (GIRK) current when the interaction between UFP-101 and N/OFQ was assessed.

2. Materials and methods

All animal experiments were approved by the Institutional Animal Care and Use Committee of College of Medicine, National Taiwan University. All efforts were made to minimize the number of animals used. The preparation of periaqueductal gray slices, electrophysiological recordings, and data analysis were similar to that described in the previous study (Chiou and Fan, 2002).

Coronal midbrain slices (400 μm) containing the periaqueductal gray were dissected from 12- to 18-day-old rats (Wistar strain) (Chiou and Huang, 1999) and equilibrated in the artificial cerebral spinal fluid (CSF) at room temperature for at least 1 h before recording. The artificial CSF consisted of (mM): NaCl 117, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and dextrose 11.4 and was oxygenated with 95% O_2/5% CO_2, (pH=7.4). Slices were mounted on a submerged recording chamber and perfused with the artificial CSF at a rate of 2–3 ml/min. Blind patch-clamp whole cell recording was conducted at 30 °C. The internal solution contained (mM): K^+ gluconate 125, KCl 5, $CaCl_2$ 0.5, BAPTA 5, Hepes 10, MgATP 5, and GTPtris 0.33 (pH=7.3). The electrode resistance was 4–8 MΩ.

To study the GIRK channel activating effect of N/OFQ, a hyperpolarization ramp protocol was applied. The cell was held at −70 mV, stepped to −60 mV for 100 ms, ramped from −60 mV to −140 mV for 400 ms and stepped back to −70 mV (Fig. 1, inset). After the whole cell configuration was formed, hyperpolarization ramps were given every 30s. Membrane currents elicited by hyperpolarization ramps were recorded through an amplifier (Axopatch 200B, Axon Instruments Inc., Union City, CA) with a PC computer running pClamp 8 (Axon Instruments Inc.) and simultaneously recorded with a chart recorder (Gould 3000) at a low frequency response of 10 Hz to monitor the time course of drug effects. The access resistance (10–15 MΩ) was monitored using the Membrane Test function of pClamp 8 software during the whole-cell recording. To make sure of no deterioration of clamp efficiency during recordings, only neurons with unchanged access resistance before and after drug treatments were accepted.

After a stable recording was obtained, the perfusion solution was switched to the artificial CSF containing the tested drugs. To quantitatively analyze the antagonism of N/OFQ-induced current by UFP-101 in the same neuron, unless stated, N/OFQ was applied first. UFP-101 was added after the response of N/OFQ had reached a steady state, being 5–10 min. The response of UFP-101 was continuously monitored until the steady state was attained, which took about 7–15 min. Given that the control membrane current of each neuron varied depending on its input resistance, the effect of N/OFQ was quantified as the percent change of the membrane current at −140 mV (I_{-140}), after normalization by reference to the control value for each neuron. Data are presented as the mean ±S.E.M. The n number indicates the number of tested neurons. Student's t-test was used for statistical analysis on the relative changes of I_{-140}. UFP-101 was synthesized as reported previously. N/OFQ was purchased from Tocris (Bristol, UK) and other chemicals were from Sigma (St. Louis, MO) or Tocris (Bristol, UK). UFP-101 and N/OFQ were dissolved in deionized water as 1000 times concentrated stock solutions of the tested concentrations. The stock solutions were subdivided into individual tubes which contained 30 μl UFP-101 or 60 μl N/OFQ and were frozen at −20 °C. A tube of stock solution was thawed and added into the artificial CSF before use.

3. Results

3.1. N/OFQ activated inwardly rectifying K^+ channels

N/OFQ shifted the holding current (I_{hold} in Fig. 1) outwardly and increased the membrane current elicited by hyperpolarization ramps from −60 to −140 mV voltage-dependently in ventrolateral periaqueductal gray neurons (Fig. 1). The currents increased at more negative potentials were greater than those at less negative

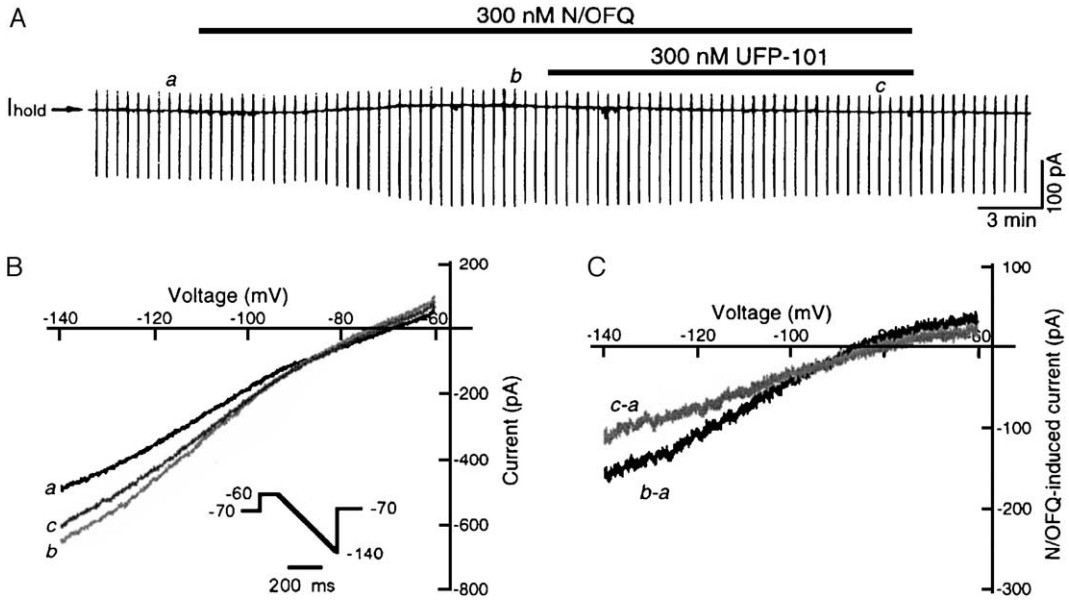

Fig. 1. Antagonism of N/OFQ-induced GIRK current by UFP-101 in a ventrolateral periaqueductal gray neuron. Membrane currents were evoked by hyperpolarization ramps from −60 to −140 mV at 0.2 mV/ms every 30 s from the holding potential of −70 mV (inset). (A) The chart recording of membrane currents of a neuron treated with 300 nM N/OFQ and further with 300 nM UFP-101. The baseline of the traces resembles the holding current (I_{hold}) of the recorded neuron. (B) Current–voltage ($I-V$) curves of the membrane current in the control (a) or the presence of N/OFQ (b) or N/OFQ+UFP-101 (c). (C) $I-V$ curves of N/OFQ-induced currents that were obtained by subtracting the current in the control from that during exposure to N/OFQ in the absence (b–a) or presence (c–a) of UFP-101.

ones. Thus, a characteristic of inward rectification was shown in the current–voltage ($I-V$) curve of N/OFQ-induced current that was obtained by subtracting the control current from that in the presence of N/OFQ (Fig. 1C). The reversal potential of N/OFQ-induced current was at −89.2±0.2 mV ($n=92$), which corresponds to the equilibrium potential of K^+ ions (−91 mV) according to the Nernst equation. Therefore, N/OFQ, as reported previously, activated GIRK channels in ventrolateral periaqueductal gray neurons. The effect of N/OFQ was quantified by the increment of I_{-140}. The increment produced by 1 and 3 μM N/OFQ were 40.3±7.1% ($n=14$) and 40.4±4.0% ($n=7$), respectively. The latter one was taken as 100% response to N/OFQ (E_{max}) in constructing the concentration–response curves (Fig. 2). The estimated EC_{50} for N/OFQ is 46±1 nM.

UFP-101 reduced the current induced by N/OFQ but did not change its reversal potential (Fig. 1). The reversal potential of N/OFQ-induced current in the presence of UFP-101 was −90.1+0.8 mV ($n=71$). The antagonistic effect of UFP-101 on N/OFQ-induced K^+ current was concentration-dependent and surmountable by increasing the concentration of N/OFQ. Thus, UFP-101 produced a parallel shift of the concentration–response curve of N/OFQ in a concentration-dependent manner (Fig. 2A). Schild plot analysis yielded a pA_2 value of 6.92±0.06 and a slope of 0.8±0.1 (Fig. 2B), compatible with a competitive type of antagonism.

3.2. UFP-101 antagonized N/OFQ effect in a competitive manner

For a quantitative comparison, the data used in constructing the concentration–response curve were obtained from neurons treated with N/OFQ first, and followed by UFP-101. To avoid possible desensitization of NOP receptors or run-down of G protein during recording period, a group of neurons were treated with 100 nM UFP-101 first and then with 100 nM N/OFQ. The increment of I_{-140} by N/OFQ in this group (55.0±3.5%, $n=8$, open diamond in Fig. 2) was not significantly different from that in the group treated with UFP-101 afterward (53.6±3.9%, $n=5$).

3.3. UFP-101 had no effect on the membrane current per se

UFP-101 was tested alone to investigate whether this compound has residual agonistic activity at NOP receptors of ventrolateral periaqueductal gray neurons. At concentrations up to 1 μM, UFP-101 had no effect on the holding current or the membrane current elicited by voltage ramps (Fig. 3). The I_{-140} after treatment with UFP-101 was 99.7±0.1% of the control value ($n=3$). Fig. 3 demonstrated that, in a neuron which was unaffected by 1 μM UFP-101, baclofen, a $GABA_B$ receptor agonist that is also a GIRK channel activator (Christie and North, 1988), increased the GIRK conductance. This demonstrates that the negative result of UFP-101 alone is not due to a deterioration or absence of GIRK channels in the recording neuron.

3.4. UFP-101 did not affect the effect of DAMGO

Activation of μ-opioid peptide (MOP) receptors, but not κ- or δ-opioid peptide (KOP or DOP) receptors (Chieng and Christie, 1994), also activates GIRK channels in 30–60% of ventrolateral periaqueductal gray neurons recorded (Behbehani et al., 1990; Chieng and Christie, 1994; Chiou and Huang, 1999; Chiou, 2001; Chiou and How, 2001). UFP-101 was, therefore, challenged against the effect of [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO), a selective MOP receptor agonist, to examine the selectivity of UFP-101. DAMGO (0.3 μM) increased the membrane current elicited by voltage ramps and shifted the holding current outwardly (Fig. 4), as previously reported (Chiou, 2001).

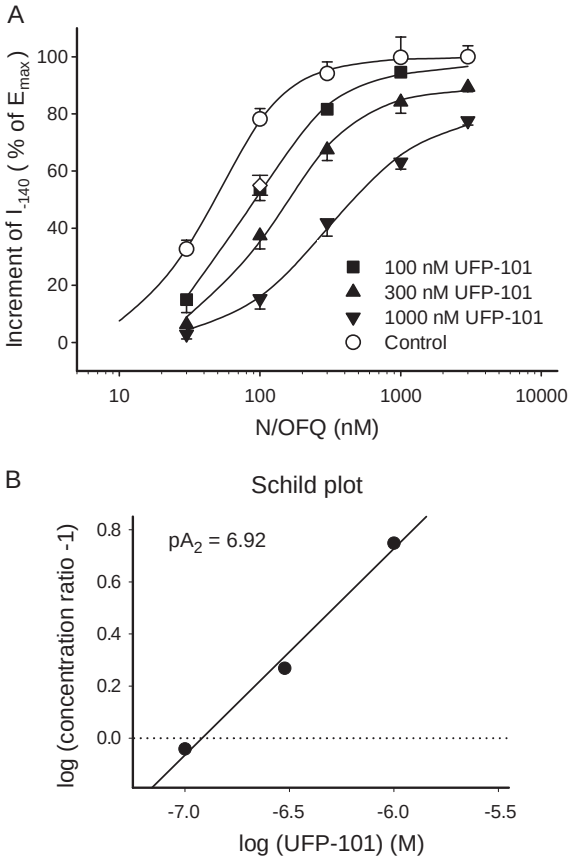

Fig. 2. UFP-101 antagonized the current induced by N/OFQ competitively. (A) Concentration–response curves to N/OFQ-induced currents in the absence (open circles) or presence (filled symbols) of various concentrations of UFP-101. The ordinate is the membrane current at −140 mV (I_{-140}) increased by N/OFQ, expressed as the percentage of the maximal effect produced by N/OFQ (E_{max}). The maximal increment was 40.4±4.0% (n=7), obtained with 3 μM N/OFQ. In each neuron, N/OFQ was applied first and then UFP-101 was further added, except the ones (open diamond) that were treated with 100 nM UFP-101 first and followed by 100 nM N/OFQ. The curves were fitted based on the logistic equation $E=E_{max}/[1+(EC_{50}/D)^n]$, where E represents the increment I_{-140}, E_{max} the maximal increment, D the concentration of N/OFQ, and n the Hill coefficient. The EC_{50}'s for N/OFQ obtained in the absence and presence of UFP-101 were used to construct the Schild plot. Mean±S.E.M., n=7–34 for N/OFQ alone or 3–12 for N/OFQ plus UFP-101. (B) The Schild plot for UFP-101, from which the estimated pA_2 is 6.92±0.06 and the slope is 0.8±0.1. The linear regression coefficient is 0.997.

The current induced by DAMGO also displayed inward rectification and reversed at potential near the equilibrium potential of K^+ ions (Fig. 4C), indicating that DAMGO also activates GIRK channels. Since DAMGO affects only a portion of ventrolateral periaqueductal gray neurons (Chiou, 2001), we challenged the effect of UFP-101 in those neurons sensitive to DAMGO. In DAMGO-sensitive neurons, further addition of UFP-101 at concentrations up to 1 μM, which almost abolished the effect of N/OFQ of 0.3 μM, did not affect the current increased by DAMGO of 0.3 μM (Fig. 4), which increased I_{-140} in an extent similar to that produced by 0.3 μM N/OFQ in ventrolateral periaqueductal gray neurons (Chiou, 2001). The I_{-140} increased by DAMGO after treatment with UFP-101 (1 μM) was 100.6±0.3% (n=3) of that before UFP-101 treatment.

4. Discussion

The present study demonstrated that UFP-101 is a potent (pA_2=6.92) and competitive antagonist of postsynaptic NOP receptors that mediate GIRK channel activation in rat ventrolateral periaqueductal gray neurons. To the best of our knowledge, this is the first quantitative study of UFP-101 on postsynaptic NOP receptors in brain slices containing a pain-relevant region.

In ventrolateral periaqueductal gray neurons, activation of MOP, but not DOP or KOP, receptors also results in membrane hyperpolarization (Chieng and Christie, 1994) due to activation of GIRK channels. Therefore, the antagonism of N/OFQ-induced GIRK channel activation by UFP-101 in the present study unlikely involves DOP or KOP receptors. The finding that UFP-101 did not affect the GIRK channel activation induced by DAMGO, a MOP receptor selective agonist, further suggests that the antagonism of UFP-101 is selective for NOP receptors among the opioid receptors.

Activation of the projection neurons of the PAG activates the descending inhibitory pathway and results in analgesic effect (Behbehani, 1995). The fact that N/OFQ activates GIRK channels in almost all ventrolateral PAG neurons (Vaughan et al., 1997; Chiou et al., 2004) would lead to an overwhelm stabilization of the neurons, including projection neurons, in the ventrolateral PAG. Thus, GIRK channel activation would contribute substantially to the anti-opioid or hyperalgesic action of N/OFQ (Mogil and Pasternak, 2001). As a selective antagonist of NOP receptors, UFP-101, hence, inhibits the hyperalgesic effect of N/OFQ (Calo' et al., 2002). In fact, UFP-101 alone had analgesic effect when given intracerebroventricularly (Calo' et al., 2002), suggesting that endogenous N/OFQ plays a significant nociceptive role in the pain regulation.

The finding that UFP-101 had no residual agonistic activity at NOP receptors indicates that it is a pure NOP receptor antagonist. This is in line with the previous reports using peripheral preparations, cerebral cortical synaptosomes (Calo' et al., 2002) and locus ceruleus and dorsal raphe slices (Gavioli et al., 2004).

The parallel shift of the concentration–response curve of N/OFQ induced by UFP-101 indicates that the antagonism is competitive. The pA_2 value for UFP-101 (6.92) obtained in the present study at native NOP receptors of rat ventrolateral periaqueductal gray slices is higher than that (6.67) of its parent peptide antagonist, [$Nphe^1$]N/OFQ-(1–13)-NH_2, in the same preparations (Chiou et al., 2002). In peripheral preparations, UFP-101 was also found to be more potent than [$Nphe^1$]N/OFQ-(1–13)-NH_2 (Calo' et al., 2000a, 2002). However, UFP-101 is 10-fold more potent than [$Nphe^1$]N/OFQ-(1–13)-NH_2 in peripheral preparations while the difference is only 2 folds in the present brain slice preparations.

Several compounds have been reported to be NOP receptor antagonists. However, some of these compounds

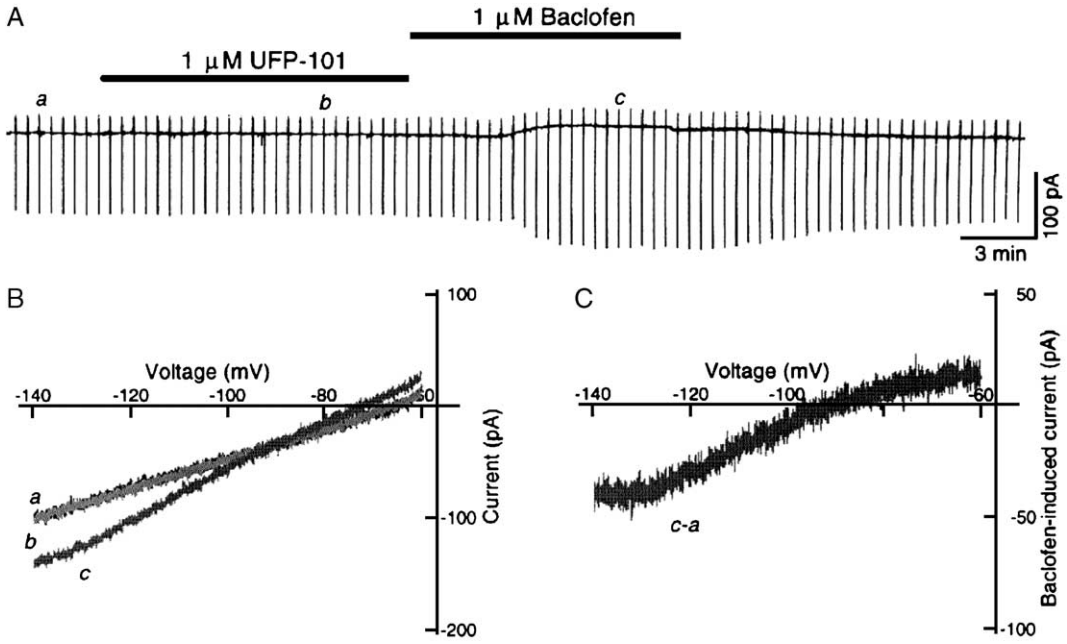

Fig. 3. Lack of effect of UFP-101 on the membrane current per se in a baclofen-sensitive neuron. Membrane currents were elicited and recorded as that presented in Fig. 1. (A) The chart recording of the membrane current of a neuron treated with 1 μM UFP-101 or 1 μM baclofen. (B) I–V curves of the membrane current in the control (a), or the presence of UFP-101 (b) or baclofen (c). (C) The *I–V* curve of baclofen-induced current that was obtained by subtracting the current in the control from that during exposure to baclofen (c–a). Note that the baclofen-induced current was also characterized by inward rectification and reversed at the potential near K^+ equilibrium potential.

are flawed by their non-specificity, such as NalBzOH (Chiou et al., 2004), σ-ligands (Kobayashi et al., 1997) and peptide III-BTD (Becker et al., 1999) or by their residual agonist activity, such as acetylated hexapeptides (Dooley et al., 1997; Berger et al., 1999) and [Phe^{1}ψ(CH$_2$-NH)Gly^2]N/OFQ-(1–13)-NH$_2$ (Chiou, 2000; Calo' et al., 2000b; Chiou et al., 2004). A selective nonpeptide antagonist, CompB (J-113397), has been developed (Ozaki et al., 2000) and was

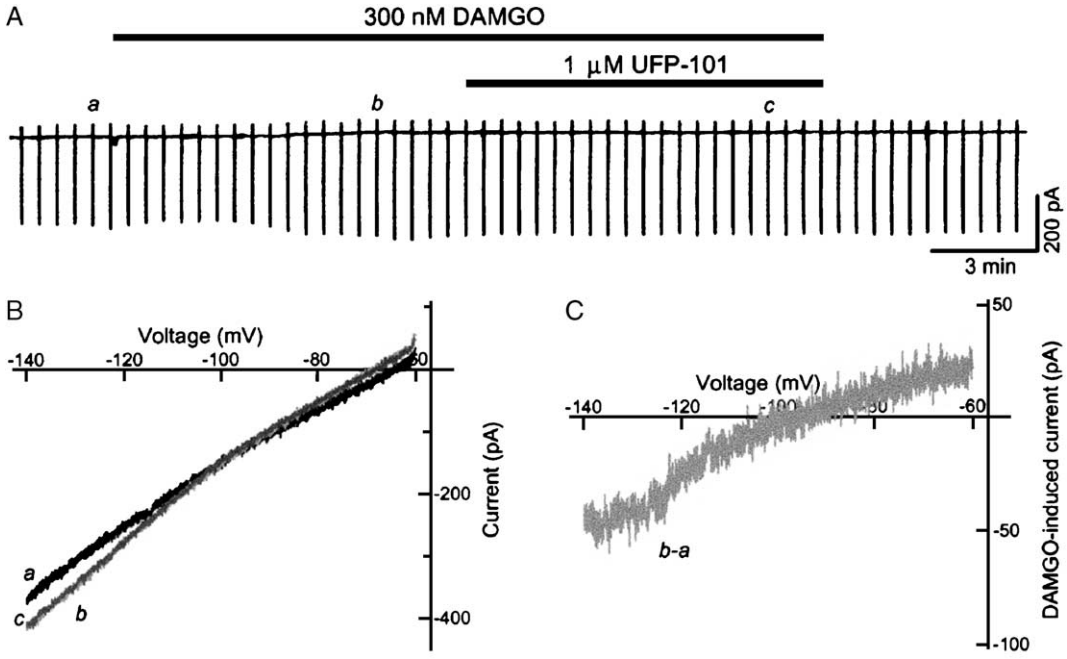

Fig. 4. UFP-101 did not antagonize DAMGO-induced GIRK current. Membrane currents were elicited and recorded as that presented in Fig. 1. (A) The chart recording of the membrane current of a neuron treated with 300 nM DAMGO and further with 1 μM UFP-101. (B) *I–V* curves of the membrane current in the control (a) or the presence of DAMGO (b) or DAMGO+UFP-101 (c). (C) The *I–V* curve of DAMGO-induced current that was obtained by subtracting the current in the control from that during exposure to DAMGO (b–a). Note that the DAMGO-induced current was also characterized by inward rectification and reversed at the potential near K^+ equilibrium potential.

shown to be a potent antagonist with pA_2 value of 8.37 in ventrolateral periaqueductal gray neurons (Chiou and Fan, 2002). However, CompB was recently shown to induce dopamine release through a mechanism not mediated by NOP receptors (Koizumi et al., 2004). Thus, despite its peptide nature might limit its usefulness especially for in vivo studies, UFP-101 still represents a very useful tool as a selective NOP receptor blocker to investigate the functional roles of endogenous N/OFQ.

Acknowledgements

This study was supported by National Health Research Institutes, Taiwan (NHRI-EX91-9005NC, NHRI-EX92-9005NC and NHRI-EX93-9005NC) and National Science Council, Taiwan (NSC 92-2320-B002-088 and NSC 93-2320-B002-117).

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