Neurotensin Excites Periaqueductal Gray Neurons Projecting to the Rostral Ventromedial Medulla

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1Department of Anesthesiology and 2Department of Neurology, Chang Gung Memorial Hospital; and 3Department of Anatomy and 4Department of Physiology, Chang Gung University School of Medicine, Kwei-San, Tao-Yuan, Taiwan, R.O.C.

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Li, Allen H., Hwa-Min Hwang, Peter P. Tan, Tony Wu, and Hung-Li Wang. Neurotensin excites periaqueductal gray neurons projecting to the rostral ventromedial medulla. J Neurophysiol 85: 1479–1488, 2001. Microinjection of neurotensin into the midbrain periaqueductal gray (PAG) produces a potent and naloxone-insensitive analgesic effect. To test the hypothesis that neurotensin induces the analgesic effect by activating the PAG-rostral ventromedial medulla (RVM) descending antinociceptive pathway, PAG neurons that project to RVM (PAG-RVM) were identified by microinjecting DiIC18, a retrograde tracing dye, into the rat RVM. Subsequently, fluorescently labeled PAG-RVM projection neurons were acutely dissociated and selected for whole cell patch-clamp recordings. During current-clamp recordings, neurotensin depolarized retrogradely labeled PAG-RVM neurons and evoked action potentials. Voltage-clamp recordings indicated that neurotensin excited PAG-RVM neurons by opening the voltage-insensitive and nonselective cation channels. Both SR 48692, a selective NTR-1 antagonist, and SR 142948A, a nonselective antagonist of NTR-1 and NTR-2, failed to prevent neurotensin from exciting PAG-RVM neurons. Neurotensin failed to evoke cationic currents after internally perfusing PAG-RVM projection neurons with GDP-β-S or anti-G_{a11} antiserum. Cellular Ca^{2+} fluorescence measurement using fura-2 indicated that neurotensin rapidly induced Ca^{2+} release from intracellular stores of PAG-RVM neurons. Neurotensin-evoked cationic currents were blocked by heparin, an IP_{3} receptor antagonist, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a fast chelator of Ca^{2+}. These results suggest that by activating a novel subtype of neurotensin receptors, neurotensin depolarizes and excites PAG-RVM projection neurons through enhancing Ca^{2+}-dependent nonselective cationic conductance. The coupling mechanism via G_{a11} proteins is likely to involve the production of IP_{3}, and subsequent IP_{3}-evoked Ca^{2+} release leads to the opening of nonselective cation channels.

INTRODUCTION

The midbrain periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM), which includes nucleus raphe magnus (NRM), nucleus reticularis paragigantocellularis pars alpha (NRGα), and nucleus reticularis paragigantocellularis (NRP), are key components of the brain stem sensory complex (Bandler and Shipley 1994; Fields et al. 1991). Antrograde and retrograde tracing studies showed that PAG neurons project monosynaptically to the NRM, NRGα, and NRP (Beitz et al. 1983; Lakos and Basbaum 1988). Electrical stimulation in the PAG evokes a monosynaptic excitatory potential in the RVM neurons (Mason et al. 1985). Furthermore, analgesia induced by the PAG stimulation is abolished by the lesion of NRM and adjacent medullary reticular formation (Sandkuhler and Gebhart 1984). Thus the antinociceptive effect produced by PAG excitation is mediated by an excitatory innervation from the PAG to the RVM, which projects to the dorsal horn of spinal cord and inhibits the pain transmission (Fields et al. 1991).

It is well known that when microinjected into PAG, opioid peptides and morphine produce the analgesic effect by activating the PAG-RVM antinociceptive pathway (Fields et al. 1991; Morgan et al. 1992). In addition to opioid-induced analgesia, an extensive body of evidence suggests that endogenous neurotensin, a tridecapeptide, regulates the pain transmission by modulating the PAG-RVM antinociceptive neuronal circuitry. When microinjected into the PAG, neurotensin produced a potent and naloxone-insensitive analgesia (Al-Rodhan et al. 1991). It has been shown that neurotensin-containing neurons and fibers are present in the ventromedial and ventrolateral columns of the PAG (Shipley et al. 1987). PAG neurons express a high density of neurotensin receptors (Uhl 1990) and are densely innervated by neurotensin-immunoreactive nerve terminals, which originate from the bed nucleus of the stria terminalis, the central nucleus of the amygdala, and the lateral hypothalamus (Behbehani et al. 1988; Gray and Magnuson 1992; Rizvi et al. 1991). Neurotensin-containing axon terminals form synapses with PAG neurons that project to the NRM and adjacent reticular formation (Williams and Beitz 1989). Intracellular recording studies showed that neurotensin exerts a direct excitatory effect on PAG neurons (Behbehani et al. 1987, 1988). Thus it is very likely that neurotensin produces the analgesic effect by directly exciting PAG neurons that project to the RVM. To test this hypothesis, in the present study whole cell patch-clamp recordings and intracellular Ca^{2+} fluorescence measurement were performed to investigate the ionic and molecular mechanisms by which neurotensin modulates the excitability of retrogradely labeled PAG-RVM projection neurons.

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METHODS

Retrograde labeling and acute isolation of rat PAG-RVM projection neurons

Rat PAG neurons that project to the RVM were retrogradely labeled by 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine (DiI(C18); Molecular Probes, Eugene, OR) as described previously (Kangrga and Loewy 1995; Osborne et al. 1996). Briefly, 11- to 13-day-old Sprague-Dawley rats were anesthetized with ether and placed in a stereotaxic frame. After exposing the dura by trephination, DiIC18 dissolved in DMSO (20 mg/ml) was pressure injected into the RVM (injection volume, ~100 nl).

Three to 4 days after the DiIC18 injection, PAG-RVM projection neurons were acutely dissociated according to the procedures described previously (Wu and Wang 1996; Wu et al. 1995). Briefly, rats were terminally anesthetized with pentobarbital sodium and decapitated. The whole brain was quickly removed, and 300-μm-thick midbrain slices containing the PAG were prepared by using a Vibratome slicer in the ice-cold PIPES-buffered Ringer solution containing (in mM) 120 NaCl, 5 KCl, 20 NaHCO3, 2 MgSO4, 2 CaCl2, 1 KH2PO4, 10 glucose, and 15 PIPES, pH 7.4. Ventrolateral and lateral segments of the PAG were excised and incubated for 20 min at 32°C in an oxygenated PIPES-buffered Ringer solution containing (in mM) 125 NaCl, 5 KCl, 2 CaCl2, 2 MgSO4, 10 glucose, and 15 PIPES, pH 7.4 containing 0.5 mg/ml pronase E (Sigma, St. Louis, MO). Then tissue segments were triturated with a Pasteur pipette, and dissociated neurons were plated onto polylysine-coated coverslips and kept at a 100% O2 atmosphere for 30 min. Subsequently, DiI(C18) retrogradely labeled PAG-RVM neurons were identified under the epifluorescence illumination (rhodamine filter) and selected for whole cell patch-clamp recordings or the measurement of intracellular calcium level.

To confirm the injection site and visualize the distribution of DiIC18-labeled neurons in the PAG, brain was quickly dissected and fixed in the phosphate-buffered saline containing 4% paraformaldehyde. Subsequently, 100-μm vibratome sections of the PAG or RVM were prepared and observed by the fluorescence microscopy. Although a small amount of DiIC18 spread laterally into the NRGp and NRPG, the majority of DiIC18 injections examined (n = 5) were applied directly into the NRM (data not shown).

Whole cell voltage- and current-clamp recordings

Acutely dissociated PAG-RVM neurons were voltage and current clamped by using the conventional whole cell version of patch-clamp techniques (Hamil et al. 1981). Patch pipettes with a resistance of 3–4 MΩ were fabricated from hard borosilicate glasses using a pipette puller (P-87, Sutter Instruments, Novato, CA). Holding potentials, data acquisition, and analysis were controlled by an on-line personal computer programmed with AxoTape 2.0 and pCLAMP 6.0 (Axon Instruments, Foster City, CA). Current and voltage signals obtained by a patch-clamp amplifier (Axopatch-200A, Axon Instruments) were filtered at 2 kHz, digitized (Digidata 1200A interface, Axon Instruments) and stored on the hard disk of the computer for a later analysis.

The extracellular solution had the following composition (in mM): 145 NaCl, 3 KCl, 2 CaCl2, 1 MgCl2, 15 glucose, and 10 HEPES (pH 7.3 with NaOH). The recording pipette was filled with (in mM) 65 KCl, 70 KF, 1 MgCl2, 0.1 CaCl2, 1.1 EGTA, 2 ATP, 0.3 GTP, and 5 HEPES (pH 7.3 with KOH). Liquid junction potentials were corrected as described previously (Barry and Lynch 1991). Series resistance was usually <0.10 MΩ, and the compensation circuitry of the amplifier was used to minimize the series resistance error. In some experiments, GTP was replaced by guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S) or guanosine-5'-O-(3-thiotriphosphate) (GTP-γ-S). Neuropeptide peptides (Peninsula, Belmont, CA) were dissolved in the external solution and applied to neurons using pressure ejections (Picospritzer, General Valve, Fairfield, NJ) from blunt micropipettes (diameter, 20–30 μm).

Intracellular administration of the anti-Gq/11 antibody

Rabbit polyclonal antiserum (QL) directed against the common carboxyl decapetide of Gq/11 and Gq/11 was purchased from Du Pont/NEON (Boston, MA). This antibody has been shown to block various Gq/11-mediated effects including phospholipase C activation, inositol(1,4,5) triphosphate (IP3)-evoked calcium release and cationic currents (Murthy and Mahlkof 1994; Wang and Wu 1996; Wang and Wago 1996).

Anti-Gq/11 antibody was dissolved in a slightly modified internal solution (1:50 dilution; final concentration was approximately 0.5 mg/ml) containing 0.2% BSA and 1 mM GTP. For whole cell patch-clamp recordings, the tip of patch electrode was filled with antiserum-free pipette solution, and electrode was backfilled with the internal solution containing the anti-Gq/11 antibody. Antiserum-free solution in the tip of patch pipette not only facilitated the formation of high-gigaohm seal but also made it possible to record neureotensin-evoked membrane currents before the antibody diffused into PAG-RVM neurons and antagonized Gq/11-mediated effects. As controls, antiserum was heated at 90°C for 10 min and then used for whole cell recordings as described above. After the whole cell patch-clamp recordings, the successful intracellular dialysis of the anti-Gq/11 antibody was confirmed by performing immunofluorescence stainings with Texas Red–conjugated goat anti-rabbit IgG (Vector, Burlingame, CA).

Single-cell reverse transcriptase-polymerase chain reaction (RT-PCR) assay

To study the expression of Gq/11 and Gq/11 mRNAs in single PAG-RVM projection neurons, single-cell RT-PCR assay was performed as described previously (Wang and Wu 1996; Wu and Wang 1996). Briefly, after finishing whole cell patch-clamp recordings, the cellular content was aspirated into the tip of patch pipette by applying a gentle suction. The first strand cDNA was synthesized in a reaction volume of 30 μl containing 3 mM MgCl2, 50 mM Tris-HCl (pH 8.3), 77 mM KCl, 10 mM dithiothreitol, 8 ng/μl random hexamers, 1 mM of each deoxynucleotide 5'-triphosphate, 20 U of ribonuclease inhibitor (Promega, Madison, WI), and 150 U of Moloney murine leukemia virus reverse transcriptase (Promega) for 1 h at 42°C. Then the reaction mixture was heated at 90°C for 5 min and used as the DNA template for the PCR.

PCR was carried out in a programmable thermal controller with the following oligonucleotide primers. 1) Sense primer for Gq/11 is 5’-GTTGTCGTCTTTTGAGAATCCATA3’ and corresponds to nucleotides 359–382 of mouse Gq/11 (Strathmann and Simon 1990). 2) Sense primer for Gq/11 is 5’-GACCCTGGAGAACATCATCTTTCCAGG3’ and corresponds to nucleotides 582–605 of mouse Gq/11 (Strathmann and Simon 1990). 3) Common antisense primer for Gq/11 and Gq/11 is 5’-GATCTCCCTTACGGTTCAGCTGACG3’ and corresponds to nucleotides 1044–1067 of mouse Gq/11 (Strathmann and Simon 1990). With these primers, the predicted sizes of PCR products for Gq/11 and Gq/11 cDNA fragments are 709 and 486 bp, respectively. Aliquots of PCR products were separated and visualized in an ethidium bromide–stained agarose gel (1.5%) by the electrophoresis. PCR DNA fragments encoding Gq/11 and Gq/11 were also gel purified and used for the dideoxy chain-termination DNA sequencing (Thermo Sequenase cycle sequencing kit, Amersham, Cleveland, OH).

Intracellular Ca2+ measurement

[Ca2+]i of PAG-RVM neuron was measured using the fluorescence ratio imaging with fura-2 AM (acetoxyethyl ester) as described
Identification of PAG-RVM projection neurons

To identify PAG neurons that project to the PAG-RVM, DiI<sub>C18</sub>, a retrograde tracing dye, was microinjected into the rat RVM. In accordance with previous retrograde-labeling studies (Beitz et al. 1983; Osborne et al. 1996; Reichling and Basbaum 1990), DiI<sub>C18</sub>-labeled neurons were found in the ventrolateral, lateral, and dorsomedial divisions of the PAG, but were scarce in the dorsolateral PAG (data not shown). In the present study, DiI<sub>C18</sub> retrogradely labeled neurons were acutely dissociated from the ventrolateral and lateral PAG, which mediate the antinociceptive effect by sending projections to the RVM (Bandler and Shipley 1994). Consistent with previous studies (Osborne et al. 1996; Reichling and Basbaum 1990), most of retrogradely labeled PAG-RVM neurons (about 70%) were small bipolar cells (diameter, 12–15 μm; Fig. 1, A and B). Other labeled PAG-RVM projection neurons were larger multipolar cells (diameter, 15–20 μm; Fig. 1, C and D).

Neurotensin excites PAG-RVM projection neurons by enhancing a nonselective cationic conductance

In agreement with previous studies (Behbehani et al. 1987; Sanchez and Ribas 1991), acutely isolated bipolar or multipolar PAG-RVM neurons exhibited spontaneous action potentials. The spontaneous firing frequency ranged between 5 and 15 Hz (mean firing rate, 6.1 ± 1.5 Hz, mean ± SE, n = 27), and the membrane input resistance was 650 ± 57 MΩ (n = 27). Sixty-five percent of bipolar or multipolar PAG-RVM neurons studied (n = 247) responded to neurotensin with the induction of inward current at the negative membrane potential and a membrane depolarization. During whole cell current-clamp recordings, neurotensin (1 μM) increased the firing rate of spontaneously active PAG-RVM neurons (data not shown) and triggered action potentials from PAG-RVM projection neurons (n = 21) hyperpolarized to prevent the spontaneous firing (Fig. 2A). Consistent with a previous study (Behbehani et al. 1987), the excitatory effect of neurotensin on...
PAG-RVM projection neurons resulted from a reversible depolarization of 12–15 mV (Fig. 2A). Under voltage clamp at a holding potential ($V_{\text{H}}$) of $-60 \text{ mV}$, neurotensin (1 $\mu$M) evoked an inward current reversibly (Fig. 2B; $21 \pm 2 \text{ pA, } n = 125 \text{ cells}$) and with a concentration-dependent manner (Fig. 2C; EC$_{50} = 84 \text{ nM}$). NT(8–13) (3 $\mu$M), the C-terminal hexapeptide fragment of neurotensin, also depolarized the PAG-RVM neurons by evoking inward currents (mean amplitude, $16 \pm 1 \text{ pA, } n = 8$, $V_{\text{H}} = -60 \text{ mV}$). These results indicate that neurotensin depolarizes and excites PAG-RVM projection neurons by evoking an inward current at negative membrane potentials.

To investigate the ionic mechanism by which neurotensin induces inward currents in PAG-RVM projection neurons, a current-voltage curve was constructed by measuring neurotensin-evoked currents at various holding potentials. Neurotensin (1 $\mu$M)-induced currents reversed the direction at $-15 \pm 2 \text{ mV}$ ($n = 12 \text{ neurons}$), a reversal potential expected for nonselective cation channels, and were linear over the holding potentials between $-100$ and 40 mV (Fig. 3). Reducing the external sodium concentration from 145 to 70 mM greatly decreased the magnitude of neurotensin-evoked inward current. When the sodium concentration was decreased to 70 mM (NaCl was replaced by Tris-HCl), the amplitude of neurotensin-induced currents was decreased to 70 mM (NaCl was replaced by Cs$^+$, neurotensin (1 $\mu$M) still evoked inward currents from PAG-RVM neurons reversibly at the holding potential of $-60 \text{ mV}$ (mean magnitude, $20 \pm 1 \text{ pA, } n = 5$). With a recording solution containing cesium ions, current-voltage ($I$-$V$) curve of neurotensin-activated current was also linear over the range of membrane potentials ($-100$ to 40 mV) studied, and the average extrapolated reversal potential was $-12 \pm 2 \text{ mV (n = 5)}$. These findings suggest that neurotensin depolarizes PAG-NRM neurons by opening voltage-insensitive and nonselective cation channels, which are permeable to Na$^+$, K$^+$, and Cs$^+$ ions.

Up to now, subtype 1 neurotensin receptor (NTR-1) and subtype 2 neurotensin receptor (NTR-2), which possess the seven transmembrane structure of G-protein–coupled receptors, have been cloned from the rat brain (Vincent et al. 1999). SR 48692, a highly potent nonpeptide antagonist of NTR-1 ($K_{d} = 3 \text{ nM}$), has been shown to inhibit neurotensin-mediated biological effects (Gully et al. 1993). However, SR 48692 (10 $\mu$M) failed to prevent neurotensin (1 $\mu$M) from evoking inward cationic currents in DiIC$_{18}$-labeled PAG-RVM neurons (Fig. 4A; control NT current, $20 \pm 2 \text{ pA; with SR 48692, NT current, } 19 \pm 2 \text{ pA, } n = 8$, $V_{\text{H}} = -60 \text{ mV}$). In the presence of 10 $\mu$M SR 142948A, a potent nonselective antagonist of NTR-1 ($K_{d} = 6 \text{ nM}$) and NTR-2 ($K_{d} = 4 \text{ nM}$) (Gully et al. 1997), neurotensin (1 $\mu$M) still evoked cationic currents (Fig. 4B; control NT current, $20 \pm 1 \text{ pA; with SR 142948A, NT current, } 19 \pm 2 \text{ pA, } n = 8$, $V_{\text{H}} = -60 \text{ mV}$). In contrast to a previous study showing that SR 48692 and SR 142948A activate NTR-2 artificially expressed in the Chinese hamster ovary (CHO) cells (Yamada et al. 1998), SR 48692 and SR 142948A did not affect the membrane potential and spontaneous firing rate of DiIC$_{18}$-labeled PAG-RVM neurons. Our investigation indicated that 100 nM SR 48692 or SR 142948A completely blocked neurotensin (5 $\mu$M)-evoked cationic currents in acutely dissociated substantia nigra dopaminergic neurons (Wu et al. 1995; Li and Wang, unpublished results). These findings propose that neurotensin excites PAG-RVM projection neurons by activating a novel subtype of neurotensin receptors.

$G_{\alpha q/11}$ proteins couple neurotensin receptors to cation channels of PAG-RVM neurons

Neurotensin receptor is a member of the superfamily of G-protein–coupled receptors (Vincent et al. 1999), suggesting

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**Fig. 2.** Neurotensin depolarizes and excites PAG-RVM projection neurons. A: during the whole cell current-clamp recording, neurotensin (NT; 1 $\mu$M) depolarized a DiIC$_{18}$-labeled PAG-RVM neuron and triggered action potentials. Spontaneous action potentials were inhibited by applying a hyperpolarizing current. B: under the whole cell current-clamp recording, neurotensin reversibly evoked an inward current from the same neuron. Holding potential ($V_{\text{H}}$) = $-60 \text{ mV}$. C: neurotensin induced the inward current from PAG-RVM projection neurons with a concentration-dependent manner. Each point shows the mean ± SE value of 13 neurons. $V_{\text{H}} = -60 \text{ mV}$. **Fig. 3.** Neurotensin depolarizes and excites DiIC$_{18}$-labeled PAG-RVM neurons by enhancing a voltage-insensitive and nonselective cationic conductance. Neurotensin (1 $\mu$M)-evoked currents were obtained at various holding potentials ($-100$ to 40 mV), and a current-voltage ($I$-$V$) curve was constructed. Note that neurotensin-induced currents reversed the polarity at about $-15 \text{ mV}$ and were linear over the membrane potentials studied. Each point shows the mean value from 12 neurons.
that G-proteins are involved in neurotensin activation of nonselective cationic conductance. This hypothesis was tested by dialyzing retrogradely labeled PAG-RVM neurons with GTP analogues, GDP-β-S and GTP-γ-S. GDP-β-S binds to G-proteins and causes an irreversible inactivation of G-proteins. When PAG-RVM projection neurons were dialyzed with 1 mM GDP-β-S, the mean amplitude of NT current obtained 1 min after starting recordings was 22 ± 1 pA (n = 10, V_h = −60 mV). However, neurotensin-induced current became significantly smaller after perfusing PAG-RVM neurons with GDP-β-S for 3–5 min (Fig. 5B, P < 0.01). Neurotensin failed to evoke cationic currents in PAG-RVM neurons dialyzed with 1 mM GDP-β-S for 7 min (Fig. 5, A and B).

One minute after initiating the recording, neurotensin evoked an inward current reversibly (mean magnitude, 23 ± 3 pA, n = 10, V_h = −60 mV) from PAG-RVM neurons dialyzed with 0.5 mM GTP-γ-S, an irreversible activator of G-proteins (Fig. 5C). When PAG-RVM projection neurons were internally perfused with 0.5 mM GTP-γ-S for 7 min, neurotensin evoked cationic currents irreversibly (Fig. 5, C and D). Following the irreversible induction of cationic currents, subsequent application of neurotensin did not induce any membrane current. Internal perfusion of GTP-γ-S or GDP-β-S did not affect the resting membrane properties of PAG-RVM neurons. When DiIC18-labeled PAG-RVM neurons were studied with control internal solution containing 0.3 mM GTP, neurotensin-evoked cationic currents were totally reversible and did not display any significant rundown for 10–15 min after the initiation of whole cell recordings (the initial amplitude of neurotensin current, 21 ± 2 pA; after 15 min, mean amplitude, 19 ± 3 pA, n = 8, V_h = −60 mV). These results suggest that G-proteins couple neurotensin receptors to nonselective cation channels of PAG-RVM projection neurons.

Neurotensin receptor has been shown to be linked to G_{a q/11}−phospholipase C pathway (Vincent et al. 1999; Wang 1997). Our previous investigation demonstrated that G_{a q/11} couples neurotensin receptors to nonselective cation and inwardly rectifying potassium channels of substantia nigra dopaminergic neurons (Wang and Wu 1996; Wu et al. 1995). To investigate the functional role of G_{a q/11} in mediating the neurotensin enhancement of cationic conductance of PAG-RVM neurons, we dialyzed DiIC18-labeled PAG-RVM neurons with a specific antibody (QL) raised against the common C-terminus of G_{aq} and G_{ar11} during the whole cell voltage-clamp recordings. Successful perfusion of PAG-RVM neurons with the anti-G_{aq/11} antiserum was confirmed by performing the immuno-
fluorescence staining after the whole cell recordings (data not shown). When PAG-RVM neurons \((n = 10)\) were perfused with the anti-\(G_{\alpha q/11}\) antiserum for 1 min, neurotensin still evoked inward currents (Fig. 6A). After dialyzing PAG-RVM neurons for 4 min, neurotensin-induced inward currents became significantly smaller (Fig. 6C, \(P < 0.01\)). Twelve minutes after the initiating whole cell recordings, neurotensin failed to evoke the inward cationic current (Fig. 6, A and C). When retrogradely labeled PAG-RVM neurons \((n = 10)\) were internally perfused with the heat-inactivated anti-\(G_{\alpha q/11}\) antibody, the initial amplitude of neurotensin-evoked cationic current was \(20 \pm 2\) pA. After internally perfusing PAG-RVM neurons with the heat-inactivated antiserum for 12 min, neurotensin-induced cationic currents were not significantly inhibited (Fig. 6, B and C; mean amplitude, \(18 \pm 1\) pA). Furthermore, internal perfusion of the anti-\(G_{\alpha q/11}\) antibody did not affect the resting electrical properties of retrogradely labeled PAG-RVM neurons. These results clearly indicate that the inhibition of neurotensin-evoked cationic current by the anti-\(G_{\alpha q/11}\) antibody is due to the specific block of neurotensin receptor-\(G_{\alpha q/11}\) coupling.

The anti-\(G_{\alpha}\) antibody used in the present study recognizes both \(G_{\alpha q}\) and \(G_{\alpha 11}\). To test the possibility that \(G_{\alpha q}\) or \(G_{\alpha 11}\) selectively mediates the neurotensin activation of cationic conductance, we also investigated the expression of mRNAs encoding \(G_{\alpha q}\) and \(G_{\alpha 11}\) in individual PAG-RVM projection neurons. In these experiments, neurotensin-induced membrane currents were recorded from PAG-RVM neurons. After finishing whole cell voltage-clamp recordings, the cellular content of each neuron was aspirated to the tip of the patch electrode and utilized for the subsequent reverse transcription. One-half of the reverse transcription product was utilized as the DNA template for the PCR amplification of \(G_{\alpha q}\) or \(G_{\alpha 11}\) cDNA fragment. Amplified cDNA fragments encoding \(G_{\alpha 11}\) (lane 1) and \(G_{\alpha q}\) (lane 2) were obtained from this neuron. The same finding was also observed from 9 other PAG-RVM projection neurons that responded to neurotensin. The locations of DNA size markers (\(\lambda\)174 DNA digested with Hind I) are indicated on the right side.

![Figure 6](http://jn.physiology.org/)

**Fig. 6.** Internal perfusion of an anti-\(G_{\alpha q/11}\) antibody blocks neurotensin-evoked cationic currents in PAG-RVM projection neurons. A: 1 min after starting the whole cell recording with a pipette solution containing the anti-\(G_{\alpha q/11}\) antiserum, neurotensin (1 \(\mu\)M) evoked an inward current (top trace). After internally perfusing this PAG-RVM neuron with the antiserum for 12 min, neurotensin failed to induce the inward current (bottom trace). \(V_{hi} = -60\) mV. B: in a PAG-RVM projection neuron dialyzed with the heat-inactivated anti-\(G_{\alpha q/11}\) antibody for 12 min, neurotensin (1 \(\mu\)M) still evoked an inward cationic current (bottom trace). C: the time course for the inhibitory effect of the anti-\(G_{\alpha q/11}\) antiserum on neurotensin-induced cationic currents. The inward current recorded 1 min after initiating whole cell recordings was used as the initial NT-evoked current. For DiIC\(_{18}\) retrogradely labeled PAG-RVM neurons dialyzed with the anti-\(G_{\alpha q/11}\) antibody (•; each point represents the mean ± SE value from 10 neurons), the amplitude of initial NT-evoked inward current was \(19 \pm 3\) pA. When PAG-RVM neurons were perfused with the heat-inactivated antiserum (○; each point is the mean ± SE value for 10 cells), the magnitude of initial NT-induced current was \(20 \pm 2\) pA.

**IP\(_3\)-evoked Ca\(^{2+}\) release mediates the neurotensin enhancement of cationic conductance**

Receptor-activated \(G_{\alpha 11}\) proteins cause the stimulation of phospholipase C (PLC), and PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate and generates second messengers, ino-
protein kinase C, neurotensin (1 μM) measurement using fura-2 shows that neurotensin (1 μM) rapidly increased the cytoplasmic Ca^2+ level (Fig. 8C; basal Ca^2+ level, 126 ± 17 nM; with neurotensin, Ca^2+ level, 322 ± 35 nM, n = 10). Neurotensin still increased the intracellular Ca^2+ level when experiments were performed in a Ca^2+-free external solution containing 1 mM EGTA (basal Ca^2+ level, 113 ± 15 nM; with neurotensin, Ca^2+ level, 226 ± 16 nM, n = 9). The functional role of Ca^2+ as the messenger of neurotensin modulation of cationic conductance was further investigated by buffering intracellular Ca^2+ with a fast Ca^2+ chelator, BAPTA. In retrogradely labeled PAG-RVM neurons dialyzed with 10 mM BAPTA for 7–9 min, neurotensin (1 μM) failed to evoke cationic currents (Fig. 8D; initial amplitude of neurotensin current, 25 ± 2 pA, n = 10, V_H = −60 mV). Together with the finding that the intracellular dialysis of heparin blocks neurotensin-induced cationic currents, these results suggest that IP_3-evoked Ca^2+ release is responsible for the neurotensin enhancement of cationic conductance of PAG-RVM projection neurons.

**DISCUSSION**

To gain insight into the cellular and molecular mechanisms by which neurotensin produces an antinociceptive effect in the PAG, we investigated the electrophysiological effect of neurotensin on retrogradely labeled PAG-RVM neurons. Consistent with published studies (Osborne et al. 1996; Reichling and Basbaum 1990), our results indicate that PAG-RVM projection neurons are small bipolar or large multipolar cells. A previous investigation showed that electrolytic lesions of RVM abolished the neurotensin-induced analgesia in the PAG and that microinjection of neurotensin into the PAG led to an excitation of NRM neurons (Behbehani and Pert 1984), suggesting that neurotensin produces the antinociceptive effect by directly exciting PAG neurons that project to the RVM. The results reported here, for the first time, provide the direct evidence that neurotensin depolarizes and excites DiIC18-retrogradely labeled PAG-RVM projection neurons.

Inflammatory pain stimulus has been shown to cause neurotensin release within the PAG (Williams et al. 1995). It has also been reported that both preproneurotensin mRNA and...
neurotensin peptide are up-regulated within PAG neurons in rat models for the chronic noception (Williams and Beitz 1993). Together with the results presented here, these findings suggest that endogenous neurotensin is released in response to the nociceptive stimulus and modulates the spinal pain transmission by exciting PAG-RVM projection neurons. In contrast to a direct excitation of PAG-RVM neurons by neurotensin, opioid peptides and morphine are believed to disinhibit PAG-RVM projection neurons by hyperpolarizing and decreasing the activity of GABAergic interneurons, which tonically inhibit descending output neurons (Vaughan et al. 1997). Interestingly, morphine and [d-Ala\(^2\),N-methyl-Phe\(^4\),Gly-ol\(^5\)] enkephalin (DAMGO), a specific \(\mu\)-opioid receptor agonist, have been reported to induce an increase of neurotensin release in the PAG (Stiller et al. 1997), proposing that the excitation of PAG-RVM projection neurons by endogenous neurotensin could also contribute to the opioid-mediated analgesia in the PAG.

Although a previous study demonstrated that neurotensin depolarizes periaqueductal gray neurons (Behbehani et al. 1987), the exact ionic mechanism of neurotensin-induced excitation in the PAG has not been investigated. Our voltage-clamp recordings suggest that neurotensin depolarizes PAG-RVM projection neurons through enhancing a voltage-independent and nonselective cationic conductance. In accordance with the present study, it has been reported that neurotensin depolarizes supraoptic nucleus neurons by opening nonselective cation channels (Kirkpatrick and Bourque 1995). On the other hand, neurotensin has been shown to excite cholinergic neurons of nucleus basalis, serotonergic neurons of the dorsal raphe nucleus, ventral tegmental and substantia nigra dopaminergic neurons through a dual ionic mechanism, an increase in the nonselective cationic conductance, and a decrease in the potassium conductance (Farkas et al. 1994; Jiang et al. 1994; Jolas and Aghajanian 1996; Wu et al. 1995).

Neurotensin receptor is believed to be a member of the family of \(G_q\)-coupled receptors (Vincent et al. 1999; Wang 1997). Among the members of \(G_q\) family, including \(G_{q1}\), \(G_{q2}\), \(G_{q3}\), and \(G_{q}\) (Helper and Gilman 1992), \(G_{q}\) and \(G_{q1}\) are highly expressed in the brain (Milligan 1993). To investigate the involvement of \(G_{aq}\) and \(G_{a11}\) in mediating the neurotensin enhancement of cationic conductance, anti-\(G_{aq/11}\) antibody raised against the common C-terminal decapeptide of \(G_{aq}\) and \(G_{a11}\) was dialyzed into Dil\(_{C18}\)-labeled PAG-RVM neurons during the whole cell recordings. Our results indicate that internal perfusion of PAG-RVM neurons with anti-\(G_{aq/11}\) antisem completely blocks neurotensin-evoked cationic currents. Although the \(\beta y\) dimer of \(G\) protein could also function as the mediator of neurotransmitter modulation of ion channels (Herlitze et al. 1996), the present study suggests that \(\alpha\)-subunits of \(G_{q/11}\) mediate the neurotensin enhancement of cationic conductance of PAG-RVM projection neurons.

The anti-\(G_{aq/11}\) antisem used in the present study recognizes both \(G_{aq}\) and \(G_{a11}\). Therefore we studied the expression of \(G_{aq}\) and \(G_{a11}\) in individual PAG-RVM neurons. Single-cell RT-PCR analysis demonstrates that both \(G_{aq}\) and \(G_{a11}\) mRNAs are present in NT-responsive PAG-RVM projection neurons. This finding suggests that neurotensin modulation of cationic conductance is mediated either by both \(G_{aq}\) and \(G_{a11}\) or by one of these two \(\alpha\)-subunits. The first hypothesis is supported by the following findings. 1) \(G_{aq}\) and \(G_{a11}\) share 97% amino acid sequence identity in C-terminal domains, which couple neurotransmitter receptors to various effectors, and indistinguishably activate phospholipase C-\(\beta\) isoforms (Helper and Gilman 1992; Sternweis and Smircka 1992). 2) Agonist stimulations of \(M_1\) muscarinic, vasopressin, and bombesin receptors lead to the activation and down-regulation of both \(G_{aq}\) and \(G_{a11}\) (Mullaney et al. 1993; Offermanns et al. 1994), indicating that these neurotransmitter receptors interact with both \(G_{aq}\) and \(G_{a11}\) equally and nonselectively. However, intranuclear injection of DNA plasmid encoding the antisense sequence of \(G_{aq}\) selectively attenuated the muscarinic inhibition of M-type potassium currents in sympathetic neurons (Haley et al. 1998). Moreover, it has been shown that pretreating ventromedial hypothalamic neurons with \(G_{a11}\) antisense oligonucleotide blocked muscarinic inhibition of delayed rectifier \(K^+\) currents (french-Mullen et al. 1994). These findings suggest that G-protein-coupled receptors could interact selectively with \(G_{aq}\) or \(G_{aq/11}\). Further studies using the specific antisense oligonucleotide to \(G_{aq}\) or \(G_{a11}\) are required to elucidate the exact coupling mode between \(G_{aq/11}\) and neurotensin receptors of PAG-RVM projection neurons.

\(G_{aq/11}\) could couple neurotensin receptors to nonselective cation channels of PAG-RVM neurons either indirectly through second messengers or directly in a membrane-delimited way. The present study suggests that activation of neurotensin receptors in PAG-RVM projection neurons results in the stimulation of phospholipase C and the generation of IP\(_3\) via \(G_{aq/11}\) proteins. The subsequent IP\(_3\)-evoked Ca\(^{2+}\) release is responsible for the opening of nonselective cation channels (Patridge et al. 1994). This transduction pathway is supported by the following findings. 1) Heparin, an antagonist of IP\(_3\) receptor, inhibited the neurotensin enhancement of cationic conductance. 2) Neurotensin rapidly induced the release of Ca\(^{2+}\) from intracellular stores. 3) Neurotensin-evoked cationic current was blocked by BAPTA. In accordance with the present study, our previous investigation showed that \(G_{aq/11}\) couples NTR-1 receptors to cation channels of substantia nigra dopaminergic neurons indirectly through the IP\(_3\)-Ca\(^{2+}\) signaling pathway (Wu et al. 1995).

SR 48629, a nonpeptide antagonist of NTR-1, has been shown to block several neurotensin-mediated pharmacological effects in the brain, which include neurotensin excitation of midbrain dopaminergic neurons, neurotensin-evoked dopamine efflux in the striatum, and turning behavior induced by neurotensin (Gully et al. 1993; Wu et al. 1995). However, the present study demonstrates that SR 48629 fails to inhibit neurotensin excitation of Dil\(_{C18}\)-labeled PAG-RVM neurons, suggesting that neurotensin-induced analgesia in the PAG is not mediated by the activation of NTR-1. In agreement with the present study, it has been reported that SR 48629 fails to antagonize neurotensin-induced antinociception in the brain (Dubuc et al. 1994). Subtype 2 neurotensin receptor (NTR-2) with a low affinity for SR 48629 has also been cloned from the brain (Vincent et al. 1999). NTR-2 has been reported to partially mediate central and SR 48629–resistant analgesic effect of neurotensin (Dubuc et al. 1999; Gully et al. 1997). Our results demonstrate that SR 142948A, a potent antagonist of NTR-1 and NTR-2, fails to prevent neurotensin from exciting retrogradely labeled PAG-RVM neurons. In contrast to the present study showing that neurotensin enhances the nonselective conductance of PAG-RVM projection neurons through
IP$_3$-Ca$^{2+}$ pathway, the exact signal transduction pathway of neurotensin-activated NTR-2 is still unknown (Vincent et al. 1999). Furthermore, several groups of investigators reported that neurotensin (1 μM) fails to induce IP$_3$ formation and intracellular Ca$^{2+}$ mobilization in CHO or HEK 293 cells expressing a high density of NTR-2 (Botto et al. 1998; Yamada et al. 1998). Therefore it is very likely that neurotensin exerts an excitatory effect on PAG-RVM neurons and produces an analgesic effect by activating a novel subtype of neurotensin receptors. Further studies using the molecular cloning method are required to prove the existence of an additional subtype of neurotensin receptors, which mediates the neurotensin-induced analgesia in the PAG.

In summary, the present study provides the evidence that the central antinociceptive action of neurotensin in part results from the excitation of PAG-RVM projection neurons. In addition to NTR-2, which has been shown to mediate the central antinociceptive effect of neurotensin (Dubuc et al. 1999; Gully et al. 1997), the results presented here suggest that an additional subtype of neurotensin receptors mediate neurotensin excitation of PAG-RVM neurons and neurotensin-induced analgesia in the PAG. Future development of specific agonists for neurotensin receptors mediating the central analgesia could lead to a better clinical management of the pain.


