Functional expression, activation and desensitization of opioid receptor-like receptor ORL_1 in neuroblastoma×glioma NG108-15 hybrid cells

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Abstract **Neuroblastoma×glioma NG108-15 hybrid cells have been examined for the expression of opioid receptor-like receptor (ORL_1). [^{3}H]Nociceptin/orphanin FQ (OFQ) bound to the cell membrane specifically ($K_d = 3.6 \pm 0.6$ nM) and inhibited forskolin-stimulated cAMP accumulation ($EC_{50} = 0.72 \pm 0.3$ nM). The responsiveness of NG108-15 cells to nociceptin/OFQ was blocked by pertussis toxin but not by naltrindole. The inhibitory activity of nociceptin/OFQ was significantly reduced after a prechallenge with the same peptide but was not influenced by DPDPE pretreatment, indicating acute and homologous desensitization of ORL_1 receptors. Naltrindole caused the overshoot of cAMP in DPDPE-pretreated cells but not in nociceptin/OFQ-pretreated cells. The results indicate that ORL_1 is functionally expressed and does not cross-interact with specific ligands of the δ opioid receptor in NG108-15 cells.**

Key words: Opioid receptor; Opioid receptor-like 1 receptor (ORL_1); Nociceptin/orphanin FQ; Functional expression; Desensitization; NG108-15 cell

1. Introduction

Opioids are clinically effective analgesics. Through interaction with endogenous opioid peptides and exogenous opioid alkaloids, opioid receptors mediate numerous physiological and pharmacological effects. Three subtypes of the opioid receptors (μ, δ, and κ) have been identified through their different affinities for various opioid ligands [1]. These opioid receptors are all coupled to the inhibitory G protein (Gi) and negatively regulate adenylyl cyclase [1]. Recently, another Gi protein coupled receptor, opioid receptor-like receptor (ORL_1), has been cloned from brain [2–8]. Its endogenous specific agonist nociceptin/orphanin FQ (OFQ) has also been identified [9,10]. Nociceptin/OFQ specifically binds to ORL_1 exogenously expressed in CHO cells and inhibits forskolin-stimulated cAMP accumulation. But in contrast to the analgesic effects of most opioids, nociceptin/OFQ induces hyperalgesia in animals [9,10]. The cellular and molecular mechanisms underlying nociceptin's anti-opioid (hyperalgesic) effects are yet to be understood. A neuronal cellular model system is needed to study nociceptin/OFQ-ORL_1 mediated signal transduction and possible interactions of ORL_1 with other opioid receptors. In the present study, we report that neuroblastoma×glioma NG108-15 hybrid cells, which express the δ opioid receptor, also endogenously express functional ORL_1. Our study demonstrated that ORL_1 can be acutely and homologously desensitized by nociceptin/OFQ and that nociceptin/OFQ does not cross-interact with the δ opioid receptor in NG108-15 cells.

2. Materials and methods

2.1. Cell cultures

Neuroblastoma×glioma NG108-15 hybrid cells were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Evergreen, Hangzhou, China), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine as described previously [11].

2.2. Membrane preparation and nociceptin/OFQ-ORL_1 binding assay

Cells were harvested and homogenized in 50 mM Tris-HCl, pH 7.4. The homogenates were centrifuged at 38 000×*g* for 15 min at 4°C and the pellets were resuspended in 50 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin (BSA, Sigma). The membrane preparations were homogenized again just before use.

Membrane protein (0.5 mg) was incubated with different concentrations of [^{3}H]nociceptin/OFQ (33 Ci/mmol, Phoenix Pharmaceuticals, Inc., California) in 0.4 ml of 50 mM Tris-HCl, pH 7.4, and 1% BSA at 30°C for 1 h. The reaction was terminated by filtering through GF-B filters presoaked with 0.1% polyethyleneimine. After washing, the nociceptin/OFQ bound to the membrane was determined by measuring radioactivity remaining on the filter in a scintillation counter. Non-specific binding was determined in the presence of 50 μM unlabelled nociceptin/OFQ (Tocris Cookson Ltd, UK) and was subtracted from the total binding.

2.3. Cyclic AMP assay

Cells were challenged with nociceptin/OFQ or DPDPE in the presence of 1 μM forskolin and 500 μM 1-methyl-3-isobutylxanthine (IBMX, Sigma) at 37°C for 10 min. The reactions were terminated with 1 N perchloric acid and neutralized with 2 N K_2CO_3. The cAMP level of each sample was determined using radioimmunoassay as described previously [11]. Data were averages of triplicate samples and are presented as a percentage of control (in the presence of forskolin alone).

2.4. Receptor desensitization and cAMP overshooting

NG108-15 cells were prechallenged with 0, 0.1, 1, or 10 μM of nociceptin/OFQ or 10 μM [D-Pen^2,D-Pen^5]-enkephalin (DPDPE, Sigma) at 37°C for 10 min. The cells were then washed twice with phosphate buffered saline and challenged again with 1 μM of nociceptin/OFQ or 1 μM DPDPE. In the experiment of overshoot of cellular cAMP levels, cells were preincubated with nothing, 1 μM of nociceptin/OFQ or DPDPE at 37°C for 2 h and washed four times with phosphate buffered saline. The cAMP accumulation was determined 10 min after addition of 10 μM of naltrindole (Sigma) or naloxone (Sigma). The cellular cAMP levels were measured as described above.

3. Results

Membrane fractions were prepared from NG108-15 cells, and the presence of ORL_1 receptors was examined using

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Abbreviations: OFQ, orphanin FQ; ORL_1, opioid receptor-like 1 receptor; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; PTX, pertussis toxin; cAMP, cyclic AMP; IBMX, 1-methyl-3-isobutylxanthine

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[^{3}H]nociceptin/OFQ, a specific agonist of ORL_1 [9,10]. As shown in Fig. 1A, binding of nociceptin/OFQ to NG108-15 was specific and saturable. Analysis of saturation binding data indicates a dissociation constant of 3.6 ± 0.6 nM and B_{max} of 155 ± 14 fmol/mg protein, comparable to the value obtained in rat brain homogenates [12]

The function of the ORL_1 receptors expressed in NG108-15 cells was also assessed. As shown in Fig. 1B, nociceptin/OFQ strongly inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner, indicating functional expression of ORL_1 receptor in NG108-15 cells. The nociceptin/OFQ induced attenuation of cAMP accumulation occurred at nanomolar concentrations of nociceptin ($EC_{50} = 0.72 \pm 0.3$ nM) and reached maximum inhibition at micromolar concentrations of nociceptin/OFQ, consistent with that observed in ORL_1 transfected CHO cells ($EC_{50} = 0.9$ and 1 nM [9,10]).

ORL_1 is negatively coupled to G-protein and its activation attenuates cellular cAMP accumulation [9,10]. As shown in

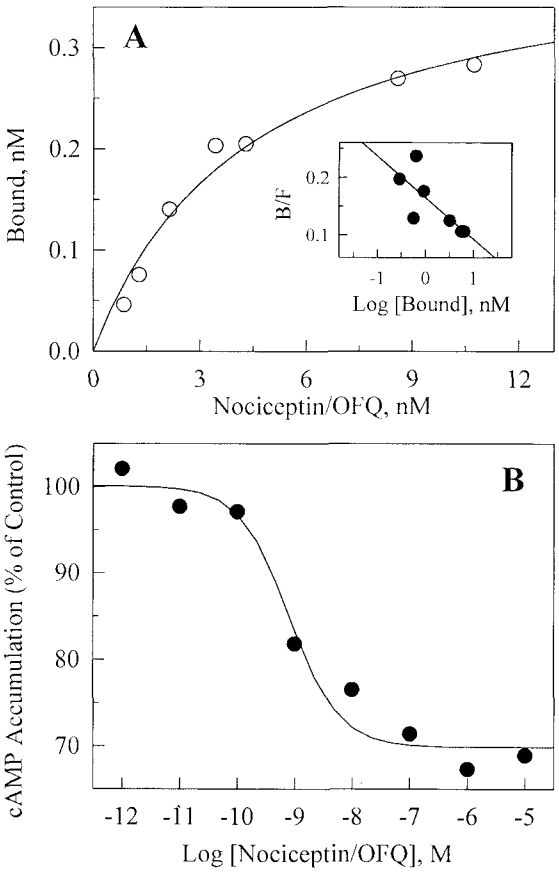

Fig. 1. Functional expression of ORL_1. A: Saturation binding of ^{3}H-labeled nociceptin/OFQ to NG108-15 cells. Membrane preparations from NG108-15 cells were incubated with various concentrations of [^{3}H]nociceptin/OFQ, and the [^{3}H]nociceptin/OFQ bound to membranes was determined as described in Section 2. The Scatchard plot obtained from the saturation curve is shown as an inset. The K_d of nociceptin/OFQ was calculated to be 3.6 ± 0.6 nM, and the B_{max} 155 ± 14 fmol/mg protein. B: The dose-response curve for inhibition of forskolin stimulated accumulation of cellular cAMP by nociceptin/OFQ. NG108-15 cells were incubated with various concentrations of nociceptin/OFQ at 37°C for 10 min and the cAMP accumulation was determined as described in Section 2. The EC_{50} of nociceptin/OFQ inhibition was 0.72 ± 0.3 nM. The figure is representative of three independent experiments. Each value is the average of triplicate samples.

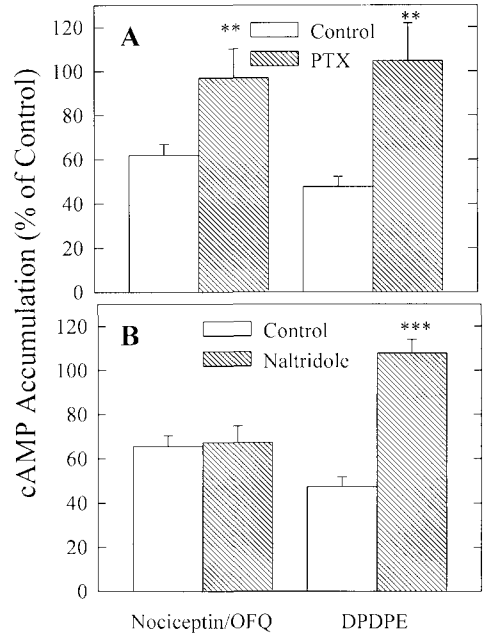

Fig. 2. Effects of PTX and naltrindole on the activity of nociceptin/OFQ. A: Cells were pretreated with or without 200 ng/ml PTX for 24 h, and then were challenged with 1 μM of nociceptin/OFQ or DPDPE at 37°C for 10 min. B: Cells were challenged, in the absence or presence of 10 μM naltrindole, with 1 μM of nociceptin/OFQ or DPDPE at 37°C for 10 min. The cAMP accumulation was determined and is presented as a percentage of control. Plotted is the percentage of cAMP accumulation from at least three experiments (means ± S.E.). Student's *t*-test was used for statistical analysis of data. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

Fig. 2A, pretreatment of the cells with pertussis toxin (PTX), a blocker of Gi/Go, completely abolished nociceptin/OFQ induced inhibition of cAMP accumulation. This suggests that PTX-sensitive G protein(s) is required for the inhibitory effect of nociceptin/OFQ in NG108-15 cells. The data also confirm that ORL_1, a putative Gi protein coupled receptor, is functionally expressed in the neuronal NG108-15 cells.

NG108-15 is a clonal, neuronal cell line which has long been used as a cellular model for opioid receptor studies. It has been demonstrated that the NG108-15 cells contain a homogeneous population of opiate receptors, the δ subtype [13,14]. Like ORL_1 and other opioid receptors, the δ opioid receptor is coupled to Gi protein [1,15]. To exclude any probable contribution of the δ opioid receptor to nociceptin/OFQ's inhibition of cAMP accumulation, we tested the ability of naltrindole, a δ-specific antagonist, to block nociceptin induced attenuation of cAMP accumulation. As shown in Fig. 2B, treatment of NG108-15 cells with the δ-specific antagonist naltrindole did not affect the ability of nociceptin to inhibit forskolin stimulated cAMP accumulation. In contrast, naltrindole completely blocked DPDPE induced inhibition of cAMP accumulation, suggesting that DPDPE does not cross-activate ORL_1 under the experimental conditions. The above data clearly indicate that nociceptin/OFQ does not activate the δ opiate receptor and the δ opioid receptor is apparently not involved in nociceptin induced attenuation of cAMP accumulation in NG108-15 cells.

Opioid receptor desensitization, the reduced responsiveness of the opioid receptor/Gi system after opioid stimulation, has been implicated as one of the molecular mechanisms under-

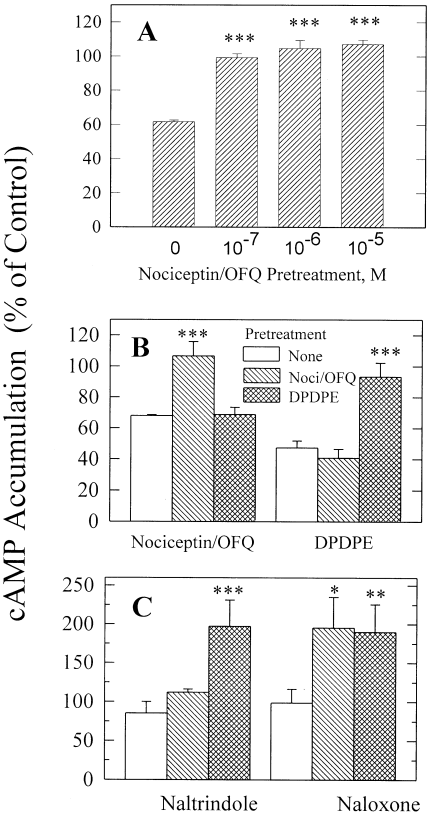

Fig. 3. Desensitization of ORL_1 and cAMP overshooting. A: Cells were pretreated with 0, 0.1, 1, or 10 μM nociceptin/OFQ at 37°C for 10 min and washed with phosphate buffered saline. The cells were then challenged again with 1 μM of nociceptin. B: Cells were pretreated with nothing, 10 μM of nociceptin/OFQ, or 10 μM DPDPE at 37°C for 10 min, and then challenged again with 1 μM of nociceptin/OFQ (Noci/OFQ) or 1 μM DPDPE. C: Cells were preincubated with nothing, 1 μM of nociceptin/OFQ, or 1 μM DPDPE at 37°C for 2 h, and washed with phosphate buffered saline. The cAMP accumulation was determined 10 min after 10 μM of naltrindole or naloxone was added to the cells. Plotted is the percentage of cAMP accumulation from at least three experiments (means ± S.E.). Student's *t*-test was used for statistical analysis of data. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

lying opioid tolerance. To examine the ability of nociceptin to induce desensitization of ORL_1 and the δ opioid receptors, NG108-15 cells were pretreated with the ORL_1 endogenous agonist nociceptin/OFQ or the δ opioid receptor-specific agonist DPDPE, and the ability of nociceptin or DPDPE to inhibit forskolin stimulated cAMP accumulation was determined. As shown in Fig. 3A, the inhibition on cAMP accumulation was abolished after a brief prechallenge with nociceptin/OFQ, indicating that ORL_1 receptor undergoes acute desensitization. However, prechallenge with nociceptin did not change inhibition of cAMP accumulation induced by DPDPE, a δ-specific agonist (Fig. 3B). Moreover, pretreatment of DPDPE desensitized only the δ opioid receptor, but did not attenuate cAMP accumulation stimulated with the ORL_1-specific agonist nociceptin/OFQ (Fig. 3B). This suggests that desensitization of ORL_1 is agonist-specific (i.e. homologous), and ORL_1 is not cross-desensitized by the δ-specific agonist in NG108-15 cells.

In addition to desensitization of opioid receptors, a compensatory increase in adenylyl cyclase activity could be observed in cells when opioid receptor was activated for an extended period of time. The compensatory increase in cellular cAMP level, cAMP overshoot, could be observed either by removal of agonist or by addition of antagonist after chronic treatment with δ opioid agonist in NG108-15 cells [13] and neuroblastoma NS20Y cells [16]. The cAMP overshooting was also observed with μ opioid receptor in human neuroblastoma SH-SY5Y cells [17]. We next investigated if administration of a non-specific opiate antagonist or a δ-specific antagonist after chronic treatment with nociceptin/OFQ causes cAMP overshoot. NG108-15 cells were prechallenged with nothing, 1 μM of nociceptin/OFQ or 1 μM DPDPE for 2 h, and cAMP accumulation was determined 10 min after addition of naltrindole or naloxone. As shown in Fig. 3C, administration of naloxone, a non-specific antagonist of opioid receptors, increased intracellular cAMP levels by approximately 100% in both DPDPE- and nociceptin/OFQ-treated cells. However, when naltrindole, a δ-specific antagonist, was added, cAMP overshoot was observed only in the cells prechallenged with the δ-specific agonist DPDPE-prechallenged cells, not in the cells treated with the ORL_1-specific agonist nociceptin/OFQ (Fig. 3C). This indicates that continuous activation of ORL_1 results in a compensatory increase of adenylyl cyclase activity, and confirms that the inhibitory activity of nociceptin/OFQ on cAMP accumulation was not mediated by the δ opioid receptor in NG108-15 cells.

4. Discussion

The anti-opioid effect mediated by ORL_1 [9,10] suggests that ORL_1, like other opioid receptors, plays an important role in pain modulation. The molecular and cellular mechanisms of the ORL_1 mediated anti-analgesic effect are not yet understood. Although it has been demonstrated that activation of ORL_1 inhibits adenylyl cyclase in CHO cells exogenously transfected with ORL_1, the function and signal transduction processes of ORL_1 have not been studied in a neuronal cellular environment. Our present study using NG108-15 cells revealed that this neuronal cell line endogenously expresses functional ORL_1. The receptor binding experiments indicated that nociceptin/OFQ binds to the NG108-15 cell membrane in a dose-dependent and saturable manner, with a dissociation constant of 3 nM (Fig. 1A). Nociceptin/OFQ also strongly inhibited forskolin-stimulated cAMP accumulation in NG108-15 cells. The EC_{50} of the inhibitory activity of nociceptin/OFQ was 0.7 nM (Fig. 1B), consistent with values determined in CHO cells previously [9,10]. Furthermore, PTX strongly blocked the inhibitory effect of nociceptin/OFQ (Fig. 2A), suggesting involvement of Gi coupled receptors. The above data indicate the endogenous and functional expression of ORL_1 in NG108-15 cells. The K_d observed for nociceptin binding to ORL_1 (3.6 ± 0.6 nM) in NG108-15 cells is comparable to the value obtained in rat brain (5 ± 1.1 nM [12]). However, the reported K_d value in non-neuronal CHO cells exogenously transfected with ORL_1 is lower (0.1 nM [10]). This may reflect differences in membrane environment of neuronal versus non-neuronal cells.

Like μ, δ, and κ opioid receptors, the newly discovered ORL_1 is also widely expressed throughout the brain and spinal cord as detected by Northern blotting, in situ hybridization and immunohistochemistry [2,3,5,18]. The expression of ORL_1 overlaps with that of other opioid receptors in many regions of the central nervous system. Furthermore, ORL_1

mediates an anti-opioid effect as demonstrated in animal experiments [9,10], suggesting that ORL_1 may interact directly or indirectly with opioid receptors. However, it has not been demonstrated that ORL_1 co-expresses endogenously with μ, δ, or κ opioid receptors in any neuronal cells. NG108-15 cells have been used as a cellular model system for opioid research. It has been previously observed that this clonal cell line endogenously expresses a homogeneous population of opioid receptors, the δ opioid receptors [13,14]. Our present study revealed that this neuronal cell line endogenously and functionally expresses both ORL_1 and the δ opioid receptors. Treatment of NG108-15 cells with naltrindole, a specific antagonist of the δ opioid receptor, did not significantly change the inhibitory effect of nociceptin/OFQ on cellular cAMP accumulation, while the same treatment abolished the inhibitory effect of DPDPE (Fig. 2B), indicating that the inhibitory function of nociceptin/OFQ on cAMP accumulation observed in NG108-15 cells is not mediated by the δ opioid receptor.

Chronic administration of opiates results in tolerance and dependence. However, the mechanisms underlying this are largely unknown. Desensitization, phosphorylation, and down-regulation of opioid receptors, and postreceptor intracellular messenger pathways including up-regulation of the cAMP pathway have been implicated in opioid tolerance and dependence [19–21]. The work of Law et al. and our group has demonstrated that the δ opioid receptors in NG108-15 cells undergo chronic and acute homologous desensitization [11,13]. Our present study provides the first report of acute and homologous desensitization of ORL_1 receptors. In addition to receptor desensitization, chronic administration of opioid increases levels of expression of adenylyl cyclase and protein kinase A in locus ceruleus neurons. Removal of the opiate leads to cAMP overshoot and this contributes to the activation of locus ceruleus neurons during withdrawal [18]. In the current study, we examined the effect of chronic treatment with nociceptin/OFQ on the activation of adenylyl cyclase in NG108-15 cells. The data showed that addition of naloxone results in overshoot of cAMP in the cells chronically exposed to nociceptin/OFQ (Fig. 3C). Administration of naltrindole caused the overshooting of cAMP levels in DPDPE-pretreated cells but not in cells pretreated with nociceptin/OFQ. Taken together, the above results show that ORL_1, upon pretreatment with nociceptin/OFQ, undergoes acute desensitization and up-regulates cellular cAMP. The results also suggest that ORL_1 does not cross-interact with or cannot be desensitized by specific ligands of the δ opioid receptor.

Nociceptin/OFQ exerts an anti-opioid effect in animal experiments [9,10]. However, it is very homologous to the opioid receptors in structure and its distribution largely overlaps with opioid receptors. Like opioid receptors, ORL_1 is negatively coupled to the G protein and activation of ORL_1 causes attenuation of cellular cAMP accumulation. The molecular and cellular mechanisms behind the anti-opioid effect mediated by ORL_1 are still a mystery. The NG108-15 cell line, as we have demonstrated, endogenously and functionally expresses ORL_1. It will serve as an excellent cellular model to study ORL_1 mediated signal transduction in neuronal cells. The presence of well characterized δ opioid receptor in NG108-15 cells allows us to study the interactions between the ORL_1 and opioid receptor mediated signal transduction pathways in a natural, neuronal cellular environment.

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