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ORIGINAL ARTICLE

Orphanin FQ Antagonizes the Inhibition of Ca^{2+} Currents Induced by μ-Opioid Receptors

Min Zhang,*[*,1,2] Xiaomin Wang,[1,3] Dabao Zhang,[4] Guoheng Xu,[1] Hongwei Dong,[1] Yingxin Yu,[1] and Jisheng Han[1]

[1]Neuroscience Research Institute, Peking University, Beijing, China; [2]Present affiliation: Department of Biological Statistics and Computational Biology, Cornell University, Ithaca, NY 14853; [3]Department of Physiology, Capital University of Medical Sciences, Beijing, China; and [4]Department of Biostatistics and Computational Biology, University of Rochester Medical Center, Rochester, NY 14642

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Abstract

Orphanin FQ (OFQ), an endogenous peptide ligand of opioid receptor-like receptors (ORLs), has properties similar to traditional opioids. This peptide inhibits adenylyl cyclase and voltage-gated calcium channels but stimulates inwardly rectifying potassium channels. Among other actions, however, OFQ also has pharmacological functions that are different from, or even opposite to, those of opioids. For example, OFQ antagonizes the behavioral analgesic effects mediated by κ- and μ-opioid receptors. In a previous paper, we reported that OFQ antagonizes inhibition of calcium channels mediated by κ-opioid receptors. We report here that OFQ also antagonizes the inhibition of calcium channels mediated by μ-opioid receptor. Further, single-cell RT-PCR reveals that the antagonistic effect of OFQ is correlated with the presence of ORL1 mRNA in individual cells.

Index Entries: Orphanin FQ; ORL1 receptor; single-cell RT-PCR; opioid receptor; calcium channel; patch clamp.

Introduction

Orphanin FQ (OFQ), a putative endogenous ligand of the opioid receptor-like receptor (ORL1) (Mollereau et al., 1994), is a 17 amino acid–long peptide (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) that was isolated in 1995 (Reinscheid et al., 1995). This peptide has high homology to the dynorphin family, especially dynorphin A (Lapalu et al., 1997). However, because of the presence of an N-terminal phenylalanine (Nphe) in place of the tyrosine found in most opioid peptides, OFQ has a relatively low affinity for traditional opioid receptors. Orphanin FQ (OFQ) and its receptor seem to be localized in neural circuits that are different from those employing other opioids and their receptors (Agius et al., 1998). Similar to other opioid ligands, OFQ is negatively coupled to adenylyl cyclase and to voltage-gated calcium channels, and is positively coupled to inwardly rectifying potassium channels. However, OFQ has some actions that are very different from those of other opioids. For example, OFQ antagonizes the behavioral analgesic effects produced by κ- and μ-opioid receptors (Mogil et al., 1996). Opioids produce analgesia by inhibiting presynaptic neurotransmitter release, which is closely related to calcium influx through voltage-gated calcium channels, specifically the N-type calcium channel. Given that both OFQ and opioids can produce a dose-dependent inhibition of calcium channel currents, it is interesting to know

*Author to whom all correspondence and reprint requests should be addressed. E- mail: mz35@cornell.edu

whether OFQ interacts with the inhibition of calcium channel currents mediated by other opioid receptors. We reported previously that OFQ could antagonize the inhibition of calcium channel mediated by κ-opioid receptor (Zhang et al., 1998). Here, we investigate the interaction of OFQ with the inhibition of calcium channel currents mediated by μ-opioid receptor. The new findings not only serve as an extension of our previous results but also provide the underlying mechanism for the opiate-modulating function of OFQ at the cellular level (Harrison and Grandy, 2000).

Materials and Methods

Cell Preparation

Single dorsal root ganglion (DRG) neurons were freshly isolated from humanely killed male Wistar rats (180–200 g, provided by Experimental Animal Center, Peking University). The ganglia were incubated with trypsin type I-S (Sigma, 0.56 mg/mL) and collagenase type IA (Sigma, 1.2 mg/mL) at 37°C for 35 min. Then soya bean trypsin inhibitor II-S was added to the enzyme solution and the preparation was incubated for 10 more min. After incubation, Dulbecco's Modified Eagle Medium (DMEM) was changed to the extracellular solution described below. When examined under a microscope, DRG neurons varied widely in size. In this study only cells with relatively small diameters were chosen for patch clamping. Generally, the recordings were made between 2 and 8 h after plating.

Patch-clamp Recording

Recordings were made in whole-cell configuration at room temperature (22–24°C). Patch pipets with resistance of 2–3 MΩ contained the following intracellular solution (100 m*M* CsCl, 2 m*M* tetraethylammonium chloride (TEACl), 5 m*M* $MgCl_2$, 10 m*M* HEPES, 10 m*M* EGTA, 2 m*M* Mg^{2+}ATP, and 0.25 m*M* cAMP. The pH value was adjusted to 7.2 with CsOH, and osmolarity adjusted to 305 with D-glucose. The solution was stored at –20°C and then thawed just before an experiment. The control extracellular solution contained 150 m*M* NaCl, 5 m*M* KCl, 2.5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* HEPES, and 10 m*M* D-glucose. The pH was adjusted to 7.4 with NaOH, and osmolarity to 320 with D-glucose. This solution was stored at 4°C. To isolate Ba^{2+} currents through Ca^{2+} channels after whole-cell recording was established, the extracellular solution was changed to one containing 140 m*M* TEACl, 5 m*M* $BaCl_2$, 5 m*M* CsCl, 1 m*M* $MgCl_2$, 10 m*M* HEPES, 10 m*M* D-glucose, and 0.001 m*M* tetrodotoxin (pH 7.4; 320 mOsm/L; stored at 4°C).

Voltage-gated calcium channel currents were recorded in standard whole-cell patch-clamp mode using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany), filtered at 3 kHz with a 4-pole Bassel filter, digitized (5 kHz), stored, and analyzed by a Power Macintosh 9600/200MP computer using Pulse+PulseFit (HEKA Elektronik). Ca^{2+} channel currents were elicited by stepping the membrane from a holding potential of –90 to –10 mV for 100 ms every 20s. Capacity and series resistance were automatically compensated by the AUTO mode of EPC-9; leak and capacity currents were subtracted by computer.

Single-Cell RT-PCR Technique

After whole-cell recording, negative pressure was applied to harvest the cytoplasm of the cell into the patch pipet. During the process, the nucleus was not drawn into the pipet to prevent contamination. The extracted mRNAs were put into an Eppendorf tube filled with the following solution: 2 μL 10 × RT buffer, 4 μL $MgCl_2$ (25 m*M*), 1 μL oligo(dT) (0.5μg/μL), 1 μL dNTP (10 mmol/L), 0.5 μL RNasin (40 U/μL), 0.7 μL avian myoblastosis virus (AMV) (25 U/μL), and 7 μL ddH_2O. The solution was then mixed completely and incubated at 42°C for 30 min. Ten microliters of the reverse transcription product was removed and placed into another PCR tube containing 2 μL 10 × buffer, GAPDH/ORL1 primer 1 μL and 2 μL, respectively, 0.5 μL *Taq* DNA polymerase, and 2 μL ddH_2O. The following protocol was employed for PCR amplification: 94°C for 40 s, 42°C for 60 s, and 72°C for 90 s for 45 cycles. The sequences of the GAPDH primer were as follows: upper, 5′-TCCCTCAAGATTGTCAGCAA-3′; lower, 5′-AGATCCACAACGGATACATT-3′. According to Wang et al. (1994), ORL1 primer sequences were as follows: upper, 5′-ACCCTGGTCTTGCTAACA-3′; lower, 5′-CAGCACCAGTCGAGTGAT-3′.

The RT-PCR products were cloned onto a pGEM vector and then cut with three groups of restriction enzymes for further identification.

Statistical Analysis

The values were presented as the mean ± S.E.M. Comparison between groups was preformed by paired or unpaired Student's *t*-test. $p < 0.05$ or less is considered statistically significant based on a two-sided hypothesis test.

Compounds and Administration

Orphanin FQ ([OFQ] obtained from Phoenix Pharmaceuticals, CA) was dissolved in the control solution described above, aliquoted, and stored at

–20°C. One aliquot was taken out before each experiment and diluted further in the control solution. Ohmefentanyl ([OMF] Shanghai Pharmacological Research Institute, Chinese Academy of Sciences, Shanghai, China) and naloxone were stored at 4°C. AMV, oligo(dT), *Taq* DNA polymerase, dNTP, RNasin, pGEM vector, and the DNA ladder were from Promega (WI). All other drugs were from Sigma Chemical Co. (St. Louis, MO). To apply different drug solutions while recording, a series of six gravity-fed microtubes were glued together, side by side, and mounted to a micromanipulator to exchange these drug solutions.

Results

Interacting Effects of OFQ and OMF on Ca^{2+} Channel Currents

As both OMF and OFQ inhibit voltage-gated calcium channel currents, we were interested in determining whether OFQ could antagonize the inhibitory effect induced by OMF. Previous work indicated that calcium channel currents are progressively reduced by OMF at concentrations ranging from 1 n*M* to 10 µ*M* (Liu et al., 1995), and the inhibitory effect could be removed completely by the nonselective opioid receptor antagonist naloxone (10 µ*M*), whereas 10 µ*M* naloxone *per se* has no effect on calcium channel currents. Because 1 and 10 µ*M* OMF showed no significant differences in their ability to inhibit calcium channel currents (data not shown), the present experiments employed 1 µ*M* OMF. Orphanin FQ (OFQ) also inhibits voltage-gated calcium channel currents in a dose-dependent manner, with a concentration of 50 n*M* being the minimum required to detect an inhibition of calcium channel currents (Zhang et al., 1998). However, naloxone did not change the inhibitory effect of OFQ, which indicates that this effect is not mediated by classic opioid receptors. We therefore tested the regulatory effect of OFQ, that is, whether OFQ could antagonize the inhibitory effect of OMF. An example of the effects of OMF and OFQ on calcium channel currents of a DRG neuron is shown in Fig. 1. In this experiment, the peak current was decreased 32% by 1 µ*M* OMF. The subsequent application of 50 n*M* OFQ reversed the inhibitory effect almost completely, despite the continued presence of OMF. Washing with peptide-free solution restored calcium current amplitude completely to its pretreatment value. Among 37 neurons with satisfactory recordings of calcium currents, peak currents were inhibited by 1 µ*M* OMF in 27 of these neurons. For the cells that responded

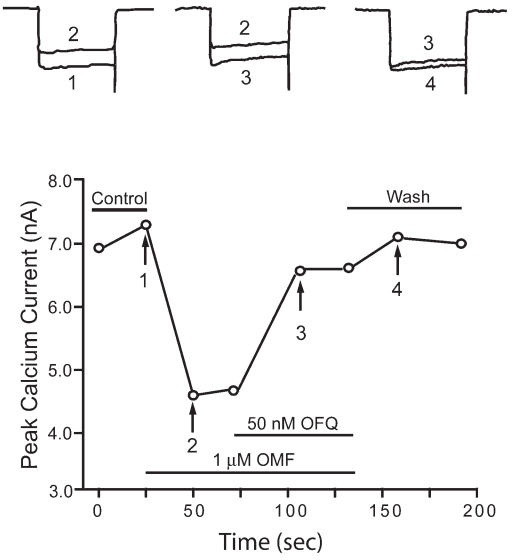

Fig. 1. OFQ reverses OMF-induced inhibition on calcium currents. The traces (1–4) shown in the upper part of the graph are the Ca^{2+} currents, which were elicited by steps to –10 mV from –90 mV at times (1–4) indicated in the respective lower part of the graph. Bars indicate the time course of drug application, and concentration of the drug is shown above the bar. Note that the first and last two time points (circles) are the vehicle effects (before and after the application of drugs, respectively), whereas the four points in between are the effects of the corresponding drugs.

to OMF, 20 of these (74.1%) also showed a reversal of this inhibitory effect by application of 50 n*M* OFQ. The range of reversal of the OMF response by OFQ varied from 27% to 100%. For those cells that did not respond to OMF, OFQ was not applied further, as the goal of this study was to investigate the regulatory effect of OFQ.

Cell-specific differences in the response to OFQ were not attributable to variations in the basal amplitude of calcium currents, because a comparison revealed no difference between current amplitudes in cells that showed a reversal response to OFQ versus those that did not (Fig. 2). This comparison also indicated that 50 n*M* OFQ reversed the effects of OMF only partially. A detailed analysis of the dose-response relationship for OFQ showed that the effect of OFQ was dose-dependent, but even a saturating concentration did not cause a complete reversal of the effect of OMF (Fig. 3).

OFQ Action Correlates with the Presence of mRNA Encoding ORL1

We next sought to determine why only a subset of OMF-sensitive DRG neurons responded to OFQ. Rather than applying an antagonist to investigate

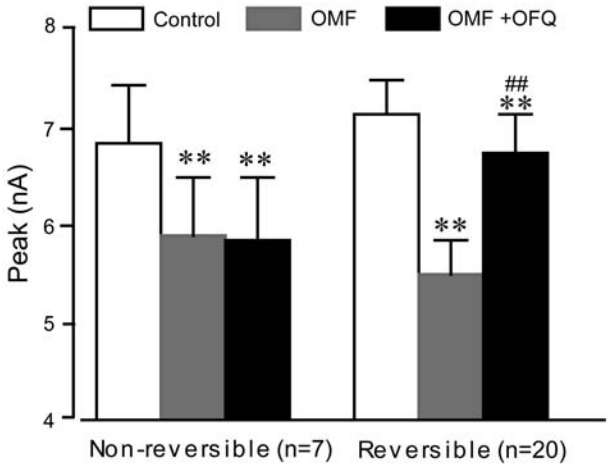

Fig. 2. Suppression of calcium current by μ-opioid receptor agonist OMF (1 μ*M*) and its reversal by OFQ (50 n*M*). In a total of 27 neurons, 20 were reversed by OFQ and 7 were nonreversible. Statistical analysis showed that there were no significant differences between the control currents of the groups. Each column represents the mean ± S.E.M. (**) $p < 0.01$ (paired *t*-test) compared with control groups; (##) $p < 0.01$ compared with the OMF group.

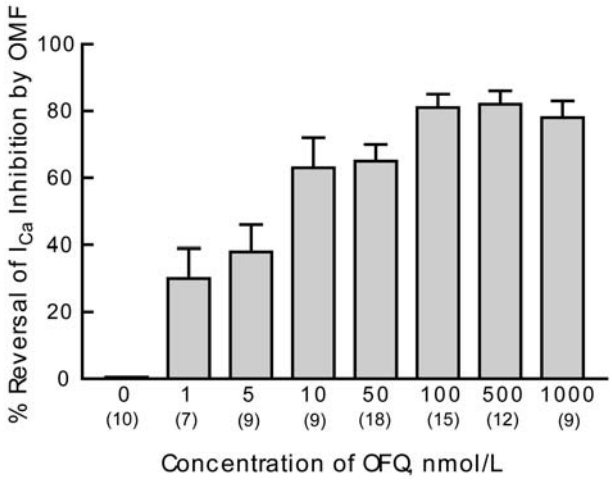

Fig. 3. The concentration-reversal relationship of OFQ on OMF-induced inhibition of voltage-gated calcium channels in rat DRG neurons. Each column represents the mean ± S.E.M. The reversal effect increases as the concentration of OFQ increases and reaches its peak at 100–500 nmol/L. Shown in parentheses along the *x*-axis are the number of cells in each group of OFQ concentration.

whether the reversal effect of OFQ was mediated by ORL1 receptor, we used the single-cell RT-PCR technique to measure the level of ORL1 mRNA in cells that had been assayed for responsiveness to OFQ. Figure 4A shows the RT-PCR products of two different cells. Lane 1 shows the results of mRNA analysis in a cell that did not show reversal of the OMF response by OFQ, whereas lane 2 shows a similar analysis performed in a cell that showed reversal of the OMF response by OFQ. Lanes 3–5 are negative controls (under the same conditions but without cytoplasm, AMV, or primer, correspondingly). Lane M is a calibration standard consisting of a 1-kb DNA ladder. Although the OFQ-reversible cell showed the presence of ORL1 mRNA, the OFQ-nonreversible cell was negative. Among the 12 cells in which we assayed for ORL1 mRNA, none (0 out of 6) of the cells that did not show an OFQ reversal contained ORL1 mRNA, whereas most (5 out of 6) of the OFQ-reversible cells contained ORL1 mRNA. To confirm that the mRNA really encodes ORL1, we cloned the resulting DNA into a pGEM vector for restriction enzyme digestion analysis. The results showed that the DNA is ORL1 receptor (Fig. 4B).

Discussion

Orphanin FQ (OFQ) not only produces analgesia (Reinscheid et al., 1995) but also antagonizes the analgesic effect mediated by μ- and κ-opioid receptors (Mogil et al., 1996). The analgesic action is easy to understand because OFQ and ORL1 receptor are similar to traditional opioid peptides and their receptors. However, the antagonistic effect of OFQ was surprising and more difficult to understand. Opioids produce analgesia by inhibiting neurotransmitter release from presynaptic terminals, where the release results from calcium influx through voltage-gated calcium channels (especially the N-type channel). The analgesic actions of opioids are mediated by inhibition of calcium channels, which has been demonstrated for opioid receptors μ (Seward et al., 1991), δ (Motin et al., 1995), and κ (Gross et al., 1990), as well as for the ORL1 receptor (Knoflach et al., 1996). Because the analgesic effects of OFQ could be explained by inhibition of calcium channels, how is it possible to explain its antagonistic effect? We applied the patch-clamp technique to examine the interaction of OFQ and other opioids on calcium channels. Previous results show that OFQ antagonizes the inhibition of calcium channel current induced by the κ-opioid receptor agonist U50,488H (Zhang et al., 1998). The present work demonstrates that OFQ can also antagonize the inhibitory effect induced by μ-opioid receptor agonist OMF.

In the present study, DRG neurons were chosen to study the interaction between the ORL1 and μ-opioid receptor. Dorsal root ganglion (DRG) neurons

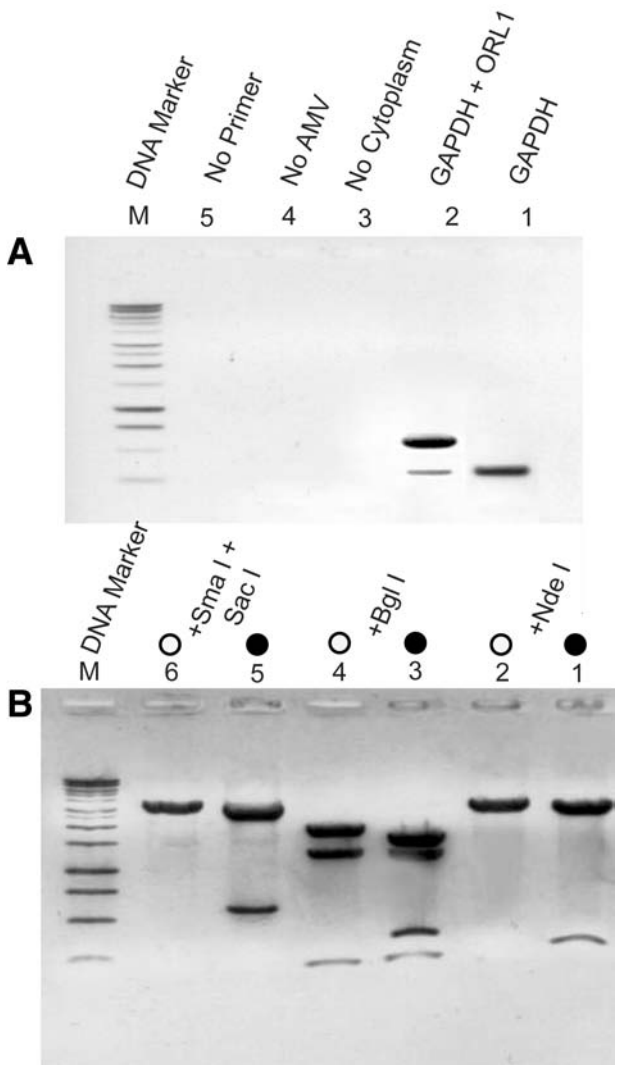

Fig. 4. **(A)** The RT-PCR products of different cells. Lane 1: Cells without ORL1 receptor mRNA. Only internal control (GAPDH; 309 bp) is detected. Lane 2: The cell with ORL1 receptor mRNA. Both internal control and ORL1 receptor (591 bp) are detected. Lanes 3–5: Negative controls (under the same condition but without cytoplasm, AMV, or primer, correspondingly). Lane M: 1-kb DNA ladder. **(B)** The enzyme identification of single-cell RT-PCR products of ORL1 receptor mRNA. Lane M: 1-kb DNA ladder. Lanes 1 and 2 are cut with NdeI; lanes 3 and 4 are cut with BglI; lanes 5 and 6 are cut with SmaI + SacI. Lanes 1, 3, and 5 (solid circles) are the products of pGEM/ORL1; lanes 2, 4, and 6 (open circles) are those of pGEM (serving as negative controls).

are primary sensory neurons whose cell bodies are aggregated in DRG. It has been reported that all three conventional opioid receptors (μ, δ, and κ) and the newly identified ORL1 receptor are present in DRG neurons (Zhang et al., 1995). Furthermore, agonists for μ- and κ-opioid receptors, as well as ORL1 receptor ligands, inhibit voltage-gated calcium channels in DRG neurons (Liu et al., 1995; Knoflach et al., 1996; Xu et al., 1996). It is known that freshly dissociated DRG neurons can be divided by size into populations with small (19–27 μm), medium (28–33 μm), and large (39–50 μm) cell body diameters. Generally, the large cells correspond to the A_α and A_β fibers, whereas the small and medium cells correspond to the A_δ and C fibers. As A_δ and C fibers conduct pain and thermal information, we chose only the small and medium cells for our experiments. We found that 73% of these neurons exhibited inhibitory effect to OMF, and in most of these responsive neurons (74%) the inhibition was reversed by OFQ. Among the antagonists that have been identified at the behavioral level (Ozaki et al., 2000b; Yamada et al., 2003), CompB (also known as J-113397) was reported to inhibit OFQ binding to the ORL1 receptor (Ozaki et al., 2000a) and also occlude the synaptic transmission induced by OFQ without obvious agonist effects (Vaughan et al., 2001). Besides, Chiou et al. (2002) presented that Nphe was able to block OFQ-evoked inwardly rectifying K^+ currents in slices of periaqueductal gray. However, Chin et al. (2002) found that both CompB and Nphe exhibit partial agonist activity on ionic conductance in an acutely dissociated diagonal band of broca neurons. Therefore, for some of the antagonists, the specificity might depend on the ionic conductance to which ORL1 is coupled (Chin et al., 2002), whereas others remain to be determined. To consider an alternative approach, we used single-cell RT-PCR (Eberwine et al., 1992) to understand the mechanism underlying the anti-opioid property of OFQ. Specifically, to determine whether the reversal effect of OFQ was mediated by the ORL1 receptor, we applied the single-cell RT-PCR technique to assay for the presence of ORL1 receptor mRNA. None of the six cells that did not show reversal of the OMF response by OFQ possessed ORL1 mRNA, whereas five of six of the cells that showed OFQ reversal were positive for ORL1 mRNA. This indicates that OFQ reverses the inhibitory effect of OMF only in cells that contain ORL1 receptor mRNA. Although we cannot exclude the possibility that the unresponsive cells contain an extraordinary low abundance of this mRNA that could not be detected by the present method, our results certainly indicate that the reversal effect of OFQ is related to ORL1 mRNA. It is not yet clear whether this mRNA is expressed as protein in these cells. Our results also provide evidence for the colocalization

of ORL1 and μ-opioid receptors in the same DRG neurons.

Diverse-Pierluissi et al. (1995) reported that the effect on Ca^{2+} channels can either be combined or attenuated when two modulators are applied simultaneously. Results of behavioral experiments indicate that intrathecal injection of OFQ can reverse the analgesic effect mediated by κ- and μ-opioid receptors. Our current results, combined with those of Zhang et al. (1998), show that OFQ can reverse the inhibition of calcium channel current mediated by both κ- and μ-opioid receptors. Similar phenomena have been observed by Polo-Parada and Pilar (1999), where the sequential activation of μ- and κ-opioids can occlude the inhibitory effect of Ca^{2+} currents induced by somatostatin in ciliary and DRG neurons. It appears that the ability of OFQ to inhibit calcium channel currents and reverse the inhibition mediated by μ-opioid receptors arises from intracellular signal transduction mechanisms rather than extracellular ones. First, a chemical interaction between OMF and OFQ is not likely attributable to the chemical structure of the two peptides. Second, the same concentrations of OFQ are needed to produce inhibition and reversal of the effects produced by OMF, which excludes the possibility that OFQ acts as a partial agonist. Third, the affinity of OFQ for μ-opioid receptors and the affinity of OMF for the ORL1 receptor are very low, so they do not appear to cross-activate these receptors. Therefore, this phenomenon can only be explained by intracellular mechanisms. As both OFQ and OMF produce their effects through G-protein signaling systems and downstream intracellular second messengers, antagonism at these levels could result in the reversal of the effect on calcium channel currents. It is known that G-protein subunits are involved (Diverse-Pierluissi et al., 1995); however, the specific intracellular pathways responsible for the reversal effect remain to be determined. A combination of pharmacological and genetic tools will lead to a deeper understanding of the underlying mechanism. The anti-opioid effect of OFQ on calcium channels not only gives more evidence for the anti-opioid property of OFQ but also provides a possible cellular mechanism for the behavioral antagonistic effects of OFQ. Therefore, OFQ might work as a regulatory peptide, similar to cholecystokinin (Liu et al., 1995), which is a well-characterized peptide that produces both analgesic effects by itself and anti-analgesic effects induced by opiates (Wiesenfeld-Hallin and Xu, 1996). With the accumulating information on the cross talk between modulators at the ion channel level (Liu et al., 1995; Xu et al., 1996; Polo-Parada and Pilar, 1999), one might consider this mechanism to play an important role in the neuronal regulatory process. The goal of this study was to investigate the modulation effect of OFQ; however, it would be interesting to determine whether OMF could reverse the inhibitory effect of OFQ. Moreover, the intracellular mechanisms for this anti-opioid action require further investigation.

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