

Nociceptin/orphanin FQ stimulates extracellular acidification and desensitization of the response involves protein kinase C

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Abstract A Chinese hamster ovary (CHO) cell line, CHO-ORL₁, stably expressing human opioid receptor-like receptor 1 (ORL₁) has been used to determine ORL₁-mediated signaling events using microphysiometry. Nociceptin/orphanin FQ (N/OFQ), a specific endogenous agonist of ORL₁, induced an increase in extracellular acidification rate (ECAR) in CHO-ORL₁ cells. The ECAR response stimulated by N/OFQ was concentration-dependent and pertussis toxin-sensitive. Repeated exposures of the cells to N/OFQ caused desensitization of ORL₁. The ECAR response was recovered at the half-life of approximately 12 min after the initial challenge. Pretreatment with inhibitor of cAMP-dependent kinase did not affect desensitization of ORL₁. However, specific inhibitors for protein kinase C almost abolished N/OFQ-induced desensitization of extracellular acidification responsiveness, indicating the involvement of protein kinase C in the process.

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1. Introduction

Opioid receptors belong to the G-protein-coupled receptor family that is characterized by the seven transmembrane spanning domains in structure. Activation of opioid receptor inhibits adenylyl cyclase activity [1]. There are three opioid receptor subtypes: μ , δ , and κ . They mediate numerous physiological and pharmacological effects through interaction with opioids. Their essential role in opioid-induced analgesia has been demonstrated in the opioid receptor knock-out mouse by Matthes et al. recently [2].

Opioid receptor-like receptor 1 (ORL₁), a new member of the family of G-protein coupled receptors, has been cloned from brain recently [3–9]. It does not bind classical opioid ligands [6] and was considered an orphan receptor until its endogenous specific agonist nociceptin/orphanin FQ (N/OFQ) was identified [10,11]. In spite of the structure similarities that ORL₁ and N/OFQ share with opioid receptors and opioid peptides, N/OFQ has been shown to reverse analgesia induced

by opioids [10,11]. The molecular and cellular basis of the anti-opioid effects mediated by ORL₁ remains to be understood.

Recent studies have demonstrated that interaction of N/OFQ with ORL₁ results in activation of membrane-associated G protein, inhibition of adenylyl cyclase activity and increase of inwardly rectifying K⁺ conductance in neuronal and non-neuronal cells [10–15]. In our earlier work, we observed that ORL₁ undergoes acute and homologous desensitization [13,15]. In the present study, we have demonstrated that activation of ORL₁ increased extracellular acidification rate rapidly by using a microphysiometer, a real-time and non-invasive system for monitoring the fast cellular response. Our results also indicate that desensitization of ORL₁-stimulated extracellular acidification involves protein kinase C (PKC).

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) cells (American Type Culture Collection) were cultured in DMEM (Gibco-BRL) containing 10% heat-inactivated fetal calf serum (Evergreen). CHO-ORL₁ cells stably expressing human ORL₁ were cultured in F12 medium (Gibco-BRL) containing 10% heat-inactivated fetal calf serum and 0.2 mg/ml Geneticin (Gibco-BRL). All culture media were supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM glutamine.

2.2. Construction of CHO-ORL₁ stable cell line

The cDNA for the human ORL₁ was isolated by PCR directly from human brain cDNA (Clontech Laboratories, CA, USA). Oligonucleotide primers, based on the published sequence [3], were used to amplify a 1.1 kb fragment containing the entire coding sequence of ORL₁. This fragment was cloned into pCDNA₃ (Invitrogen) and nucleotide sequence analysis was performed using the Sequenase 2.0 DNA sequencing kit (Amersham) according to the manufacturer's suggested protocols. CHO cells were transfected with pCDNA₃ carrying human ORL₁ insert by calcium phosphate precipitation [16]. A Geneticin-resistant clone expressing human ORL₁ (CHO-ORL₁) was isolated and used in this study.

2.3. Microphysiometry

Extracellular acidification rates were measured with the silicon-based light addressable potentiometer Cytosensor Microphysiometer (Molecular Devices Corp., CA, USA). CHO-ORL₁ cells were seeded at 1×10^5 cells/capsule cup (Molecular Devices Corp.). After the cells adhered to the capsule membrane and were cultured overnight, a spacer gasket and capsule insert (Molecular Devices Corp.) were placed into each capsule cup. The assembled capsule cups were loaded into Cytosensor chambers. Low buffering, carbonate-free DMEM (Gibco-BRL) was used as medium in the Cytosensor, and the extracellular acidification rates were monitored, collecting a rate measurement every 90 s. Cells were exposed to N/OFQ in running medium (0.1 ml/min) for 335 s, and the effects on the extracellular acidification rate were measured by the instrument. The basal acidification rate was normalized to 100%.

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Abbreviations: N/OFQ, nociceptin/orphanin FQ; ORL₁, opioid receptor-like receptor 1; PTX, pertussis toxin; ECAR, extracellular acidification rate; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; BSA, bovine serum albumin; LPA, lysophosphatidic acid

3. Results and discussion

CHO-ORL₁, a CHO cell line stably expressing human ORL₁, was used in this study. Expression of ORL₁ was confirmed by Northern analysis with ORL₁-specific cDNA probe, radioligand binding and agonist-dependent [³⁵S]GTPγS binding assays (data not shown). Functional responses mediated by ORL₁ in CHO-ORL₁ cells were accessed in real-time scale by a microphysiometer which measures changes in the rate at which cells acidify their environment. Perfusion of 10 nM N/OFQ increased the extracellular acidification rate of CHO-ORL₁ cells to approximately 150% of the unstimulated rate within 5–10 min (Fig. 1A). The effect of N/OFQ on the extracellular acidification rate measured in the microphysiometer was concentration-dependent. As shown in Fig. 2A, the extracellular acidification response to N/OFQ occurred at 0.1 nM concentration of N/OFQ and reached maximum at 10 nM. The EC₅₀ (0.6 ± 0.1 nM) for N/OFQ-stimulated extracellular acidification was determined. This value is comparable to those for N/OFQ-stimulated GTPγS binding (0.5 nM) measured in this study and for N/OFQ-stimulated attenuation of cellular cAMP production (0.7–1 nM) reported previously [11–13,15] using CHO cells transfected with ORL₁. Pertussis toxin, a specific inhibitor of Gi/Go protein, completely blocked the cellular response to N/OFQ (Fig. 1B), indicating

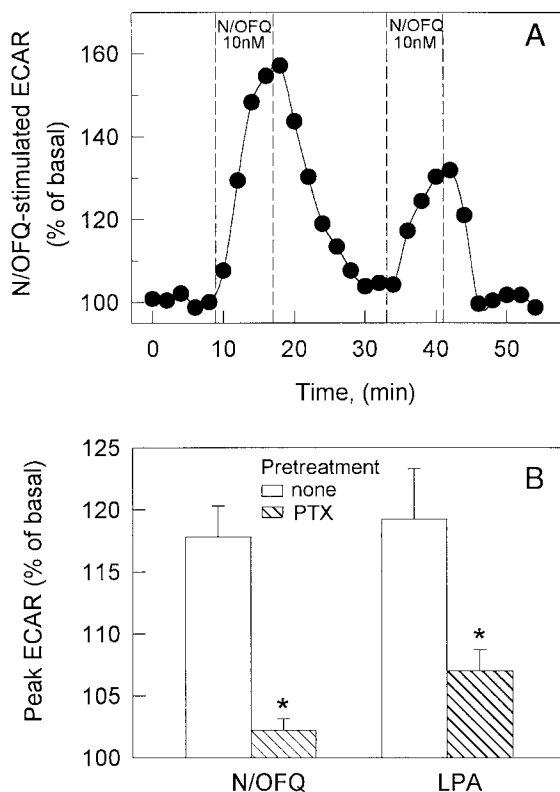


Fig. 1. Changes in ECAR in response to N/OFQ. A: CHO-ORL₁ cells were exposed to 10 nM N/OFQ for 6 min twice with a time interval of 12 min. The figure is representative of three independent experiments. B: Cells in capsules were pretreated with or without 100 ng/ml PTX for 24 h, and then exposed to 10 nM of N/OFQ or 100 nM LPA for 6 min in the microphysiometer. The data are averages from at least three experiments. Student's *t*-test was used for statistical analysis of data. **P* < 0.05 as compared with the value without treatment. The basal acidification rate was normalized to 100%.

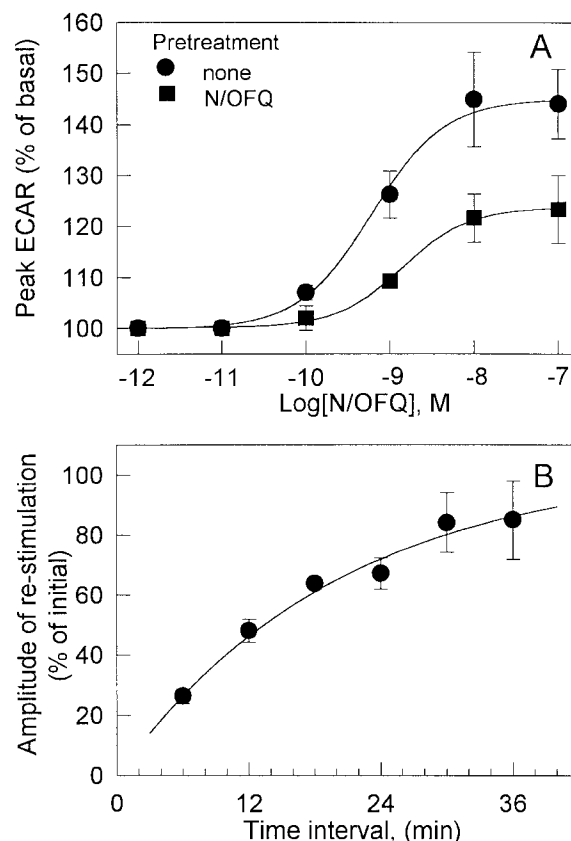


Fig. 2. Desensitization of N/OFQ-stimulated ECAR response. A: The peak values of N/OFQ-induced ECAR change with or without 10 nM N/OFQ pretreatment were plotted in the figure separately with an EC₅₀ of 1.6 ± 0.1 nM and 0.6 ± 0.1 nM respectively. B: CHO-ORL₁ cells were challenged twice with 10 nM N/OFQ at various time intervals as indicated in the figure. The peak ECAR values in response to the second agonist treatment were plotted as % of peak ECAR of the initial treatment in the figure. The values on the plot represent the averages of at least three experiments.

that the metabolic response stimulated by N/OFQ was mediated through Gi/Go protein.

Desensitization of opioid receptors, the reduced responsiveness of opioid receptor/Gi system after opioid stimulation, has been implicated in the molecular mechanisms underlying opioid tolerance. We have demonstrated recently, using cAMP assay, that ORL₁ undergoes homologous desensitization in neuronal cells [15]. However, the time dependence and the mechanism of desensitization of ORL₁ need to be further investigated. A microphysiometer monitors extracellular acidification rate in real-time scale. Cellular response to stimuli can be repeatedly determined from the same sample in a microphysiometer. We have investigated the time course of desensitization of ORL₁ receptor using microphysiometry in this study. As shown in Fig. 1A, ECAR response to N/OFQ decreased significantly after pretreatment with the same agonist at the same dosage while the time needed to reach the maximum response remained the same. After prechallenge with ORL₁ agonist, the concentration–response curve for N/OFQ-stimulated ECAR response shifted toward the right (EC₅₀ increased from 0.6 ± 0.1 nM to 1.6 ± 0.1 nM) and the N/OFQ-stimulated ECAR response reduced approximately 50% (Fig. 2A). These data suggest that ORL₁ undergoes acute desensitization after agonist pretreatment.

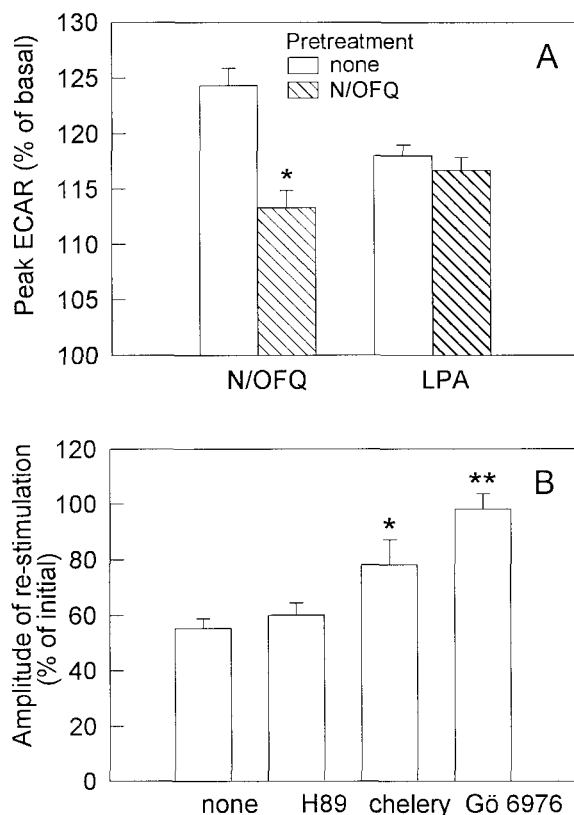


Fig. 3. Agonist-dependent desensitization of ORL₁ and involvement of PKC. A: Cells were pretreated with or without 10 nM of N/OFQ, and then challenged again with 10 nM of N/OFQ or 100 nM LPA. B: Cells were treated twice with 10 nM of N/OFQ in a 12 min interval after preincubation with none, 480 nM H89, 6.6 μ M chelerythrine chloride, or 80 nM Gö 6976 for 15 min in the microphysiometer. The peak ECAR values in response to the second agonist treatment were plotted as % of peak ECAR of the initial treatment in the figure. The values on the plot represent the averages from at least three experiments. Student's *t*-test was used for statistical analysis of data. **P* < 0.05, ***P* < 0.01, as compared with the value without N/OFQ pretreatment.

The desensitization of ORL₁ was reversible and the time course for ORL₁ recovering from desensitization was determined. As shown in Fig. 2B, the ECAR response induced by N/OFQ was recovered rapidly after initial exposure to the ORL₁ agonist. The half-life of the functional recovery of ORL₁ was approximately 12 min and about 90% of the ECAR response was recovered within 36 min after the initial exposure. Lysophosphatidic acid (LPA), agonist of another G_i-protein-coupled receptor which is endogenously expressed in CHO cells, caused a pertussis toxin-sensitive increase in extracellular acidification in CHO-ORL₁ cells (Fig. 1B). However, pretreatment of N/OFQ did not affect LPA-stimulated extracellular acidification (Fig. 3A). This indicates that the desensitization of ORL₁ is agonist-specific.

Agonist-dependent desensitization of receptor-effector coupling could involve several mechanisms at the cellular level. However, receptor sequestration and down-regulation may not account for the acute desensitization while protein phosphorylation could be a major determinant for uncoupling the system, as in the well studied β_2 -adrenergic receptor [17] and the δ -opioid receptor systems [18]. Receptor phosphorylation

has been implicated as one mechanism of receptor desensitization [19]. It has been shown that the inhibitor of protein kinases attenuates the development of tolerance to opioids, and activation of PKC significantly reduces the development of morphine dependence [20]. Our previous research has shown that the desensitization of the δ -opioid receptor requires activation of PKC [18]. Therefore, the involvement of protein kinases in the process of desensitization of ORL₁ was investigated using cell-permeable protein kinase inhibitors. As shown in Fig. 3B, when cells were exposed to N/OFQ a second time in a 12 min interval, the extracellular acidification response reduced to 50% of the initial agonist treatment at the same dosage. Pretreatment with H89 [21], a potent specific inhibitor of cAMP-dependent kinase (PKA) (K_i = 48 nM), had no effect on the desensitization of ORL₁. However, after pretreatment with the selective inhibitors of PKC, either chelerythrine chloride (K_i = 0.66 μ M) [22] or Gö 6976 (K_i = 8 nM) [23], the extracellular acidification in response to the second exposure of N/OFQ was partially or completely restored (Fig. 3B). These results clearly indicate that N/OFQ desensitizes ORL₁ through a mechanism that requires PKC not PKA. The PKC substrate involved in the desensitization of ORL₁ signaling is to be identified. Our previous research has demonstrated that the agonist-induced acute desensitization is temporally parallel to the opioid receptor phosphorylation [19,24]. The phosphorylation of ORL₁ has not been reported. However, based on the similarities of ORL₁ and opioid receptors in the receptor-mediated signal transduction processes, it is conceivable to consider the ORL₁ receptor as a likely substrate of PKC. Work is currently underway in our laboratory to look into the mechanism of ORL₁ desensitization and the possible role of receptor phosphorylation.

A cytosensor microphysiometer measures the proton excretion of cells accurately in real time [25]. Proton excretion, or extracellular acidification, depends largely on the energy consumption of a cell. When cell surface receptors are triggered by ligands, the cellular metabolism is activated by second messenger pathways resulting in increased energy metabolism and extracellular acidification which can be measured by a microphysiometer. Activation of a wide variety of cell surface receptor types including β -adrenergic receptor, nerve growth factor, etc., has been studied using microphysiometry [25]. Microphysiometry can give not only valuable and rapid data regarding receptor activation but also information on the intracellular events that are stimulated upon agonist occupation of a receptor. However, the effect of activation of an opioid receptor or opioid receptor homologue on extracellular acidification has not been documented. This is the first report showing that activation of a member of the opioid receptor/opioid receptor-like receptor family causes an increase of extracellular acidification rate. We have also determined, using this method, that ORL₁ undergoes acute desensitization and that PKC is required for the desensitization of the ORL₁-mediated ECAR responsiveness.

Opioids are clinically effective analgesic drugs while N/OFQ mediates an anti-opioid effect. Studying the interactions between those receptors and their endogenous ligands has great pharmaceutical and clinical importance but no antagonist of ORL₁ has been reported yet. Our finding that activation of ORL₁ stimulated rapid ECAR response therefore provides a useful screening tool for evaluating ligands for potential therapeutic purposes.

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