Biophysical and Pharmacological Characterization of Voltage-Dependent Ca\textsuperscript{2+} Channels in Neurons Isolated From Rat Nucleus Accumbens

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Churchill, Dennis and Brian A. MacVicar. Biophysical and pharmacological characterization of voltage-dependent Ca\textsuperscript{2+} channels in neurons isolated from rat nucleus accumbens. J. Neurophysiol. 79: 635–647, 1998. The nucleus accumbens (NA) has an integrative role in behavior and may mediate addictive and psychopharmacological drug action. Whole cell recording techniques were used to characterize electrophysiologically and pharmacologically high- and low-threshold voltage-dependent Ca\textsuperscript{2+} currents in isolated NA neurons. High-threshold Ca\textsuperscript{2+} currents, which were found in all neurons studied and include both sustained and inactivating components, activated at potentials greater than −50 mV and reached maximal activation at ∼0 mV. In contrast, low-threshold Ca\textsuperscript{2+} currents activated at voltages greater than −64 mV with maximal activation occurring at −30 mV. These were observed in 42% of acutely isolated neurons. Further pharmacological characterization of high-threshold Ca\textsuperscript{2+} currents was attempted using nimodipine (Nim), ω-conotoxin-GVIA (ω-CgTx) and ω-agatoxin-IVA (ω-Aga), which are thought to identify the L, N, and P/Q subtypes of Ca\textsuperscript{2+} currents, respectively. Nim (5–10 μM) blocked 18%, ω-CgTx (1–2 μM) blocked 25%, and ω-Aga (200 nM) blocked 17% of total Ca\textsuperscript{2+} current. Nim primarily blocked a sustained high-threshold Ca\textsuperscript{2+} current in a partially reversible manner. In contrast, ω-CgTx irreversibly blocked both sustained and inactivating components. ω-Aga irreversibly blocked only a sustained component. In all three of these Ca\textsuperscript{2+} channel blockers, plus 5 μM ω-conotoxin-MVIIC to eliminate a small unblocked Q-type Ca\textsuperscript{2+} current (7%), a toxin-resistant high-threshold Ca\textsuperscript{2+} current remained that was 32% of total Ca\textsuperscript{2+} current. This current inactivated much more rapidly than the other high-threshold Ca\textsuperscript{2+} currents, was depressed in 50 μM Ni\textsuperscript{2+} and reached maximal activation 5–10 mV negative to the toxin-sensitive high-threshold Ca\textsuperscript{2+} currents. Thus NA neurons have multiple types of high-threshold Ca\textsuperscript{2+} currents with a large component being the toxin-resistant "R" component.

INTRODUCTION

The entry of Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels performs many neuronal functions including the regulation of synaptic transmission, membrane excitability, cell growth/apoptosis, enzyme activity, and gene expression (Augustine et al. 1987; Choi 1995; Ghosh et al. 1994; McCleskey 1994). Neurons express a variety of Ca\textsuperscript{2+} currents each having distinct physiological properties (Eliot and Johnston 1994; Foehring and Scroggs 1994; Forti et al. 1994; Fox et al. 1987; Miller 1987; Nowycky et al. 1985; Tsien et al. 1988), distribution among and within neuronal types and display a wide range of responses to neuromodulators (Berridge and DuPont 1994; Snutch and Reiner 1992; Tsien et al. 1988, 1991).

Ca\textsuperscript{2+} currents have been classified into low-threshold (Akaie 1991; Huguenard 1996), and high-threshold sustained and inactivating currents (Bean 1989; Carbone and Swandulla 1989; Kostyk 1989; Tsien et al. 1988). More recently, high-threshold Ca\textsuperscript{2+} currents have been grouped into L, N, and P/Q types using biophysical and pharmacological tools; the latter includes peptide toxins found in invertebrate venom in addition to dihydropyridines (DHPs) that have been well-characterized (Tsien et al. 1991). It has been demonstrated in many neurons that DHPs primarily block L-type, ω-conotoxin-GVIA blocks N-type, ω-agatoxin-IVA blocks P/Q-type, and ω-conotoxin-MVIIC blocks N/P/Q-type Ca\textsuperscript{2+} currents (Hillyard et al. 1992; Mintz et al. 1992b; Sher and Clementi 1991; Tsien et al. 1991). An additional class of neuronal high-threshold Ca\textsuperscript{2+} current has been named the R-type current by some investigators due to its resistance to block by the available toxins (Brown et al. 1994; Eliot and Johnston 1994; Magee and Johnston 1995; Randall and Tsien 1995).

The nucleus accumbens (NA), as an interface between the limbic and extrapyramidal system, has an integrative role in behavior. It functions in selective attention, secondary reinforcement, and reward (Carlsson and Carlsson 1990a, 1990b; Meltzer 1991). Both the NA and the related striatum (involved in motor integration) are composed of GABAergic medium-spiny neurons and receive cortical excitatory input and modulatory dopaminergic input from the ventral midbrain (Gerfen 1988) as well as input of a wide variety of endogenous neuromodulators (Angulo and McEwen 1994).

Neurotransmitter modulation of Ca\textsuperscript{2+} channel subtypes has been observed in many CNS neurons including the striatum (Surmeier et al. 1995; Tsien et al. 1991; Yan and Surmeier 1996). Therefore, it is possible that addictive drugs and neuroleptics that act on several neurotransmitter systems in the NA may at least partially influence the properties of NA neurons by affecting Ca\textsuperscript{2+} currents. We used whole cell voltage-clamp techniques to characterize some basic biophysical and pharmacological properties of the Ca\textsuperscript{2+} currents in cultured and acutely dissociated rat
NA neurons. Similar experimental approaches to those used here have been applied on Ca$^{2+}$ currents in the striatum and several other neuronal types (Bargas et al. 1994; Hoehn et al. 1993; Randall and Tsien 1995; Tsien et al. 1991). We found that NA neurons express T-, L-, N-, P/Q-type Ca$^{2+}$ currents as well as a rapidly inactivating toxin-resistant high-threshold Ca$^{2+}$ current. The present study is a necessary step for further investigations into the roles of Ca$^{2+}$ currents in these neurons.

**METHODS**

**Cell isolation and culture**

NA neurons used in this study were isolated using a technique modified from Kay and Wong (1986) and similar to that used on rat striatal neurons (Hoehn et al. 1993). Five- to 11- or 24- to 32-day-old ether-anesthetized Charles River rat pups (P5–11 or P24–32) of either sex were decapitated, and their brains removed and immersed in ice-cold artificial cerebral spinal fluid (ACSF) that contained (in mM) 124 NaCl, 5 KCl, 1.3 MgCl$_2$, 26.2 NaHCO$_3$, 10 glucose, and 2 CaCl$_2$ [pH 7.35–7.40 and equilibrated with carbogen (95% O$_2$, 5% CO$_2$)]. The cerebrum was bisected in the coronal plane with a razor blade, and 400–μm coronal vibratome slices were made. Three to five slices per brain were kept based on identification of the landmarks shown in Fig. 1A. During slicing, the brain and slices were kept in 4°C ACSF. The NA was punched out (see Fig. 1A) with a 16-gauge needle on a 1-ml syringe and transferred to a spinner flask containing 30 μl of ACSF, 1 mM kynurenic acid, 26–28 U/ml papain, and 0.27–0.28 mg/ml 1-cysteine (at 32–33°C). Plugs of tissue were stirred gently to prevent settling and bubbled with carbogen for 30–75 min. The tissue was removed to 5 ml of N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-buffered Eagle’s minimal essential medium (MEM) (cat No. 11590-040; Gibco BRL, Burlington, ON) and triturated using a fire-polished Pasteur pipette (1-mm tip diam). The cells were centrifuged at 800 rpm for 5 min, and the supernatant replaced with modified SF1C medium (McCarthy and De Vellis 1980), which contained (components from Gibco BRL and Sigma, St. Louis, MO) 47% MEM, 40% Dulbecco’s modified Eagle’s medium (DMEM), 10% Hams F-12 nutrient medium (cat No. 11550-027; Gibco BRL), 0.25% albumin, 10 μg/ml transferrin, 30 nM Na$_2$SeO$_3$, 30 nM triiodothyronine, 25 μg/ml insulin, 200 nM progesterone, 125 nM hydrocortisone, 5 μg/ml superoxide dismutase, 10 μg/ml catalase, 10 μl penicillin/streptomycin, and 1% fetal calf serum (FCS) (pH 7.3). The cells then were plated on Cell Tak-coated (cat No. 40240; Collaborative Biomedical Products, Chicago, IL) glass coverslips (cat No. 12 CIR No. 1 D; VWR, Edmonton, AB) in a multwell plate (cat No. 3534; Costar, Toronto, ON) and allowed to settle and stick for 30–60 min before using them for ≤8 h.

For long-term culture, P5–11 neurons were plated on glass coverslips on a confluent monolayer of glia that were prepared using a method adapted from Rayport et al. (1992) and McCarthy and De Vellis (1980). Glia were isolated from the cerebral cortex of 1-day-old rat pups. The cortex was removed, chopped up, and triturated with a fire-polished Pasteur pipette (1-mm tip diam) and plated onto polyornithine-coated coverslips into modified DMEM (cat No. 310-4080A; Gibco BRL) containing: 0.7 mM NaHCO$_3$, 20 mM HEPES, 10% heat inactivated FCS, and 10 μl of penicillin/streptomycin (cat No. 15070-014; Gibco BRL) at pH 7.3. Medium was changed twice weekly and then neurons (5–14 days old) were plated onto the confluent glial cultures in modified SF1C medium. The culture medium then was changed only once after 1 day in culture at which time 25 μM 5-fluoro-deoxyuridine was added to halt glial growth (Rayport et al. 1992). Cells were maintained under 5% CO$_2$ at 37°C and used within 10 days.

**Immunohistochemistry**

Immunohistochemistry was performed on P5–11 cells cultured for 4–7 days with antibodies to β-tubulin (1:1,000) and γ-aminobutyric acid (GABA) (GABA; 1:5,000). Cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for 20 min at 4°C, washed three times in phosphate-buffered saline (PBS) for 10 min each, and then incubated with the primary sera in PBS with 10% normal goat serum (cat No. 16210-015; Gibco BRL) and 0.3% tritonX with the Ab in PBS for 2 h at 37°C and then washed three times with PBS. The appropriate secondary anti-IgG antibody (anti-rabbit rhodamine-labeled for GABA and anti-mouse fluorescein isothiocyanate (FITC; β-tubulin) secondary antibodies. These neurons are typical of those used for electrophysiological recordings made on cultured cells.
computer that was controlled using Axon Imaging Workbench software (Axon Instruments, Foster City, CA).

**Electrophysiology**

For electrophysiology, one coverslip of cells was placed into a 30 mm Petri dish with HEPES-buffered MEM or modified SF1C. The coverslip was broken into pie-shaped pieces by pressing the blunt end of forceps on its center. One piece was removed for electrophysiology, and the rest were replaced in the incubator for later use. The coverslip was placed into a recording chamber composed of a 3-mm-thick sheet of Plexiglas with a 13-mm-diam hole drilled in the middle to form a 400-μl well with a glass coverslip bottom fixed in place with vacuum grease. Extracellular recording solution was gravity-fed into the chamber and spined from the bath by suction maintaining a 200-μl volume. The extracellular recording solution for isolating Ca²⁺ currents contained (in mM) 130 tetraethylammonium (TEA)–Cl, 10 HEPES, 5 4-AP, 5 CaCl₂, 10 glucose, 3 KCl, and 300–600 mM tetrodotoxin (pH 7.35 with HCl). This level of external Ca²⁺ provided good stability of the whole cell patch recordings and increased the size of the measurable currents in these small cells. Standard patch-clamp methods were performed (Hamill et al. 1981) with an Axopatch-1D amplifier (Axon Instruments) and a 486 AT computer. Patch pipettes were pulled from thin-walled 1.5 mm OD capillary tubing (cat No. TW150F-6; WPI, Sarasota, FL) and had ~1-μm tip openings when pulled on a horizontal multistage pipette puller (model P-87; Sutter Instruments, Novato, CA). Some pipettes were coated with a thick coat of beeswax to within 100 μm from the tip and were firepolished (see figure legends for details). These were filled with a solution containing (in mM) 72 Tris-PO₄, 25 Tris-base, 11 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 40 TEA-Cl, and 2 Mg-ATP (pH 7.35 with TEA-OH) and yielded pipette resistances of 3–8 MΩ. Gigaseals were formed with suction and fast capacitive transients were subtracted before the whole cell configuration was obtained with more suction. After a stable whole cell recording was obtained, the cell capacitance was recorded digitally and then canceled with the analog circuitry. Series resistance compensation only had a small impact on recordings of peak amplitude and slow inactivation times and was not used. Experiments were conducted at 19–23°C.

**Data acquisition and analysis**

Data were acquired using an Axon TL-1 DMA interface (Axon Instruments) at a frequency of 83.33 kHz for capacitive transient acquisition and 2.6 kHz for all other data and then filtered with the amplifier’s built in three-pole Bessel filter. Ten kilohertz filtering was used to record the capacitance transient example for each cell, and 500 Hz was used for all other data recording. Holding current averaged 20 pA, rarely exceeding 50 pA. The leak current was left unsubtracted or was cadmium subtracted. In all traces shown, the dotted lines on traces mark zero current. Data were saved to disk and analyzed and displayed using Pclamp version 6.0 (Axon Instruments), spreadsheet software (Excel ver.5.0; Microsoft), and a graphing program (Prism ver.2.0; GraphPad, San Diego, CA). Fits were done using the nonlinear regression routine of Prism. All data are presented as means ± SE, and where appropriate the Student’s t-test was used to determine statistical significance (P < 0.05).

The average capacitance (Cₘ) and series resistance (Rₛ) for 228 cells was 6.7 ± 3 pF and 15.7 ± 0.3 MΩ. These were determined by measuring the area and height (Iₚeak) of capacitive transients (Cₘ = area/VSTEP, Rₛ = VSTEP/Iₚeak) recorded using a hyperpolarizing 25-ms-long, 10-mV voltage step (VSTEP) from a holding potential of −100 mV applied every 500 ms (analogue measurement of these values gave similar results). Of the 67 long-term cultured cells from which recordings were made, 32 were judged to have adequate space clamp based on a smoothly increasing rate of activation with depolarization, no obvious jumps in I-V plots or the current during a voltage pulse that could be attributed to delays in activation of the Ca²⁺ current in different regions of the cell, and good capacitive transient subtraction using the amplifier circuitry. Data from the other 35 cells were not analyzed.

Ca²⁺ currents in these cells exhibited a slow, progressive decay with repeated pulses (50% decay in 10 min with 5 s between pulses) that resembled rundown but was reduced only partly by having ATP in the electrode. It was reduced significantly by lengthening the time between voltage pulses (~7 s) and by using shorter pulse lengths (<50 ms). This observation is similar to the slow inactivation process as described by Murchison and Griffith (1996) in basal forebrain neurons. In addition to this rundown-like decay, a somewhat faster decay occurred on switching the holding voltage (V₀) between −100 and −50 mV, recovering on return to V₀ = −100, that also resembled that described in other neurons (Kay 1991; Keller and Nusinovitch 1996; Murchison and Griffith 1996). Slow inactivation in the nucleus accumbens stabilized in 40–60 s and was taken into account when doing two I-V protocols first at V₀ = −100 and then at V₀ = −50 (as in Figs. 2, 5, and 7D) by leaving time for the current to stabilize between the two protocols.

In pharmacological experiments, the effect of each antagonist was assessed as quickly as possible after obtaining a whole cell recording and getting 1–2 min of control responses. To calculate toxin effects, the average peak current of the two to three traces at the end of the toxin application period were measured and normalized to the average of the last two or three traces just before the antagonist was applied. Peak current was taken as the maximal calcium current during a voltage pulse and end-pulse current as the average current during the last 10 ms of the pulse.

**Drugs and their delivery**

Cell superfusion was achieved with a multichannel, gravity-based, fast-perfusion system with feed from up to eight different solutions fed through PE160 tubing from 60- or 30-ml syringes into a common stainless steel tube of ~200 μm open diameter and ~0.5 μl. The superfusion tube was placed within 500–1,000 μm of the cell and supplied a flow rate of ~0.5 ml/min that achieved solenoid-controlled solution changes of well less than 1 s. Nimodipine (Nim; cat No. N-149; RBI, Natick, MA) was made up as 0.3 M stored in single use containers at 40 °C and aliquots were thawed and mixed with solutions just before use. ω-Conotoxin-MVIIC (ω-CmTx) was a generous gift from George P. Miljanich, PhD (Neurex, Menlo, Park, CA) and was made up as a 5- or 1-MM stock. ω-Conotoxin-GVIA (ω-CgTx; cat No. C9915, Sigma) was made up as a 1-MM stock. ω-Agatoxin-IVA (ω-Aga) was a generous gift from Nicholas A. Saccamano, PhD (Pfizer, Groton, CT), came as a 91-μM stock in double-distilled H₂O, and was stored as 91 or 10-μM stocks. All solutions including control solutions during toxin experiments contained 0.01–0.02% ethanol and 0.1 μg/ml cytochrome c. All other chemicals used in this study were analytic or research grade from BDH (Toronto, ON) and Sigma.

**Results**

**Cell preparation**

Figure 1A shows a brightfield micrograph of a coronal slice from a P5–11 rat showing the landmarks used when making tissue punches. The NA has been divided functionally into the shell and the core regions using a variety of
Biophysical properties of whole cell Ca\(^{2+}\) current

Figure 2B illustrates typical whole cell currents in an acutely dissociated NA neuron from a P5–11 rat. In other experiments (e.g., Figs. 4 and 6) in which 1 mM cadmium (Cd\(^{2+}\)) was used to block Ca\(^{2+}\) current, little outward or inward current was apparent showing that contaminating currents were blocked adequately in our experiments. The rate of activation of Ca\(^{2+}\) current increased with depolarization, and the maximally activated inward current occurred at 0–10 mV. In this and many cells, both low- and high-threshold Ca\(^{2+}\) currents were evident. The threshold of activation for low-threshold current was −64.3 ± 0.9 mV, n = 40.

In this study, we also have separated high-threshold current into its sustained and inactivating components (HTS and HTI, respectively). This was done so that the Ca\(^{2+}\) current components blocked by the different toxins could be described in more detail within an historical context and so that a detailed comparison to the voltage-dependent activation of the toxin-resistant current could be made. In acutely isolated neurons, HTI and HTS components had very similar thresholds of activation of −50 mV (−51.4 ± 0.7 mV, n = 66 and −53.3 ± 0.8 mV, n = 48, respectively). The voltage-dependent properties were examined by running paired current-voltage (I–V) protocols in which currents were elicited by voltage steps of −120 to 50 mV first from a V\(_{H}\) of −100 mV and then from −50 mV (Fig. 2A, B). The inactivating current was obtained by subtracting currents obtained at V\(_{H}\) = −50 mV from those obtained at V\(_{H}\) = −100 mV (Fig. 2A, ■ and traces in Fig. 2C). In the cell in Fig. 2, A–C, both low-threshold inactivating (LTI) and HTI inactivate by V\(_{H}\) = −50 mV (Fig. 2A, ○). The currents remaining at −50 mV were composed of an HTS component (Fig. 2B, bottom). The average voltage at which each component of Ca\(^{2+}\) current was maximal and the average magnitude of the current respectively were as follows: LTI: −34.7 ± 0.9 mV, −13.6 ± 1.1 pA, n = 45 (peak estimated at −30 or −40 mV); HTS: 3.1 ± 0.6 mV, −94.7 ± 7.5 pA, n = 106 (from I–V plot at V\(_{H}\) = −50 mV); and HTI: 3.3 ± 0.6 mV, −93.8 ± 5.8 pA, n = 104 (from I–V plot of inactivating current).

Differences have been reported previously in the expression of the various components of Ca\(^{2+}\) currents among acutely dissociated neurons, cultured neurons, and neurons isolated from different aged rats (Lorenzon and Foehring 1995b; Rossi et al. 1994; Thompson and Wong 1991). This was also true for studies conducted on isolated striatal neurons, a structure related to the NA, in which LTI was expressed in young and cultured neurons but not in striatal neurons isolated from older rats (Bargasta et al. 1991, 1994; Hoehn et al. 1993). To see if the expression of LTI followed this same pattern in the NA, we also recorded from 9 acutely isolated neurons from P24–32 rats and 32 cultured neurons from P5–11 rats. Figure 2D shows an I–V plot from a typical cultured NA neuron from a P5–11 rat, and Fig. 2E shows a typical result from acutely dissociated NA neurons from a P24–32 rat. The data were acquired and displayed as for the acutely isolated cell from P5–11 rats (Fig. 2, A–C).

The cultured neurons (Fig. 2D) showed the same range of high-threshold currents and an LTI current that were found with the acutely isolated cells from P5–11 rats except that the LTI was found in a higher percentage of cells (Table 1). The average voltage at which each component of Ca\(^{2+}\) current was maximal and the average size of the current respectively were as follows: LTI: −31.7 ± 4.1 mV, −51.1 ± 7.6 pA, n = 24; HTS: 3.0 ± 0.9 mV, −185.5 ± 24.9 pA, n = 32; and HTI: 0.2 ± 1.6 mV, −93.5 ± 15.3 pA, n = 31. Cultured cells, unlike acutely dissociated cells, could be identified occasionally as spiny (SP) or aspiny (ASP). Of 25 cells where such an identification could be performed, 15 were ASP (10–20-μm diam cell bodies) and 10 were SP (8–18 μm diam). No difference was found between the ASP and SP morphological types regarding the expression of HTI and HTS (100% occurrence each) and LTI expression (60 and 70% occurrence, respectively). Acutely isolated cells from P24–32 rats (Fig. 2E) also exhibited a similar range of Ca\(^{2+}\) current components except, in this case, it was more difficult to identify LTI given the relatively larger density of the high-threshold components (Table 1). The average voltage at which each component of Ca\(^{2+}\) current was maximal and the average size of the current respectively were as follows: LTI: −35.0 ± 2.2 mV, −26.3 ± 7.7 pA, n = 5; HTS: 4.4 ± 1.8 mV, −173.9 ± 50.7 pA, n = 9; and HTI: 1.7 ± 3.1 mV, −76.8 ± 27.3 pA, n = 9.

Table 1 summarizes the average densities of the various biophysically identified currents and the percentages at which the different components were found in the three preparations. The average cell capacitances for P5–11 acutely dissociated, P5–11 cultured, and P24–32 acutely dissociated cells respectively were as follows: 6.0 ± 0.4, 7.0 ± 0.5, and 4.0 ± 0.6 pF. In the rest of the paper, cultured neurons mostly were used to acquire data on LTI (Figs. 3 and 7) because LTI was generally larger and more consistently present in these cells, and acutely isolated NA neurons from P5–11 rats were used for the pharmacological studies because the cells were the most easily acquired and voltage clamped.
To describe the LTI current in these neurons in more detail, the voltage dependence of inactivation and the time dependence of recovery from inactivation of LTI was examined in Fig. 3. To determine the voltage dependence of inactivation of LTI, −110- to −40-mV conditioning voltages in 5-mV increments preceded a 200-ms test pulse to −30 mV that was applied every 5 s (Fig. 3A). LTI current was measured as the difference between the peak and end-pulse current for each trace and was normalized to the value at −10 mV (○). This averaged normalized current was graphed against the conditioning voltage and then fit with a Boltzmann curve (Fig. 3B). Complete inactivation occurred at around −50 mV and maximal activation occurred just negative to −100 mV. The voltage at half-maximal conductance ($V_{1/2}$) and slope of the Boltzmann fit were −80 and 6.7 mV, respectively. These values are typical of central neurons (Akaike 1991; Huguenard 1996) and similar to those found for striatal neurons (Hoehn et al. 1993).

Figure 3, C and D, illustrates the time dependence of recovery of LTI from voltage-dependent inactivation. These data were acquired using a voltage protocol in which voltage was held first at −50 mV to inactivate LTI and then stepped to −100 mV for 50–1,850 ms (in 100-ms increments) to reactivate it before a −30-mV test pulse was applied to determine the magnitude of the recovered current. On graphing the average results from 10 cultured cells and then fitting the data with a single exponential curve, the time constant

**TABLE 1.** Current densities of the various biophysically identified components of Ca$^{2+}$ current in different neuronal preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>n</th>
<th>LTI</th>
<th>HTI</th>
<th>HTS</th>
<th>LTI</th>
<th>HTI</th>
<th>HTS</th>
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<tbody>
<tr>
<td>P5–11 acute</td>
<td>106</td>
<td>9.57 ± 0.83</td>
<td>21.39 ± 1.13</td>
<td>20.74 ± 1.48</td>
<td>42</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>P5–11 cultured</td>
<td>32</td>
<td>5.14 ± 0.63</td>
<td>13.91 ± 1.81</td>
<td>27.7 ± 3.99</td>
<td>77</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>P24–32 acute</td>
<td>9</td>
<td>8.09 ± 1.8</td>
<td>31.13 ± 6.59</td>
<td>58.15 ± 13</td>
<td>55</td>
<td>66</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are means ± SE. LTI, low-threshold inactivating; HTI, high-threshold inactivating; HTS, high-threshold sustained.
of the toxin-resistant component that was found to be distinct from the toxin-sensitive components.

**Individual Nim or toxin application—trains of pulses**

Figure 4 illustrates results from the individual application of each of the toxins tested (Fig. 4, A–D) as well as 50 μM Ni²⁺ (Fig. 4E). The blocking effects were monitored using 0-mV voltage pulses from $V_h = -100$ mV that were applied every 7 s.

The DHP Nim (5–10 μM) blocks L-type Ca²⁺ channels in a variety of central neurons (Aosaki and Kasai 1989; Bean 1989; Hille 1992; Marchetti et al. 1995; Miller 1987; Nowycky et al. 1985; Tsien et al. 1988) including the striatum (Bargàs et al. 1994; Hoehn et al. 1993). In NA neurons, 10 μM Nim caused a partially reversible block of Ca²⁺ current. Nim reduced the Ca²⁺ current by 17.8 ± 1.5% ($n = 21$) of the total in 22.5 ± 5.4 s (Fig. 4A). On removal of Nim from the recording chamber, 88.4 ± 1.4% of the blocked current recovered in 34.6 ± 4.8 s ($n = 10$). A control trace (Fig. 4Aa) was subtracted from a trace obtained in the presence of Nim (Fig. 4Ab), to show the kinetics of the Nim-sensitive current. For the pulse duration used (150 ms), the kinetics of the Nim-sensitive current was largely nonactivating. This aspect of the toxin-sensitive currents is discussed in more detail in the next section.

N-type Ca²⁺ channels in a variety of central neurons, including the striatum (Bargàs et al. 1994; Hoehn et al. 1993), are blocked by the peptide toxin ω-CgTx (Kasai et al. 1987; McCleskey et al. 1987; Olivera et al. 1985; Plummer et al. 1989; Tsien et al. 1991). In NA neurons, 1 μM ω-CgTx caused an irreversible block of Ca²⁺ current (Fig. 4B). ω-CgTx reduced the total Ca²⁺ current by 36.0 ± 2.5% ($n = 21$) in 41.9 ± 4.5 s; 2 μM ω-CgTx only blocked an additional 8 ± 4% of current ($n = 6$), suggesting that 1 μM ω-CgTx was probably at saturating concentration. On removal of ω-CgTx from the recording chamber, only an average 2.0 ± 0.7% of the blocked current recovered after several minutes of washout. The kinetics of the ω-CgTx-sensitive current was partially nonactivating.

ω-Aga is a potent blocker (~ $K_p = 20$ nM) of P-type Ca²⁺ channels (Llinsàs et al. 1992; Mintz et al. 1992a,b) and at higher concentrations is thought to block Q-type channels (Randall and Tsien 1995; Sather et al. 1994). ω-Aga has been shown to block current in the striatum (Bargàs et al. 1994). Figure 4C illustrates the effect on a single NA cell of 200 nM ω-Aga, a concentration that may block P- and Q-type channels (Randall and Tsien 1995), and of 20 nM ω-Aga, which should affect mostly P-type channels. Twenty nanomolar ω-Aga only blocked 7.0 ± 2.0% ($n = 5$) of the Ca²⁺ current in 42.4 ± 12.0 s, whereas further addition of 200 nM ω-Aga resulted in a 19.5 ± 2.0% ($n = 28$) reduction in Ca²⁺ current in 84.1 ± 9.8 s. With removal of 200 nM ω-Aga from the recording chamber, there was no recovery of blocked Ca²⁺ current (1.8 ± 1.2%) after several minutes of superfusion with control solution. The ω-Aga-sensitive current had nonactivating kinetics regardless of the concentration used.

ω-CmTx is a broader spectrum toxin that blocks N-, P-, and Q-type channels (Hillery et al. 1992; Liu et al. 1996; McDonough et al. 1996; Stea et al. 1994; Wu and Saggau...
Block of whole cell Ca\(^{2+}\)/currents by separate application of each of the Ca\(^{2+}\)/channel toxins (A–D) and 50 \(\mu\)M Ni\(^{2+}\) (E). Ca\(^{2+}\)/current was monitored with a train of 150-ms-long, 0-mV voltage pulses applied from \(V_\text{H} = -100\) mV every 7 s. Toxins were applied using rapid perfusion after \(\approx 30\) s of stable control responses. Once a stable toxin effect was achieved, the toxin was washed from the recording chamber, and then 0.5–1 mM Cd\(^{2+}\) was applied to block all the current. A measurement of peak (○) and end-pulse (●) current was graphed against time of recording (left). ○ periods of control solution application; ■ periods of toxin or Cd\(^{2+}\) application (as labeled). Illustrated for each treatment (right) are a control trace (a), a trace from a period where a stable toxin effect was achieved (b), and the difference between the 2 (a–b) to show the kinetics of the toxin-sensitive current.

A: nimodipine (Nim, 10 \(\mu\)M) effect was reversible, and the Nim-sensitive current was largely noninactivating.

B: \(\omega\)-conotoxin-GVIA (\(\omega\)CgTx, 1 \(\mu\)M) irreversibly blocked a large partially inactivating component of the Ca\(^{2+}\)/current.

C: \(\omega\)-agatoxin-IVA (\(\omega\)Aga; 20 and 200 nM) irreversibly blocked a mostly noninactivating component of Ca\(^{2+}\)/current.

D: \(\omega\)-conotoxin-MVIIC (\(\omega\)CmTx, 5 \(\mu\)M) blocked a partially inactivating component in a somewhat reversible manner.

E: Ni\(^{2+}\) (50 \(\mu\)M) rapidly and reversibly blocked a component of Ca\(^{2+}\)/current, which, in this cell, almost completely inactivated during the 150-ms voltage pulse.

Ni\(^{2+}\) rapidly blocked a mostly inactivating current. This is evident from the graph and traces shown in Fig. 4E, which shows a large reduction in the peak current but only a small reduction in end-pulse current. Ni\(^{2+}\) caused a 25.9 \(\pm\) 3.0% reduction in peak current within 7 s in the nine cells tested. The effect was mostly reversible with recovery of 95.5 \(\pm\) 2.3% of the blocked current occurring in 62.1 \(\pm\) 12.3 s. The percent block data shown in this figure (Fig. 4) have been summarized as part of Fig. 6C.

**Individual toxin application—current-voltage relationships**

Figure 5 illustrates the voltage-dependence properties of the of the Nim-, \(\omega\)CgTx- and \(\omega\)Aga-sensitive currents that remain at \(V_\text{H} = -50\) mV. All three averaged and normalized I-V curves are very similar in their voltage dependence. To
mV minus traces at V had no effect on LTI in five LTI-expressing cells, however, shown by the solid bars in Fig. 6. Toxin concentrations were selected that generally are considered saturating for their respective currents. [ωCmTx (2–5 μM) was included because it is considered to block N- and P-type channels as well as Q-type channels (Hillyard et al. 1992; Randall and Tsien 1995; Sather et al. 1993; Zhang et al. 1993)]. When ωCgTx and ωAga are used at saturating concentrations, any further blocked current is considered to be of the Q type.

Figure 6 shows a typical example of one such toxin experiment on an acutely isolated NA neuron from a P5–11 rat. Figure 6A shows whole cell Ca2+ current traces selected from the stabilized period during the application. Currents were elicited using a train of 120-ms step commands to 0 mV from Vh = −100 mV applied every 5 s. The peak (●), end-pulse (□), and baseline (○) currents were graphed against time of recording in seconds in Fig. 6B. There was no change in the baseline current showing that the recording was stable. The current was allowed to reach an apparent steady state before the addition of the next toxin. The percent block was calculated by comparing the steady-state periods before and after each toxin application. In this cell, Nim blocked 31%, ωCgTx 15%, ωAga 24% and ωCmTx 6% of the total current. This left a resistant component of 24%. Ni2+ reduced the toxin-resistant component by a further 12%. A final component of 12% remained that was resistant to all these treatments.

Figure 6D shows average subtracted current traces from 37 cells obtained by subtracting a trace from the steady-state period before each toxin application from a trace from the steady-state period after that toxin’s application in experiments as in Figs. 6, A and B. All traces were first Cd2+ subtracted and normalized to the control current so that the relative magnitude of the averaged traces reflects the relative magnitude of the blocked current. The percent decay for each current was as follows: 5–10 μM Nim-sensitive, 11% (n = 21); 1–2 μM ωCgTx-sensitive, 16% (n = 37); 200 nM ωAga-sensitive, 24% (n = 37); 5 μM ωCmTx-sensitive, 50% (n = 19); toxin-resistant, 66% (n = 19); toxin-resistant/Ni2+-sensitive, 88%; and toxin and Ni2+-resistant, 56%. These results are consistent with known properties of L-, N-, and P-type channels (Tsien et al. 1991). ωCmTx blocked a small partially inactivating component that is probably an additional part of a Q-type current that remained unblocked by 200 nM ωAga. The remaining toxin-resistant current and that portion suppressed by Ni2+ were distinct from the toxin-sensitive currents by inactivating much more rapidly.

The average amount of each toxin-sensitive and resistant current obtained from the data in Fig. 6, A, B, and D, is shown by the solid bars in Fig. 6C, which summarizes all the toxin data. In addition, the open bars are results from single and separate application of each toxin from experiments as in Fig. 4 (except for Nim, which is from Fig. 6). The solid bar for Nim is for the effect of Nim after the application of ωCgTx. These results give some information on the selectivity of the toxins and Ni2+. Nim blocked about the same amount of current whether ωCmTx was applied before it or not (18.8 ± 2.2% vs. 17.6 ± 1.6%, respectively). ωCgTx applied alone blocked statistically (P < 0.05) more

**Group application of Nim and all the toxins**

In the remaining experiments, the drugs were applied sequentially and cumulatively to more accurately determine the ratio of pharmacologically separated high-threshold current components and to examine the toxin-resistant current.
Voltage dependence of the toxin-resistant Ca$^{2+}$ current

Figure 7 examines the voltage dependence of the toxin-resistant current and compares it with the toxin-sensitive current and LTI. Current traces from an acutely isolated NA neuron in response to 150-ms voltage pulses are shown in Fig. 7A. The top set of traces show the current sensitive to combined application of 10 μM Nim, 1 μM ωCgTx, 200 nM ωAga, and 2 μM ωCmTx. These were obtained by subtracting the control traces from toxin-resistant traces shown in the middle. The toxin-sensitive current has very distinct kinetics compared with the toxin-resistant current in this cell. In Fig. 7B, voltage ramp currents for the same cell in Fig. 7A are shown (obtained using voltage ramps of −120 to 50 mV at 0.33 mV/s applied from $V_{th} = −100$ mV). The bars on the traces mark the peak current showing that the toxin-resistant and toxin-resistant/Ni$^{2+}$-sensitive currents peak at voltages more negative than the toxin-sensitive component.

A rather large example of an LTI is shown in Fig. 7C (for a different cell than in the rest of this figure) along with an I-V of the toxin-resistant current (Fox et al. 1987; Hille 1992) for the cell shown in Fig. 7, A and B. The I-V for LTI was obtained by isolating LTI from high-threshold currents using its relative resistance to 25 μM Cd$^{2+}$. The estimated normalized conductance curves for LTI (●), the toxin-resistant current (○), and the toxin-sensitive current (■) are shown in Fig. 7D. These conductance curves are useful for comparing the voltage dependence of the individual currents. There may be errors in estimating the true conductance relationship because of contamination of non-Ca$^{2+}$ channel outward currents as described earlier for Fig. 5. The estimated conductance curves were fit with single Boltzmann curves, which yielded slopes and $V_{0.5}$ of 4.6, −42.8 (n = 14); 6.6, −10.3 (n = 6) and 5.7, −3.1 mV (n = 6), respectively.

**Discussion**

This study demonstrates that NA neurons express multiple Ca$^{2+}$ current subtypes including Nim-, ωCgTx- and ωAga-sensitive Ca$^{2+}$ currents, plus a more rapidly inactivating high-threshold toxin-resistant current. There is also a low-
threshold rapidly inactivating T-type current (LTI). Identification of high-threshold current subtypes was largely based on generally accepted pharmacological tools. We confirmed other studies that the biophysical differences between high-threshold subtypes are modest (McCleskey 1994; Olivera et al. 1994; Tsien et al. 1991). However, some biophysical characterization was carried out (Fig. 5) to show that the toxin-resistant current differed from LTI (Fig. 7) and the other high-threshold components not only in its kinetics but also in its voltage dependence.

Most NA neurons (90%) are medium spiny GABAergic neurons, the remainder are larger aspiny neurons (Gerfen 1988; Smith and Bolam 1990). Both types were seen in culture and expressed similar currents. NA neuronal cultures were stained with anti-GABA to show that the majority of neurons used here were GABAergic. Acutely isolated cells were mostly spherically shaped without processes, with only short processes, or only short newly sprouted processes and could not be further subclassified. The region of brain tissue removed for cell isolation (Fig. 1A) roughly corresponded to the central core region of the NA. A variety of techniques has been used to separate the NA into the inner core and outer shell regions (Jongen-Rêlo et al. 1994; Meredith et al. 1992, 1993; Zahm and Brog 1992). Because each technique yields different maps of the core versus shell location within the NA, it is difficult to further state the functional identity of the neurons used here.

NA neurons isolated from P5–11 rats were chosen for most of this study because these were easily isolated and voltage clamped. However, because work on the related striatal neurons suggested that LTI was expressed only in neurons isolated from young rats (Hoehn et al. 1993) or in cultured cells (Bargas et al. 1991) but not in neurons from >P28 (Bargas et al. 1994), we also examined cultured P5–11 NA neurons and NA neurons from P24–32 rats. Evidence for loss of LTI during development has been found for some neurons (Thompson and Wong 1991) but not others (Fisher et al. 1990; Takahashi et al. 1989). LTI was found in all three NA preparations. It was seen most easily in neurons from P5–11 rats, but also was identified in neurons from P24–32 rats even though it was relatively smaller. The difference between the NA and striatum is borne out by studies in brain slices from adult rats in which low-threshold Ca2+ spikes, which are generated by low-threshold Ca2+ currents, were found in the NA (O’Donnell and Grace 1993, 1995) but not the striatum (Bargas et al. 1989; Pineda et al. 1992).

**Toxin-sensitive currents**

Saturating Nim blocked 18% of total current in NA neurons in a partially reversible manner. Most of the blocked current was HTS and decayed 11% during a 150-ms voltage pulse similar to the L-type currents examined in other neurons (Bean 1989, 1991; Carbone and Swandulla 1989; Hille 1992; Kostyuk 1989; McCarthy and TanPiengco 1992; Regan et al. 1991). The concentration of Nim used in this study (5–10 μM) was not completely selective for L-type channels. It was found here that Nim reduced LTI by 20%. Similar Nim effects were reported for other neurons (Akaike et al. 1989; Huguenard 1996; McCleskey et al. 1987). Nim also appeared to partially block CgTx-sensitive channels. CgTx block was reduced significantly after Nim addition, suggesting a partial overlap similar to that found for other neurons (Akaike et al. 1989; McCleskey et al. 1987).

Saturating CgTx irreversibly blocked 25% of total NA Ca2+ current that decayed 16% during a voltage pulse and was composed of both HTS and HTI. These properties are typical of those expected for a N-type Ca2+ current that generally is accepted as CgTx-sensitive (Aosaki and Kasai 1989; Hess 1990; Kasai et al. 1987; McCleskey et al. 1987;
neurons, 1992), and on the basis of single-channel studies, it has been suggested that DHP-resistant but CgTx-sensitive channels can have both sustained and inactivating kinetics (Aosaki and Kasai 1989; Plummer and Hess 1991; Plummer et al. 1989).

ωAga (200 nM) was used to test for P/Q-type channels. It irreversibly blocked 17% of total current (mostly HTS with a small inactivating component). In cerebellar Purkinje neurons, ωAga irreversibly blocks a noninactivating current with high affinity (Llinàs et al. 1992; Mintz et al. 1992a,b). More recently it has been shown that Q-type current is blocked reversibly with lower affinity (Randall and Tsien 1995; Zhang et al. 1996). Both currents are identified with class A α subunits, although α1A-expressing oocytes give a more Q-like current with inactivating kinetics and low-affinity ωAga block (Mori et al. 1991; Sather et al. 1993). Cerebellar granule cells have been clearly shown to express both a small P- and larger Q-type currents (Randall and Tsien 1995). In our experiments, we applied both 20 and 200 nM ωAga to see if we could determine whether the current expressed in these cells was a Purkinje cell-like P-type current, a Q-type current, or both. ωAga at 20 nM only blocked a small component of Ca2+ current, which did not vary in its kinetics from the larger current blocked by 200 nM ωAga. This, plus the further block of current after 200 nM ωAga by ωCmTx (Fig. 6), suggests that the majority if not all of the ωAga-sensitive current is probably Q type. If a P-type current similar to that found in Purkinje cells (i.e., blocked with very high affinity) is present in NA neurons, it is a small component of total current. The rundown/slow inactivation of Ca2+ current in these cells made it impractical to pursue the possibility of such a small P-type current.

**Toxin-resistant Ca2+ current**

The largest single fraction of total current was resistant to the toxins and Nim (32%). It rapidly inactivated (66% decay), had a high-threshold activation range, and had a voltage of maximal activation 5–10 mV more negative than the toxin-sensitive currents. A rapidly inactivating toxin-resistant and Ni2+-sensitive component, which some investigators have named R type, has been a common finding in a variety of neuronal preparations (Brown et al. 1994; Eliot and Johnston 1994; Lorenzon and Foehring 1995a; Magee and Johnston 1995; Pearson et al. 1995; Randall and Tsien 1995). These unique properties support the possibility that this is a distinct current. Indeed, the resistant current in this and other neurons resembles the toxin-resistant current found in α1A-expressing oocytes and HEK cells (Soong et al. 1993; Williams et al. 1994; Zhang et al. 1993). The message for class E α-subunit clones is found throughout the CNS including in the NA (Soong et al. 1993). The resistant current is clearly high threshold and thus cannot be the T current. It is unlikely that a portion of the resistant current was composed of unblocked Q-type current because the toxins were applied for 5–10 min in experiments shown in Fig. 7. Presently, the definition of the toxin-resistant current remains one of exclusion. The toxin-resistant current could have different molecular identities in different neurons and possibly be composed of multiple subtypes in individual neurons.

In summary, NA neurons express a variety of high-threshold Ca2+ currents and a LTI Ca2+ current. The high-threshold currents in NA neurons include four pharmacologically distinct subtypes. It is not well understood why neurons express such a wide variety of Ca2+ channels. Certainly these different types, which are being identified increasingly with distinct molecular subtypes (Snutch and Reiner 1992; Snutch et al. 1991), can have different localization within and among cells and can undergo a wide variety of modulation (Berridge and Dupont 1994; Snutch and Reiner 1992; Tsien et al. 1988, 1991). In light of this, we have conducted this study to form a base for more detailed examination of NA Ca2+ current modulation. The goal is to provide further insight into the role of Ca2+ currents in normal NA function and possibly in NA involvement in drug addiction and the treatment of diseases of the basal ganglia such as schizophrenia.

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