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-ORL_1, stably expressing human opioid receptor-like receptor 1 (ORL_1) has been used to determine ORL_1-mediated signaling events using microphysiometry. Nociceptin/orphanin FQ (N/OFQ), a specific endogenous agonist of ORL_1, induced an increase in extracellular acidification rate (ECAR) in CHO-ORL_1 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 ORL_1. 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 ORL_1. 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.

Key words: Opioid receptor-like receptor 1 (ORL_1); Nociceptin/orphanin FQ; Extracellular acidification; Opioid receptor; Receptor desensitization; Protein kinase C

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 (ORL_1), 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 ORL_1 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 ORL_1 remains to be understood.

Recent studies have demonstrated that interaction of N/OFQ with ORL_1 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 ORL_1 undergoes acute and homologous desensitization [13,15]. In the present study, we have demonstrated that activation of ORL_1 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 ORL_1-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-ORL_1 cells stably expressing human ORL_1 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 μg/ml streptomycin and 2 mM glutamine.

2.2. Construction of CHO-ORL_1 stable cell line

The cDNA for the human ORL_1 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 ORL_1. This fragment was cloned into $pCDNA_3$ (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 ORL_1 insert by calcium phosphate precipitation [16]. A Geneticin-resistant clone expressing human ORL_1 (CHO-ORL_1) 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-ORL_1 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; ORL_1, 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

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3. Results and discussion

CHO-ORL_1, a CHO cell line stably expressing human ORL_1, was used in this study. Expression of ORL_1 was confirmed by Northern analysis with ORL_1-specific cDNA probe, radioligand binding and agonist-dependent [^{35}S]GTPγS binding assays (data not shown). Functional responses mediated by ORL_1 in CHO-ORL_1 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-ORL_1 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 EC_{50} (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 ORL_1. Pertussis toxin, a specific inhibitor of Gi/Go protein, completely blocked the cellular response to N/OFQ (Fig. 1B), indicating that the metabolic response stimulated by N/OFQ was mediated through Gi/Go protein.

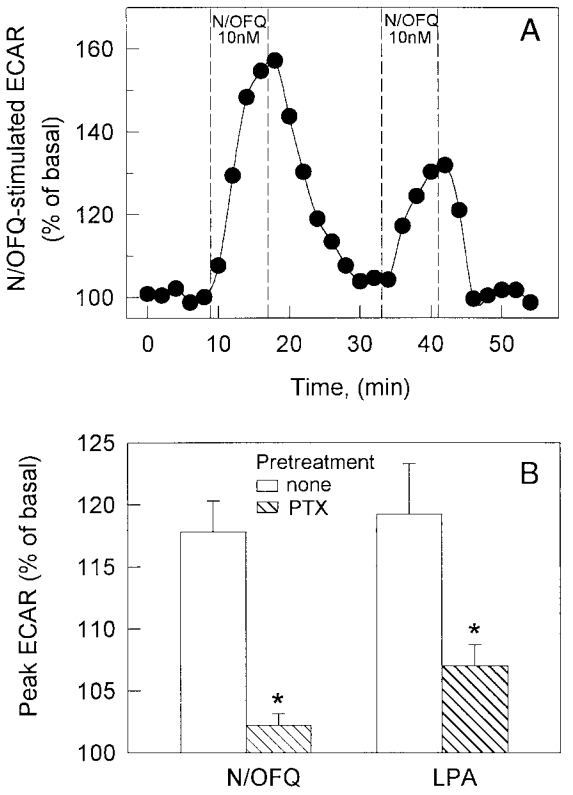

Fig. 1. Changes in ECAR in response to N/OFQ. A: CHO-ORL_1 cells were exposed to 10 nM N/OFQ for 6 min twice with a time interval of 12 min. The figure is representative of three independent experiments. B: Cells in capsules were pretreated with or without 100 ng/ml PTX for 24 h, and then exposed to 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%.

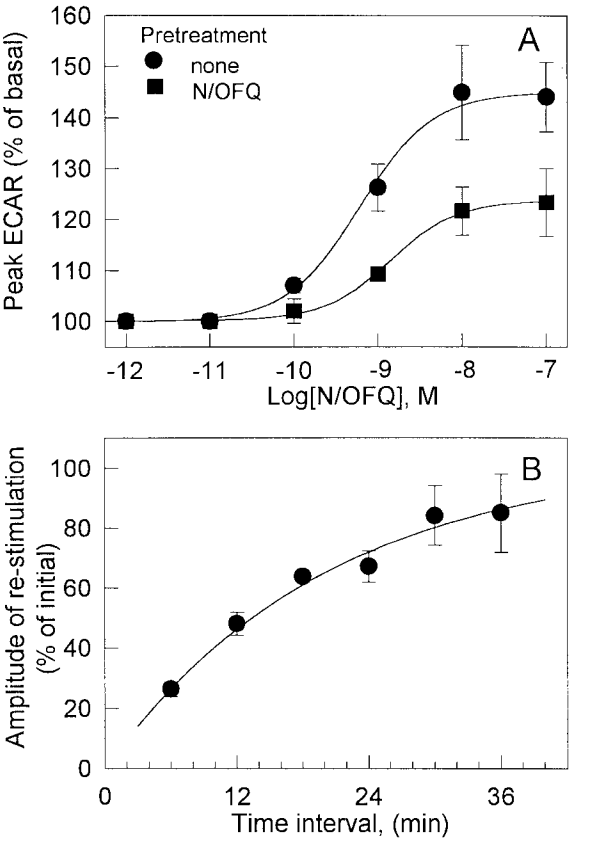

Fig. 2. Desensitization of N/OFQ-stimulated ECAR response. A: The peak values of N/OFQ-induced EACR change with or without 10 nM N/OFQ pretreatment were plotted in the figure separately with an EC_{50} of 1.6±0.1 nM and 0.6±0.1 nM respectively. B: CHO-ORL_1 cells were challenged twice with 10 nM N/OFQ 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.

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 ORL_1 undergoes homologous desensitization in neuronal cells [15]. However, the time dependence and the mechanism of desensitization of ORL_1 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 ORL_1 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 ORL_1 agonist, the concentration–response curve for N/OFQ-stimulated ECAR response shifted toward the right (EC_{50} 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 ORL_1 undergoes acute desensitization after agonist pretreatment.

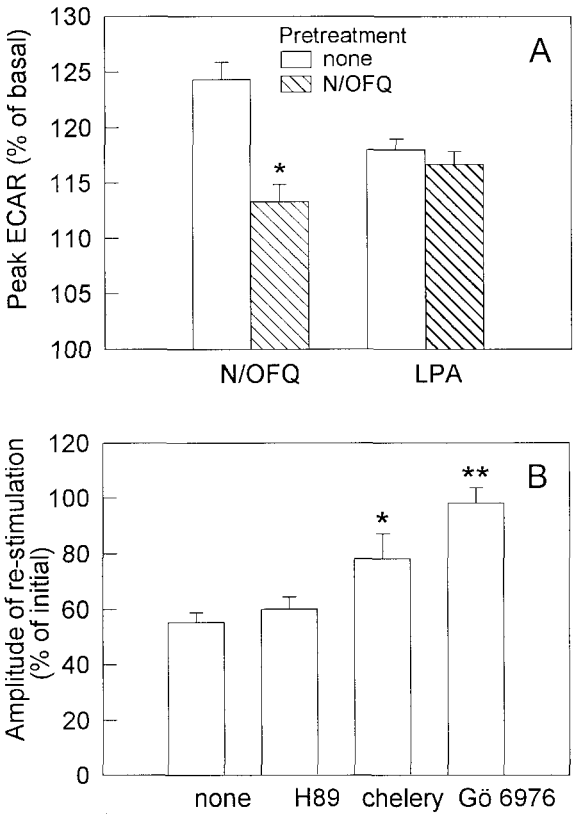

Fig. 3. Agonist-dependent desensitization of ORL_1 and involvement of PKC. A: Cells were pretreated with or without 10 nM of N/OFQ, and then challenged again with 10 nM of N/OFQ or 100 nM LPA. B: Cells were treated twice with 10 nM of N/OFQ in a 12 min interval after preincubation with none, 480 nM H89, 6.6 μM 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 ORL_1 was reversible and the time course for ORL_1 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 ORL_1 agonist. The half-life of the functional recovery of ORL_1 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-ORL_1 cells (Fig. 1B). However, pretreatment of N/OFQ did not affect LPA-stimulated extracellular acidification (Fig. 3A). This indicates that the desensitization of ORL_1 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 ORL_1 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 ORL_1. However, after pretreatment with the selective inhibitors of PKC, either chelerythrine chloride ($K_i = 0.66$ μM) [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 ORL_1 through a mechanism that requires PKC not PKA. The PKC substrate involved in the desensitization of ORL_1 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 ORL_1 has not been reported. However, based on the similarities of ORL_1 and opioid receptors in the receptor-mediated signal transduction processes, it is conceivable to consider the ORL_1 receptor as a likely substrate of PKC. Work is currently underway in our laboratory to look into the mechanism of ORL_1 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 ORL_1 undergoes acute desensitization and that PKC is required for the desensitization of the ORL_1-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 ORL_1 has been reported yet. Our finding that activation of ORL_1 stimulated rapid ECAR response therefore provides a useful screening tool for evaluating ligands for potential therapeutic purposes.

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References

[1] Reisine, T. (1995) Neuropharmacology 34, 463–472.
[2] Matthes, H.W.D., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le Meur, M., Dolle, P., Tzavara, E., Hanoune, J., Roques, B.P. and Kieffer, B. (1996) Nature 383, 819–823.
[3] Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.-L., Moisand, C., Chalon, P., Caput, D., Vassart, G. and Meunier, J.-C. (1994) FEBS Lett. 341, 33–38.
[4] Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., Miyata, T., Houtani, T. and Sugimoto, T. (1994) FEBS Lett. 343, 42–46.
[5] Chen, Y., Fan, Y., Liu, J., Mestek, A., Tian, M., Kozak, C.A. and Yu, L. (1994) FEBS Lett. 347, 279–283.
[6] Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M. and Grandy, D.K. (1994) FEBS Lett. 347, 284–288.
[7] Wang, J.B., Johnson, P.S., Imai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M. and Uhl, G.R. (1994) FEBS Lett. 348, 75–79.
[8] Wick, M.J., Minnerah, S.R., Lin, X., Elde, R., Law, P.Y. and Loh, H.H. (1994) Mol. Brain Res. 27, 37–44.
[9] Lachowicz, J.E., Shen, Y., Monsma Jr., F.J. and Sibley, D.R. (1995) J. Neurochem. 64, 34–40.
[10] Meunier, J.-C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerle, P., Butour, J.-L., Guillemot, J.-C., Ferrara, P., Monsarrat, B., Mazargull, H., Vassart, G., Parmentier, M. and Costentin, J. (1995) Nature 377, 532–535.
[11] Reinscheid, R., Nothacker, H.-P., Bourson, A., Ardati, A., Henningson, R.A., Bunzow, J.R., Grandy, D., Langen, H., Monsma Jr., F.J. and Civelli, O. (1995) Science 270, 792–794.
[12] Sim, L.J., Xiao, R. and Childers, S.R. (1996) NeuroReport 7, 729–733.
[13] Ma, L., Cheng, Z.-J., Fan, G.-H., Cai, Y.-C. and Jiang, L.-Z. (1997) FEBS Lett. 403, 91–94.
[14] Vaughan, C.W. and Christie, M.J. (1996) Br. J. Pharmacol. 117, 1609–1611.
[15] Cheng, Z.-J., Fan, G.-H., Zhao, J., Zhang, Z., Wu, Y.-L., Jiang, L.Z., Zhu, Y., Pei, G. and Ma, L., NeuroReport (in press).
[16] Velu, T.J., Beguinot, L., Vas, W.C., Zhang, K., Pastan, I. and Lowy, D.R. (1989) J. Biol. Chem. 39, 153–166.
[17] Lefkowitz, R.J. (1993) Cell 74, 409–412.
[18] Cai, Y.-C., Zhang, Y., Wu, Y. and Pei, G. (1996) Biochem. Biophys. Res. Commun. 219, 342–347.
[19] Pei, G., Kieffer, B.L., Lefkowitz, R.Z. and Freeman, N.J. (1995) Mol. Pharmacol. 48, 173–177.
[20] Fundytus, M.E. and Coderre, T.S. (1996) Eur. J. Pharmacol. 300, 173–181.
[21] Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Tishioka, T. and Hidaka, H. (1990) J. Biol. Chem. 265, 5267–5272.
[22] Herbert, J.M., Augereau, J.M., Gleye, J. and Maffrand, J.P. (1990) Biochem. Biophys. Res. Commun. 172, 993–999.
[23] Qatsha, K.A., Rudolph, C., Marme, D., Schachtele, C. and May, W.S. (1993) Proc. Natl. Acad. Sci. USA 90, 4674–4678.
[24] Cai, Y.-C., Ma, L., Fan, G.-H., Zhao, J., Jiang, L.-Z. and Pei, G. (1997) Mol. Pharmacol. 51, 583–587.
[25] McConnel, H.M., Owicki, J.C., Perce, J.W., Miller, O.L., Baxter, C.T., Wada, H.G. and Pitchford, S. (1992) Science 257, 1905–1912.