Molitor, Scott C. and Paul B. Manis. Voltage-gated Ca$^{2+}$ conductances in acutely isolated guinea pig dorsal cochlear nucleus neurons. *J. Neurophysiol.* 81: 985–998, 1999. Although it is known that voltage-gated Ca$^{2+}$ conductances (VGCCs) contribute to the responses of dorsal cochlear nucleus (DCN) neurons, little is known about the properties of VGCCs in the DCN. In this study, the whole cell voltage-clamp technique was used to examine the pharmacology and voltage dependence of VGCCs in unidentified DCN neurons acutely isolated from guinea pig brain stem. The majority of cells responded to depolarization with sustained inward currents that were enhanced when Ca$^{2+}$ was replaced by Ba$^{2+}$, were blocked partially by Ni$^{2+}$ (100 μM), and were blocked almost completely by Cd$^{2+}$ (50 μM). Experiments using nifedipine (10 μM), α-Aga IVA (100 nM) and α-CTX GVIA (500 nM) demonstrated that a variety of VGCC subtypes contributed to the Ba$^{2+}$ current in most cells, including the L, N, and P/Q types and antagonist-insensitive R type. Although a large depolarization from rest was required to activate VGCCs in DCN neurons, VGCC activation was rapid at depolarized levels, having time constants <1 ms at 22°C. No fast low-threshold inactivation was observed, and a slow high-threshold inactivation was observed at voltages more positive than ~20 mV, indicating that Ba$^{2+}$ currents were carried by high-voltage activated VGCCs. The VGCC subtypes contributing to the overall Ba$^{2+}$ current had similar voltage-dependent properties, with the exception of the antagonist-insensitive R-type component, which had a slower activation and a more pronounced inactivation than the other components. These data suggest that a variety of VGCCs is present in DCN neurons, and these conductances generate a rapid Ca$^{2+}$ influx in response to depolarizing stimuli.

**INTRODUCTION**

Postsynaptic Ca$^{2+}$ plays an important role in many neuronal processes, including the activation of ionic conductances (McManus 1991; Sah 1996) and synaptic plasticity (Linden 1994; Nicoll and Malenka 1995). Voltage-gated Ca$^{2+}$ conductances (VGCCs) provide a major source of postsynaptic Ca$^{2+}$ influx in response to synaptic input (Eilers et al. 1995; Jaffe et al. 1994; Miyakawa et al. 1992) and during action potentials (Jaffe et al. 1992; Lev-Ram et al. 1992; Markram et al. 1995). Ca$^{2+}$ imaging experiments in dorsal cochlear nucleus (DCN) have revealed that action potentials elicit a VGCC-mediated Ca$^{2+}$ influx in the soma and proximal dendrites of two major populations of DCN neurons (Manis and Molitor 1996; Molitor and Manis 1996). VGCCs also contribute to the electrogenic responses that determine the discharge pattern of neurons during depolarization (Llinás 1988) and may be involved in generating the intrinsic discharge patterns of DCN neurons. A slow depolarization resulting from VGCC activation may be responsible for the complex spiking behavior of cartwheel cells (Agar et al. 1996; Manis et al. 1994; Waller and Godfrey 1994; Zhang and Oertel 1993). In addition, K$^{+}$ channels activated by a Ca$^{2+}$ influx through VGCCs may be responsible for the slow afterhyperpolarization observed in pyramidal cells after sustained depolarization (Hirsch and Oertel 1988; Manis 1990). However, no studies have been performed that investigate the biophysical and pharmacological properties of VGCCs that contribute to Ca$^{2+}$ influx and discharge patterns in DCN neurons.

Mammalian neurons possess a variety of VGCC subtypes that can be distinguished by their voltage dependence and their sensitivity to various pharmacological agents. VGCCs can be classified broadly in terms of the voltage-dependent properties they exhibit: low-voltage activated (LVA), which activate near the resting potential and exhibit a rapid inactivation over this voltage range (Huguenard 1996), and high-voltage activated (HVA), which activate at more depolarized voltages and exhibit a slow inactivation at these depolarized voltages (Bean 1989; Tsien et al. 1988). At the present, at least five HVA subtypes have been found in the CNS: the dihydropyridine-sensitive L type, the α-CTX GVI-A-sensitive N type (Fox et al. 1987), the α-Aga IVA and α-CTX MVIIC-sensitive P and Q types (Hillyard et al. 1992; Llinás et al. 1989; Mintz et al. 1992b), and a dihydropyridine- and peptide toxin-insensitive R type, which may be a collection of one or more additional VGCC subtypes (Randall and Tsien 1995; Tottene et al. 1996). The existence of multiple VGCC subtypes within a single neuron could generate differential responses at the synaptic level, having differences in their voltage dependence (Forti et al. 1994; Fox et al. 1987) or in their responses to neurotransmitter modulation (Bean 1989).

The present study addresses two objectives: to survey the VGCC subtypes present in DCN neurons and to estimate the types of stimuli that will generate a Ca$^{2+}$ influx through VGCCs. To this end, we have investigated the pharmacology and voltage dependence of VGCCs in acutely isolated guinea pig DCN neurons using the whole cell voltage-clamp technique. Our results indicate that various HVA VGCCs are present in DCN neurons, and these conductances are capable of generating a large Ca$^{2+}$ influx in response to action potentials. The Ca$^{2+}$ influx and discharge patterns produced by VGCCs
may play important roles in the neuronal processing that occurs in the DCN.

**METHODS**

**Acute cell isolation**

Pigmented guinea pigs weighing 150–300 g were anesthetized with pentobarbital (35–40 mg/kg), decapitated, and the brainstem was removed quickly and placed into an oxygenated piperazine-N, N'-bis-(2-ethanesulfonic acid) (PIPES)-buffered dissection solution at 30°C (see composition in *Solutions*). Using an oscillating tissue slicer, the cochlear nuclei were cut along the strial axis, parallel to the orientation of the parallel fibers, into 400-μm-thick slices. The DCN was isolated from the remainder of the brain stem using Castroviejo scissors. DCN slices were placed in a spinner flask containing oxygenated dissection solution with 0.67 mg/ml of bovine pancreatic trypsin (Sigma, type XI) and 0.5 mg/ml bovine serum albumin (BSA). Sigma, A7638) and were spun slowly (90 rpm) for 30 min. Enzymatically treated slices were thoroughly rinsed in enzyme-free dissection solution with BSA and allowed to incubate for 1 h in this solution. Before recording, two or three slices were triturated gently in 0.3–0.5 ml of the dissection/BSA solution with a sequence of three or four fire-polished pipettes having gradually decreasing diameters (2–0.5 mm). Cells were plated on 35-mm culture dishes coated with 10 μg/ml of poly-d-lysine to promote cell adherence (Yavin and Yavin 1974). After waiting 10–15 min for cells to adhere, a continuous flow of a N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)-buffered recording solution (0.5 ml/min) was established to clear away debris and for the exchange of extracellular fluids. Fluid flow was maintained with gravity-fed lines and solution exchange was performed using solenoid valves. Isolated cells were maintained at room temperature (–22°C), and were viable for 2–3 h after plating.

**Solutions**

The dissection solution contained (in mM) 110 NaCl, 5 KCl, 25 glucose, 0.2 CaCl₂, 4 MgCl₂, and 20 PIPES, pH 7.0 with 5 M NaOH. A Na⁺/K⁺ recording solution contained (in mM) 130 NaCl, 5 KCl, 25 glucose, 2.5 CaCl₂, 1.3 MgCl₂, and 10 HEPES, pH 7.35 with 5 M NaOH. To isolate VGCCs pharmacologically from voltage-gated Na⁺ and K⁺ conductances, a Ca²⁺ recording solution containing (in mM) 120 choline Cl, 20 tetraethylammonium chloride (TEA CI), 4 4-aminoipyridine (4-AP), 25 glucose, 2.5 CaCl₂, 1.3 MgCl₂, and 10 HEPES, pH 7.35 with 1 M TEA OH, and a Ba²⁺ recording solution containing (in mM) 115 choline Cl, 20 TEA CI, 4 4-AP, 25 glucose, 10 BaCl₂, 1.3 MgCl₂, and 10 HEPES, pH 7.35 with 1 M TEA OH, were used. In some experiments, the choline CI was replaced with NaCl and 1 μM TTX was added to block Na⁺ conductances. Currents are referred to as Ca²⁺ or Ba²⁺ currents to indicate which recording solution was used. The VGCC antagonists NiCl₂ (100 μM), CdCl₂ (50 μM), nifedipine (10 μM), ωAgA IV (100 nM), and ωCTX GVIA (500 nM) were diluted from concentrated stock solutions and added to small aliquots of the Ba²⁺ recording solution before recording. A Ca²⁺ electrode solution containing (in mM) 130 CsCl, 4 NaCl, 11 EGTA, and 10 HEPES, pH 7.20 with 1 M CsOH and a Tris electrode solution containing (in mM) 90 Tris PO₄, 108 Tris base, 20 TEA CI, 11 EGTA, and 10 sucrose, pH 7.20 with 2 M Tris base were used to provide further block of outward K⁺ conductances. In addition, 2 mM MgATP, 100 μM leupeptin, 100 μM GTP, 10 mM creatine phosphate, and 100 U/ml creatine phosphokinase were added to the electrode solutions to promote the stability of whole cell recordings and to retard rundown of VGCCs (Horn and Korn 1992). ωAgA IV was a generous gift of Pfizer, and ωCTX GVIA was obtained from Alomone Labs; the remaining chemicals were obtained from Sigma or Aldrich.

**Whole cell recording**

Dissociated cells were visualized on an inverted scope (IM-35, Carl Zeiss) with Hoffman modulation contrast optics using ×25, 0.45 N. A. or ×40, 0.65 N. A. objectives. Electrodes were pulled from borosilicate glass capillaries (TW150F-4 glass, World Precision Instruments), fire-polished, and coated with silicone elastomer (Sylgard, Dow Corning) and had a resistance of 3–5 MΩ with either the Cs⁺ or the Tris electrode solution. Before seal formation, a small amount of enzyme-free electrode solution was sucked into the tip of the pipette to facilitate gigohm seal formation. The whole cell recording configuration (Hamill et al. 1981) was obtained in the Na⁺/K⁺ recording solution. Junction potentials for both the Cs⁺ and Tris electrode solutions were measured to be <2 mV in all recording solutions and were not included in any voltage measures. There was a significant increase in series resistance when using the Tris electrode solution (125 ± 1.6 (SE) MΩ in Cs⁺, n = 18; compared with 24.3 ± 1.2 MΩ in Tris, n = 85); however, the Tris solution generally produced more stable recordings and provided a better block of K⁺ currents at depolarized potentials. Recordings in either the Ba²⁺ or Ca²⁺ recording solution were obtained if a rapidly inactivating choline- or TTX-sensitive current was observed on depolarization above ~45 mV in the Na⁺/K⁺ recording solution. Whole cell recordings were obtained using an Axopatch 200 amplifier (Axon Instruments), filtered at 1–50 kHz and digitized at 1–100 kHz with a 12 bit A/D converter (Digidata 1200, Axon Instruments).

**Data analysis**

Currents elicited over a range of voltages were used to assess the voltage-dependent properties of VGCCs. Averages of four current traces were obtained at a given command step, which were presented sequentially in 5- or 10-mV intervals ranging from −150 to +50 mV. The rate at which command steps were presented was dependent on their duration: 10-ms steps were presented every 250 ms, 100-ms steps were presented every 1 s, and 2- to 4-s steps were presented every 10–15 s. Command steps were presented directly from the holding potential or from a prepulse step; tail currents were elicited by repolarizing steps immediately after the command step. Leakage and residual capacitive currents were eliminated by subtracting scaled versions of the average current generated by a sequence of hyperpolarizing command steps. Current-voltage relationships were constructed by plotting the average or peak current magnitudes against corresponding transmembrane voltage values (see APPENDIX) over specified time windows. Time constants were obtained by performing exponential fits of current traces using a Marquardt-Levenberg minimization algorithm. Exponential fits were limited so that the transmembrane voltage did not deviate >5 mV from the steady-state voltage over the duration of the fit window. Although the same command potentials were used, the transmembrane voltage varied between cells, and a uniform voltage scale was needed to average current magnitudes and time constants across experiments. Therefore the measured currents and time constants were interpolated linearly onto a standardized voltage scale before averaging data across experiments.

VGCC subtype-specific antagonists were used to assess the contribution of VGCC subtypes to the overall current. Currents were elicited with 100-ms steps to −10 mV once every 10 s during exposure to various VGCC antagonists. Despite the inclusion of ATP and an ATP-regenerating system in the pipette solution, a slow rundown of VGCCs was observed that could produce time-dependent errors in estimating the effects of a particular antagonist. To minimize the effects of rundown, an exponential was fit to the time course of the current magnitudes before bath infusion of any antagonists (typically 3–5 min), and the resulting fit was extrapolated over the duration of the experiment (Mintz et al. 1992a). Estimates of antagonist sensitivity were obtained by averaging steady-state current magnitudes over
pA (n = 74) in the Ba²⁺ recording solution. When normalized to membrane capacitance, peak Ca²⁺ current densities averaged −22.8 ± 3.0 pA/pF and peak Ba²⁺ current densities averaged −48.7 ± 4.4 pA/pF. Ba²⁺ currents initially activated between −50 and −40 mV, and the largest inward currents were evoked with command steps between −15 and 0 mV (Fig. 1A). After the activation of inward currents with depolarization, large and rapidly deactivating inward tail currents were observed immediately on repolarization after the command step (arrowhead in Fig. 1A). The magnitude of these tail currents increased monotonically with command step voltage and reached maximal amplitudes with command steps between +20 and +35 mV.

The deviation of the transmembrane voltage from the desired command potential complicates the interpretation of voltage-clamp data. During Ba²⁺ current activation, the transmembrane voltage time course is determined by the flow of current across the uncompensated series resistance. Current flow into the pipette due to VGCC activation (Fig. 1A) produces a voltage drop across the finite series resistance, resulting in transmembrane voltages (Fig. 1B, ---) that are positive relative to the command voltage (Fig. 1B, -- -). Although the difference between the command and transmembrane voltage is usually a few millivolts once steady state is attained (0.5–4 mV in Fig. 1B), the delay to steady state can be ≥1 ms, depending on the membrane properties and the magnitude of the Ba²⁺ currents. The voltage errors during Ba²⁺ current deactivation are more pronounced (arrowhead in Fig. 1B; see also Figs. 5B and 6B), partially due to the large magnitude of the tail currents elicited during repolarization. The rapid time course of these tail currents augments these voltage errors: the voltage errors due to current flow across the uncompensated series resistance are superimposed on the errors due to the filtering of the repolarizing step by the uncompensated membrane capacitance. Because the dependence of activating and deactivating Ba²⁺ current magnitudes on the transmembrane voltage is a critical measure of VGCC function, a method for estimating the transmembrane voltage was developed to more accurately assess the biophysical properties of VGCCs (see APPENDIX).

Divalent ion pharmacology

The divalent ion pharmacology of the Ca²⁺ and Ba²⁺ currents recorded from isolated DCN neurons suggests that these inward currents were generated by VGCCs. Peak inward currents recorded with Ca²⁺ (2.5 mM) as the charge carrier were 73.5 ± 3.5% (n = 6) smaller than the corresponding peak inward currents recorded when Ba²⁺ (10 mM) was the charge carrier (Fig. 2A). Adding Ni²⁺ (100 μM) to the Ba²⁺ solution reduced the peak inward current by 55.6 ± 6.5% (n = 6), whereas Cd²⁺ (50 μM) reduced the peak Ba²⁺ current by 98.4 ± 0.4% (n = 4). Although Ca²⁺ is the primary charge carrier through VGCCs in vivo, Ba²⁺ was used in the majority of these experiments. The substitution of Ba²⁺ for Ca²⁺ increased current magnitudes, allowing for a more accurate characterization of VGCC properties in most cells. In addition, Ba²⁺ blocked residual outward currents, providing a more complete isolation of currents carried by VGCCs. However, with the exception of the outward currents in the Ca²⁺ solution at depolarized voltages, the steady-state voltage dependence of the inward currents was not altered when Ba²⁺ replaced Ca²⁺.

FIG. 1. Acutely isolated dorsal cochlear nucleus (DCN) neurons respond to depolarization with sustained inward currents in the absence of voltage-gated Na⁺ and K⁺ currents. A: averages of 4 current traces elicited by 10-ms command steps ranging from −40 mV (smallest current) to −15 mV (largest current) from a holding potential of −60 mV during recording in the Ba²⁺ solution.  , large inward tail currents were evoked during repolarization to −55 mV after the command step. Calibration bar: 2 ms, 400 pA. B: calculated transmembrane voltage traces corresponding to the current traces shown in A. —, transmembrane voltages (Vₜₚ); -- , command potentials (Vc) for comparison. , transmembrane voltages corresponding to the deactivating tail currents shown in A. Noninteger scale is used for the transmembrane voltages (−39 to −11 mV in 5.6-mV steps) to indicate the magnitude of the difference between command potentials and transmembrane voltages at steady state. Parameters used to calculate the transmembrane voltage for this neuron are: Rₑ = 11.7 MΩ; τᵣ = 0.926; τᵩ = 167.5 µs; τᵣ = 481.9 µs; Kₑ = 10.5 MΩ; cₑ = 14.1 pF; τᵩᵣ = 10 µs; p = 0.80; and c = 0.80. Refer to APPENDIX for more details regarding the calculation of transmembrane voltage. Experiments were performed with Tris electrodes.

the last minute of antagonist application and normalizing these averages to the extrapolated exponential fit over the same period. This procedure assumes that the rate of rundown was equal among all VGCC subtypes; this is unlikely to be a valid assumption. If one subtype has a faster rundown rate, its contribution would be underestimated and the contribution of the remaining subtypes would be overestimated.

RESULTS

General properties

The vast majority of isolated DCN neurons respond to depolarization with sustained inward currents in the presence of voltage-gated Na⁺ and K⁺ channel antagonists. During recording in either the Ca²⁺ or the Ba²⁺ solution, depolarizing command steps elicited measurable inward currents in 97 of 103 neurons (Fig. 1A). Peak inward currents averaged −286 ± 35 pA (n = 25) in the Ca²⁺ recording solution and −550 ± 54
as the primary charge carrier (Fig. 2B). Therefore the remaining data were obtained in the Ba\(^{2+}\) recording solution to assess the pharmacological and voltage-dependent properties of VGCCs.

**VGCC subtypes**

The Ba\(^{2+}\) currents recorded from acutely isolated DCN neurons were carried by a variety of VGCC subtypes. The VGCC subtype-specific antagonists nifedipine (10 \(\mu\)M), \(\omega\)AgA IVA (100 nM), and \(\omega\)CTX GVIA (500 nM) were used to assay for the presence of known VGCC subtypes. The L-type antagonist nifedipine produced a consistent reduction in Ba\(^{2+}\) current magnitude in all cells tested (Fig. 3, A–D). The P/Q-type antagonist \(\omega\)AgA IVA blocked a significant portion of VGCCs in some neurons (Fig. 3, A and C) but had little or no effect in others (Fig. 3, B and D). Similarly, the N-type antagonist \(\omega\)CTX GVIA blocked a substantial fraction of the Ba\(^{2+}\) current in some cells (Fig. 3, B and C) and had little or no effect in others (Fig. 3, A and D). In most cells, a portion of the Ba\(^{2+}\) current was resistant to nifedipine, \(\omega\)AgA IVA, and \(\omega\)CTX GVIA but could be blocked by 50 \(\mu\)M Cd\(^{2+}\) (Fig. 3, A–D). Thus the Ba\(^{2+}\) currents in most isolated DCN neurons were carried by three or more known HVA VGCC subtypes.

With the exception of the component insensitive to nifedipine, \(\omega\)AgA IVA, and \(\omega\)CTX GVIA, VGCC subtypes contributing to the overall Ba\(^{2+}\) current appeared to possess similar biophysical properties. Current traces were averaged before and during exposure to a particular antagonist and were subtracted to estimate the portion of the Ba\(^{2+}\) current blocked by that antagonist (Fig. 3, A–D, insets). Although the rundown of VGCCs could confound these results, similarities were observed between difference traces from cells in which rundown was not prominent (Figs. 3, A and C, insets). Measures of activation rates and inactivation extent were obtained from difference currents to quantify the biophysical properties of the individual VGCC subtypes. The average activation time constants were 1.4 \(\pm\) 0.2 ms for the \(\omega\)AgA IVA-sensitive component (n = 10), 1.6 \(\pm\) 0.2 ms for the \(\omega\)CTX GVIA-sensitive component (n = 10), and 1.5 \(\pm\) 0.2 ms for the nifedipine-sensitive component (n = 10). The antagonist-insensitive component had an average activation time constant of 2.7 \(\pm\) 0.4 ms (n = 10) and was significantly slower than the other three components (unpaired Student’s t-test, P < 0.02 for all comparisons). The magnitude of inactivation was 6.8 \(\pm\) 2.0% for the \(\omega\)AgA IVA-sensitive component (n = 11), 6.0 \(\pm\) 1.5% for the \(\omega\)CTX GVIA-sensitive component (n = 12), and 0.1 \(\pm\) 0.1% for the nifedipine-sensitive component (n = 13). The antagonist-insensitive component exhibited 16.5 \(\pm\) 2.9% inactivation (n = 13), which was significantly larger than the other three components (unpaired Student’s t-test, P < 0.02 for all comparisons). The nifedipine-sensitive component also had significantly less inactivation than the \(\omega\)AgA IVA- and \(\omega\)CTX GVIA-sensitive components (unpaired Student’s t-test, P < 0.001 for both comparisons).

The order in which antagonists were applied was varied to minimize the effects of rundown in determining the relative contributions of VGCC subtypes to the overall Ba\(^{2+}\) current. Two different antagonist application sequences were developed under the assumption that the effects of nifedipine, but not those of \(\omega\)AgA IVA or \(\omega\)CTX GVIA, were reversible over the time course of these experiments. The first sequence consisted of applying nifedipine after the peptide toxins \(\omega\)AgA IVA and \(\omega\)CTX GVIA had been applied (Fig. 3A); the second sequence consisted of applying the peptide toxins during a continuous application of nifedipine (Fig. 3, B–D). No statistically significant differences could be detected in the relative proportions of VGCC subtypes observed in cells in which nifedipine was presented before and during presentation of the peptide toxins (Fig. 4A) when compared with cells in which nifedipine was presented after \(\omega\)AgA IVA and \(\omega\)CTX GVIA had been applied (Fig. 4B). Across all experiments, nifedipine...
blocked 32.5 ± 3.6% (n = 13) of the Ba\textsuperscript{2+} current, \(\omega\)Aga IVA blocked 15.5 ± 4.2% (n = 11), and \(\omega\)CTX GVIA blocked 23.2 ± 6.3% (n = 12). Some of the Ba\textsuperscript{2+} current was insensitive to nifedipine, \(\omega\)Aga IVA, and \(\omega\)CTX GVIA but still could be blocked by Cd\textsuperscript{2+} (23.3 ± 4.1%, n = 11), whereas a small fraction of the overall Ba\textsuperscript{2+} current was not blocked by any antagonists, including Cd\textsuperscript{2+} (5.5 ± 1.0%, n = 12).

**Voltage dependence of Ba\textsuperscript{2+} currents**

Open-channel Ba\textsuperscript{2+} current-voltage relationships were constructed by measuring the magnitudes of tail currents elicited on return to various repolarizing voltage steps after the activation of VGCCs with a command step to +50 mV. Under the conditions of these experiments, there are two problems with the interpretation of these data. First, the transmembrane voltage (Fig. 5B, ---) does not follow the repolarizing step potential (Fig. 5B, •••) over the duration of the tail current. Large differences between the repolarizing step potential and the estimated transmembrane voltage could be observed at the tail current peak (estimated transmembrane voltages at the tail current peak are indicated in Fig. 5B, ---). To minimize errors due to the difference between repolarizing and transmembrane voltages, tail current magnitudes (\(I_{\text{tail}}\) in Fig. 5A) were plotted as a function of the estimated transmembrane voltage at the tail current peak (\(V_{\text{tail}}\) in Fig. 5B). Second, tail current peaks are delayed relative to the onset of the repolarizing step (peak times indicated in Fig. 5A, •--•). A significant number of channels could close during the delay to the tail current peak, resulting in an underestimation of the open-channel current (Taylor 1988). Unfortunately, accurate exponential fits of tail current decay could not be obtained to correct for the underestimation of open-channel current magnitudes. The decay of larger tail currents did not follow an exponential time course, presumably due to a changing transmembrane voltage throughout the duration of the tail current (Fig. 5B). Despite the likely underestimation of open-channel currents, large inward open-channel Ba\textsuperscript{2+} currents were observed at hyperpolarized voltages (Fig. 5C). Open-channel Ba\textsuperscript{2+} current magnitudes decreased monotonically with voltage; an inward rectification at depolarized voltages prevented the reversal to an outward current.

Similar to the open-channel Ba\textsuperscript{2+} current, the voltage dependence of steady-state VGCC open probability could be estimated by measuring the voltage dependence of tail current magnitudes. Tail currents were elicited at a repolarizing potential of −80 or −55 mV after the activation of VGCCs with command steps ranging from −100 to +50 mV.
steady-state voltage \( V_{ss} \). Because of variations in the transmembrane voltage at each tail current peak (Fig. 6B), an alternative method for estimating the steady-state open probability was required.

To correct for variations in the transmembrane voltages at each tail current peak, the steady-state VGCC open probability was estimated as a ratio of tail currents elicited at the same

**FIG. 4.** Summary of Ba\(^{2+}\) current responses to the VGCC subtype-specific antagonists oAgA IVA (100 nM), oCTX GVIA (500 nM), and nifedipine (10 \( \mu \text{M} \)). Relative contribution of each VGCC subtype to the overall Ba\(^{2+}\) current was estimated by the reduction in Ba\(^{2+}\) current during the bath infusion of a particular antagonist. Relative contribution of VGCC subtypes resistant to the 3 subtype-specific antagonists used in these experiments was estimated by the reduction of Ba\(^{2+}\) current during exposure to CdCl\(_2\) (50 \( \mu \text{M} \)), after the other antagonists had been applied. Pharmacological presentations were designed under the assumption that the effects of nifedipine, but not those of oAgA IVA or oCTX GVIA, were reversible over the time course of these experiments. Ba\(^{2+}\) current magnitudes (data points in Fig. 3, A–D) were averaged over the last minute of antagonist application and normalized to the extrapolated control period fit (Fig. 3, A–D, - - -) to account for the rundown of VGCCs over the duration of these experiments. A: relative contributions of VGCC subtypes sensitive to nifedipine (37.3 \( \pm \) 3.4%, \( n = 8 \)), oAgA IVA (11.7 \( \pm \) 4.0%, \( n = 6 \)) and oCTX GVIA (18.5 \( \pm \) 6.0%, \( n = 7 \)) from experiments in which nifedipine was presented before and during applications of oAgA IVA and oCTX GVIA (e.g., Fig. 3, B–D). Most of the Ba\(^{2+}\) current resistant to the subtype-specific antagonists was blocked by Cd\(^{2+}\) (25.6 \( \pm \) 3.1%, \( n = 6 \)), whereas a small fraction of the overall Ba\(^{2+}\) current was resistant to all applied antagonists (6.0 \( \pm \) 1.0%, \( n = 7 \)). B: relative contributions of VGCC subtypes sensitive to oAgA IVA (20.1 \( \pm \) 7.9%, \( n = 5 \)), oCTX GVIA (29.7 \( \pm \) 12.8%, \( n = 5 \)) and nifedipine (24.9 \( \pm \) 6.8%, \( n = 5 \)) from experiments in which nifedipine was presented after application of oAgA IVA and oCTX GVIA (e.g., Fig. 3A). Again most of the Ba\(^{2+}\) current resistant to the subtype-specific antagonists was blocked by Cd\(^{2+}\) (20.5 \( \pm \) 8.7%, \( n = 5 \)), and a small fraction of the overall Ba\(^{2+}\) current was resistant to all applied antagonists (4.8 \( \pm \) 1.9%, \( n = 5 \)). Experiments were performed using Tris electrodes.

**FIG. 5.** Voltage dependence of open-channel Ba\(^{2+}\) currents. A: averages of 4 Ba\(^{2+}\) tail currents elicited by repolarizing steps ranging from \(-110\) mV (largest tail current) to \(-20\) mV (smallest tail current) after a 5-ms command step to \(+50\) mV. Residual capacitive transients at the offset of the command step have been blanked for clarity. ---, times of tail current peaks; the magnitudes of these peaks (\( I_{tail} \)) were used to construct the open-channel Ba\(^{2+}\) current-voltage relationship shown in C. Calibration bar: 0.3 ms, 2 nA. B: transmembrane voltage traces corresponding to the current traces shown in A. ---, transmembrane voltages; --, command potentials for comparison; - - -, indicates times of the tail current peaks shown in A; transmembrane voltage at the tail current peaks (\( V_{tail} \)) were used to construct the open-channel Ba\(^{2+}\) current-voltage relationship shown in C. Parameters used to calculate the transmembrane voltage for this neuron are: \( R_e = 13.0 \text{ M\Omega}; A_f = 0.924; \tau_f = 200.2 \mu\text{s}; \tau_v = 665.9 \mu\text{s}; R_p = 12.5 \text{ M\Omega}; C_{m,t} = 11.2 \text{ pF}; \tau_{fast} = 10 \mu\text{s}; p = 0.70; c = 0.70; C: voltage dependence of open-channel Ba\(^{2+}\) currents. Averages of 4 tail current traces were elicited by repolarizing potentials ranging from \(-150\) to \(+45\) mV after the activation of VGCCs with a 5-ms command step to \(+50\) mV. Open-channel current-voltage relationships were constructed by plotting the magnitudes of tail currents elicited during the repolarizing step (\( I_{tail} \)) in A as a function of the transmembrane voltage at the tail current peak (\( V_{tail} \)) in B. Open-channel currents were normalized to their magnitude at 0 mV and averaged across 6 cells. Although the same repolarizing voltage steps were used in each experiment, the transmembrane voltage at tail current peaks varied between cells. Therefore tail current magnitudes were interpolated linearly onto a standardized voltage base before averaging across cells. Experiments were performed with Tris electrodes.
transmembrane voltage. Assuming that the VGCC open probability is maximal at +50 mV, the steady-state open probability $P_o(V_{ss})$ was calculated by comparing the magnitude of a tail current elicited after a step to $V_{ss}$ to the magnitude of a tail elicited after a step to +50 mV

$$P_o(V_{ss}) = \frac{I_{tail}(V_{ss}, V_{tail})}{I_{tail}(+50 \text{ mV}, V_{tail})}$$

$I_{tail}(V_{ss}, V_{tail})$ is the magnitude of the tail current ($I_{tail}$ in Fig. 6A) elicited by the transition from the steady-state transmembrane voltage ($V_{ss}$ in Fig. 6B) to the transmembrane voltage measured at the tail current peak ($V_{tail}$ in Fig. 6B). $I_{tail}(+50 \text{ mV}, V_{tail})$, obtained from the open-channel Ba$^{2+}$ current data in Fig. 5, is the magnitude of a tail current ($I_{tail}$ in Fig. 5A) evoked by the transition from +50 mV to the same transmembrane voltage at the tail current peak ($V_{tail}$ in Fig. 5B). The resulting steady-state open probability-voltage relationship (Fig. 6C) shows that a large depolarization was required to activate VGCCs: <5% of maximal activation was achieved at ~40 mV. However, VGCCs were steeply voltage dependent once activated: 80% of the increase in open probability was achieved at 20 mV. However, VGCCs were steeply voltage dependent once activated: 80% of the increase in open probability was achieved at 20 mV.
The voltage dependence of steady-state Ba$^{2+}$ current magnitudes was described accurately by a product of the open-channel Ba$^{2+}$ current and the steady-state open probability. Steady-state current-voltage relationships were constructed by plotting the average current during the last 1 ms of a 10-ms command step ($I_{ss}$ in Fig. 6A) against the average transmembrane voltage over the same period ($V_{ss}$ in Fig. 6B). Measurable inward currents initially were observed at transmembrane voltages above $-50$ mV, and the largest inward currents were obtained around $-8$ mV (Fig. 6D, ○). Outward currents were sometimes observed around or above $+50$ mV; however, the lack of intracellular Ba$^{2+}$ requires that any outward currents be carried by other ions present in the intracellular solution. Steady-state Ba$^{2+}$ current magnitudes were reconstructed as a product of the steady-state open probability (Fig. 6C) and open-channel Ba$^{2+}$ current (Fig. 5C) to confirm the accuracy of these estimates (Fig. 6D, —). The reconstructed current magnitudes compare favorably to the measured values over a wide voltage range. Differences between the measured and reconstructed values at hyperpolarized voltages can be attributed to slightly positive steady-state open probability values and large inward open-channel current magnitudes over this voltage range.

Ba$^{2+}$ currents activated rapidly over the range of voltages at which these currents could be elicited. The time course of VGCC activation in response to a voltage step will contain multiple components that can be described by a sum of exponentials. However, an imperfect voltage clamp can prevent the accurate measurement of these components: if there is any uncompensated series resistance, changes in current magnitude will produce a corresponding change in transmembrane voltage. Because the time course of VGCC activation is voltage dependent, changes in transmembrane voltage subsequently will alter the current time course. Rather than extending over the entire duration of the 10-ms command step, exponential fits were limited so that the transmembrane voltage at the beginning of the fit did not deviate $>5$ mV from steady-state values (Fig. 7A, thick lines). This procedure precludes the use of higher-order kinetic models: only the slowest components of VGCC activation could be measured; faster components would have decayed before the beginning of the fit window. The time constants from these exponential fits had a bell-shaped voltage dependence, with the slowest time constants occurring around $-20$ mV, and the fastest values occurring at the most depolarized voltages (Fig. 7B). It is not possible to correct for the filtering of membrane currents by any remaining uncompensated membrane time constant (Sigworth 1983), which can distort the time course of faster currents (Armstrong and Gilly 1992). It is likely that the time course of VGCC activation is affected by this filtering and would occur more rapidly under an ideal voltage clamp.

On longer time scales, a slower component of VGCC activation could be observed in some Ba$^{2+}$ current traces. This slow activation sometimes was present during the first 100 ms of the 2- to 4-s command steps used to investigate the time course of inactivation (Fig. 8B, △). In addition, a slowly developing inward current could be elicited by 100-ms command steps (αCTX-sensitive difference traces in Fig. 3, B and C; nifedipine-sensitive difference traces in Fig. 3, B and D). In most cases, a slowly decaying tail current was associated with the slowly developing inward current. This slow component of VGCC activation and deactivation may correspond to the "mode 2" gating augmented by dihydropyridine agonists during single-channel recordings of L-type VGCCs (Tsien et al. 1986). However, the conditions required to evoke this slow component were not investigated in this study, and it is not clear if this component would be prominent under physiological conditions.

A slow inactivation was observed at depolarized voltages, suggesting that a majority of VGCCs recorded from acutely isolated DCN neurons were of the HVA subtype. Unlike LVA VGCCs, the inward current did not appear to have any significant low-threshold inactivation. No significant changes in the peak current magnitude elicited by a 100-ms command step to 0 mV were observed with 50-ms precommand steps to either $-100$ or $-40$ mV from the holding potential of $-70$ mV (Fig. 8A). Across experiments, 50-ms precommand steps to $-100$ mV resulted in peak current magnitudes that were 99.6 ± 2.0% ($n = 8$) of the peak current magnitudes elicited directly from the holding potential of $-70$ mV; and 50-ms precommand steps to $-40$ mV resulted in peak current magnitudes that were...
CA\textsuperscript{2+} CHANNELS IN THE DCN

The results of this study show that the majority of neurons isolated from guinea pig DCN possess VGCCs. There are many neuronal populations residing within the guinea pig DCN, and our results indicate that a variety of VGCC subtypes are present in these neurons. Given these conditions, it is reasonable to expect VGCCs to exhibit a diverse range of biophysical properties, and the apparent uniformity of VGCC properties in isolated DCN neurons is an intriguing result. In all neurons from which Ba\textsuperscript{2+} currents were observed, VGCCs had similar steady-state voltage dependence, activated and deactivated rapidly, and exhibited no voltage-dependent inactivation on physiologically relevant time scales. Regardless of subtype, the VGCCs present in isolated DCN neurons are poised to produce a rapid Ca\textsuperscript{2+} influx in response to a large depolarization.

The use of the isolated cell preparation is advantageous for the characterization of voltage-gated conductances in adult mammalian neurons (Kay and Wong 1986); however, the acute isolation procedure poses some potential problems for the interpretation of the results obtained in the present study. First, the trauma resulting from the enzymatic and mechanical dispersion of neuronal tissue could alter the expression of proteins contributing to the electrophysiologic properties of neurons, resulting in patterns of conductances that would not be observed in recordings from intact preparations. Acutely isolated ventral cochlear nucleus neurons produce responses to depolarizing current injection that are similar to responses obtained from in vitro slice preparations (Manis and Marx 1991), indicating that these neurons do not express different sets of biophysical properties, and the apparent uniformity of VGCC properties in isolated DCN neurons is an intriguing result. In all neurons from which Ba\textsuperscript{2+} currents were observed, VGCCs had similar steady-state voltage dependence, activated and deactivated rapidly, and exhibited no voltage-dependent inactivation on physiologically relevant time scales. Regardless of subtype, the VGCCs present in isolated DCN neurons are poised to produce a rapid Ca\textsuperscript{2+} influx in response to a large depolarization.

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conductances as a result of the acute isolation procedure. Second, the acute isolation procedure may modify the properties of existing membrane proteins, including voltage-gated conductances. Trypsin, the enzyme used in the dissociation procedure, is known to inactivate N-methyl-D-aspartate receptors (Allen et al. 1988). However, the use of trypsin in previous studies did not appear to adversely affect VGCCs (Kay and Wong 1987; Thompson and Wong 1991). In addition, the biophysical and pharmacological properties of VGCCs in the present study are similar to those found in neurons dissociated without the use of trypsin (Regan 1991; Zidanic and Fuchs 1995). Therefore the use of the acute dissociation procedure is not likely to adversely affect VGCCs in the present study.

VGCC subtypes

Acutely isolated guinea pig DCN neurons possess a variety of HVA VGCC subtypes. The divalent ion pharmacology is more typical of HVA rather than LVA VGCCs: unlike their LVA counterparts, HVA VGCCs are usually more selective for Ba\(^{2+}\) than for Ca\(^{2+}\) and are blocked more potently by Cd\(^{2+}\) than by Ni\(^{2+}\) (Bean 1989; Huguenard 1996). Responses to subtype-specific antagonists also confirmed the presence of HVA VGCCs. All neurons tested contained a significant L-type component; only some neurons contained N- and P/Q-type components, and these appeared in varying amounts. The observation of a consistent nifedipine-sensitive component and variable \(\alpha\)Aga IVA- and \(\alpha\)CTX GVIA-sensitive components could be explained by the differential localization of VGCC subtypes within a single cell. In hippocampal pyramidal cells, L-type VGCCs cluster in the proximal dendrites (Westenbroek et al. 1990); whereas N-type VGCCs are distributed over the entire dendritic arbor (Westenbroek et al. 1992). Because the acute cell isolation procedure produces neurons that only possess a cell body and proximal processes, it is expected that such a distribution of VGCCs would result in a consistent nifedipine-sensitive component, and a reduced, if not erratic, \(\alpha\)CTX GVIA-sensitive component.

Most neurons possess a significant inward current that remains in the presence of nifedipine after exposure to \(\alpha\)Aga IVA and \(\alpha\)CTX GVIA or during a simultaneous exposure to all

FIG. 9. Voltage-dependent properties of nifedipine-sensitive and insensitive currents. Data in A–D are measures obtained from nifedipine-sensitive (○) and nifedipine-insensitive (□) currents from an individual cell. Averages of 4 Ba\(^{2+}\) currents were obtained before and during a 5-min exposure to nifedipine (10 \(\mu\)M); nifedipine-sensitive currents were estimated subsequently as the difference between the overall Ba\(^{2+}\) and nifedipine-insensitive currents. Rather than directly subtracting 2 sets of current traces elicited by the same series of command steps, nifedipine-insensitive currents were interpolated linearly onto the transmembrane voltages associated with the overall Ba\(^{2+}\) currents before subtraction. A: voltage dependence of open-channel Ba\(^{2+}\) currents. Averages of 4 tail currents elicited by the transition to repolarizing potentials ranging from \(-150\) to \(+45\) mV after a 5-ms command step to \(+50\) mV were obtained before and during exposure to nifedipine. Open-channel Ba\(^{2+}\) current-voltage relationships were constructed as described in Fig. 5C. B: voltage dependence of steady-state VGCC open probability. Averages of 4 tail currents elicited by the transition to a repolarizing voltage of \(-80\) mV after 10-ms command steps ranging from \(-100\) to \(+50\) mV were obtained before and during exposure to nifedipine. Steady-state open-probability voltage relationships were constructed as described in Fig. 6C. C: voltage dependence of steady-state Ba\(^{2+}\) current magnitudes. Averages of 4 activating currents elicited by 10-ms command steps ranging from \(-100\) to \(+50\) mV from a holding potential of \(-60\) mV were obtained before and during exposure to nifedipine. Steady-state current-voltage relationships were constructed as described in Fig. 6D. D: voltage dependence of the time course of Ba\(^{2+}\) current activation. Exponential fits of the activating currents used in C were performed as described in Fig. 7B. Time constants from the nifedipine-insensitive current data (□) are missing at more depolarized voltage levels because the current amplitudes over the specified fit windows were too small to generate accurate values. Experiments were performed using Tris electrodes.

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three antagonists. The majority of this remaining inward current is blocked by 50 µM Cd^{2+} and may correspond to R-type VGCCs (Ellinor et al. 1993; Zhang et al. 1993). In the present study, the antagonist-insensitive component exhibits a slower activation and a more pronounced inactivation than the L-, N-, and P/Q-type components. This is consistent with the voltage-dependent properties of R-type VGCCs, which exhibit many similarities to LVA VGCCs (Bourinet et al. 1996; Ellinor et al. 1993; Soong et al. 1993). It is possible that this antagonist-insensitive component could consist of LVA VGCCs as well; however, the high sensitivity of residual currents to Cd^{2+} and the insensitivity of the overall current to a hyperpolarized prepulse voltage suggest that these channels belong to the HVA family. It is also possible that Q-type VGCCs could contribute to this antagonist-insensitive component. Although the concentrations of the antagonists used should provide a complete block of L-, N-, and P-type VGCCs, the concentration of α-Aga IVA used in these experiments was only slightly greater than the IC_{50} for Q-type channels (Randall and Tsien 1995). In some neurons, it is possible that additional α-Aga IVA would have blocked a larger portion of the antagonist-insensitive current by providing a more complete block of Q-type channels. However, several neurons exhibited little or no response to 100 nM α-Aga IVA, and it is unlikely that additional α-Aga IVA would have provided a further block of the remaining current in these cells.

The results of the study do not provide any information about the distribution of VGCCs throughout the DCN. Various cell populations reside within the guinea pig DCN (Hackney et al. 1990), and the use of the isolated cell preparation does not permit positive morphological identification of cell types. Without morphological identification, there is no way to determine whether the various VGCC subtypes observed in the present study are distributed differentially among the individual DCN cell populations. In addition, the results of this study do not provide any information about the distribution of VGCC subtypes throughout the dendritic arbors of the various cell types. A VGCC-mediated Ca^{2+} influx into the dendrites of DCN cartwheel and pyramidal cells can be elicited by somatic action potentials (Manis and Molitor 1996; Molitor and Manis 1996); however, these dendritic processes inevitably are destroyed by the cell-isolation procedure. Recordings from hippocampal pyramidal and cerebellar Purkinje neurons have shown that the relative levels of VGCC subtypes differed between the soma and the dendrites of these neurons (Kavalali et al. 1997; Mouginot et al. 1997). Therefore it is possible that the dendrites of DCN neurons possess VGCCs that differ from those characterized in this study.

**Voltage-dependent properties**

The voltage-dependent properties of VGCCs suggest that these conductances are suited to respond to action potentials. A large depolarization from rest is required to activate VGCCs in isolated DCN neurons: <10% of maximal activation is achieved below −40 mV, whereas >90% of maximal activation is achieved above +10 mV. An action potential would maximally activate VGCCs, whereas a subthreshold synaptic depolarization would result in little or no VGCC activation. In addition, the activation time course is rapid enough at depolarized voltages to reach steady-state levels within the duration of an action potential. The lack of any low-threshold inactivation should prevent previous neuronal activity from altering the response of VGCCs to action potentials; the sustained depolarization required to produce any voltage-dependent inactivation is not likely to occur during periods of normal neuronal activity. However, VGCCs are known to inactivate due to elevated internal Ca^{2+} (de Leon et al. 1995; Imredy and Yue 1994). It is not clear whether elevated intracellular Ca^{2+} inactivates VGCCs in DCN neurons; the use of Ba^{2+} as the charge carrier minimized this effect so that voltage-dependent inactivation could be investigated in isolation.

Once activated, VGCCs are capable of producing a large Ca^{2+} influx. An asymmetric distribution of Ba^{2+} in these experiments (10 mM Ba^{2+} outside, 11 mM EGTA inside) results in large inward currents at hyperpolarized voltages and produces an inward rectification at depolarized voltages, which prevents the reversal to an outward current. A similar asymmetric Ca^{2+} distribution exists in vivo, and the voltage dependence of the Ca^{2+} influx should exhibit similar properties under physiological conditions. The voltage dependence of open-channel currents suggests that the bulk of the Ca^{2+} influx will occur after the action potential peak during the repolarizing phase before VGCCs deactivate in response to hyperpolarized voltage levels (Llinàs et al. 1981). However, imaging studies at the parallel fiber-stellate cell synapse in the rat cerebellum indicate that the Ca^{2+} influx in presynaptic terminals becomes more prominent during the rising phase of parallel fiber-mediated action potentials at physiological temperatures (Sabatini and Regehr 1996). It is possible that VGCCs in DCN neurons behave in a similar fashion at physiological temperatures, resulting in a Ca^{2+} influx that occurs more rapidly with respect to action potential initiation.

**Functional implications**

VGCCs may be involved in producing different discharge patterns in DCN neurons. VGCCs are likely to be involved in the evoked responses of cartwheel cells, which are a major population of inhibitory interneurons in the superficial DCN. Cartwheel cells respond to depolarizing current injection with a burst of fast action potentials superimposed on a slow depolarization that has been attributed to VGCCs (Agar et al. 1996; Manis et al. 1994; Zhang and Oertel 1993). In contrast, VGCCs may contribute indirectly to the evoked responses of pyramidal cells, which form the main projection pathway from the DCN to the inferior colliculus. Pyramidal cells are only capable of generating Ca^{2+}-dependent action potentials in the presence of voltage-gated Na^{+} and K^{+} conductance antagonists (Hirsch and Oertel 1988), and models incorporating only voltage-gated Na^{+} and K^{+} conductances are capable of reproducing many pyramidal cell responses to intracellular current injection (Hewitt and Meddis 1995; Kim et al. 1994). However, a slow afterhyperpolarization observed in pyramidal cells after a sustained discharge of action potentials (Hirsch and Oertel 1988; Manis 1990) may be attributed to Ca^{2+}-activated K^{+} conductances, which would be activated by the Ca^{2+} influx through VGCCs. Thus VGCCs may play different roles in determining the patterns of evoked responses across neuronal populations of the DCN.

VGCCs are also capable of regulating neuronal responses through the actions of Ca^{2+} as an intracellular second messen-
ger. Despite the differential involvement of VGCCs in the generation of evoked responses, somatically evoked action potentials result in a VGCC-mediated Ca\textsuperscript{2+} influx into the soma and proximal dendrites of both pyramidal and cartwheel cells (Manis and Molitor 1996; Molitor and Manis 1996). Because Ca\textsuperscript{2+} is an important intracellular second messenger (Tsien and Tsien 1990), an evoked Ca\textsuperscript{2+} influx through VGCCs could play a role in regulating neuronal responses. Ca\textsuperscript{2+} can interact with and regulate other signaling pathways in the DCN, such as protein kinase C, which regulates the strength of synaptic transmission at parallel fiber synapses (Francis and Manis 1995; Scott and Manis 1992); and intracellular Ca\textsuperscript{2+} release through inositol 1,4,5-trisphosphate receptors (Mignery et al. 1989; Ryugo et al. 1995), which may be activated through metabotropic glutamate receptors present at parallel fiber and auditory nerve synapses (Molitor and Manis 1997). A Ca\textsuperscript{2+} influx through VGCCs could act on many different time scales, from the short-term activation of ionic conductances (such as Ca\textsuperscript{2+}-activated K\textsuperscript{+} conductances), to the long-term regulation of synaptic responses. Regardless of the contribution of VGCCs to discharge patterns, an influx of Ca\textsuperscript{2+} through VGCCs may be an important indicator of neuronal activity and may have significant implications for information processing in this nucleus.

APPENDIX

The study of voltage-dependent conductances requires the accurate control of transmembrane voltage. In many circumstances, the single-electrode, whole cell voltage-clamp technique can be used to provide this control. However, the experimental conditions in the present study resulted in deviations of the transmembrane voltage from the presented command potential. This discrepancy arises from two sources: the voltage drop due to the flow of ionic current across a finite series resistance and the filtering of the command waveform by the membrane capacitance and series resistance. Therefore accurate estimates of the transmembrane voltage are needed to assess the voltage dependence of the Ba\textsuperscript{2+} currents presented in this study.

A method for calculating the transmembrane voltage can be derived from a simple circuit used to model the isolated neurons in these experiments. The cell is modeled as a membrane resistance \( R_m \), in parallel with a membrane capacitance \( C_m \) and a current source \( I_m(t) \); an additional resistance \( R_e \) is added in series to represent the access between the pipette tip and cell interior. Under the conditions of these experiments, it is assumed that \( R_m \gg R_e \), which effectively eliminates \( R_e \) and reduces the model circuit to \( R_m \) in series with \( C_m \) and \( I_m(t) \). The solution to the differential equation governing this reduced circuit can be expressed in the Laplace domain as a function of complex frequency (s)

\[
V_m(s) = \frac{V_c(s) - RI_m(s)}{1 + s\tau_m} \tag{A1}
\]

where \( V_m(s) \) is the transmembrane voltage, \( V_c(s) \) is the command potential, \( I_m(s) \) is the sum of all ionic currents flowing across the membrane, and \( \tau_m \) is the membrane time constant, which is a product of \( R_m \) and \( C_m \). The physical interpretation of Eq. A1 is that \( V_m(t) \) can be obtained by filtering the difference \( V_c(t) - RI_m(t) \) with a single-pole low-pass filter having a \(-3\) dB cutoff frequency of \(1/2\pi\tau_m\). In practice, the transmembrane voltage is calculated by convolving \( V_c(t) - RI_m(t) \) with the impulse response of the membrane filter, which is a decaying exponential with amplitude 1/\( \tau_m \) and time constant \( \tau_m \).

The properties of the membrane filter must be known to calculate the transmembrane voltage. The values of \( R_m \) and \( \tau_m \) can be obtained from currents elicited by command steps that do not activate ionic currents. In the absence of any on-line amplifier compensation or off-line capacitive current subtraction, the response of the model circuit to a hyperpolarizing command step of magnitude \( \Delta V \) will be a decaying exponential with amplitude \( \Delta V/R_m \) and time constant \( \tau_m \). For the majority of neurons used in the present study, the capacitive currents evoked by hyperpolarizing command steps were more accurately described by a sum of two exponentials

\[
I_m(t) = \frac{\Delta V}{R_m} [A_1 \exp(-t/\tau_1) + (1 - A_1) \exp(-t/\tau_2)] \tag{A2}
\]

where \( 0 \leq A_1 \leq 1 \) and \( \tau_1 < \tau_2 \). For these neurons, the impulse response of the membrane filter is also a sum of two exponentials, the first with amplitude \( A_1/\tau_1 \) and time constant \( \tau_1 \) and the second with amplitude \( (1 - A_1)/\tau_2 \) and time constant \( \tau_2 \). A biexponential capacitive current suggests that the isolated neurons in these experiments did not have a uniform transmembrane voltage and were more accurately modeled as two isopotential compartments joined in series with a finite resistance. However, typical values for the amplitudes \( A_1 > 0.9 \) and time constants \( \tau_1 \approx 3 \cdot \tau_2 \) of capacitive currents suggest that in most cells this second compartment possessed only a small fraction of the total membrane capacitance and, hence, a small fraction of the total membrane surface. Therefore it is reasonable to assume that the majority of VGCCs contributing to the overall Ba\textsuperscript{2+} current in the present study reside in one compartment and are responding to a uniform transmembrane voltage.

The true command potential also must be known to calculate the transmembrane voltage. Many voltage-clamp amplifiers provide compensation circuitry that use estimates of \( R_m \) and \( C_m \) to modify the command potential so that the transmembrane voltage will more faithfully replicate the desired command potential. The Axopatch 200 amplifier used in these experiments provides two separate compensation pathways by which the command potential is modified: prediction and correction compensation. Prediction compensation reduces low-pass filtering of the membrane by boosting the high-frequency components of the command potential

\[
V_c^\prime(s) = V_c(s) \frac{1 + s\tau_p}{1 + s\tau_s(1 - \rho)} \tag{A3}
\]

where \( \tau_p \) is the prediction compensation time constant, which is a product of the series resistance \( R_{sc} \) and membrane capacitance \( C_{mc} \) amplifier settings; and \( \rho \) is the fraction of prediction compensation (%/100) amplifier setting. If the amplifier settings are such that \( \tau_p \approx \tau_m \), the prediction compensation circuitry effectively reduces the membrane time constant from \( \tau_m \) to \( \tau_p \) \((1 - \rho)\). Correction compensation reduces the voltage drop due to the flow of ionic current across a finite series resistance by adding a scaled and filtered version of this current to the command potential

\[
V_{c,e}(s) = V_c(s) + cR_{sc} \frac{I_m(s)}{1 + s\tau_{sc}} \tag{A4}
\]

where \( \tau_{sc} \) is the lag filter time constant and c is the fraction of correction compensation (%/100) amplifier setting. If \( I_m(t) \) is not significantly attenuated by the lag filter, the correction compensation circuitry effectively reduces the series resistance from \( R_m \) to \( R_m - cR_{sc} \). The modifications to the command voltage by the prediction and correction compensation superimpose, so that the modified command potential \( V_c^\prime(t) \) is obtained by filtering the original command potential \( V_c(t) \) with the prediction compensation circuitry (Eq. A3), and adding the lag-filtered product of \( cR_{sc} \cdot I_m(t) \) (Eq. A4). The difference between the modified command potential \( V_{c,e}(t) \) and the product \( R_m \cdot I_m(t) \) then can be attenuated by the membrane filter to obtain the transmembrane voltage \( V_m(t) \) (Eq. A1).
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