Ro 64-6198 [(1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one] Acts Differently from Nociceptin/Orphanin FQ in Rat Periaqueductal Gray Slices

Lih-Chu Chiou, Kuang-Chieh Chuang, Juergen Wichmann, and Geo Adam

Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan (L.-C. C., K.-C. C.); and Discovery Chemistry, Pharmaceutical Division, F. Hoffmann-La Roche AG, Basel, Switzerland (J.W., G.A.)

Received April 19, 2004; accepted July 8, 2004

ABSTRACT

Ro 64-6198 [(1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one] was developed as a nonpeptide agonist of nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptors, using bioassays at cloned receptors expressed in cell cultures. We have investigated the actions of Ro 64-6198 at native NOP receptors of the ventrolateral periaqueductal gray (PAG), a crucial site for N/OFQ-induced reversal of opioid analgesia, using the patch-clamp recording technique in rat brain slices. Ro 64-6198, like N/OFQ, activated G protein-coupled inwardly rectifying potassium (GIRK) channels (GIRK) in ventrolateral PAG neurons but displayed only 60% efficacy and 22% potency of N/OFQ. Unlike N/OFQ that activated GIRK through NOP receptors in almost all tested neurons, Ro 64-6198 affected only 62% (114/185) of the neurons recorded, among which 57% were sensitive to CompB (J-113397), a selective NOP receptor antagonist. The effect of Ro 64-6198 was not affected by naloxone (1 μM), sulpiride (10 μM), and 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine (NAN-190) (1 μM), respectively, the antagonist of opioid, dopamine D2, and 5-HT1A receptors. In Ro 64-6198-unresponsive neurons, N/OFQ activated GIRK through NOP receptors. It is concluded that Ro 64-6198 is a weak agonist of NOP receptors both in terms of potency and efficacy in ventrolateral PAG neurons. Heterogeneity of NOP receptors has been proposed from binding studies and in vivo functional studies. The possibility was discussed that two subsets of NOP receptors exist in ventrolateral PAG neurons, and Ro 64-6198 activates only one subset but N/OFQ activates both of them.

A novel family of G protein-coupled receptors was cloned and named initially as opioid receptor-like 1 orphan receptors because it is highly homologous to classical opioid receptors but displays little affinity to traditional opioids (Molleau et al., 1994). After the identification of its endogenous ligand that was known as nociceptin (Meunier et al., 1995) or orphanin FQ (Reinscheid et al., 1995), this receptor family was named nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptors (Alexander et al., 2001) and was classified as a nonopioid branch of the opioid peptide receptor family (Nolemenature Committee-International Union of Pharmacology, http://www.iuphar-db.org/iuphar-rd/). 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; Molig and Pasternak, 2001; Fiset et al., 2003). Among these, the effects of N/OFQ on pain regulation were actively explored. Different from traditional 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; Molig and Pasternak, 2001). Nevertheless, N/OFQ shares similar cellular actions with traditional opioids, such as inhibition of cAMP formation, activation of K+ channels, and inhibition of
Ca\(^{2+}\) channels (Darland et al., 1998). Heterogeneity of NOP receptors has been implicated (Mathis et al., 1997; 1999), and the splicing variants of NOP receptors have been reported (Peluso et al., 1998). Hence, the development and characterization of NOP receptor ligands would be of help in revealing the physiological roles of N/OFQ and in clarifying the possible diversity of NOP receptors.

Ro 64-6198 was developed as a nonpeptide agonist of NOP receptors with the potency and efficacy similar to N/OFQ at cloned NOP receptors expressed in cell cultures (Jenck et al., 2000; Wichmann et al., 2000). However, at native NOP receptors of peripheral tissues, Ro 64-6198 acts differently in different tissues. It is equally potent to N/OFQ at NOP receptors of the rat vas deferens (Rizzi et al., 2001). However, in the mouse vas deferens where N/OFQ is a full agonist of NOP receptors (Calo et al., 1996), Ro 64-6198 inhibits the contraction through an NOP receptor-independent mechanism (Rizzi et al., 2001). At the NOP receptors of the guinea pig ileum, Ro 64-6198 is 66 times less potent than N/OFQ (Rizzi et al., 2001). In vivo studies also showed that intraventricular injection of Ro 64-6198 mimics some, but not all, of the effects produced by N/OFQ (Jenck et al., 2000; Dayan et al., 2001; Higgins et al., 2001; Kuzmin et al., 2004). It seems that Ro 64-6198 might act differently at native NOP receptors among tissues or species. The action of Ro 64-6198 at native NOP receptors of brain tissues has not been characterized in vitro, although the effects of Ro 64-6198 on neurological functions of rodents have been reported in vivo (Jenck et al., 2000; Higgins et al., 2001; Ciccioppo et al., 2002; Le Pen et al., 2002). Its potential as a novel anxiolytic agent was especially noted (Jenck et al., 2000). Therefore, we investigated the effect of Ro 64-6198 quantitatively in brain slices containing the midbrain ventrolateral periaqueductal gray (PAG), an area enriched in NOP receptors (Anton et al., 1996) and involved in the antipodiid effect of N/OFQ (Morgan et al., 1997) and morphine-induced supraspinal analgesia (Yaksh et al., 1976).

In ventrolateral PAG neurons, N/OFQ has been shown to activate inwardly rectifying K\(^+\) channels through NOP receptors (Vaughan and Christie, 1997; Chio, 1999, 2001; Chio and Fan, 2002; Chio et al., 2002) that are coupled to G proteins (Ikeda et al., 1997). Therefore, the effect of Ro 64-6198 was evaluated by its activation of these G protein-coupled inwardly rectifying K\(^+\) channels (GIRK) through NOP receptors, and its effect was compared with that of N/OFQ. The preliminary results were presented at the 32nd Annual Meeting of Society for Neuroscience, Orlando, FL (Chio and Chuang, 2002).

### Materials and Methods

All animal experiments conformed to the guidelines of 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 dissection of PAG slices, electrophysiological recordings, and data analysis were similar to that described in the previous report (Chio and Fan, 2002).

Coronal midbrain slices (400 \(\mu m\)) containing the PAG were dissected from 12- to 18-day-old rats (Wistar strain) and equilibrated in artificial cerebral spinal fluid (ACSF) at room temperature for at least 1 h before recording. The ACSF consisted of: 117 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 1.2 mM NaH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), and 11.4 mM dextrose 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 ACSF at a rate of 2 to 3 ml/min. Blind patch-clamp whole cell recording was conducted at 30°C. The internal solution contained: 125 mM K\(^+\) gluconate, 5 mM KCl, 0.5 mM CaCl\(_2\), 5 mM BAPTA, 10 mM Hepes, 5 mM MgATP, and 0.33 mM GTP-Tris, pH 7.3. The electrode resistance was 4 to 8 M\(\Omega\). After whole-cell configuration was formed and a stable recording was obtained, the perfusion solution was switched to tested drug-containing ACSF.

To elucidate if Ro 64-6198 would, like N/OFQ, activate GIRK, a hyperpolarization ramp protocol was used (Chio and Fan, 2002). Cell were held at -70 mV, stepped to -60 mV for 100 ms, and stepped back to -70 mV (Fig. 1, inset). Membrane currents elicited by voltage 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 M\(\Omega\)) was monitored using the membrane test function of pClamp 8 software during the whole-cell recording. Series resistance compensation was not employed. To make sure of no deterioration of clamp efficiency during recordings, only neurons with unchanged access resistance before and after drug treatments were accepted.

The effect of N/OFQ or Ro 64-6198 in each neuron was quantified by the percent increment of the membrane current at -140 mV (I\(_{140}\)), taking its own I\(_{140}\) before drug treatment as 100%. To quantitatively evaluate the possible antagonism produced by the tested receptor antagonist (CompB, sulpiride, NAN-190, or naloxone), the interaction between Ro 64-6198 and the intended antagonist was examined in the same neuron. Given that not all neurons were sensitive to Ro 64-6198 (see Results), it was not practical to apply the intended antagonist before the application of Ro 64-6198 in the same neuron. Therefore, the receptor antagonists were applied to Ro 64-6198-sensitive neurons after the response of Ro 64-6198 had

---

**Fig. 1.** Effects of CompB on Ro 64-6198-induced GIRK activation. A and D, chart recordings of membrane currents evoked by hyperpolarization ramps from -60 to -140 mV (holding potential = -70 mV, inset) in two Ro 64-6198-responsive neurons that are sensitive (A) and insensitive (D), respectively, to CompB, a selective NOP receptor antagonist. The baseline indicates the holding current. Neurons were treated with Ro 64-6198 first and followed by CompB. B, I-V curves of membrane currents of neuron A in the control (a), the presence of Ro 64-6198 (b), or Ro 64-6198 plus CompB (c). C, I-V curves of Ro 64-6198-induced current of neuron A in the absence (b-a) or presence (c-a) of CompB.
reached a steady state, being about 20 to 25 min. The responses of the antagonists were continuously monitored thereafter. In the study verifying the function of NOP receptor-mediated GIRK activation in those Ro 64-6198-unresponsive neurons (Fig. 3), N/OFQ was added after Ro-646198 had been applied for at least 20 min. When the effects of Ro 64-6198 on N/OFQ-induced GIRK activation were studied (Fig. 4), Ro 64-6198 was added after the response of N/OFQ had reached the steady state. Data are presented as the mean ± S.E.M. with a number indicating the number of neurons tested. Usually one neuron was recorded per slice, and three to four slices were used per rat. Student's t test was used for statistical analysis.

Ro 64-6198 was synthesized as reported previously (Wichmann et al., 2000). CompB was a generous gift from Banyu Pharmaceutical Company (Tokyo, Japan). Other chemicals were purchased from Tocris Cookson Inc. (Bristol, UK) or Sigma-Aldrich (St. Louis, MO).

Results

Ro 64-6198 Activated GIRK in Ventrolateral PAG Neurons. Ro 64-6198 (0.03–30 μM) shifted the holding current (the baseline in Fig. 1A) outwardly and increased the membrane current elicited by hyperpolarization ramps (Fig. 1A) in a voltage-dependent manner (Fig. 1B). The current-voltage (I-V) curve of the current increased by Ro 64-6198, obtained by subtracting the current in the control from that in the presence of Ro 64-6198, was characterized by inward rectification (Fig. 1C). The reversal potential was at -89.3 ± 0.8 mV (n = 74), which is close to the equilibrium potential of K⁺ ions, -91 mV, according to the Nernst equation. When recording electrodes were filled with GTP-free internal solution, Ro 64-6198 failed to affect the membrane currents elicited by voltage ramps. The I-140 values after treatment with Ro 64-6198 were 100 ± 6% of the control (n = 10). Therefore, Ro 64-6198, like N/OFQ (Vaughan and Christie, 1997; Chiou, 1999; 2001; Chiou and Fan, 2002; Chiou et al., 2002), activates inwardly rectifying K⁺ channels that are coupled to G proteins (Ikeda et al., 1997).

Ro 64-6198 Was Less Potent, Less Efficacious, and Slower Acting Than N/OFQ. Compared with the effect of N/OFQ, which usually takes 5 to 7 min to reach the steady state (Chiou et al., 2002), the effect of Ro 64-6198 took about 20 to 25 min to reach the steady state. However, the onset time for Ro 64-6198 was not more than that for N/OFQ (Fig. 1A and D) (Chiou et al., 2002). At higher concentrations, Ro 64-6198 had a faster onset time, but its effect still took more than 15 min to reach the steady state. This slow action kinetic of Ro 64-6198 was also found when peripheral tissue preparations were used (Rizzi et al., 2001).

To quantitatively compare the effects of Ro 64-6198 and N/OFQ, the effect of Ro 64-6198 on K⁺ channel activation was quantified by the increment of I-140 and expressed as the percentage of the maximal effect induced by N/OFQ, which has been found to be produced by 1 μM N/OFQ and be 39 ± 4% (n = 26) increment of I-140 (Chiou et al., 2002). Similar increment was reproduced by 1 μM N/OFQ in the present study (37 ± 7%, n = 5). The concentration-response curve of Ro 64-6198 was shown in Fig. 2 and compared with that of N/OFQ (Chiou et al., 2002). The EC50 of Ro 64-6198 was 2.3 ± 1.1 μM, being 45 times higher than that of N/OFQ, 52 ± 6.8 nM (Chiou et al., 2002). The efficacy of Ro 64-6198 was lower than that of N/OFQ, being 61.5 ± 5.6% of the maximal increment induced by N/OFQ (Fig. 2).

Ro 64-6198 Affected Two-Thirds of the Neurons Recorded. Among 185 neurons tested, Ro 64-6198 (0.03–30 μM) activated GIRK in only 114 (62%) neurons and had no effect in the remaining 71 neurons. The neurons unresponsive to Ro 64-6198 were not limited to those treated with lower concentrations of Ro 64-6198. The numbers of Ro 64-6198-sensitive neurons over the total numbers of neurons tested with various concentrations of Ro 64-6198 were depicted in Fig. 2. Even at the highest concentration (30 μM) tested, there were still two of seven recorded neurons unresponsive to Ro 64-6198 (Fig. 2). The Ro 64-6198-unresponsive neurons are also independent of their locations, at either superficial or deeper layer of the slice.

The Effect of Ro 64-6198 Was Blocked by CompB in a Portion of Neurons. The effect of Ro 64-6198 (0.03–30 μM) in 31 of 54 (57%) of the neurons recorded was antagonized by CompB, a potent and selective nonpeptide NOP receptor antagonist (Ozaki et al., 2000). However, in the remaining 23 neurons, the effects of Ro 64-6198 were not affected by CompB at the concentrations up to 0.3 to 1 μM, which are much higher than its IC50 (4 nM) at NOP receptors of ventrolateral PAG neurons (Chiou and Fan, 2002). Figure 1, A to C, demonstrates one of the neurons, on which the effect of Ro 64-6198 was effectively antagonized by CompB, whereas Fig. 1D is the one resistant to the blockade of CompB.

Electrical Membrane Properties of Neurons Classified by Their Sensitivity to Ro 64-6198 or CompB. The electrical membrane properties of neurons classified based on their sensitivity to Ro 64-6198 or CompB were compared in Table 1. Neither the membrane input resistance nor the resting membrane potential is different between Ro 64-6198-
sensitive and insensitive groups. However, the capacitances of Ro 64-6198-sensitive and CompB-insensitive neurons were significantly lower, indicating that the sizes of these neurons are slightly smaller. However, it is not evident that projection neurons or interneurons in the PAG could be differentiated from their sizes or electrical membrane properties (Reichling et al., 1984; Beitz and Shepard, 1985).

N/OFQ Activated GIRK in Ro 64-6198-Unresponsive Neurons through NOP Receptors. In those Ro 64-6198-nonresponsive neurons, N/OFQ was applied to see if NOP receptors exist and are functional there. Figure 3A demonstrates one of these neurons, in which Ro 64-6198 (1 μM) was ineffective, but N/OFQ (0.3 μM) activated the GIRK conductance. The effect of N/OFQ was blocked by further addition of CompB (0.3–1 μM) (data not shown). The same result was also observed in five, four, seven, eight, seven, and three neurons that had shown no response to 0.03, 0.1, 0.3, 1, 3, and 10 μM of Ro 64-6198, respectively; further addition of N/OFQ (0.1–0.3 μM) activated GIRK channels. It is, therefore, suggested that the NOP receptors in these neurons are functional but were unaffected by Ro 64-6198. In a separate experiment, in those neurons that had shown no response to Ro 64-6198 but were affected by N/OFQ, further addition of Ro 64-6198 (3 μM) did not alter the response of N/OFQ (Fig. 3B). It is suggested that there is indeed a portion of neurons that is sensitive to N/OFQ but not to Ro 64-6198, either pre- or post-treatment.

Interactions between Ro 64-6198 and N/OFQ. Ro 64-6198 has been found to induce desensitization of NOP receptors (Dautzenberg et al., 2001). The low efficacy nature of Ro 64-6198 might make it act like an antagonist of NOP receptors. Therefore, we further investigated the possible interaction between Ro 64-6198 and N/OFQ in the same neuron. In nine neurons that have been perfused with N/OFQ first (0.1 or 0.3 μM), further addition of Ro 64-6198 (0.3–3 μM) in the presence of N/OFQ did not significantly reduce the effect of N/OFQ in any of the neurons tested (Fig. 4). On the contrary, the current increased by N/OFQ was slightly enhanced by Ro 64-6198 in certain neurons (Fig. 4). These could be those neurons on which the effect of Ro 64-6198 was not mediated by NOP receptors. It could also be due to that additive effect of Ro 64-6198 in these neurons where N/OFQ did not exert its full effect. Again, there are neurons that were unaffected by Ro 64-6198 even in the presence of N/OFQ (Fig. 4).

The Effect of Ro 64-6198 Was Unaffected by Dopamine D2, 5-HT1A, or Opioid Receptor Antagonist. Ro 64-6198 at high concentrations could act at dopamine D2 receptors (Jenck et al., 2000) and opioid receptors (Dautzenberg et al., 2001; Rizzi et al., 2001). Besides, the structure of Ro 64-6198 is similar to the 5-HT1A agonist, spiroxatrine (Herrick-Davis and Titeler, 1988), from which a nonpeptide NOP receptor agonist, NNC 63-0532, has been developed (Thomsen and Hohlweg, 2000). We further examined if sulpiride, naloxone, and NAN-190, the antagonists of dopamine D2, opioid, and 5-HT1A receptors, respectively, would reverse the effect of Ro 64-6198. The results show that none of these antagonists altered the effect of Ro 64-6198 (Fig. 5). After treatment with sulpiride (10 μM), naloxone (1 μM), and NAN-190 (1 μM), the I$_{140}$ values increased by Ro 64-6198 (1–3 μM) were 100 ± 1.9% (n = 3), 102 ± 2.7% (n = 9), and

<table>
<thead>
<tr>
<th>Neuron No.</th>
<th>N/OFQ 0.1 + Ro 0.3</th>
<th>N/OFQ 0.1 + Ro 0.3</th>
<th>N/OFQ 0.1 + Ro 1</th>
<th>N/OFQ 0.3 + Ro 1</th>
<th>N/OFQ 0.3 + Ro 3</th>
<th>N/OFQ 0.3 + Ro 3</th>
<th>N/OFQ 0.3 + Ro 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. N/OFQ activated GIRK in Ro 64-6198-unresponsive neurons. Chart recordings of membrane currents evoked by hyperpolarization ramps in two Ro 64-6198-unresponsive neurons. A, the neuron was treated with Ro 64-6198, 1 μM, followed by N/OFQ, 0.3 μM. B, the neuron was treated with Ro 64-6198, 3 μM, then with N/OFQ, 0.3 μM, and further with Ro 64-6198, 3 μM.

Fig. 4. Effects of Ro 64-6198 on N/OFQ-induced GIRK activation. Shown are the percent increments of I$_{140}$ induced by N/OFQ (0.1 or 0.3 μM) before and after further addition of Ro 64-6198, 0.3 to 3 μM. Each symbol represents the data from one individual neuron, which was treated with N/OFQ and followed by N/OFQ plus Ro 64-6198, at the concentration (in micromolar) depicted. Note that the effect of N/OFQ was not decreased in any of the nine neurons tested.
part of inward rectification. Therefore, this synthetic
free internal solution excludes the possibility that Ro-64-6198
ineffectiveness of Ro-64-6198 in neurons recorded with GTP-
which are coupled to G proteins (Ikeda et al., 1997). The
in ventrolateral PAG neurons (Vaughan and Christie, 1997;
pared with N/OFQ, Ro 64-6198 is a slower acting weak ago-
visited NOP receptors but Not by D2, 5-HT1A, or Opioid Receptors.
ist both in terms of potency and efficacy.
pared with N/OFQ, Ro 64-6198 is a slower acting weak ago-
dopamine D2, serotonin 5-HT1A, or opioid receptors. Com-
ected to G proteins (Ikeda et al., 1997). The
ineffectiveness of Ro-64-6198 in neurons recorded with GTP-
free internal solution excludes the possibility that Ro-64-6198 could activate K+ channels directly.
However, the effects of Ro 64-6198 in only 57% of the
recorded neurons were antagonized by CompB, which
can effectively antagonize the effect of N/OFQ in all the ventro-
lateral PAG neurons tested (Chiou and Fan, 2002). This
suggests that the effect of Ro 64-6198 in ventrolateral PAG
neurons is partly mediated by NOP receptors. Ro 64-6198
has been found to have non-NOP receptor-mediated effects
in vivo (Higgins et al., 2001) and in vitro (Jenck et al., 2000;
Dautzenberg et al., 2001; Rizzi et al., 2001). However, the
ineffectiveness of sulpiride, NAN-190, and naloxone in an-
tagonizing the effect of Ro 64-6198 indicates that the effect of
Ro 64-6198 in ventrolateral PAG neurons is not mediated
dough dopamine D2, 5-HT1A, or opioid receptors.
Ro 64-6198 Is a Weak Agonist of NOP Receptors in
Ventrolateral PAG Neurons. The finding that Ro 64-6198
is 45 times less potent than N/OFQ and has only 62% efficacy
of N/OFQ suggests that, compared with N/OFQ, Ro 64-6198
is a less potent and efficacious agonist of NOP receptors
in ventrolateral PAG neurons. It is a full agonist, as potent as
N/OFQ, at expressed NOP receptors in culture cells (Jenck et
al., 2000; Dautzenberg et al., 2001; Hashiba et al., 2002) and
native NOP receptors of rat vas deferens but is 66 times less
potent than N/OFQ at NOP receptors of the guinea pig ileum
(Rizzi et al., 2001). The lower potency and slower kinetics
of the action of Ro 64-6198 observed in brain slices and periph-
eral preparations (Rizzi et al., 2001) might result from more
penetration barriers in intact tissues, compared with culture
cells. The reason for the difference in the action kinetics
between N/OFQ and Ro 64-6198 is not clear. It might be
attributed to the differences in the modality of binding to
receptors or in the cascades after receptor binding. Dautzen-
berg et al. (2001) found that Ro 64-6198, but not N/OFQ,
caused NOP receptor internalization. The lower efficacy of Ro
64-6198 displayed in brain slices and rat vas deferens (Rizzi
et al., 2001) might be attributed to the different densities of
NOP receptors in different preparations. Other NOP receptor
ligands, naloxone benzoylhydrazone, Ac-RYYRIK-NH2, and
[Phe1Phe2(CH2-NH)Gly3]-nociceptin-(1-13)-NH2, also have
different pharmacological profiles (agonist, partial agonist, or
antagonist) at NOP receptors of different preparations (Calo
et al., 2000a; Chiou, 2001; Bigoni et al., 2002).
Ro 64-6198 Activates a Subset of NOP Receptors. In
previous studies, we have shown that N/OFQ activated GIRK
in 96% of the neurons recorded, and this effect was blocked by
the specific peptide antagonist of NOP receptors, [Nphe1]N/
OFQ-(1-13)-NH2 (Chiou et al., 2002), or the nonpeptide one,
CompB (Chiou and Fan, 2002). This suggests that NOP
receptors are expressed in 96% of the ventrolateral PAG neu-
rons recorded. In the present study, we found Ro 64-6198
activated GIRK in 62% of the neurons recorded, among which
only 57% were sensitive to the blockade of CompB. This
suggests that the effect of Ro 64-6198 is mediated through
NOP receptors in only 35% (57% of 62%) of the neurons
recorded. In those Ro 64-6198-insensitive neurons, NOP re-
ceptors are functional since N/OFQ still activated GIRK
in the remaining 61%, of the ventrolateral PAG neurons
recorded.

There are several explanations for the finding of Ro 64-
6198-insensitive but N/OFQ-sensitive neurons. First, Ro 64-
6198-unresponsive neurons might be those neurons with
lower density of NOP receptors so that the weak agonist-like
Ro 64-6198 could only produce a subtle change in the re-
response, whereas the full agonist, N/OFQ, could induce a
significant effect. If this is the case, the ineffectiveness of Ro
64-6198 should only be observed when lower concentrations
of Ro 64-6198 were used. However, Ro 64-6198-unresponsive
neurons were observed at every concentration (up to 30 μM)
tested (Fig. 2). Second, the low efficacy property of Ro 64-
6198 might make it act like an NOP receptor antagonist. This
possibility can be ruled out since Ro 64-6198 did not reduce
the effect of N/OFQ in any of the neurons that were treated
with N/OFQ first and further with Ro 64-6198 (Fig. 4). Fur-
thermore, there are neurons that were consistently unre-
sponsive to Ro 64-6198, regardless if Ro 64-6198 was added
before or after N/OFQ (Figs. 3 and 4). Third, Ro 64-6198 is a
hydrophobic compound and might bind to nonspecific sites

Fig. 5. Opioid, dopamine D2, or 5-HT1A receptor antagonist failed to affect Ro 64-6198-induced GIRK activation. Chart recordings of membrane currents evoked by hyperpolarization ramps in neurons treated with 1 μM Ro 64-6198 first and then further with 1 μM NAN-190 (5-HT1A receptor antagonist) (A) or with 10 μM sulpiride (dopamine D2 receptor antagonist) or 1 μM naloxone (opioid receptor antagonist) (B). CompB, 1 μM, was applied further and blocked the effect of Ro 64-6198 as shown in both A and B.

101 ± 2.2% (n = 12), respectively, of the before the treatment.

Discussion
In the present study, we found that Ro 64-6198 activated
GIRK in two-thirds of the ventrolateral PAG neurons re-
corded. In those Ro 64-6198-unresponsive neurons, N/OFQ
activated GIRK through activating NOP receptors. The effect of
Ro 64-6198 is partly mediated by NOP receptors but not by
dopamine D2, serotonin 5-HT1A, or opioid receptors. Com-
pared with N/OFQ, Ro 64-6198 is a slower acting weak ago-
nist both in terms of potency and efficacy.
Heterogeneity of NOP Receptors. Heterogeneity of NOP receptors has been implicated from binding studies (Mogil and Pasternak, 2001), and the splicing variants of NOP receptors have been reported (Peluso et al., 1998). Functional heterogeneity of NOP receptors was also proposed by the findings from two in vivo studies that Ro 64-6198 produced some but not all responses generated by NOFQ. Ro 64-6198 mimicked high doses of NOFQ that induced hypolocomotion in mice, but it failed to induce hyperlocomotion as that produced by low doses of NOFQ (Kuzmin et al., 2004). Intracerebral injection of Ro 64-6198 induced hypotension, diuresis, and hyperphagia but not bradycardia or renal sympathetic depression, as that induced by NOFQ (Dayan et al., 2001). The present in vitro study further provides functional cell lineage evidence for the heterogeneity of NOP receptors in the brain tissue relevant to pain regulation. Ro 64-6198 was also found to affect the NOP receptors of vas deferens in rats but not that in mice (Rizzi et al., 2001). However, NOFQ is a potent NOP receptor agonist in mouse vas deferens preparations (Calo et al., 1996). Therefore, it seems that the Ro 64-6198-insensitive subset of NOP receptors exists in both central and peripheral tissues. Nevertheless, the weak and slow-acting agonist properties of Ro 64-6198 at NOP receptors would limit its application. Further development of more potent and selective antagonists that can distinguish NOP receptor subtypes would be of help in clarifying the heterogeneity of NOP receptors.

Acknowledgments

We thank Dr. Satoshi Ozaki at Banyu Pharmaceutical Company for the generous gift of CompB.

References


Reichling DB, Lakos S, and Bassbaum AI (1984) Intracellular electrophysiological during its penetration into deeper neurons in the slice. As a result, the concentration of Ro 64-6198 in the deeper neurons might be too low to be effective in activating NOP receptors. This is unlikely since the Ro 64-6198-unresponsive neurons are not restricted to the deeper neurons. Fourth, the ineffectiveness of Ro 64-6198 might be due to its slow action kinetics in the tissue preparations. This is also unlikely since the effects of Ro 64-6198 were examined in those Ro 64-6198-unresponsive neurons for at least 20 min (Fig. 3), at which time the effect of Ro 64-6198 had almost reached the steady state in Ro 64-6198-sensitive neurons (Fig. 1). Therefore, although Ro 64-6198 is a weak agonist of NOP receptors in ventrolateral PAG neurons, the possibility cannot be ruled out that there is one subset of NOP receptors that is unaffected by Ro 64-6198 but sensitive to NOFQ. It is suggested that two subsets of NOP receptors exist in the ventrolateral PAG, one of which (in 35% of the recorded neurons) is sensitive to both NOFQ and Ro 64-6198, and the other (in 61% of the recorded neurons) is only sensitive to NOFQ but not to Ro 64-6198.
and Golgi analysis of the midbrain periaqueductal gray (PAG) of the rat (Abstract).


Address correspondence to: Professor Lih-Chu Chiou, Department of Pharmacology, College of Medicine, National Taiwan University, 1 Jen-Ai Road, Section 1, Taipei 100, Taiwan. E-mail: lechiou@ha.mc.ntu.edu.tw