Nociceptin/Orphanin FQ Modulation of Ionic Conductances in Rat Basal Forebrain Neurons

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ABSTRACT

Nociceptin/orphanin FQ (N/OFQ) is an endogenous opioid-like heptadecapeptide that plays an important role in a variety of physiological functions. N/OFQ and its receptor opioid receptor-like orphan receptor-1 are abundant in the diagonal band of Broca (DBB), a basal forebrain nucleus where the loss of cholinergic neurons is linked to memory and spatial learning deficits. In the whole animal, central injections of N/OFQ have been shown to disrupt spatial learning. In this study, we investigated the basis for these behavioral observations by examining the cellular effects of N/OFQ on chemically identified DBB neurons. Whole cell patch-clamp recordings were performed on enzymatically dissociated DBB neurons. Under voltage-clamp conditions, bath application of N/OFQ (10 pM–1 μM) resulted in a dose-dependent depression of whole cell currents. Single cell reverse transcription-polymerase chain reaction analysis identified cholinergic and fewer GABAergic cells to be N/OFQ-responsive. [Nphe1]nociceptin-(1-13)-NH2 and CompB (J-113397) antagonized the N/OFQ response, but both compounds also displayed partial agonist activity. Using a combination of channel blockers we determined that the effects of N/OFQ were mediated via a suite of Ca2+ (N- and L-type) and Ca2+-dependent K+ (iberiotoxin-sensitive) conductances. In addition, biophysical analysis of voltage subtraction protocols revealed that N/OFQ reduces transient outward and the delayed rectifier K+ currents. Because N-type and L-type Ca2+ channels are important in the context of neurotransmitter release, our observations indicate that N/OFQ inhibition of Ca2+ dependent conductances in cholinergic neurons would be expected to result in depression of acetylcholine release, which may explain the behavioral actions of N/OFQ in the brain.

Nociceptin/orphanin FQ (N/OFQ) is an endogenous heptadecapeptide that binds to a receptor (ORL-1), which is homologous to the classical opioid receptors (Henderson and McKnight, 1997). N/OFQ and its receptor play an important role in a variety of physiological functions, including cardiac and renal control (Kapusta, 2000), locomotion (Florin et al., 1996), and nociception (Darland et al., 1998). A role for N/OFQ in memory and learning has also been reported on the basis of in vivo and in vitro observations. Microinjection of N/OFQ into the hippocampus has been shown to disrupt spatial learning. In this study, we investigated the basis for these behavioral observations by examining the cellular effects of N/OFQ on chemically identified DBB neurons. Whole cell patch-clamp recordings were performed on enzymatically dissociated DBB neurons. Under voltage-clamp conditions, bath application of N/OFQ (10 pM–1 μM) resulted in a dose-dependent depression of whole cell currents. Single cell reverse transcription-polymerase chain reaction analysis identified cholinergic and fewer GABAergic cells to be N/OFQ-responsive. [Nphe1]nociceptin-(1-13)-NH2 and CompB (J-113397) antagonized the N/OFQ response, but both compounds also displayed partial agonist activity. Using a combination of channel blockers we determined that the effects of N/OFQ were mediated via a suite of Ca2+ (N- and L-type) and Ca2+-dependent K+ (iberiotoxin-sensitive) conductances. In addition, biophysical analysis of voltage subtraction protocols revealed that N/OFQ reduces transient outward and the delayed rectifier K+ currents. Because N-type and L-type Ca2+ channels are important in the context of neurotransmitter release, our observations indicate that N/OFQ inhibition of Ca2+ dependent conductances in cholinergic neurons would be expected to result in depression of acetylcholine release, which may explain the behavioral actions of N/OFQ in the brain.

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ABBREVIATIONS: N/OFQ, nociceptin; ORL-1, opioid receptor-like orphan receptor; DBB, diagonal band of Broca; VSCC, voltage-sensitive calcium channel; RT-PCR, reverse transcription-polymerase chain reaction; TEA, tetraethylammonium; ChAT, choline acetyltransferase; GAD, glutamate decarboxylase; Nphe, [Nphe1]nociceptin-(1-13)-NH2; I-V, current-voltage; iCa, calcium-activated potassium current; iK or BK channels, larger conductance calcium-activated potassium current; IBTX, iberiotoxin; iBa, barium current; iO, transient outward current; iD, delayed rectifier potassium current.
of an inwardly rectifying K⁺ current (Vaughan et al., 1997; Chiou, 1999; Slugg et al., 1999). N/OFQ also has been shown in the hippocampus and periaqueductal gray to modulate voltage-sensitive Ca²⁺ channels (VSCC; Knořlach et al., 1996; Connor and Christie, 1998). These actions of N/OFQ at a cellular level have been linked to its role in important physiological processes such as pain control and release of neurohormones such as vasopressin (Doi et al., 1998; Pan et al., 2000).

Given that the DBB plays a central role in memory processes and that DBB is enriched with N/OFQ, we examined the ionic mechanisms of N/OFQ actions on acutely dissociated DBB neurons, whose chemical phenotype was determined using single cell RT-PCR technique. We also assessed the effectiveness of recently developed antagonists of N/OFQ in attenuating the N/OFQ-evoked responses in DBB neurons.

Materials and Methods

**Dissociation Procedures.** Details of the procedure for acute dissociation of neurons from the DBB have been described previously (Jassar et al., 1999). Briefly, brains were quickly removed from decapitated male Sprague-Dawley rats (15–25 days postnatal) and placed in cold artificial cerebrospinal fluid that contained 140 mM NaCl, 2.5 mM KCl, 1.4 mM CaCl₂, 5 mM MgCl₂, 10 mM HEPES, and 33 mM d-glucose, pH 7.4. Brain slices (350 µm in thickness) were cut on a vibratome, and the area containing the DBB was dissected out. Although most of the tissue contained the horizontal limb of the DBB, some slices may have included a portion of the vertical limb of the DBB. Acutely dissociated neurons were prepared by the enzymatic treatment of slices with trypsin (0.65 mg/ml) at 30 °C, followed by mechanical trituration for dispersion of individual cells. Cells were then plated on poly-l-lysine (0.005 wt/vol)-coated coverslips and viewed under an inverted microscope (Axiovert 35; Carl Zeiss, Thornwood, NY). All solutions were kept oxygenated by continuous bubbling with pure oxygen.

**Electrophysiological Recordings.** Whole cell patch-clamp recordings were performed at room temperature (20–22 °C) using an Axopatch-1D amplifier (Axon Instruments, Union City, CA). Junction potential was nullified with the pipette tip immersed in the bath. Patch electrodes (thin wall with filament, 1.5 mm in diameter; World Precision Instruments, New Haven, CT) were flame polished to yield resistances of 4 to 5 M. Internal patch pipette solution contained 140 mM KCl, 2.5 mM MgCl₂, 5 mM MgCl₂, 10 mM HEPES, and 33 mM d-glucose, pH 7.4. Brain slices (350 µm in thickness) were cut on a vibratome, and the area containing the DBB was dissected out. Although most of the tissue contained the horizontal limb of the DBB, some slices may have included a portion of the vertical limb of the DBB. Acutely dissociated neurons were prepared by the enzymatic treatment of slices with trypsin (0.65 mg/ml) at 30 °C, followed by mechanical trituration for dispersion of individual cells. Cells were then plated on poly-l-lysine (0.005 wt/vol)-coated coverslips and viewed under an inverted microscope (Axiovert 35; Carl Zeiss, Thornwood, NY). All solutions were kept oxygenated by continuous bubbling with pure oxygen.

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Cell size was estimated electronically using the whole cell capacitance compensation circuit on the Axopatch-1D amplifier. Series resistance compensation was continuously adjusted to >80% and monitored and readjusted as necessary during the course of each experiment. The average access resistance was 8.5 ± 0.3 MΩ (n = 130). The electrode resistance was between 4 and 5 MΩ. Maximum voltage-clamp error in recording a current of 10 nA using a patch electrode with an electrode resistance of 5 MΩ was 10 mV. This reflects the average maximum error because the currents recorded were usually smaller than 10 nA.

To examine the effects of N/OFQ on the contribution of voltage-dependent Ca²⁺ currents, we used an external solution that was nominally Ca²⁺ -free and contained 50 μM Cd²⁺. In this solution CaCl₂ was replaced with an equimolar concentration of MgCl₂. To record currents through Ca²⁺ channels, we used Ba²⁺ as a charge carrier, ibed previously (Easaw et al., 1999). The external solution contained 150 mM tetraethylammonium-chloride, 2 mM BaCl₂, 10 mM HEPES, and 30 mM glucose (pH to 7.4 with TEA-OH). The internal patch pipette solution consisted of 130 mM Cs-methanesulfonate, 2 mM MgCl₂, 10 mM HEPES, 10 mM BAPTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, and 0.1 mM leupeptin (pH to 7.2 with CsOH). Depolarizing voltage steps from −80 to +70 mV (increment 10 mV/step; 20-ms duration) were applied to voltage-clamped DBB neurons under control conditions and in the presence of N/OFQ. Leak currents were minimal under our recording conditions. They did not change during the recordings and were not affected by application of N/OFQ. Therefore, we did not subtract these in subsequent measurements of steady-state barium currents.

**Single-Cell RT-PCR for Chemical Phenotyping.** Neurons were harvested after electrophysiological recordings were completed and readied for RT-PCR according to a protocol described previously (Surmeier et al., 1996). In brief, contents of the electrode containing the cell and 5 µl of internal solution were expelled into a 0.2-ml PCR tube containing 5 µl of sterile water (W-4502; Sigma-Aldrich, St. Louis, MO), 0.5 µl of 0.1 M dithiothreitol, 0.5 µl of RNasin (10 U/µl), and 1 µl of oligo(dT) (0.5 g/µl). The tube was then placed on ice. Single-stranded cDNA was then synthesized from mRNA by adding a solution containing 1 µl of SuperScript II RT (200 U/µl), 2 µl of 10× PCR buffer, 2 µl of 25 mM MgCl₂, 1.5 µl of 0.1 M dithiothreitol, 1 µl of 10 mM dNTPs, and 0.5 µl of RNasin (10 U/µl). The PCR tube was gently mixed and incubated in a Progene thermal cycler (Technne, Princeton, NJ) at 42°C for 50 min. The process was then terminated by heating to 72°C for 15 min and the tube cooled to 4°C. Subsequently, 2 µl of the RT product was taken and combined with 5 µl of 10× PCR buffer, 5 µl of 25 mM MgCl₂, 0.5 µl of Taq polymerase (5 U/µl), 31.5 µl of sterile water (W-4502; Sigma-Aldrich), 1 µl of 25 mM dNTP mixture, and 1.5 µl of a specific set of primers (15 µM). All reagents were purchased from Invitrogen (Carlsbad, CA). Primer sequences for choline acetyltransferase (ChAT) and for glutamate decarboxylase (GAD) have been described previously (Surmeier et al., 1996; Tkatch et al., 1998) and that for α1-actin was obtained from GenBank (the lower primer 5'-GAT AGA GCC ACC AAT CCA C; the upper primer 5'-CCA TGT ACG TAG CCA TCC A). All primers were synthesized at the Department of Biochemistry (University of Alberta, Edmonton, AB, Canada). The contents were mixed together and placed in the thermal cycler. The PCR amplification protocol was as follows: step 1, 94°C 4 min; step 2, 94°C 1 min, 53°C 1 min, 72°C 45 s (step 2 was repeated 35 times); step 3, 72°C 15 min; and step 4, held at 4°C. A portion of the product was then run on a 2% TEA agarose gel and the gel was then placed in a bath containing 2 µg/ml ethidium bromide. After 10 min, DNA bands were visualized with UV light box and photographed with a Polaroid camera.

**Pharmacological Agents Used.** The following compounds used in this study were purchased from Sigma-Aldrich: ibetitoxin, TEA-CI, nimodipine, and α-conotoxin GVIA. N/OFQ was purchased from Bachem California (Torrance, CA). CompB (J-113397) and [Phe²]nociceptin-(1-13)-NH₂ (Nphe) were kind gifts from Dr. Satoshi Ozaki (Banyu Pharmaceutical, Okubo, Japan) and Dr. Girolamo
Calo (University of Ferrara, Ferrara, Italy), respectively. All the agents were dissolved in distilled water to make 1000× stock solution (stored at −70°C) and diluted in external perfusing medium just before the time of application. All drugs and chemicals were applied via bath perfusion at the rate of 3 to 5 ml/min, which allowed complete exchange in less than half a minute. Data are presented as mean ± standard error of mean. Student’s paired two-tailed t test was used for determining significance of effect, with a significance value of p < 0.05.

**Results**

Most of the acutely dissociated neurons from the DBB had neuron-like morphology (i.e., large cells with a conspicuous nucleus, nucleolus, and few blunt processes, which were truncated axon/dendrites). The average membrane capacitance estimated electronically on the Axopatch-1D amplifier was 15.7 ± 0.2 pF (n = 131). Under our recording conditions, the average input conductance measured from the slope of the current-voltage (I-V) relationships between −60 and −110 mV was 1.29 ± 0.12 nanosiemens (n = 98).

**N/OFQ Response of Whole Cell Currents in Cholinergic and GABAergic Cells.** Based on the previous observations (Jassar et al., 1999), we used a voltage-ramp protocol where the cells were held at −80 mV and subjected to voltage ramps from −110 to +30 mV at the rate of 20 mV/s after conditioning at −110 mV for 1 s. N/OFQ inhibited whole cell currents in the −30 to +30 mV range (Fig. 1A). At a voltage of +30 mV, application of 1 M N/OFQ significantly decreased whole cell currents from 7.2 ± 0.8 to 6.0 ± 0.6 nA, a reduction of 15.4 ± 1.7% (n = 14, p < 0.05; Fig. 1B). The average whole cell current at +30 mV after washout of 1 µM N/OFQ was 6.9 ± 0.5 nA, which represented a partial recovery of the responses. We did not observe any differences in N/OFQ responses obtained either 1 or up to 10 h post-trypsinization.

The average input conductance before N/OFQ treatment was 1.19 ± 0.23 nanosiemens, which was not significantly different from the input conductance of 1.21 ± 0.24 nS observed during N/OFQ application (n = 14, p < 0.05). N/OFQ inhibited peak whole cell currents of DBB neurons in a dose-dependent manner with an EC50 value of 1.2 nM (Fig. 1C). Thus, to ensure maximal responses, either 100 nM or 1 µM N/OFQ was used in all subsequent experiments.

**Chemical Identity of N/OFQ Responsive DBB Neurons.** The neurons of DBB can be divided into two major chemical phenotypic groups based upon whether they synthesize and release the neurotransmitter acetylcholine or GABA (Paolini and McKenzie, 1993). Determination of the chemical phenotype was done by RT-PCR analysis, which provides information on the presence of mRNA in a particular cell that has been recorded from. ChAT was used as a specific marker for cholinergic neurons and GAD was used as a specific marker for GABAergic neurons. Figure 1D shows the photograph of a gel indicating RT-PCR product from three N/OFQ-responsive cells. The two cells on the left are cholinergic (band corresponding to the molecular weight of the ChAT primer) and the neuron on the right is GABAergic (band corresponding to the molecular weight of GAD primer). Forty-four cells that responded to N/OFQ were collected for single cell RT-PCR analysis and of these, 20 cells displayed a specific marker for GABAergic neurons. Figure 1D shows bands from the top corresponding to 516, 394, and 298 kDa as identified by the arrows.
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positive β-actin band. Of the 20 cells where the RT-PCR reaction was successful (as judged by the presence of a β-actin band), nine cells were identified as cholinergic and three cells were GABAergic. The remaining eight cells did not show a band for either ChAT or GAD. The lack of detectable RT-PCR product in these cells may be due to prolonged whole cell recordings in many cells, which could result in a dialysis of the intracellular contents of the cell, thereby diminishing or degrading detectable mRNA for ChAT or GAD.

Pharmacological Antagonism of N/OFQ Response in DBB Neurons. The N/OFQ antagonist Nphe attenuated the N/OFQ response to whole cell currents (Fig. 2A). This attenuation was found to be dose-dependent (Fig. 2B). At a concentration of 1 μM Nphe reduced the N/OFQ response to nearly zero (n = 7; Fig. 2B). The IC50 value for Nphe was 16 pM (Fig. 2B). However, Nphe also seemed to act as a partial agonist because it depressed whole cell currents (Fig. 2A). The maximal reduction of whole cell currents evoked by Nphe (11.3 ± 1.8%; n = 5) was not significantly different from that induced by 1 μM N/OFQ (p > 0.05). The recently synthesized nonpeptide antagonist called CompB was also examined. At a concentration of 1 μM CompB, the 100 nM N/OFQ response was reduced to nearly zero (n = 4; Fig. 2C). The IC50 value for CompB was 8.7 nM (Fig. 2C). However, 1 μM CompB also significantly decreased the whole cell current from 5.6 ± 0.6 to 4.6 ± 0.5 nA (at +30 mV), which is a decrease of 18.2 ± 3.7% (p < 0.05; n = 4). This degree of inhibition of current is not significantly different from that observed with treatment of 1 μM N/OFQ (15.2 ± 1.7%; n = 14; p > 0.05).

N/OFQ Effects on Whole Cell Currents Are Attenuated by a Blockade of BK Channels. Under our recording conditions, currents that are activated in the voltage range in which N/OFQ exerts its effects, i.e., −30 to +30 mV, include Ca2+-activated K+ currents (I KCa). We have previously shown that in DBB neurons the majority of the current flowing through the I KCa channels is attributed to the larger conductance I KCa channels, which are referred to as IC or BK channels (Jassar et al., 1999; Jhamandas et al., 2001). Ibexitoxin (IBTX) is a selective inhibitor of the family of IC or BK channels and was used to determine whether the effects of the N/OFQ in DBB neurons are in fact mediated through this species of Ca2+-activated K+ conductances. Figure 2D shows the I–V relationship under control conditions, in the presence of 50 nM IBTX, and N/OFQ (1 μM) in the presence of IBTX. The amplitude of outward currents at +30 mV was significantly reduced from 5.7 ± 0.5 nA under control conditions to 4.8 ± 0.7 nA in the presence of IBTX, which is a 23.1 ± 5.0% reduction (n = 6; p < 0.05). Subsequent application of N/OFQ in the presence of IBTX did not result in a further significant reduction in the mean whole cell current (4.9 ± 0.7 nA; n = 6; p > 0.05). This occlusion of the N/OFQ response in the presence of IBTX results suggests that effects of N/OFQ on whole cell currents are mediated by BK channels.

Effect of N/OFQ on Voltage-Dependent Ca2+ Channels. Because N/OFQ blocks BK channels, one possible target of its action can be the VSCC through which the Ca2+ influx responsible for the activation of BK channels may occur. To address this possibility, we examined whether N/OFQ affected voltage-dependent Ca2+ channels. Currents

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**Fig. 2.** Dose-response relationship of N/OFQ antagonists. A, sample I–V plots of whole cell currents from a sample cell evoked under control conditions, in the presence of 1 μM N/OFQ, during the application of 1 μM Nphe, and after washout. B, dose-response curve for Nphe using an application of 100 nM N/OFQ. Each data point represents the average of three to seven cells. C, dose-response curve for CompB in 100 nM N/OFQ. Each data point represents the average of three to seven cells. D, I–V relationship for whole cell currents evoked under control conditions, in the presence of 50 nM IBTX, 1 μM N/OFQ, and both IBTX and N/OFQ. Inset shows graph showing 1 μM IBTX block of N/OFQ.
through Ca\textsuperscript{2+} channels were recorded using barium (\(I_{\text{Ba}}\)) as a charge carrier. Previous studies have shown that N-type and L-type Ca\textsuperscript{2+} conductances account for the majority of current flowing through the Ca\textsuperscript{2+} channels in DBB neurons (Easaw et al., 1999). Therefore, we examined the effects of N/OFQ on Ca\textsuperscript{2+} conductances using \(\omega\)-conotoxin, a selective blocker of N-type Ca\textsuperscript{2+} channel currents. A, sample trace of Ba\textsuperscript{2+} currents evoked under control conditions. B, I-V plot for Ba\textsuperscript{2+} currents evoked under control conditions, in the presence of 1 \(\mu\)M N/OFQ, and upon recovery. C to E, sample traces showing 100 nM \(\omega\)-conotoxin, 10 \(\mu\)M nimodipine, and a combination of 100 nM \(\omega\)-conotoxin and 10 \(\mu\)M nimodipine, respectively, blocking N/OFQ response in peak Ba\textsuperscript{2+} currents. On the right, histograms depict percentage of reductions in the presence of the blocker and the blocker plus N/OFQ.

**Fig. 3.** Effect of N/OFQ on N- and L-type voltage-dependent Ca\textsuperscript{2+} channels. A, sample trace of Ba\textsuperscript{2+} currents evoked under control conditions. B, I-V plot for Ba\textsuperscript{2+} currents evoked under control conditions, in the presence of 1 \(\mu\)M N/OFQ, and upon recovery. C to E, sample traces showing 100 nM \(\omega\)-conotoxin, 10 \(\mu\)M nimodipine, and a combination of 100 nM \(\omega\)-conotoxin and 10 \(\mu\)M nimodipine, respectively, blocking N/OFQ response in peak Ba\textsuperscript{2+} currents. On the right, histograms depict percentage of reductions in the presence of the blocker and the blocker plus N/OFQ.

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Effects of N/OFQ on Transient Outward (\(I_A\)) and Delayed Rectifier (\(I_K\)) K\textsuperscript{+} Currents. \(I_A\) and \(I_K\) are voltage-sensitive currents, and their activation and inactivation are strongly voltage-dependent. \(I_A\) requires the holding potential to be relatively hyperpolarized (approximately \(-110\) mV) for removal of its inactivation, whereas it is inactivated at \(-40\) mV. On the other hand, \(I_K\) is not inactivated at \(-40\) mV. These biophysical properties of \(I_A\) and \(I_K\) can, thus be used to isolate these currents. Therefore, a conditioning pulse to \(-40\) mV will activate \(I_K\) without any significant contamination by \(I_A\) (Connor and Stevens, 1971; Jhamandas et al., 2002). A conditioning pulse to \(-120\) mV will activate both \(I_A\) and \(I_K\). The difference currents obtained by subtracting the currents evoked by depolarizing pulses after a conditioning pulse to \(-40\) mV from those evoked after a conditioning pulse to \(-120\) mV provide an accurate estimate of \(I_A\). Figure 3B shows the currents recorded from a DBB neuron with a conditioning pulse to \(-40\) mV for 150 ms, representing mainly \(I_K\), under control conditions, in the presence of N/OFQ and recovery on washout of N/OFQ. N/OFQ depressed peak \(I_K\) from 9.6 \(\pm\) 2.0 to 8.4 \(\pm\) 2.0 nA which is a decrease of 12.8 \(\pm\) 4.0% (\(n = 6; p < 0.05\)). Figure 4B shows the different currents recorded from the same neuron representing mainly \(I_K\), under control conditions, in the presence of N/OFQ, and on washout. In the presence of N/OFQ, peak \(I_A\) currents were decreased from 3.7 \(\pm\) 0.6 to 3.2 \(\pm\) 0.6 nA, which is a decrease of 16.9 \(\pm\) 4.0%
We have previously shown that the residual sustained current remaining at the end of the 100-ms test pulse (Fig. 4B) consists mainly of $I_K$ and $I_C$ (Easaw et al., 1999), both of which are also reduced by N/OFQ. Figure 4, C and D, shows the current-voltage relationships of averaged peak $I_K$ ($n = 6$) and $I_A$ ($n = 6$), respectively, using the voltage step protocol shown in Fig. 4, A and B.

Discussion

In the present study, we demonstrate that N/OFQ inhibits whole cell currents in both cholinergic and GABAergic acutely dissociated neurons of the DBB, a basal forebrain nucleus that is important in the context of memory and learning mechanisms. The N/OFQ effects on whole cell currents were antagonized by two N/OFQ antagonists used in this study. N/OFQ blocks currents through N- and L-type Ca$^{2+}$ channels and the IBTX-sensitive Ca$^{2+}$-activated K$^+$ channels. In addition, N/OFQ also inhibited $I_A$ and $I_K$.

Pharmacological Antagonism of N/OFQ Effects on Whole Cell Currents. Under voltage-clamp conditions, N/OFQ caused a dose-dependent decrease in outward currents in the voltage range from $-30$ to $+30$ mV. Although trypsinization has been reported to inhibit activity of certain receptor types, the time-independent preservation of N/OFQ responses in our study suggests that N/OFQ receptors on DBB cells are not affected by the enzymatic dissociation procedure. The EC$_{50}$ value for N/OFQ in inhibiting whole cell currents in our study was 1.2 nM and is consistent with previous data from acutely dissociated periaqueductal gray neurons, where an EC$_{50}$ value of 5 nM was reported for Ca$^{2+}$ conductances (Connor and Christie, 1998). We chose to use higher concentrations of N/OFQ to ensure maximal effects on other ionic conductances ($I_K$, $I_A$, and $I_C$) that may display different sensitivities to the peptide. One of the difficulties in studying the actions of N/OFQ at a cellular level is the lack of an antagonist that does not also exhibit partial agonist activity. Previously, a tridecapeptide analog of N/OFQ, $\text{[Phe}^1\text{CH}^2\text{(NH)}\text{Gly}_2\text{]}\text{N/OFQ-(1-13)-NH}_2$, was identified as a potential selective antagonist for N/OFQ on the basis of its actions in guinea pig ileum (Guerrini et al., 1998). However, electrophysiological studies examining the effects of N/OFQ on the periaqueductal gray and locus coeruleus neurons revealed that $\text{[Phe}^4\text{CH}_2\text{(NH)}\text{Gly}_2\text{]}\text{N/OFQ-(1-13)-NH}_2$ also displayed partial agonist activity by inhibiting inwardly rectifying K$^+$ currents (Chiou, 1999; Connor et al., 1999).

In this study, we examined the ability of Nphe, a peptide antagonist, to block N/OFQ effects on basal forebrain neurons. Nphe has been identified as a pure antagonist for N/OFQ on the basis of its ability to block N/OFQ-evoked inwardly rectifying K$^+$ currents in slices from the periaqueductal gray (Chiou et al., 2002). However, in acutely dissociated DBB neurons, Nphe exhibited partial agonist activity,
although it was able to block N/OFQ actions on whole-cell currents with a maximal inhibition of 95.9 ± 22.6%.

The differences concerning antagonist properties of Npe between our study and previously published data may be explicable by the type of conductances that were examined. In our study, we examined the effect of Npe on whole cell currents, which are an ensemble of K⁺ and Ca²⁺-dependent currents, whereas Chiou et al. (2002) only examined inwardly rectifying K⁺ currents. Thus, the specificity of antagonism of the ORL-1 receptor may be dependent on the intrinsic conductances to which this receptor is coupled.

We also examined CompB, a recently synthesized nonpeptide antagonist for N/OFQ, previously known as J-113397. This nonpeptide antagonist has been shown to inhibit N/OFQ binding to ORL-1 in Chinese hamster ovary cells (Ozaki et al., 2000) and also to block N/OFQ effects on synaptic transmission without any apparent agonist effects (Vaughan et al., 2001). Our data indicate that CompB blocks N/OFQ induced reduction of whole cell currents in DBB neurons with an IC₅₀ value of 8.7 nM, but this compound also demonstrates partial agonist activity as judged by its ability to depress outward currents in a manner similar to N/OFQ. The partial agonist activity may be because at higher doses, CompB may activate N/OFQ or related receptors in DBB neurons.

N/OFQ Modulation of Ca²⁺ and K⁺ Conductances. In the locus coeruleus, the supraoptic nucleus, and hippocampal CA1 neurons, N/OFQ has been shown to increase inwardly rectifying K⁺ conductance (Connor et al., 1996; Madamba et al., 1999; Slugg et al., 1999). Acutely dissociated DBB neurons do not display an inwardly rectifying K⁺ conductance (Jassar et al., 1999). However, in this study we report, for the first time, the ability of N/OFQ to block voltage- and Ca²⁺-activated K⁺ conductance (BK channels). BK channels are composed of a pore-forming α subunit and modulatory β subunits (Meera et al., 2000). The β4 subunit is abundantly expressed in the brain and renders the BK channel subunit resistant to IBTX (Meera et al., 2000). The BK channels we observed are sensitive to IBTX, suggesting that β4 subunit was not contributing to channel formation in DBB neurons. Since BK channels are involved in repolarization phase of the action potential and the phenomenon of accommodation (Vergara et al., 1998), N/OFQ may through its effects on this Ca²⁺-dependent K⁺ conductance play an important role in governing the excitability of DBB neurons. Because activation of BK channels requires an influx of Ca²⁺ into the cell, we also examined the actions of N/OFQ on VSICC.

Previous studies of the effect of N/OFQ on Ca²⁺ channels in acutely dissociated neurons have shown that N/OFQ inhibits Ca²⁺ conductances. In both periaqueductal gray and the hippocampal neurons, N/OFQ mainly inhibited N- and P/Q-types Ca²⁺ conductances (Knoflach et al., 1996; Connor and Christie, 1998). In our preparation, N/OFQ-caused a significant depression of Ca²⁺ currents. In DBB neurons, a majority of the Ca²⁺ current flows through high-voltage-activated N- and L-type of Ca²⁺ conductances with a relatively small contribution from the ω-agatoxin-sensitive P-type conductance (Easaw et al., 1999). In the presence of either ω-conotoxin, a selective blocker of N-type Ca²⁺ channels or nimodipine, an L-type channel blocker, residual N/OFQ effects were observed. However, the N/OFQ response was nearly completely occluded in the presence of blockers of L-type and N-type Ca²⁺ conductances. In hippocampal neurons, activity of both N- and L-type Ca²⁺ channels is coupled to a specific activation of either the BK or the SK types of Ca²⁺-activated K⁺ channels, respectively (Marriott and Tavalin, 1998). Thus, the N/OFQ effects on Ca²⁺ entry through N-type channels may account for the downstream effects of the peptide that we observed on BK channels.

Iₖ, a transient outward K⁺ current and the slower inactivating IₖA are also important in modulating neuronal excitability, in part, through their effects on the early phase of spike repolarization (Storm, 1990). Physiological inhibition of Iₖ decreases the frequency of action potentials by decreasing the interspike interval, whereas inhibition of IₖA lengthens the duration of action potentials (Yao and Chun-Fang, 2001). Thus, N/OFQ of inhibition of Iₖ and IₖA may affect the frequency and duration of action potentials. Mutations in which Iₖ is diminished have shown impairment of learning and memory (Dudai, 1988).

Functional Considerations. N-type Ca²⁺ channels play a significant role in neurotransmitter release (Meir et al., 1999). Single cell RT-PCR analysis of DBB neurons that responded to N/OFQ revealed a majority of these to be cholinergic. Loss of cholinergic tone in basal forebrain neurons is linked to spatial memory and cognitive deficits observed in Alzheimer’s disease (Yankner, 1996). An inhibition of N- and L-type Ca²⁺ channels by N/OFQ would result in a diminished release of acetylcholine from terminals of DBB neurons that project densely to the hippocampus.

Thus, our findings indicate that N/OFQ is capable of diminishing central cholinergic tone and may affect aspects of neuronal excitability, which could help explain the disruption of memory and spatial learning that follows central injections of N/OFQ (Sandin et al., 1997) and the improved performance in memory tasks that is seen in ORL-1 knockout mice (Manabe et al., 1998).

In conclusion, we have identified dose-dependent inhibitory effects of N/OFQ on whole cell currents in cholinergic basal forebrain neurons. N/OFQ antagonists, Npe, and CompB are capable of blocking N/OFQ effects, however, both compounds exhibit partial agonist activity. An analysis of the underlying ionic mechanisms of N/OFQ effects reveals that these are mediated via specific Ca²⁺-dependent conductances that play an important role in transmitter release and neuronal excitability. Behavioral effects of N/OFQ on memory and learning may be explicable by our observations on the cellular effects of N/OFQ in cholinergic basal forebrain neurons.

References


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