Neurotensin and Substance P Inhibit Low- and High-Voltage-Activated Ca\(^{2+}\) Channels in Cultured Newborn Rat Nucleus Basalis Neurons

MARTA MARGETA-MITROVIC, JOHN J. GRIGG, KONOMI KOYANO, YASUKO NAKAJIMA, AND SHIGEHIRO NAKAJIMA

Departments of Pharmacology and Anatomy and Cell Biology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612

Marjeta-Mitrovic, Marta, John J. Grigg, Konomi Koyano, Yasuko Nakajima, and Shigehiro Nakajima. Neurotensin and substance P inhibit low- and high-voltage-activated Ca\(^{2+}\) channels in cultured newborn rat nucleus basalis neurons. J. Neurophysiol. 78: 1341–1352, 1997. Inhibition of Ca\(^{2+}\) currents by the excitatory neurotransmitters neurotensin and substance P was investigated in cultured nucleus basalis neurons with the use of the whole cell patch-clamp technique. The whole cell Ca\(^{2+}\) current, elicited from a holding potential of −80 mV by a step pulse to 0 mV and measured at 100 ms, was inhibited 67.9% by neurotensin and 57.6% by substance P. Low-voltage-activated (LVA) Ca\(^{2+}\) current, elicited by a step pulse to −40 mV from a holding potential of −90 mV, was inhibited by both neurotensin (26.2%) and substance P (24.1%). High-voltage-activated Ca\(^{2+}\) currents were separated with the use of the Ca\(^{2+}\) channel antagonists. Nimodipine (3 μM) inhibited 24.2% of the whole cell Ca\(^{2+}\) current elicited by a step to 0 or +10 mV and measured at 100 ms. Under the same conditions, ω-conotoxin (ω-CgTx)-GVIA (0.5 μM) inhibited 46.4%, ω-CgTx-GVIA + nimodipine 58.7%, and ω-CgTx-MVIIIC (5 μM) + nimodipine 75.7% of the current. ω-Aga-toxin (ω-Aga)-IVA (100 nM) did not produce any effect. Neurotensin inhibition of the whole cell Ca\(^{2+}\) current was attenuated by each of these treatments except for the ω-Aga-IVA treatment, which did not change the neurotensin effect. In contrast, neither the ω-Aga-IVA nor the nimodipine treatment had any effect on the substance-P-induced inhibition; the rest of the treatments attenuated the substance-P-induced response. Thus the data indicate that nucleus basalis neurons express LVA as well as L-, N-, and Q-type, but not the P-type, Ca\(^{2+}\) currents. N- and Q-type HVA Ca\(^{2+}\) currents, as well as LVA Ca\(^{2+}\) currents, are inhibited by both neurotensin and substance P. In contrast, L-type current is inhibited by neurotensin but not by substance P. In addition, a fraction of the total whole cell current was resistant to all Ca\(^{2+}\) channel antagonists and thus may correspond to the R-type Ca\(^{2+}\) current. This residual current was inhibited by both neurotensin and substance P. The inhibition of the whole cell Ca\(^{2+}\) current produced by both neurotransmitters was voltage independent, because a large depolarization (+70 mV) was not able to relieve either effect. In cells loaded with 0.1 mM guanosine 5′-[(γ-thio)triphosphate, response to both neurotensin and substance P became irreversible, indicating that the effects of both neurotransmitters were mediated through G proteins. However, pertussis toxin did not affect either the neurotensin or the substance P response.

INTRODUCTION

Nucleus basalis cholinergic neurons, the main source of cortical cholinergic innervation (Mesulam et al. 1983), degenerate in the course of Alzheimer’s disease. This degeneration likely underlies some of the cognitive deficits present in patients with Alzheimer’s disease (Whitehouse et al. 1982). A disturbance in intracellular Ca\(^{2+}\) homeostasis may be an important factor in the pathophysiology of this disorder, because an excessive increase in the intracellular Ca\(^{2+}\) concentration can lead to cellular degeneration and death (Choi 1988).

Voltage-gated Ca\(^{2+}\) channels are important for various vital cellular functions (Bertolino and Llinás 1992; Dunlap et al. 1995), but may also play a role in Ca\(^{2+}\)-mediated cell degeneration (Weiss et al. 1990). On the basis of the voltage dependence of activation, Ca\(^{2+}\) channels are classified into the low-voltage-activated (LVA) (Carbone and Lux 1984; Fedulova et al. 1985) and the high-voltage-activated (HVA) channels (Bertolino and Llinás 1992; McCleskey et al. 1986), and various functional roles of these Ca\(^{2+}\) channels have been suggested (Bertolino and Llinás 1992; Dunlap et al. 1995). HVA channels are further categorized into the L, N, P, and Q type by their sensitivity to the Ca\(^{2+}\) channel antagonists (Aosaki and Kasai 1989; Dunlap et al. 1995; Mintz et al. 1992b; Randall and Tsien 1995), and in this paper we use these pharmacological definitions of the HVA Ca\(^{2+}\) current subtypes.

Neurotensin (NT), discovered by Carraway and Leeman (1973), is a peptide neurotransmitter widely distributed in the brain. This interesting neuropeptide plays an important part in the regulation of the brain dopaminergic system. NT counteracts the effect of dopamine on D\(_1\) receptors, and behavioral studies suggest that NT has effects similar to those of antipsychotic agents (Jolicoeur et al. 1993; Nemeroff et al. 1992). NT-immunoreactive fibers are abundant in the basal forebrain (Jennes et al. 1982), and cholinergic neurons from this area express NT receptors (Szigethy et al. 1989). In nucleus basalis neurons, NT inhibits an inwardly rectifying K\(^+\) channel through a pertussis toxin (PTX)-insensitive G-protein-mediated mechanism (Farkas et al. 1994). Also, NT activates nonselective cation channels (Farkas et al. 1994). However, the effects of NT on the Ca\(^{2+}\) currents have not yet been reported.

Axons containing the tachykinin substance P (SP) also contact nucleus basalis neurons (Bolam et al. 1986). NK\(_1\) receptors (SP receptors) are expressed throughout the somatodendritic membrane of nucleus basalis cholinergic neurons (Kowall et al. 1993). SP produces a slow excitation in cultured brain cholinergic neurons through inhibition of an inwardly rectifying K\(^+\) channel (Stanfield et al. 1985). SP enhances Ca\(^{2+}\) currents in spinal dorsal horn neurons (Ryu and Randic 1990) but inhibits them in superior cervical ganglion sympathetic neurons (Shapiro and Hille 1993).

The objective of the investigations reported here was to analyze the effects of NT on the Ca\(^{2+}\) channels in nucleus
basalis cholinergic neurons and to compare them with the effects of SP on the same channels. We show that NT as well as SP inhibit both LVA and HVA Ca$^{2+}$ currents through PTX-insensitive G proteins and in a voltage-independent fashion. However, there is an important difference between the NT and SP effects: SP does not affect the L-type Ca$^{2+}$ channels, whereas NT does. Preliminary accounts of these findings have been published in abstract form (Grigg et al. 1991; Margeta-Mitrovic et al. 1995).

METHODS

Cell culture

Nucleus basalis neurons were cultured with the use of the method described previously (Nakajima et al. 1985), with two major modifications: papain (12 U/ml) was used for enzymatic treatment instead of trypsin and the culture medium contained rat serum instead of Earle’s salt (GIBCO, Grand Island, NY). Components of capacitative and leak currents were subtracted with the use of the standard P/4 protocol. Data were acquired and subsequently analyzed with the use of PCLAMP programs (version 5.5.1, Axon Instruments).

Electrophysiology

Calcium currents were recorded with the use of the whole cell version of the tight-seal patch-clamp method. Internal solution contained (in mM) 104.8 CsCl, 10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid-CSOH, 10 N-2-hydroxyethylpiperazine-N′-N′-2-ethanesulfonic acid (HEPES)-CSOH, 3 MgCl$_2$, 2 Na$_2$ATP, 0.1 Na$_3$GTP, 20 phosphocreatine (disodium salt), and 0.1 leupeptin, pH adjusted to 7.2 with CsOH. In guanosine 5′-[γ-thio]triphosphate (GTPγS) experiments GTP was replaced with 0.1 mM GTPγS. Standard external solution was composed of 130.8 mM NaCl, 20 mM tetrathyamine chloride, 2.5 mM CaCl$_2$, 1 mM MgCl$_2$, 11 mM glucose, 5 mM HEPES-NaOH, and 0.5 μM tetrodotoxin, pH 7.4. In experiments in which we investigated the nature of the slow tail current, we used low-CI$^-$ external and internal solutions or low-Na$^+$ external solution in combination with the standard internal solution. For experiments performed under low-Cl$^-$ conditions, CsCl in the standard internal solution was replaced with cesium methanesulfonate, whereas NaCl in the standard external solution was replaced with sodium methanesulfonate.

Low-Na$^+$ external solution contained 2 mM NaCl and 128.8 mM N-methyl-D-glucamine chloride, with other ingredients as in the standard external solution. In the experiments in which various Ca$^{2+}$ channel antagonists were used, cytochrome c (Sigma) was added to the external solution at a specified concentration binding to plastic (1 mg/ml when antagonists were applied by bath exchange and 0.1 mg/ml when sewer pipe perfusion system was used; see below). A stainless steel ring inserted in the culture dish, together with the well, formed the recording chamber with a volume of 0.45 ml. The external solution was perfused continuously at a rate of ~1 ml/min. All experiments were performed at room temperature (21.2 ± 1.4°C, mean ± SD, n = 262). In most experiments more than one culture batch was used.

Patch electrodes were pulled from thoroughly washed glass tubing, coated with Sylgard (Dow Corning), and fire polished before use. Electrodes had a resistance of ~2 MΩ. In the beginning of experiments the average series resistance was 6.6 ± 2.8 (SD) MΩ (n = 262) electronically compensated to 2.0 ± 1.1 (SD) MΩ (n = 262). The series resistance of the electrodes tended to increase during the experiments. Thus the series resistance was measured every 2–3 min during experiments and in the subsequent data analysis the effects of the series resistance on the currents were mathematically corrected (Koyano et al. 1994). Membrane potentials were corrected for the liquid junction potential between the external and patch pipette solution (8 mV with standard external and internal solutions or low-Na$^+$ external solution in combination with the standard internal solution, and 9 mV with low-Cl$^-$ external and internal solutions; external solution positive).

Currents were measured with the use of a List EPC-7 amplifier. The data were digitized with a sampling frequency of 4–10 kHz. Linear components of capacitative and leak currents were subtracted with the use of the standard P/4 protocol. Data were acquired and subsequently analyzed with the use of PCLAMP programs (version 5.5.1, Axon Instruments).

Drugs

In most of the experiments, Ca$^{2+}$ channel antagonists were applied by exchanging the bathing solution; the exchange (~4 bath volumes) was accomplished in 2–3 min. SP and NT were pressure ejected from thoroughly washed soft glass capillaries (R6, Garner Glass, tip diameter 3–5 μm) with a pressure of 6.1 ± 0.6 (SD) kPa (n = 234). The capillaries were positioned ~80 μm from the cell. In some experiments (for example, when using apamin or ω-agatoxin (ω-Aga)-IVA in high concentration), all drugs were applied through computer-controlled “sewer pipe” superfusion system whose final outlet had a diameter of 100 μm and was positioned ~180 μm from the cell.

All drugs were prepared as stock solutions and frozen. Polyethylene glycol 400 was used to dissolve nimodipine for the stock solution. The final concentration of polyethylene glycol was 0.3%. This concentration did not have any effects on the Ca$^{2+}$ currents. However, as a precaution, we added 0.3% polyethylene glycol together with cytochrome c to the control external solution in the experiments in which Ca$^{2+}$ channel antagonists were used. (The reservoir containing the nimodipine solution was protected from light throughout the experiments.) Sources of chemicals were as follows: apamin; Peninsula; GTPγS, Boehringer Mannheim; NT, Peninsula; nimodipine, RBI; ω-Aga-IVA, a gift from Pfizer; ω-conotoxin (ω-CgTx)-GVIA, Peninsula; ω-CgTx-MVIIC, Peninsula; PTX, List Biological Laboratories; somatostatin, Peninsula; SP, Peninsula.

RESULTS

NT and SP modulate whole cell Ca$^{2+}$ currents in nucleus basalis neurons

Neurons cultured from the nucleus basalis, bathed in 2.5 mM Ca$^{2+}$ and held at ~80 mV, were subjected to a series
of step depolarizations with 10-mV increments and 100 ms in duration. This protocol resulted in the set of currents shown in Fig. 1A1. The inward currents were completely abolished by application of 500 μM Cd²⁺, indicating that they resulted from the activation of Ca²⁺ channels (but see Sources of error for additional discussion). Current amplitudes were measured 95 ms after the start of depolarization and plotted against the membrane potential, generating the current-voltage plot shown in Fig. 1A2. The current-voltage curve showed a hump at approximately −40 mV and peaked at −10 mV, corresponding to previously described LVA and HVA current components. A ramp pulse from −100 to +60 mV (ramp speed 0.5 mV/ms) elicited a current trace with similar properties (Fig. 1A3). When Ca²⁺ currents were blocked by 500 μM Cd²⁺, a small outward current was unmasked at positive membrane potentials (see Sources of Error). The presence of this outward current resulted in a reversal potential error when leak current was subtracted with the use of positive membrane currents (same cell as in Fig. 1B, bottom traces). To estimate the true reversal potential of Ca²⁺ channel currents under our experimental conditions, the non-Ca²⁺ currents were subtracted with the use of records obtained in 500 μM Cd²⁺, resulting in the current-voltage plot shown in Fig. 1B. Indeed, this leak subtraction protocol shifted the Ca²⁺ current reversal potential to −10–15 mV to the right. In contrast, there was no shift in the peaks of LVA and HVA Ca²⁺ currents. Similar current-voltage relationships were recorded previously from acutely isolated cholinergic neurons from both rat and guinea pig (Allen et al. 1993; Griffith et al. 1994).

In subsequent experiments, unless otherwise specified, Ca²⁺ currents were elicited by a step depolarization to 0 or +10 mV from a holding potential of −80 mV (as in Fig. 2, A and B) and leak was subtracted with the use of the P/4 protocol. Application of NT (200 nM) or SP (200 nM) for 15 s caused a substantial inhibition of the total Ca²⁺ current (Fig. 2). As shown in Fig. 2, C and D, we measured the Ca²⁺ current only once every 12 s (more frequent stimulations were avoided because the frequent stimulation tended to accelerate the rundown of the Ca²⁺ currents) and thus we were unable to obtain a precise measurement of the drug effect onsets. Nevertheless, >20 s was usually necessary for the maximal inhibition to be reached even with a saturating concentration of the agonist. Recovery was much slower, on
the order of 3–7 min, with recovery from the SP-induced inhibition taking somewhat longer than that from the NT-induced inhibition. Because of the current rundown, the recovery was rarely complete. Thus NT and SP effects were relatively slow both in onset and in the subsequent recovery. Although in the majority of experiments NT and SP were applied by pressure ejection from glass capillaries, in a few cases we used the sewer pipe perfusion system, which allows for very rapid drug application and removal. The time courses of NT and SP effects were very similar under both conditions, indicating that the slowness of drug effects was inherent and not an artifact of the drug application system.

The average inhibition produced by NT was 67.9 ± 2.9% (mean ± SE, n = 16). The SP-induced inhibition (57.6 ± 2.2%, mean ± SE, n = 28) was smaller than the inhibition induced by NT (P < 0.01, Student’s t-test). Ninety-two percent of neurons tested were sensitive to NT and 94% were sensitive to SP. Most commonly, neurons not sensitive to NT were not sensitive to SP either. Desensitization of responses to both transmitters was observed (see for example Fig. 7), and the degree of desensitization varied greatly from neuron to neuron. However, responses to the first application of each drug were quite consistent between the culture batches and individual neurons. Because of the incomplete recovery and because of desensitization, we compared the populations of neurons, with only the first response to a particular drug analyzed statistically.

Inhibition induced by NT and SP was concentration dependent, as shown in the dose-response curves (Fig. 3). Data were fitted with the equation \([ (A_2 - A_1)x^p/(K^p + x^p)] + A_1\), where \(x\) is the neurotransmitter concentration, \(A_1\) and \(A_2\) are the minimal and maximal values of the neurotransmitter-induced inhibition, \(K^p\) is the half-effective dose (ED\(_{50}\)), and \(p\) is Hill’s coefficient. The ED\(_{50}\) was 6.1 nM for NT and 4.2 nM for SP. The NT dose-response curve was steeper than the dose-response curve of SP (Hill’s coefficients were 1.1 for NT and 0.5 for SP). This phenomenon was not studied in detail, however. For the subsequent experiments, a concentration of 200 nM was chosen for both transmitters; this is a saturating but not very high concentration that enabled a fast washing of the drug from the bath.

**LVA Ca\(^{2+}\) current is inhibited by NT and SP**

Nucleus basalis neurons express LVA channels (Allen et al. 1993; Griffith et al. 1994). Almost all neurons tested in this study showed a noticeable LVA current as assessed from the current-voltage relationship, which revealed a hump at approximately −40 mV (see Fig. 1). This current was more sensitive to Ni\(^{2+}\) and less sensitive to Cd\(^{2+}\) than the rest of the Ca\(^{2+}\) current (n = 2, data not shown), in agreement with previous observations (Allen et al. 1993). To determine whether the LVA component of the Ca\(^{2+}\) current was modulated by NT and SP, we activated it selectively by applying pulses to −40 mV from a holding potential of −90 mV with a duration of 100 ms. Figure 4, A and B, illustrates modulation of the LVA Ca\(^{2+}\) current by NT and SP in representative cells. Time courses of the NT and SP effects are shown in Fig. 4, C and D. They are similar to the time courses of the total Ca\(^{2+}\) current modulation by NT and SP (Fig. 2); however, the LVA current did not show a rundown. NT inhibited 26.2 ± 3.7% (mean ± SE, n = 12) and SP inhibited 24.1 ± 3.0% (mean ± SE, n = 10) of the LVA current. The NT- and SP-inhibited currents, obtained by digital subtraction, are shown in Fig. 4, A and B, insets. These currents inactivated to a large extent during 100-ms pulses, suggesting that we indeed recorded the inhibition of LVA Ca\(^{2+}\) current. However, to examine the possibility that we were observing the inhibition of small HVA current activated by this protocol, we performed a series of experiments in which LVA Ca\(^{2+}\) channels were inactivated by holding neurons at −60 mV. In basal forebrain cholinergic neurons, holding cells at −60 mV would inactivate the LVA channels completely, leaving HVA currents almost intact (Griffith et al. 1994). Thus 100-ms pulses to −40 mV from a holding potential of −60 mV reveal the HVA current fraction. The current inhibited by NT in the cells held at −90 mV was 388 ± 87 (SE) pA (n = 12). This was significantly larger (P < 0.02, Student’s t-test) than the current inhibited in cells held at −60 mV (128 ± 36 pA, mean ± SE, n = 6). Similarly, SP inhibited 332 ± 76 (SE) pA (n = 10) in cells held at −90 mV and 111 ± 23 (SE) pA (n = 6) in cells held at −60 mV (P = 0.02, Student’s t-test). Therefore both NT and SP modulate the LVA Ca\(^{2+}\) current.
rupture. Horizontal bars: drug applications lasting 15 s.
in each trace and plotted against time, NT and SP effects; same cells as in A.
the formation of a gigaohm seal, rupture of the cell membrane and stabilization of the whole cell recording configuration, the solution was switched to the control solution containing 1 mg/ml of cytochrome c and 0.3% of polyethylene glycol 400 (the latter was used as the nimodipine solvent).

This moment is represented as time 0 in Fig. 5A. After ≈3 min to perform the current-voltage protocol, we started the application of standard voltage pulses to 0 mV (in some cells to +10 mV) that continued throughout the duration of the experiment. Control solution was superfused for the next 8–10 min (phase 1 of the experiment) and was then switched to a solution containing one or more Ca²⁺ channel antagonists (for example, ω-CgTx-GVIA in Fig. 5A). This solution was superfused for the next 8–10 min (phase 2 of the experiment) and then NT or SP was applied for 15 s. Recording then continued for an additional 8–10 min (phase 3 of the experiment) until the neurotransmitter response partially recovered.

Data from these experiments are summarized in Fig. 5B. Five different Ca²⁺ channel antagonist combinations were tested: ω-Aga-IVA (100 nM), nimodipine (3 μM), ω-CgTx-GVIA (0.5 μM), nimodipine + ω-CgTx-GVIA, and nimodipine + ω-CgTx-MVIIC (5 μM). We selected these concentrations of Ca²⁺ channel antagonists on the basis of the pharmacological properties of Ca²⁺ channels present in other preparations (Mintz et al. 1992a; Randall and Tsien 1995; Regan et al. 1991), trying to avoid very high concentrations that may not be selective for particular channel types. For example, dihydropyridines are considered specific L-type channel antagonists, but at concentrations of ≈10 μM they partially block N-type (Jones and Jacobs 1990) and LVA Ca²⁺ channels (Akaïke et al. 1989). Current amplitudes were measured and averaged in the last minute of each experimental phase. The antagonist effect was calculated as a difference between current amplitude at the end of phases 1 and 2, and it was expressed as a fraction of the amplitude at the end of phase 1 (control amplitude). The average current rundown during the same period, measured in the control experiments, was 7.6 ± 1.2% (n = 15).

ω-Aga-IVA inhibited 0.3 ± 4.2% (n = 12), nimodipine 24.2 ± 2.5% (n = 15), ω-CgTx-GVIA 46.4 ± 2.0% (n = 13), ω-CgTx-GVIA and nimodipine 58.7 ± 2.2% (n = 15), and ω-CgTx-MVIIC with nimodipine 75.7 ± 1.9% (n = 12) of the control Ca²⁺ current. Analysis of variance (ANOVA) showed that all of these treatments with the exception of 5. Which HVA Ca²⁺ channels are expressed in nucleus basalis neurons?

Using various Ca²⁺ channel antagonists, we examined which HVA Ca²⁺ channels are present in cultured nucleus basalis neurons. The same experimental paradigm, shown in Fig. 5A, was used to assess which HVA Ca²⁺ channels are modulated by NT and SP, as described below. Cells were initially superfused with the regular external solution. After the formation of a gigaohm seal, rupture of the cell membrane and stabilization of the whole cell recording configuration, the solution was switched to the control solution containing 1 mg/ml of cytochrome c and 0.3% of polyethylene glycol 400 (the latter was used as the nimodipine solvent). This moment is represented as time 0 in Fig. 5A. After ≈3 min to perform the current-voltage protocol, we started the application of standard voltage pulses to −40 mV from holding potential of −90 mV; pulses were applied every 12 s. A and B: bottom traces were recorded just before drug application, top traces when maximal inhibition was achieved (30–40 s later). Insets: currents inhibited by NT (A) or SP (B), obtained by digital subtraction. C and D: time courses of NT and SP effects; same cells as in A and B. Peak current was determined in each trace and plotted against time, time 0 being moment of membrane rupture. Horizontal bars: drug applications lasting 15 s.

Fig. 4. Inhibition of LVA current by 200 nM NT and SP. LVA currents were elicited by 100-ms depolarizing pulses to −40 mV from holding potential of −90 mV; pulses were applied every 12 s. A and B: bottom traces were recorded just before drug application, top traces when maximal inhibition was achieved (30–40 s later). Insets: currents inhibited by NT (A) or SP (B), obtained by digital subtraction. C and D: time courses of NT and SP effects; same cells as in A and B. Peak current was determined in each trace and plotted against time, time 0 being moment of membrane rupture. Horizontal bars: drug applications lasting 15 s.

Fig. 5. Attenuation of NT and SP effects by Ca²⁺ channel antagonists. A: whole cell Ca²⁺ currents were elicited by standard protocol (holding potential −80 mV, steps to 0 or +10 mV, duration 100 ms). Pulses were applied every 30 s throughout, except every 20 s for 2 min at end of each phase of experiment and every 12 s during transmitter application. Current amplitudes were measured at end of each depolarizing pulse (100 ms). Time 0: start of perfusion with solution containing 1 mg/ml cytochrome c and 0.3% polyethylene glycol, which took place ≈3 min after break of patch. Horizontal bars: time of drug applications. ω-Conotoxin (ω-CgTx)-GVIA (0.5 μM) inhibited fraction of current; subsequent application of SP produced inhibition of Ca²⁺ current. B: effects of Ca²⁺ channel antagonists on the whole cell Ca²⁺ current. For each experiment, effect of Ca²⁺ channel antagonist was measured during 1 min preceding transmitter application (when channel block reached steady state) and was expressed as percentage of control current amplitude, which was determined during 1 min before antagonist application. Control solution contained 0.3% polyethylene glycol (PEG) and 1 mg/ml cytochrome c (cyc. c). C and D: attenuation of NT and SP by Ca²⁺ channel antagonists. For each experiment, current inhibited by NT (C) or SP (D) was expressed as percentage of control current amplitude (measured during 1 min preceding Ca²⁺ channel antagonist application). For B–D, data are represented as means ± SE, and sample sizes are indicated below bars. ω-agtx, ω-Aga toxin; nim, nimodipine.
100 nM \( \omega \)-Aga-IVA treatment were significantly different from the control and from each other \((P < 0.05)\).

The data suggest that P-type channel is not present in this neuronal type, because 100 nM \( \omega \)-Aga-IVA (the concentration saturating for P-type channels) (Mintz et al. 1992b; Randall and Tsien 1995) did not have any effect. The L-type current fraction in nucleus basalis neurons is \( \approx 17\% \) (nimodipine effect minus rundown), whereas the N-type current fraction is \( \approx 39\% \) \((\omega \)-CgTx-GVIA effect minus rundown). Thus L- and N-type current together should make up \( \approx 56\% \) of the total Ca\(^{2+}\) current in these neurons (measured at 100 ms). In fact, the combination of \( \omega \)-CgTx-GVIA and nimodipine inhibited \( \approx 51\% \) of the current (58.7% minus rundown), indicating that the inhibition induced by two antagonists is largely additive, each antagonist inhibiting a separate current fraction. It has been reported that \( \omega \)-CgTx-MVIIC at 5 \( \mu \)M inhibits N-, P-, and Q-type Ca\(^{2+}\) channels (Hillyard et al. 1992; Randall and Tsien 1995), whereas \( \omega \)-CgTx-GVIA (at 0.5 \( \mu \)M) completely blocks N-type channels (Allen et al. 1993). With P-type channels absent, the difference between the block induced by \( \omega \)-CgTx-GVIA in the presence of nimodipine and the block induced by 5 \( \mu \)M \( \omega \)-CgTx-MVIIC in the presence of nimodipine (75.7–58.7 = 17.0\%) could represent the Q-type current fraction, assuming the same pharmacological properties that were observed in other preparations (Hillyard et al. 1992; Mackie et al. 1995; Randall and Tsien 1995) apply to our cultured nucleus basalis neurons. To confirm the presence of Q-type channels in nucleus basalis neurons, six neurons were exposed to 1 \( \mu \)M \( \omega \)-Aga-IVA, a concentration that should completely inhibit the Q-type channels (Randall and Tsien 1995). The treatment with 1 \( \mu \)M \( \omega \)-Aga-IVA resulted in inhibition of 36.6 ± 6.3\% of Ca\(^{2+}\) current after 8–9 min of drug application. If the same rate of rundown as in the rest of the experiments \((\approx 1\% /\text{min})\) is assumed, this result would indicate that \( \approx 29\% \) of the current was flowing through the Q-type channels, which is comparable with the Q-type current fraction calculated from the experiments in which \( \omega \)-CgTx-MVIIC was used (17\%). To conserve the toxin, \( \omega \)-Aga-IVA at this high concentration was applied through the sewer pipe perfusion system instead of the customary bath exchange (to prevent clogging, the concentration of cytotoxic was decreased to 0.1 ng/ml). Interestingly, even with this very fast drug application system, and despite the high concentration of the toxin used, the steady-state block was achieved very slowly \((3–4 \text{ min}, \text{data not shown})\). In contrast, when \( \omega \)-CgTx-GVIA (0.5 \( \mu \)M) or nimodipine (3 \( \mu \)M) were applied in this manner, the maximal block was usually achieved in \( <30 \text{ s} \) (not shown). This slowness of the \( \omega \)-Aga-IVA-produced block further indicates the absence of P-type Ca\(^{2+}\) channels, which have high affinity for this toxin and are thus blocked rapidly (Mintz and Bean 1993). In summary, we can conclude that L-, N-, and Q-type HVA Ca\(^{2+}\) channels are present and that P-type channels are absent in nucleus basalis neurons.

Even in the presence of saturating concentrations of nimodipine and \( \omega \)-CgTx-MVIIC, 24.3\% of the steady-state current \((\text{measured at 100 ms})\) remained unblocked. When recorded under those conditions, current-voltage relationships generally showed a single peak at approximately \(-40 \text{ mV} \) (not shown). Thus it is likely that a fraction of the residual Ca\(^{2+}\) current was carried through the LVA Ca\(^{2+}\) channels, because the LVA current did not inactivate completely at 100 ms (see Figs. 1B and 4A and B). In addition, HVA Ca\(^{2+}\) current resistant to the Ca\(^{2+}\) channel antagonists \((R\) type\) \((\text{Randall and Tsien 1995})\) might be present. However, the experiments we performed do not allow us to assess quantitatively the contributions of LVA and HVA currents to this residual current.

Which HVA Ca\(^{2+}\) channels are modulated by NT and SP?

This section describes the modulation of HVA Ca\(^{2+}\) currents by NT and SP; an example of the experimental protocol is shown in Fig. 5A. Because responses to the transmitters were partially irreversible (probably because of the rundown) and the second application showed desensitization, we sampled data from the first transmitter application only.

The effects of the Ca\(^{2+}\) channel antagonists on the NT-induced inhibition are summarized in Fig. 5C. Current inhibited by NT \((\text{measured at the moment of maximal inhibition})\) is expressed as a fraction of the control current, i.e., the current measured at the end of phase 1 \((\text{see previous section for the protocol description})\). Under control conditions NT inhibited 72.7 ± 2.3\% of the current \((n = 8)\). A similar NT-induced inhibition was observed in \( \omega \)-Aga-IVA-treated cells \((70.3 ± 5.7\%, n = 6)\). Nimodipine attenuated the NT-induced inhibition to 41.5 ± 4.2\% \((n = 8)\). \( \omega \)-CgTx-GVIA to 34.8 ± 3.5\% \((n = 7)\), \( \omega \)-CgTx-GVIA + nimodipine to 27.1 ± 2.7\% \((n = 8)\), and \( \omega \)-CgTx-MVIIC + nimodipine to 15.0 ± 1.6\% \((n = 6)\). ANOVA indicated that all treatments were significantly different from the control treatment \((P < 0.05)\). The treatment with \( \omega \)-CgTx-MVIIC together with nimodipine produced a significantly larger attenuation of NT response from the combination of \( \omega \)-CgTx-GVIA + nimodipine \((P < 0.05)\). These results indicate that NT modulates L-, N-, and Q-type HVA Ca\(^{2+}\) channels in the nucleus basalis cholinergic neurons.

Figure 5D shows the data for SP. SP inhibited 55.8 ± 1.8\% of the current in the control cells \((n = 7)\). Treatment with \( \omega \)-Aga-IVA did not alter the size of the SP response \((56.4 ± 6.2\%; n = 6)\). In contrast to the effect of NT, the presence of nimodipine did not affect the SP response either \((58.4 ± 3.5\%, n = 7)\). In cells treated with \( \omega \)-CgTx-GVIA, the SP response was diminished to 32.3 ± 2.2\% \((n = 6)\). A similar reduction of the SP response was seen in cells treated with the combination of \( \omega \)-CgTx-GVIA + nimodipine \((28.7 ± 1.4\%, n = 7)\). Further attenuation of the SP response \((16.2 ± 0.6\%, n = 6)\) was seen after treatment with the mixture of \( \omega \)-CgTx-MVIIC + nimodipine. ANOVA showed that SP responses were different \((P < 0.05)\) from the control SP response after all treatments except the \( \omega \)-Aga-IVA treatment and the nimodipine treatment. These data indicate that the L-type channels (as well as P-type channels, which are not present) were not modulated by SP. The SP response in cells treated with \( \omega \)-CgTx-GVIA + nimodipine was not different from the response in the cells treated with \( \omega \)-CgTx-GVIA alone, further strengthening the idea that the L-type channels are not affected by SP. The combination of \( \omega \)-CgTx-GVIA + nimodipine diminished the SP response significantly less than the combination of \( \omega \)-CgTx-MVIIC + nimodipine \((P < 0.05)\). Taken together, these data suggest that SP modulates the N- and Q-type but not the L-type channels.
NT and SP inhibition of whole cell Ca\textsuperscript{2+} currents is voltage independent

Many neurotransmitters modulate Ca\textsuperscript{2+} currents in a voltage-dependent manner; modulation (mostly inhibition) is smaller at more depolarized potentials and can be relieved by application of a large depolarization. Thus the double-pulse protocol was used to test the voltage dependency of the NT and SP inhibitions in nucleus basalis neurons. As shown in Fig. 6 (voltage protocol, inset), from a holding potential of −80 mV the voltage was stepped to 0 mV for 35 ms (control), followed immediately by 35 ms of a large depolarization (+70 mV, conditioning pulse). The membrane potential was then returned to −80 mV for 15 ms and stepped again to 0 mV for 35 ms (test pulse). Inhibition was measured isochronally with the time of the peak current in the control trace. For each cell the first depolarization to 0 mV (before the conditioning pulse) served as a control for the test pulse (after the conditioning pulse). Results are shown in Fig. 6, A and B. There was no significant difference in prepulse and postpulse inhibition for either NT or SP.

As a positive control, we tested the somatostatin inhibition of Ca\textsuperscript{2+} currents in cultured noradrenergic locus coeruleus neurons with the use of the same protocol (Fig. 6, A and B, SOM). Somatostatin inhibition was relieved to a large extent after the conditioning pulse. It is interesting to note that in the locus coeruleus neurons before the somatostatin application, the postpulse Ca\textsuperscript{2+} current was slightly larger than the prepulse current; this facilitation could reflect the relief of tonic G protein inhibition present in these cells (Scott and Dolphin 1990). In contrast, in NB neurons the postpulse current was somewhat smaller than the prepulse current; this may be due to a lack of tonic modulation of the Ca\textsuperscript{2+} currents by G proteins and an incomplete recovery of the LVA channels from the inactivation induced by the conditioning pulse.

NT and SP inhibition of whole cell Ca\textsuperscript{2+} currents is mediated through PTX-insensitive G proteins

NT and SP receptors belong to the family of seven-transmembrane-domain G protein-coupled receptors. To determine whether the modulation of Ca\textsuperscript{2+} channels is G protein mediated, we loaded cells with the nonhydrolyzable GTP analogue GTP\textsubscript{Y}S (0.1 mM) instead of the 0.1 mM GTP used for the normal patch pipette solution. The results are shown in Fig. 7. Cells were dialyzed for 10 min before transmitter application. GTP\textsubscript{Y}S by itself did not have any effect on the baseline current, indicating that the spontaneous activity of the involved G proteins is very low in these cells. However, the application of either NT or SP (Fig. 7, C and D) irreversibly inhibited the current, with a second application of each drug producing no effect. In contrast, in the control (GTP-loaded) cells, the NT- or SP-induced inhibition was reversible and the second application of each drug produced a significant response (Fig. 7, A and B). The second response was smaller than the first because of the desensitization. The results are summarized in Fig. 7E. In control (GTP-loaded) cells, the NT-induced inhibition recovered substantially after 10 min, whereas in the GTP\textsubscript{Y}S-loaded neurons NT effect hardly recovered (P < 0.001, Student’s t-test). Similar data were obtained with SP (P < 0.001, Student’s t-test). Although we already showed that NT and SP modulate different types of Ca\textsuperscript{2+} channels, it seems that all of these effects are G protein mediated, because none of the components of the induced inhibition recovered.

The most common pathway of Ca\textsuperscript{2+} channel inhibition involves the PTX-sensitive G proteins G\textsubscript{o} or G\textsubscript{i} (Dolphin 1990). To determine whether these G proteins mediate the NT- and SP-induced inhibition of Ca\textsuperscript{2+} currents in nucleus basalis neurons, we treated the cultures overnight with 500 ng/ml of PTX or heat-inactivated PTX. As a positive control, somatostatin modulation of Ca\textsuperscript{2+} currents in locus coeruleus neurons was used again. Results are summarized in Fig. 8. Although the somatostatin effect was completely eliminated after the PTX treatment (P < 0.002, Student’s t-test), neither the NT nor the SP inhibition was affected by the same treatment. NT and SP modulation of Ca\textsuperscript{2+} currents is therefore mediated by a PTX-insensitive G protein in nucleus basalis cholinergic neurons.

Sources of error

After the end of each depolarization, a slow inward current (tail current) remained; this current returned to the baseline very slowly, in 1–2 s (Fig. 9A; see also Figs. 1A1, 2, A and B, and 6A). This rate of decay is much slower than the one expected for voltage-gated Ca\textsuperscript{2+} channels (deactivation time constants reported for HVA and LVA channels are, respectively, between 0.1 and 0.4 ms, and between 3 and 3.4 ms) (Armstrong and Matteson 1985; Avery and Johnston 1996; Carbone and Lux 1987). To find the origin of the tail current, we tested the effect of apamin, an inhibitor of some subtypes of small-conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels...
completely abolished second response to each drug. In control experiments To investigate the ionic character of the SK channel cur-
small inhibition of the Ca$_{2+}$ current as plateau current ) . Figure 9 shows the time course
slower (n) and this inhibition was partially reversible ( Fig. 9A ). A lower concentration of apamin (0.1 µM) was equally effective, but the onset of block was slower (n = 2, data not shown). Apamin also induced a small inhibition of the Ca$_{2+}$ inward current (we refer to this current as plateau current). Figure 9B shows the time course of the apamin-induced block. The block of the tail current (○) was complete and did not recover in 10 min after apamin was washed from the cell vicinity. In contrast, the plateau current was inhibited by apamin to a much smaller degree, and this inhibition was partially reversible (○). In addition, the apamin-induced inhibition of the plateau current was reproducible: if apamin was applied for the second time after several minutes of washing, it reversibly inhibited the plateau current (20.6 ± 0.6% at the end of the depolarizing pulse, n = 2, data not shown) without any further effect on the already irreversibly inhibited tail current. The current reversibly inhibited by apamin showed prominent inactiva-
tion during 100-ms voltage pulses and activated at negative membrane potentials (not shown), suggesting that the apamin-sensitive plateau current was likely to be the LVA Ca$_{2+}$ current.

The results are summarized in Fig. 9C. Apamin inhibited 92.4 ± 3.2% of the slow tail current, and 18.2 ± 4.4% (mean ± SE) of the plateau current (n = 5, P < 0.0001, Student’s t-test). We also measured the inhibition of the tail current by N-type channel antagonist ω-CgTx-GVIA to test the possibility that the inhibition of the Ca$_{2+}$-dependent tail current was just a consequence of the Ca$_{2+}$ channel inhibition and corresponding decrease in the Ca$_{2+}$ influx. In contrast with the apamin effect, the inhibitions of the tail and the plateau currents by ω-CgTx-GVIA were almost the same (Fig. 9C). Similar proportional decreases in the tail current were observed with other treatments that inhibited Ca$_{2+}$ currents (for example, with NT and SP; see Figs. 2, A and B, and 6A), but only apamin selectively inhibited the slow tail current, indicating that this current flowed through SK channels. To evaluate the involvement of the Ca$_{2+}$-dependent Cl$^-$ current, the same experiment was performed in the low-Cl$^-$ external and internal solutions. Under those experimental conditions apamin effects were not different from its effects in the regular solutions (it inhibited 79.4 ± 13.5% of the tail and 9.3 ± 6.6% of the plateau current, mean ± SE, n = 3), indicating the absence of the significant involvement of Ca$_{2+}$-dependent Cl$^-$ current.

The selective susceptibility of the slow tail current to inhibition by apamin excludes the possibility that this current resulted from the insufficient voltage clamp. After the slow tail current was blocked by apamin, the deactivation of Ca$_{2+}$ currents was well fitted to an exponential function with time constants $\tau_1 = 0.47 ± 0.05$ (SE) ms and $\tau_2 = 2.47 ± 0.50$ (SE) ms (n = 5). These time constants are comparable with the Ca$_{2+}$ channel decay constants reported in the literature (Armstrong and Matteson 1985; Avery and Johnston 1996; Carbone and Lux 1987).

To investigate the ionic character of the SK channel current under our experimental conditions (K$^+$ absent from...
NT AND SP MODULATION OF Ca\(^{2+}\) CHANNELS

In seven randomly chosen cells, tail current amplitude was 573 ± 127 (SE) pA, whereas plateau current amplitude at the end of the 0 mV voltage step was 2,209 ± 141 (SE) pA. The calculated SK channel current amplitude at 0 mV was 126 ± 28 pA or 5.5 ± 0.9% (mean ± SE) of the plateau current. Thus the error caused by SK channel current at 0 mV was relatively small (~6% overestimation of the Ca\(^{2+}\) current).

In all the experiments in this study (except for the current-voltage plot in Fig. 1B), leak subtraction was performed with the use of the P/4 protocol, which assumes linearity of the leak current. To test this assumption, we blocked all Ca\(^{2+}\) currents and Ca\(^{2+}\)-dependent currents by 500 µM Cd\(^{2+}\). Under these conditions, a small outwardly rectifying current was observed at potentials positive to −10 mV (not shown); most likely this current resulted from the Cs\(^{+}\) efflux through the delayed rectifier K\(^{+}\) channels. The difference between the net outward current and the extrapolated leak current was only 84 ± 24 (SE) pA at 0 mV and 155 ± 39 (SE) pA at +10 mV (n = 7), indicating that at these potentials the relative error caused by the outward current was small (~5% underestimation for typical 2-nA current).

**DISCUSSION**

*Types of voltage-gated Ca\(^{2+}\) channels expressed in nucleus basalis cholinergic neurons*

Similarly to many central and peripheral neurons (Regan et al. 1991), brain cholinergic neurons express more than one type of Ca\(^{2+}\) channel. Cultured nucleus basalis neurons described in this study express LVA Ca\(^{2+}\) channels as well as L-, N-, and Q-type, but not P-type, HVA Ca\(^{2+}\) channels; the R-type channels may also be present. LVA Ca\(^{2+}\) channels have been previously described in cholinergic neurons from the basal forebrain, both from the medial septum and the diagonal band (Griffith et al. 1994) and from the nucleus basalis (Allen et al. 1993). The LVA current we recorded had the same properties as the previously described LVA (T-type) Ca\(^{2+}\) current: it was more sensitive to block by Ni\(^{2+}\) than by Cd\(^{2+}\) and activated by relatively small depolarizations.

HVA current components present in this neuronal type were dissected only partially in previously published studies. The presence of both L- and N-type Ca\(^{2+}\) currents was demonstrated in acutely dissociated basal forebrain neurons of the guinea pig (Griffith et al. 1994; Pegna et al. 1994). Similarly, the N-type current was expressed in rat nucleus basalis neurons cultured overnight (Allen et al. 1993), representing ≈40% of the total cell Ca\(^{2+}\) current. In that study, however, the presence of the L-type current was controversial. Low micromolar concentrations of dihydropyridine Ca\(^{2+}\) channel antagonists (<10 µM), which are selective for the L-type current, did not induce a significant inhibition of the Ca\(^{2+}\) current. However, high concentrations of the same compounds (for example, 100 µM nifedipine) inhibited almost all of the current, including the ω-CgTx-GVIA-sensitive and the LVA component. These investigators were able to induce additional Ca\(^{2+}\) current with the use of 3 µM
Bay K 8644 (a dihydropyridine L-type channel agonist), and this current was sensitive to 1 μM nifedipine, probably representing the “true” L-type current, whose fraction was too small to detect in basal conditions. We found that L- and N-type HVA Ca^{2+} channels are indeed expressed in the cultured rat nucleus basalis neurons, representing ≈17% and ≈39% of the Ca^{2+} current, respectively.

The P-type channels, discovered originally in cerebellar Purkinje neurons (Llinás et al. 1992), are blocked by micromolar concentrations of funnel-web spider toxin (Llinás et al. 1992) and by low nanomolar concentrations of peptide ω-Aga-IVA (Mintz et al. 1992b; Randall and Tsien 1995). Ca^{2+} channels with these pharmacological properties were later found in many different central and peripheral neurons in varying proportions (Mintz et al. 1992a). In Purkinje neurons, the P-type current represents ≈90% of the whole cell Ca^{2+} current. At the other extreme, hippocampal CA3 neurons and sympathetic neurons do not express the P-type channels (Mintz et al. 1992a). We did not detect the presence of P-type Ca^{2+} channels in our preparation either: 100 nM ω-Aga-IVA did not have any effect on the Ca^{2+} currents. However, the combination of 5 μM ω-CgTx-MVIIC and 3 μM nimodipine blocked ≈17% more of the Ca^{2+} current than the combination of 0.5 μM ω-CgTx-GVIA and 3 μM nimodipine did. Thus a large fraction of the non-L, non-N type current in nucleus basalis neurons (∼41%) was blocked by 5 μM ω-CgTx-MVIIC. This peptide toxin blocks N-, P-, and Q-type Ca^{2+} channels (Hillery et al. 1992; Randall and Tsien 1995). Because P-type channels were absent, ≈17% of the total cell Ca^{2+} current in nucleus basalis neurons is likely flowing through the Q-type channels. In cerebellar granule neurons, Q-type current was also blocked by ω-Aga-IVA with a half-inhibiting concentration of ≈90 nM (Randall and Tsien 1995). Thus 100 nM ω-Aga-IVA, used in our experiments, would be expected to inhibit approximately half of the Q-type current, or ≈8% of the total cell Ca^{2+} current, but we did not observe any inhibition. It is likely that the onset of inhibition under the experimental conditions described (low concentration of toxin and the slow bath exchange) was too slow to be distinguished from the rundown. However, when ω-Aga-IVA was applied at higher concentration (1 μM) with the rapid perfusion system, we were able to inhibit ≈29% of the total cell Ca^{2+} current, confirming the presence of the Q-type Ca^{2+} channels in nucleus basalis cholinergic neurons. The HVA current profile reported here differs somewhat from the one published by Pegna et al. (1994); they observed that the non-L, non-N HVA component in the guinea pig nucleus basalis neurons was completely blocked by 100 nM ω-Aga-IVA and therefore represents the P-type Ca^{2+} channel. Because P- and Q-type channels are probably both derived from the class A α₁ subunit gene (Stea et al. 1994), the observed difference might represent a variation between species.

**NT and SP modulation of voltage-gated Ca^{2+} channels in nucleus basalis neurons**

SP inhibits N-type Ca^{2+} channels in rat sympathetic neurons through a slow membrane-delimited pathway that is voltage independent and mediated through a PTX-insensitive G protein (Shapiro and Hille 1993). In this study we have demonstrated that SP inhibits N- and Q-type HVA channels as well as LVA Ca^{2+} channels. This modulation is slow, voltage independent, and mediated through PTX-insensitive G proteins, similar to the N-type channel inhibition in sympathetic neurons (Shapiro and Hille 1993); we have yet to test for the involvement of a diffusible second messenger. Modulation of Q-type channels has not been studied much. Cannabinoids, acting through the CB-1 receptor expressed in AtT20 cells, inhibited the Q-type Ca^{2+} current through a voltage-dependent, PTX-sensitive pathway (Mackie et al. 1995). Also, muscarinic M₂ agonists inhibited both the ω-CgTx-GVIA-sensitive and the ω-CgTx-GVIA-resistant current in nucleus basalis neurons through the PTX-sensitive and voltage-dependent pathway (Allen and Brown 1993). In the light of our results, the inhibition of the ω-CgTx-GVIA-resistant fraction might have represented the modulation of the Q-type Ca^{2+} channels, because they make up the majority of the ω-CgTx-GVIA-resistant current in these neurons. However, the PTX-sensitive, voltage-dependent mode of signal transduction reported by Allen and Brown (1993) and Mackie et al. (1995) is clearly different from the PTX-insensitive, voltage-independent modulatory pathway employed by SP in nucleus basalis neurons.

Similarly to SP, NT inhibited LVA, N-type, and Q-type Ca^{2+} channels in nucleus basalis neurons. The modulation of whole cell Ca^{2+} currents was slow, voltage independent, and mediated through a PTX-insensitive G protein, just as in the case of SP. Possibly, the inhibition of LVA and N- and Q-type Ca^{2+} channels by NT involves the same modulatory pathways as the SP-induced inhibition. In contrast to SP, however, NT inhibited the L-type Ca^{2+} channels as well, indicating that there is a difference in the signal transduction pathways of these two peptides. NT might activate a separate pathway in addition to the ones activated by SP, or all of the modulatory pathways may be different, despite the observed similarity of the responses. To the best of our knowledge, modulation of Ca^{2+} channels by NT has not yet been described. It is very interesting that NT can inhibit so many different channel types, suggesting the possibility of profound modification of cell function.

The ability of NT, as opposed to SP, to inhibit L-type Ca^{2+} channels may be of particular importance. Several groups reported the inhibition of L-type channels by a PTX-insensitive G protein. In sympathetic (Mathie et al. 1992) as well as striatal neurons (Howe and Surmeier 1995), L-type channels are inhibited by muscarinic agonists through a Ca^{2+}-dependent, second-messenger-mediated pathway. This pathway is slow, voltage independent, and PTX insensitive. Also, activation of the metabotropic glutamate receptor(s) inhibits L-type Ca^{2+} channels in isolated neocortical neurons through a Ca^{2+}-dependent, diffusible messenger pathway (Sayer et al. 1992). It remains to be seen whether a similar mechanism is employed in the NT-induced inhibition of L-type channels in nucleus basalis neurons. NT was shown to activate adenylly cyclase, increasing the intracellular adenosine 3',5'-cyclic monophosphate (cAMP) concentration (Yamada et al. 1994), as well as to increase the inositol phosphatase metabolism, thereby releasing inositol triphosphate (IP₃) and activating protein kinase C (Goedert et al. 1984). In the heart muscle, the cAMP-mediated phosphorylation activates L-type Ca^{2+} channels (Trautwein and Heschler 1990). Thus the observed inhibition of L-type channels by NT in nucleus basalis neurons, which occurred under the
condition of increased intracellular cAMP, is somewhat surprising. However, L-type channels are molecularly diverse (Snutch et al. 1990), and different channel classes may be subject to fundamentally different ways of modulation.

Modulation of Ca$^{2+}$ channels in nucleus basalis neurons by both NT and SP involves PTX-insensitive G proteins. PTX-insensitive G proteins include $G_i$, $G_q$, $G_{11}$, $G_{12}$, $G_{13}$, and $G_o$ (Hepler and Gilman 1992). In neuroblastoma-glioma hybrid cell line, bradykinin inhibition of Ca$^{2+}$ current is mediated by $G_{13}$ (Wilk-Blaszczyk et al. 1994b), whereas bradykinin activation of the Ca$^{2+}$-dependent K$^+$ current involves the $G_{q/11}$ G protein (Wilk-Blaszczyk et al. 1994a). $G_{q/11}$ mediates muscarinic inhibition of M current in rat sympathetic neurons (Caulfield et al. 1994) as well as the SP-induced inhibition of inwardly rectifying K$^+$ current in nucleus basalis neurons (Takano et al. 1996). It remains to be seen which PTX-insensitive G proteins are involved in the NT and SP inhibition of Ca$^{2+}$ currents in this preparation, and whether they differ from the G proteins that mediate the modulation of K$^+$ currents.

**Functional significance of NT and SP effects**

Although inhibition of Ca$^{2+}$ channels by slow inhibitory neurotransmitters, such as somatostatin, will potentiate the inhibitory effect on the cell, the role of the inhibition induced by excitatory transmitters, such as NT, SP, or angiotensin, is not clear and appears paradoxical. It was proposed that the Ca$^{2+}$ channel inhibition by excitatory transmitters will decrease the activation of a Ca$^{2+}$-dependent K$^+$ conductance and thus increase the cell excitability and prevent adaptation (Bley and Tsien 1990). We have indeed observed that NT and SP inhibit the slow tail current, which flows through the SK channels. However, we have not investigated what effects the NT- and SP-induced inhibitions of SK channels would have on the cellular excitability under the physiological conditions. On the other hand, inhibition of Ca$^{2+}$ channels might prevent a large increase in the intracellular Ca$^{2+}$ concentration during prolonged depolarizations induced by excitatory transmitters and thus promote neuronal survival (Nakajima and Nakajima 1994). In agreement with this idea, Ca$^{2+}$ channels have been implicated in Ca$^{2+}$-mediated cell toxicity (Weiss et al. 1990).

In summary, we have shown that nucleus basalis cholinergic neurons express LVA Ca$^{2+}$ channels and L-, N-, and Q-type HVA Ca$^{2+}$ channels. LVA as well as N- and Q-type channels are inhibited by both NT and SP, whereas L-type channels are inhibited by NT only. Modulation of Ca$^{2+}$ currents by both neurotransmitters was mediated by PTX-insensitive G proteins and in a voltage-independent fashion. These findings will help in understanding the actions of these neuropeptides and their roles in the physiology of the nucleus basalis neurons. Also, they establish the nucleus basalis neurons as a good preparation for future investigations into the signal transduction mechanisms of slow excitatory neurotransmitters.

We thank Drs. Igor Mitrovic, John P. Leonard, and Byron A. Heidenreich for critical reading of the manuscript and helpful suggestions and L. Johnstoon for technical and editorial help. Also, we are indebted to Pfizer for the gift of $\omega$-Aga-IVA.

This work was supported by National Institute on Aging Grant AG-06093 to Y. Nakajima and by American Federation for Aging Research Scholarship to M. Margeta-Mitrovic.

Address for reprint requests: M. Margeta-Mitrovic, Dept. of Pharmacology, M/C 868, University of Illinois at Chicago, 835 S. Wolcott Ave., Chicago, IL 60612.

Received 6 June 1996; accepted in final form 13 May 1997.

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