British Journal of Pharmacology (1999) 128, 103–107
http://www.stockton-press.co.uk/bjp

[Phe$^1\psi$(CH$_2$-NH)Gly^2]nociceptin-(1–13)-NH$_2$ activation of an inward rectifier as a partial agonist of ORL1 receptors in rat periaqueductal gray

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1 [Phe$^1\psi$(CH$_2$-NH)Gly^2]nociceptin-(1–13)-NH$_2$ (Pheψ), a tridecapeptide analogue of orphanin FQ/nociceptin (OFQ/N), was introduced as a competitive antagonist of opioid receptor-like orphan receptor (ORL1) in guinea-pig ileum and mouse vas deferens preparations *in vitro* but was recently found to act as an agonist *in vivo*.

2 In the periaqueductal gray, a site enriched with both OFQ/N and ORL1 and involved in OFQ/N-induced hyperalgesia and anti-analgesia, the effects of Pheψ and OFQ/N on the membrane current were studied using whole cell patch clamp recording technique in rat brain slices.

3 OFQ/N (0.01–1 μM) activated an inwardly rectifying type of K^+ channels in ventrolateral neurons of PAG. Pheψ (0.03–1 μM), like OFQ/N, also activated this inward rectifier but had only 30% efficacy of OFQ/N.

4 At maximal effective concentration (1 μM), Pheψ reversed the increment of K^+ conductance induced by OFQ/N (300 nM) by 46%. On the other hand, Pheψ also prevented the effect of OFQ/N if pretreated before OFQ/N.

5 It is suggested that Pheψ acts as a partial agonist of ORL1 that mediates the activation of inwardly rectifying K^+ channels in ventrolateral neurons of rat periaqueductal gray.

Keywords: [Phe$^1\psi$(CH$_2$-NH)Gly^2]nociceptin-(1–13)-NH$_2$; orphanin FQ/nociceptin; ORL1; K^+ channels; patch clamp; periaqueductal gray; brain slices

Abbreviations: OFQ/N, orphanin FQ/nociceptin; ORL1, opioid receptor-like orphan receptor; PAG, periaqueductal gray; Pheψ, [Phe$^1\psi$(CH$_2$-NH)Gly^2]nociceptin-(1–13)-NH$_2$

Introduction

Nociceptin, a heptadecapeptide (Meunier *et al*., 1995) also termed orphanin FQ (Reinscheid *et al*., 1995), has been identified as the endogenous ligand for the novel opioid receptor-like orphan receptor (ORL1), which is highly homologous to classical opioid receptors but with little affinity for traditional opioids (Mollereau *et al*., 1994). Orphanin FQ/nociceptin (OFQ/N) and ORL1 are densely distributed in brain areas relevant to nociception (Anton *et al*., 1996; Nothacker *et al*., 1996). Moreover, it exerts several cellular actions in common with classical opioids, such as inhibiting cyclic AMP production, increasing inwardly rectifying K^+ current and depressing Ca^{2+} current (see Darland *et al*., 1998). The effects of OFQ/N on the nociception, however, were highly diversified, including analgesia, hyperalgesia and an antagonism of morphine-induced analgesia (see Darland *et al*., 1998; Rossi *et al*., 1998). To further reveal the physiological roles of OFQ/N, specific antagonists are urgently needed. A tridecapeptide [Phe$^1\psi$(CH$_2$-NH)Gly^2]nociceptin-(1–13)-NH$_2$ (hereinafter called Pheψ) was introduced as a competitive antagonist of ORL1 in guinea-pig ileum and mouse vas deferens preparations (Guerrini *et al*., 1998). Thereafter, it was concluded to be a pure agonist *in vitro* for human cloned ORL1 (Butour *et al*., 1998) and *in vivo* for rat ORL1 involved in nociception (Calo *et al*., 1998; Carpenter & Dickenson, 1998; Grisel *et al*., 1998; Xu *et al*., 1998) and cardiovascular and renal functions (Kapusta *et al*., 1999).

The periaqueductal gray (PAG) is one of the areas with dense distribution of ORL1 and OFQ/N as well as opioids and their receptors (Schulz *et al*., 1996; Monteilletagius *et al*., 1998). It was proposed to be the site where OFQ/N attenuates opioid-induced antinociception (Morgan *et al*., 1997). In the ventrolateral neurons of PAG, that are involved in opioid-induced supraspinal analgesia (Yaksh *et al*., 1976), OFQ/N has been found to activate an inwardly rectifying type of K^+ channels (Vaughan *et al*., 1997). This study investigated the effects of Pheψ and OFQ/N on this inward rectifier to see if Pheψ is an agonist or antagonist for the ORL1 in the postsynaptic site of central neurons.

Methods

After decapitation, the midbrain containing PAG was quickly dissected from 12–18 day-old Wistar rats and put in the ice-cold ACSF. The ACSF contained (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 and 5% CO_2 (pH = 7.4; osmolarity = 290–295 mOsm). When the external K^+ concentration was changed, the corresponding amount of NaCl was replaced. Coronal slices of 400 μm were cut with a Microslicer (DTK-1000, D.S.K., Japan) and equilibrated in the ACSF at room temperature for at least 1 h. The slices were then transferred to a submerged recording chamber and

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perfused with the ACSF at a rate of 2–3 ml min^{-1}. Blind patch whole cell recording (Blanton *et al*., 1989) was conducted using pClamp 7.0 software on a Pentium II PC *via* a DigiData 1200A interface through an Axopatch 200A amplifier (Axon Instrument, Foster City, CA U.S.A.) Recordings were performed in ventrolateral neurons of PAG with 4–8 MΩ microelectrodes. The internal solution consisted of (mM): K gluconate 125, KCl 5, $CaCl_2$ 0.5, BAPTA 5, HEPES 10, MgATP 5, and GTPtris 0.33 (pH = 7.3; osmolarity = 275–280 mOsm). Experiments were conducted at 30°C. After whole cell configuration formation, cells were held at −70 mV and ramp-voltage commands from −140 mV to −60 mV were applied at the rate of 0.2 mV ms^{-1} every 30 s. Membrane currents were recorded at 10 KHz and low pass filtered at 2 KHz. A liquid junction potential of 11 mV was corrected in off-line analysis (Neher, 1992). Data are presented as the mean ± s.e.mean. The Student's *t*-test was used for statistical analysis. OFQ/N was purchased from RBI (Natick, MA, U.S.A.), Pheψ from Tocris (Bristol, U.K.) and other chemicals from Sigma (St. Louis, MO, U.S.A.).

Results

Experiments were performed in 55 ventrolateral PAG neurons. Only cells responsive to drug treatment were included. In 51 responsive cells, the input resistance was 420 ± 30 MΩ and the resting membrane potential was −67 ± 2 mV. A ramp depolarization from −140 to −60 mV evoked a membrane current showing inward rectification (Figure 1). The slope conductance of inward current from −140 to −120 mV was 3.9 ± 0.3 nS, which is greater than that of outward conductance, being 2.8 ± 0.4 nS, from −90 to −70 mV ($n = 17$, $P < 0.05$, paired *t*-test). OFQ/N induced an outward current at holding potential of −70 mV and hence cause hyperpolarization (Figure 1Aa). The I-V relationship of OFQ/N-elicited current displayed an inward rectification (Figure 1Ab and Table 1). The reversal potential was −91 ± 3 mV ($n = 9$) (Figure 1Ab), resembling the equilibrium potential of K^+ channels (E_K: −91 mV) estimated from the Nerst equation. Changing extracellular K^+ concentrations shifted the reversal potentials in a manner as predicted from the Nerst equation, being −120 ± 6 mV ($n = 3$; E_K: −113 mV) and −78 ± 5 mV ($n = 3$; E_K: −73 mV), respectively, in 2 and 9 mM K^+-ACSF. The effects of OFQ/N were abolished by 1 mM $BaCl_2$ (data not shown). These results indicate that OFQ/N activates an inwardly rectifying type of K^+ channels.

Pheψ also induced an outward current and increased the membrane conductance evoked by ramp commands (Figure 1Ba). The current elicited by Pheψ also showed an inward rectification and reversed polarity at −92 ± 4 mV ($n = 8$) (Figure 1Bb, Table 1). The effects of Pheψ were not affected by 1 μM naloxone (data not shown). It is likely that Pheψ, like OFQ/N, activates inwardly rectifying K^+ channels through a non-opioid receptor. The concentration-response curves for the outward currents induced by OFQ/N and Pheψ show that Pheψ is much less efficacious and slightly less potent than OFQ/N (Figure 2). The maximal efficacy of Pheψ was only

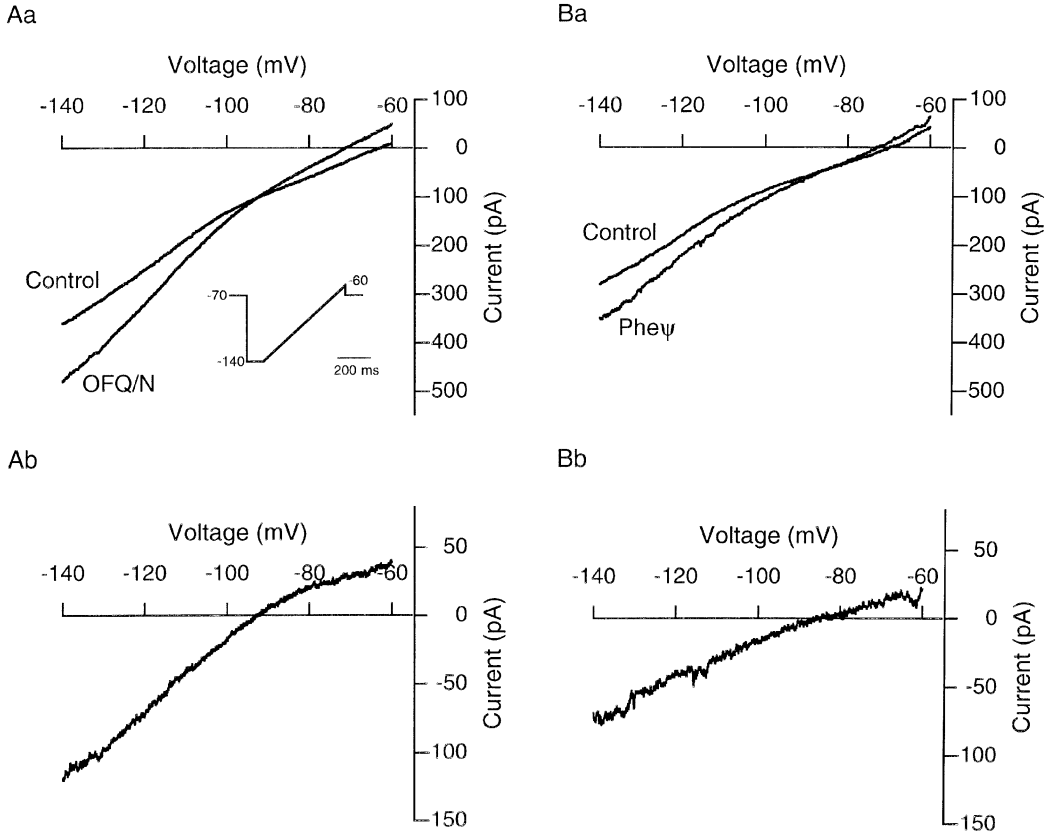

Figure 1 Both OFQ/N and Pheψ activated an inwardly rectifying type of K^+ channels. Cell was held at −70 mV and step to −140 mV for 100 ms followed by a ramp depolarization from −140 to −60 mV at a rate of 0.2 mV ms^{-1} (inset). I-V curves of membrane currents evoked by ramp commands before and after treatment with 300 nM OFQ/N (Aa) or 1 μM Pheψ (Ba). The I-V curve of peptide-elicited current was obtained by subtracting the control current from that in the presence of OFQ/N (Ab) or Pheψ (Bb). Both I-V curves show an inward rectification and reverse polarity at −94 mV (A) and −88 mV (B), which are close to the presumed K^+ equilibrium potential (−91 mV).

30% of OFQ/N. The EC_{50} for OFQ/N was 55 ± 12 and 77 ± 10 nM for Pheψ, respectively.

The interactions between Pheψ and OFQ/N were further studied. In the presence 300 nM OFQ/N, 1 μM Pheψ reversed the increment of K^+ conductance induced by OFQ/N by 46% but did not change its reversal potential (Figure 3A, Table 1). No further reversal was seen when further increasing Pheψ concentrations (data not shown). To avoid the possible bias from desensitization or tolerance to OFQ/N during treatment with both OFQ/N and Pheψ, slices were treated in a reverse order. Figure 3B shows that OFQ/N (300 nM) further increased the inwardly rectifying K^+ conductance in a cell pretreated with 1 μM Pheψ, which had activated this inward rectifier moderately. However, the increment of K^+ conductance by OFQ/N was attenuated significantly when pretreated with 1 μM Pheψ (Table 1). It indicates that Pheψ not only reversed but also prevented the effect of OFQ/N.

Table 1 The increment of slope conductances induced by OFQ/N and Pheψ

Treatment	*Conductance changes* *Inward* (nS)	*Outward* (nS)
A:		
OFQ/N	3.39 ± 0.57	2.02 ± 0.42*
OFQ/N + Pheψ	1.84 ± 0.63†	1.09 ± 0.43*†
B:		
Pheψ	0.78 ± 0.29	0.42 ± 0.26*
Pheψ + OFQ/N	1.71 ± 0.31†#	0.92 ± 0.21*†#

The inward conductance was measured from the slope conductance between -140 and -120 mV and the outward one between -90 and -80 mV. Data in Group A were obtained from seven cells treated as in Figure 3A and from six cell in group B as in Figure 3B. *$P<0.05$ vs inward conductance by paired t-test. †$P<0.05$ vs single treatment by paired t-test. #$P<0.05$ vs OFQ/N alone by group t-test.

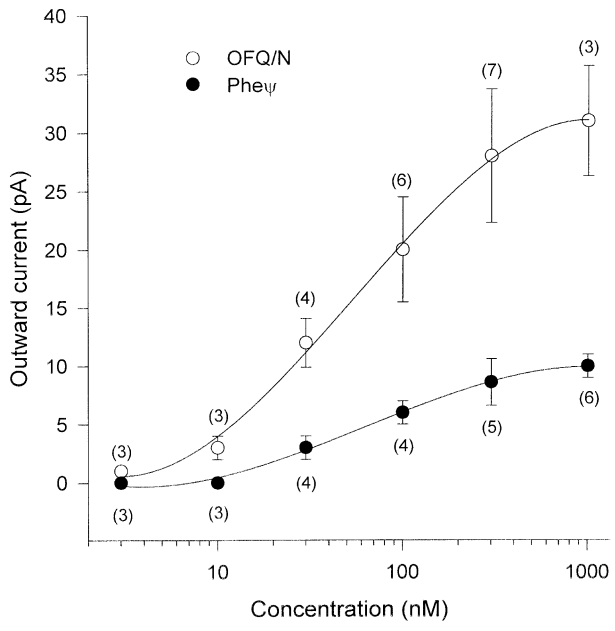

Figure 2 Concentration-response curves of outward currents induced by OFQ/N and Pheψ. Cells were held at -70 mV. The changes of holding currents induced by OFQ/N and Pheψ were plotted against the peptide concentrations. The curves were fitted based on the equation $I=Imax/[1+(EC_{50}/D)^n]$, where I represents the outward current, Imax the maximal current, D the concentration of peptide and n the Hill coefficient. The EC_{50} and n obtained from the curves are, respectively, 54 ± 12 nM and 0.98 for OFQ/N and are 77 ± 10 nM and 0.79 for Pheψ. Figures in the parenthesis are the number of cells tested.

Discussion

In this study, the finding that OFQ/N activates an inwardly rectifying type of K^+ channels in ventrolateral PAG neurons using voltage ramp protocol is in agreement with the previous report using voltage step commands (Vaughan *et al*., 1997). Pheψ was found to also activate these K^+ channels in a naloxone-insensitive manner. Although OFQ/N was reported to decrease enkephalin release at low concentrations but increase it at high concentrations (Gintzler *et al*., 1997), no depression of K^+ conductance by OFQ/N was seen in the range of concentrations used (3–1000 nM). Therefore, the effect of Pheψ cannot be attributed to an antagonism of endogenous OFQ/N.

The finding that Pheψ displayed the agonistic action with limited activity and antagonized the effect of OFQ/N indicates that this tridecapeptide acts as a partial agonist of the postsynaptic ORL1 that mediates the activation of inwardly

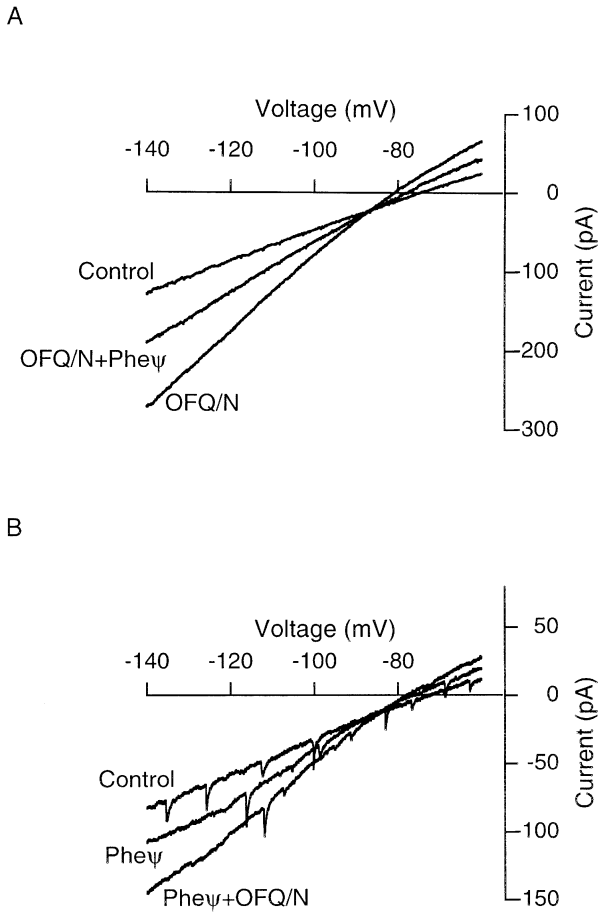

Figure 3 Interactions between OFQ/N and Pheψ in eliciting the inwardly rectifying K^+ conductance. Membrane currents were evoked by the same protocol as in Figure 1. (A) Cell was treated with 300 nM OFQ/N followed by further treatment with 1 μM Pheψ. (B) Cell was treated with 1 μM Pheψ followed by further treatment with 300 nM OFQ/N. The cell in (B) showed frequent spontaneous excitatory postsynaptic currents. Note that Pheψ partially reversed the effect of OFQ/N (A) and was unable to completely prevent its effect (B).

rectifying K^+ channels in ventrolateral neurons of rat PAG. Interestingly, in the presynaptic ORL1, Pheψ was also found to display partial agonism in guinea-pig airway (Shah *et al*., 1998) and mouse brain cortex (Schlicker *et al*., 1998) preparations. The efficacy of Pheψ (30%) found here is similar to that in guinea-pig airway preparations but slightly lower than that (45%) in mouse brain cortex.

Pheψ was initially introduced as a selective ORL1 antagonist in mouse vas deferens and guinea-pig ileum preparations (Guerrini *et al*., 1998). However, it was later reported to be a pure agonist of ORL1 receptors mediating hyperalgesia and anti-analgesia (Calo *et al*., 1998; Grisel *et al*., 1998), spinal neuron inhibition (Carpenter & Dikenson, 1998; Xu *et al*., 1998), cyclic AMP depression (Butour *et al*., 1998) and cardiovascular and renal functions (Kapusta *et al*., 1999). Although Pheψ was claimed to antagonize OFQ/N actions in amygdaloid (Meis & Pape, 1998) and medulla (Chu *et al*., 1999) neurons, without an examination of the intrinsic activity of Pheψ, it is hard to negate it as a partial agonist. The reason for the discrepancy among studies is unclear. Heterogenic ORL1 receptors have been reported in mouse brain (Mathis *et al*., 1997). It might be that the receptor subtype, density or coupling efficacy of ORL1 varies for different tissues and/or different responses. Interestingly, all the findings for the pure agonism were established *in vivo* (Calo *et al*., 1998; Carpenter & Dickenson, 1998; Grisel *et al*., 1998; Xu *et al*., 1998; Kapusta *et al*., 1999) except the cyclic AMP assay in cloned human ORL1 (Butour *et al*., 1998). Furthermore, Pheψ can inhibit the aminopeptidase that hydrolyses OFQ/N in the CSF (Montiel *et al*., 1997; Guerrini *et al*., 1998). An inhibition of OFQ/N degradation enzyme *in vivo* might have enhanced the agonistic activity of OFQ/N and then masked the antagonistic activity of Pheψ.

In this study, OFQ/N or Pheψ, unlike μ-opioids (Osborne *et al*., 1996) hyperpolarized nearly all ventrolateral PAG neurons as found previously (Vaughan *et al*., 1997). OFQ/N also inhibits the synaptic transmission (Vaughan *et al*., 1997) and Ca channels (Conner *et al*., 1998) in PAG. Morgan *et al*. (1997) suggested that the antagonism by OFQ/N of opioid analgesia is mediated by its inhibition of projecting neurons in PAG. The contribution of K^+ channel activation by OFQ/N to this neuronal inhibitory effect remains to be elucidated. Although μ-opioids inhibit both inhibitory and excitatory synaptic transmission as well as cause cell hyperpolarization (Osborne *et al*., 1996; Vaughan & Christie, 1997), we recently proposed that the inhibition by μ-opioids of the GABAergic transmission overcomes their hyperpolarizing and glutamatergic inhibitory effects and hence contributes to their analgesic action (Chiou & Huang, 1999). The possibility that OFQ/N, in contrast to μ-opioids, by a dominant hyperpolarization rather than the synaptic depression, exerts its pronociception is under investigation.

In conclusion, this study demonstrates that Pheψ acts as a partial agonist of ORL1 that mediates the activation of inwardly rectifying K^+ channels in ventrolateral neurons of rat PAG although it was reported to be a selective antagonist in peripheral tissues and a pure agonist *in vivo*.

I appreciate the advice and comments from Dr C. C. Chang (National Taiwan University) and Dr L.-Y. M. Huang (University of Texas Medical Branch at Galveston). This work was supported by the grant NSC 88-2314-B002-019 from National Science Council, R.O.C.

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(Received March 29, 1999
Revised April 23, 1999
Accepted June 1, 1999)