Activation of Metabotropic Glutamate Receptors Inhibits High-Voltage-Gated Calcium Channel Currents of Chicken Nucleus Magnocellularis Neurons

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Lu, Yong and Edwin W Rubel. Activation of metabotropic glutamate receptors inhibits high-voltage-gated calcium channel currents of chicken nucleus magnocellularis neurons. J Neurophysiol 93: 1418–1428, 2005. First published September 15, 2004; doi:10.1152/jn.00659.2004. Using whole cell patch-clamp recordings, we pharmacologically characterized the voltage-gated Ca2+ channel (VGCC) currents of chicken nucleus magnocellularis (NM) neurons using barium as the charge carrier. NM neurons possessed both low- and high-voltage-activated Ca2+ channel currents (HVA $I_{Na^{+}}$). The N-type channel blocker ($\omega$-conotoxin-GVIA) inhibited more than half of the total HVA $I_{Na^{+}}$, whereas blockers of L- and P/Q-type channels each inhibited a small fraction of the current. Metabotropic glutamate receptor (mGluR)-mediated modulation of the HVA $I_{Na^{+}}$ was examined by bath application of glutamate (100 μM), which inhibited the HVA $I_{Na^{+}}$ by an average of 16%. The inhibitory effect was dose dependent and was partially blocked by $\omega$-conotoxin-GVIA, indicating that mGluRs modulate N and other type HVA $I_{Na^{+}}$. The nonselective mGluR agonist, (1S,3R)-1-amino-3-cyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), mimicked the inhibitory effect of glutamate on HVA $I_{Na^{+}}$. Group I–III mGluR agonists showed inhibition of the HVA current with the most potent being the group III agonist ((+)-2-amino-4-phosphonobutyric acid) 1S,3R-ACPD (200 μM) had no effect on K+ or Na+ currents. The firing properties of NM neurons were also not altered by 1S,3R-ACPD. We propose that the inhibition of VGCC currents by mGluRs limits depolarization-induced Ca2+ entry into these highly active NM neurons and regulates their Ca2+ homeostasis.

INTRODUCTION

Neurons vary widely their activity patterns. Primary and secondary neurons in the auditory and vestibular systems are characterized by unusually high rates of sustained activity modulated by brief bursts of even higher spike rates to signal changes in environmental events (Born et al. 1991; Koppl 1997; Rubel and Parks 1975; Rubsamen et al. 1995; Stopp and Whitfield 1961; Sullivan and Konishi 1984; Warchol and Dallos 1990). Some motor neurons share these characteristics. Other neurons appear to have relatively low rates of action potential generation. This diversity correlates with great diversity in intracellular homeostatic and signaling mechanisms, particularly those involving Ca2+ homeostasis (Lips and Keller 1999; Vansonel and Keller 2000).

Nucleus magnocellularis (NM) neurons, the secondary afferent neurons in the avian central auditory pathways, are homologous to the bushy cells of the mammalian cochlear nucleus. NM neurons receive an excitatory glutamatergic input from the auditory nerve and inhibitory GABAergic inputs primarily from the superior olivary nucleus (reviewed in Rubel and Fritzsch 2002; Rubel et al. 2004). NM neurons are unusually active, showing unstimulated firing rates between 50 and 100 Hz and rates of 250 Hz or more in response to acoustic stimulation. Ionotropic glutamate receptors (iGluRs) in NM are specialized for fast and temporally precise firing properties, whereas metabotropic glutamate receptors (mGluRs) appear critical for Ca2+ homeostasis (Zirpel and Rubel 1996; Zirpel et al. 1995, 1998).

Ca2+ homeostasis is critically important for cellular survival, and very active neurons appear to have mechanisms for maintaining Ca2+ homeostasis that are different or more abundant than found in other neuron types (Lips and Keller 1999; Vansonel and Keller 2000; Yu et al. 2001; Zirpel et al. 2000). Elimination of the excitatory input to NM in young chicks results in death of ~30% of NM neurons and atrophy of remaining neurons (reviewed in Rubel and Fritzsch 2002). One of the early deafferentation-induced changes in NM neurons is a threefold increase in [Ca2+]i and prevents early metabolic degenerative changes (reviewed in Rubel and Fritzsch 2002). This unique role of mGluRs appears to allow NM neurons to avoid excitotoxicity under normal conditions, while processing very high rates of information flow.

Modulation of Ca2+ influx via voltage-gated Ca2+ channels (VGCCs) by mGluRs is one of the processes described in NM neurons that is hypothesized to play a role in the normal [Ca2+]i regulation. Using Ca2+ imaging techniques, Lachica et al. (1995) showed that activation of mGluRs inhibits KCl-induced Ca2+ influx presumably through VGCCs in NM neurons. However, mGluR-mediated modulation of VGCC currents in NM neurons has not been investigated nor do we understand which group(s) mGluRs mediate the modulation and whether other voltage-gated conductances and firing properties of NM neurons are affected by mGluRs.

In the present series of studies, we use whole cell recording techniques to examine the role of mGluR-mediated signaling pathways in Ca2+ regulation by NM neurons. Electrophysiological studies indicate that NM neurons possess both low-voltage-activated (LVA) and high-voltage-activated (HVA) Ca2+ channel currents ($I_{Na^{+}}$) and N-type channels are the...

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dominant HVA VGCCs; activation of mGluRs by glutamate or 1S,3R-ACP in inhibits HVA $I_{Na^{+}}$; the modulation seems involving groups I–III mGluRs; and voltage-gated Na$^{+}$ channel currents ($I_{Na^{+}}$), voltage-gated K$^{+}$ channel currents ($I_{K^{+}}$), and firing properties of NM neurons are not altered by mGluR activation. We propose that activation of mGluRs mediates a net reduction of Ca$^{2+}$ influx through VGCCs without affecting firing properties of NM neurons, and this mGluR-mediated inhibition of VGCC currents may be one mechanism whereby NM neurons are protected from Ca$^{2+}$ overload-induced excitotoxicity. An abstract of this work has appeared (Lu and Rubel 2002).

**METHODS**

**Slice preparation**

White Leghorn chicken (*Gallus domesticus*) embryos (E17–E20) were used. After rapid decapitation, a thick coronal block containing the entire brain stem was dissected free into artificial cerebrospinal fluid (ACSF). The components of ACSF were (in mM) 130 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 26 NaHCO$_3$, 1 MgCl$_2$, 10 glucose, and 2 CaCl$_2$. The pH value of aerated (95% O$_2$, 5% CO$_2$) ACSF was maintained at 7.4 and osmolality was 280–310 mosM/l. Five to seven transverse slices (200 μm in thickness) containing bilateral NM were cut by using a vibratome (Technical Products International, St. Louis, MO). Slices were then equilibrated in oxygenated ACSF at 33–36°C for 40–60 min. For recordings, slices were transferred to a 0.5-ml chamber mounted on a Zeiss Axioskop FS (Zeiss, Germany) with a ×40 water-immersion objective and infrared differential interference contrast (IR-DIC) optics and continuously superfused with room temperature (22–23°C) ACSF at a rate of 1–2 ml/min. Only one cell from each slice was studied when drugs with irreversible effects were used.

**Electrophysiology**

Voltage-clamp experiments were performed with an Axopatch 200B and current clamp experiments with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Whole cell Ca$^{2+}$ channel currents were recorded using barium as the charge carrier ($I_{Ba^{2+}}$). The external solution for recording whole cell $I_{Ba^{2+}}$ consisted of (in mM) 115 TEA-Cl, 10 BaCl$_2$, 1 MgCl$_2$, 5 NaCl, 3 CsCl, 10 HEPES, and 10 glucose, with pH 7.4 adjusted by TEA-OH and osmolarity of 295 mosM/l. The external solution was gassed with 100% oxygen. The junction potential was 4 mV and was not corrected in data analyses. Cells were held at –60 mV and a depolarizing voltage command to –30 mV was applied to activate $I_{K^{+}}$. To record voltage-gated K$^{+}$ channel currents ($I_{K^{+}}$), the recording electrodes were filled with (in mM) 105 K-gluconate, 35 KCl, 5 EGTA, 10 HEPES(K), and 1 MgCl$_2$, with pH7.2 adjusted with KOH and osmolality of 285 mosM/l. In ACSF, TTX (1 μM) and CdCl$_2$ (400 μM) were included to block $I_{Na^{+}}$ and $I_{Ca^{2+}}$. The junction potential was 11 mV and was not corrected in data analyses. Cells were held at –60 mV and a depolarizing voltage command to +10 mV was applied to activate $I_{K^{+}}$. Current-clamp experiments were done using the same internal solution as in $I_{K^{+}}$ recordings.

**Data analysis**

The peak values of $I_{Ba^{2+}}$ during the voltage pulses were measured. For each neuron, the leak current was determined by either of the two following methods: an equation describing the best fit linear current-voltage (I-V) relation was obtained by stepping to a voltage 20 mV more hyperpolarized than the holding potential (–50 mV) in 5-mV steps, and then the equation was used to calculate the leak current for a specific voltage command (the same method was also used to perform leak subtraction for $I_{K^{+}}$ and $I_{Ca^{2+}}$ except that the holding potential for $I_{K^{+}}$ and $I_{Ca^{2+}}$ was –60 mV); or second, leak current was considered as the residual current after applying CdCl$_2$ (400 μM) to block all HVA Ca$^{2+}$ channels at the end of the experiment. To control for rundown and runup of the currents examined, the amplitude of $I_{Ba^{2+}}$ obtained before drug application and after washout was averaged and used as the control value for comparisons to the amplitude during drug application (except for Fig. 2 in which the predrug values are used as control values due to the difficulty of washing out the drugs). Percent inhibition of $I_{Ba^{2+}}$ by different drugs was calculated as (1–X)100, where X equals $I_{Ba^{2+}}$ obtained in the test condition divided by the control value. Statistical analyses were done using Abacus Concepts Statview 5.0 and Microsoft Excel 8.0. Means ± SE along with the number of cells studied (n) are reported unless otherwise indicated.

**Drugs and chemicals**

All drugs were bath applied and the standard drug application time was 5 min. In some cases, shorter (2–4 min) or longer (6–8 min) applications were used. (1S,3R)-1-aminoaclopropionate-1,3-dicarboxylic acid (1S,3R-ACP). (RS)-alpha-cyclopropyl-4-phosphonobutyryl-glycine (CPPG). (RS)-alpha-methyl-4-carboxyphenoxyglycine (MCPG). (RS)-3,5-dihydroxyphenylglycine (3,5-DHPG). (2S,2'R,3'R)-2-(2',3'-di-carboxycyclopropyl)glycine (DCG-IV). (t+)-2-amino-4-phosphonobutyric acid (t+AP4) were obtained from Tocris Cookson (Ballwin, MO). ω-Agatoxin-IVA (ω-Aga-IVA) was obtained from Peptides International (Louisville, KY). Chemicals and other drugs including 1,4-dihy-
We recorded whole cell VGCC currents of NM neurons under voltage-clamp conditions after blocking voltage-gated Na⁺ and K⁺ conductances. Both LVA and HVA \( I_{\text{Ba}^2+} \) were observed, similar to what has been reported previously (Koyano et al. 1996). The LVA and HVA \( I_{\text{Ba}^2+} \) were distinguished from each other physiologically. Figure 1A shows six superimposed individual current traces from a representative NM neuron in response to voltage steps from the holding potential of \(-90\) mV. When the cell was held at \(-50\) mV (Fig. 1B), the LVA component was inactivated and the HVA \( I_{\text{Ba}^2+} \) was recorded in response to depolarizing steps. The LVA \( I_{\text{Ba}^2+} \) was rapidly inactivating and the HVA \( I_{\text{Ba}^2+} \) was noninactivating under our recording conditions (100 ms depolarizing steps). Extracellular Ba²⁺ can block K⁺ channels, argument currents via Ca²⁺ channels, and help achieve sufficient voltage clamp, but does not support Ca²⁺-dependent inactivation of Ca²⁺ channels (Hille 2001). When using Ca²⁺ as the charge carrier, we observed a LVA \( I_{\text{Ca}^2+} \) with similar kinetics to LVA \( I_{\text{Ba}^2+} \) (data not shown), indicating that the inactivation of the LVA \( I_{\text{Ba}^2+} \) is not due to Ca²⁺-dependent inactivation of Ca²⁺ channels. Due to its kinetics and sensitivity to low concentration of Ni⁺, the LVA \( I_{\text{Ba}^2+} \) might be a T-type Ca²⁺ channel current (Koyano et al. 1996).

Figure 1C shows the average current-voltage (I-V) relation (n = 5 cells) of the total peak \( I_{\text{Ba}^2+} \). The total \( I_{\text{Ba}^2+} \) was observed at potentials more depolarizing than \(-55\) mV and peaked at \(-15\) mV. Figure 1D shows the average I-V relation of the peak HVA \( I_{\text{Ba}^2+} \). The HVA \( I_{\text{Ba}^2+} \) was observed at potentials more depolarizing than \(-40\) mV and peaked at \(-10\) mV. Extracellular data of the total \( I_{\text{Ba}^2+} \) were plotted and fitted to a Boltzmann relationship of the form \( I/I_{\text{max}} = 1/[1 + \exp(V_h - V/k)] \), giving a half activation (\( V_h \)) of \(-33\) mV and a slope (\( k \)) of \(8\) mV (Fig. 1E). Extracellular data of the HVA \( I_{\text{Ba}^2+} \) were fitted to the Boltzmann relationship with a \( V_h \) of \(-24\) mV and a slope of \(4\) mV (Fig. 1F).

Rundown of the Ca²⁺ channel currents, especially the HVA component, is a common observation. Some previous studies have attempted to determine the factors, such as ATP, magnesium, and calpastatin (an inhibitor of the Ca²⁺-activated protease calpain), that influence the rundown process (Byerly and Yazejian 1986; Hao et al. 1999). Although ATP, magnesium, an ATP regenerating solution, and a protease inhibitor (leupeptin) were included in our internal solution, some rundown usually persisted. To quantify the typical rundown of the HVA \( I_{\text{Ba}^2+} \) in NM neurons, we measured the current once every 20 s for 25 min without any manipulations in a sample of cells. The averaged current decreased by <20% in 25 min (data not shown). Run-up of the HVA \( I_{\text{Ba}^2+} \) in the

**RESULTS**

**Global \( I_{\text{Ba}^2+} \) of NM neurons**

Pharmacological experiments using selective Ca²⁺ channel inhibitors were carried out to determine the type(s) of HVA VGCCs in NM neurons and how much each current contributes to the total HVA \( I_{\text{Ba}^2+} \) (Fig. 2). Nimodipine (10 μM), ω-conotoxin-GVIA (ω-CTx-GVIA, 1 μM), and ω-agatoxin-IVA (ω-Aga-IVA, 100 nM) were used to block L-, N-, and P/Q-type HVA VGCCs, respectively. The residual current resistant to a cocktail of all three inhibitors is designated as R-type \( I_{\text{Ba}^2+} \). HVA \( I_{\text{Ba}^2+} \) was recorded by applying a voltage step to \(-10\) mV from the holding potential of \(-50\) mV once every 20 s. \( I_{\text{Ba}^2+} \) values in this and subsequent figures are the absolute values of the rundown usually persisted. To quantify the typical rundown of the HVA \( I_{\text{Ba}^2+} \) in NM neurons, we measured the current once every 20 s for 25 min without any manipulations in a sample of cells. The averaged current decreased by <20% in 25 min (data not shown). Run-up of the HVA \( I_{\text{Ba}^2+} \) in the

**Types of HVA VGCCs in NM neurons**

Whole cell barium currents \( (I_{\text{Ba}^2+}) \) through Ca²⁺ channels of nucleus magnocellularis (NM) neurons. In all experiments, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 50 μM), 2-amino-5-phosphonovaleric acid (AP-5, 100 μM), and bicuculline (20 μM) were included in the external recording solution to block spontaneous synaptic currents. A: 6 superimposed current traces obtained by voltage steps to \(-90\), \(-45\), \(-35\), \(-25\), \(-20\), and \(-10\) mV from the holding potential of \(-90\) mV. The 1st trace serves as the baseline. B: the same cell shown in A was held at \(-50\) mV to inactivate the low-voltage-activated (LVA) \( I_{\text{Ba}^2+} \). Five individual current traces in response to voltage steps from \(-50\) to \(-30\), \(-25\), \(-20\), and \(-10\) mV are superimposed. The high-voltage-activated (HVA) \( I_{\text{Ba}^2+} \) was noninactivating under our recording conditions (100-ms depolarizing pulses). C and D: average current-voltage (I-V) relations (n = 5 cells) of the total peak \( I_{\text{Ba}^2+} \) and the peak HVA \( I_{\text{Ba}^2+} \). The HVA \( I_{\text{Ba}^2+} \) was observed at potentials more depolarizing than \(-40\) mV and peaked at \(-10\) mV. E: average data of the total \( I_{\text{Ba}^2+} \) were plotted and fitted to a Boltzmann relationship of the form \( I/I_{\text{max}} = 1/[1 + \exp(V_h - V/k)] \), giving a half activation (\( V_h \)) of \(-33\) mV and a slope (\( k \)) of \(8\) mV. F: average data of the HVA \( I_{\text{Ba}^2+} \) were fitted to the Boltzmann relationship with a \( V_h \) of \(-24\) mV and a slope of \(4\) mV. Bars represent mean ± SE.

The beginning of the recordings was observed in a small sample of cells.
Ca2⁺-A–D point of 0 min represents 5–10 min after accessing whole cell configuration. NM neuron. Bars represent means (H11005n respectively) show that type) were not further distinguished. The percentage of each

FIG. 2. Effects of L-, N-, P/Q-type Ca2⁺ channel inhibitors on HVA I_Ba²⁺ of NM neurons. Peak HVA I_Ba²⁺ generated by a voltage step to −10 mV from the holding potential of −50 mV was plotted against time. Note that in this and subsequent figures, the plots show the absolute values of I_Ba²⁺, and the time point of 0 min represents 5–10 min after accessing whole cell configuration. A–D: effects of application of nimodipine (10 µM), ω-CTx-GVIA (1 µM), ω-Aga-IVA (100 nM), and a cocktail containing all 3 blockers on the peak HVA I_Ba²⁺ in 4 different NM neurons. Note that each drug blocked a portion of the HVA I_Ba²⁺. Nimodipine, but not ω-CTx-GVIA or ω-Aga-IVA was washed out, and the cocktail did not block the HVA I_Ba²⁺ completely. E: pooled data (n = 4, 6, 5, 5 cells for L-, N-, P/Q-, and R-type HVA I_Ba²⁺ respectively) show that ω-CTx-GVIA-sensitive (N-type) currents dominate in NM neuron. Bars represent means ± SE.

and the zero time point represents 5–10 min after accessing whole cell configuration. We applied only one of the blockers or only the cocktail of all three blockers to any one cell. Because of the difficulty of washing out some of the blockers, we recorded only one cell from each brain slice to avoid drug contamination.

Predrug recordings were obtained for 5 min. Nimodipine (10 µM), a dihydropyridine compound which is a selective L-type Ca²⁺ channel blocker (McCarthy and TanPiencco 1992; Randall and Tsien 1995), blocked a small fraction of the HVA I_Ba²⁺, and the blocker was readily washed out (Fig. 2A). ω-CTx-GVIA (1 µM), a cone snail toxin that blocks the N-type Ca²⁺ channels irreversibly (Aosaki and Kasai 1989; Boland et al. 1994; Randall and Tsien 1995; Regan 1991), blocked more than half of the total HVA I_Ba²⁺, indicating that N-type HVA I_Ba²⁺ is the dominant component of the total HVA I_Ba²⁺ in NM neurons (Fig. 2B). ω-Aga-IVA (100 nM), a P/Q-type Ca²⁺ channel blocker, generated a small reduction of the HVA I_Ba²⁺. P/Q-type I_Ba²⁺ was small and the two components (P and Q type) were not further distinguished. The percentage of each type HVA I_Ba²⁺ of the total HVA I_Ba²⁺ was calculated as the fraction of the I_Ba²⁺ at the end of each channel blocker application of the total I_Ba²⁺ measured before any blocker applica-

tion. The cocktail did not block the HVA I_Ba²⁺ completely, suggesting the existence of R-type I_Ba²⁺ conductance (Fig. 2D, note the washout of nimodipine in this figure as well as in A). The percentages of each type HVA I_Ba²⁺ calculated from these experiments were 17.9 ± 4.2% (n = 4 cells), 71.7 ± 1.9% (n = 6 cells), 11.9 ± 2.2% (n = 5 cells), and 18.3 ± 4.4% (n = 5 cells) for L, N, P/Q, and R type, respectively (Fig. 2E). The sum of the four percentages was slightly over 100%, presumably due to nonspecific blocking and different rundown rates of the currents in different cells.

The validity of the conclusions presented in the preceding text (and in the following text) depends on the effectiveness of the Ca²⁺ channel blockers on NM neurons; the concentration of each blocker we used should have been able to completely and selectively block a specific type of HVA VGCCs. We confirmed the effectiveness of each blocker in the following experiments (Fig. 3). We used FPL 64176 (2 µM) to test the effectiveness of nimodipine (10 µM) on blocking L-type channels. FPL 64176 is an effective L-type channel activator, which acts at a site different from the binding site of dihydropyridine compounds (Zheng et al. 1991). As shown in Fig. 3A (n = 3 cells), FPL 64176 enhanced the HVA I_Ba²⁺ in the absence of nimodipine, with possibly high rundown during a short period after the enhancement reached the peak. This accelerated rundown might be expected when L-type channels are fully activated by FPL 64176 due to the fact that maintenance of Ca²⁺ channels requires many factors. Note that by ~1 min after termination of FPL 64176 application the rundown rate returned to about the same as before drug application. When nimodipine (10 µM) was included in the bath, the enhance-

FIG. 3. Further confirmation of effectiveness of L-, N-, P/Q-type Ca²⁺ channel blockers. A: FPL 64176 (2 µM), an L-type channel activator, was used to test the effectiveness of nimodipine (10 µM) for blocking L-type channels. FPL 64176 enhanced the HVA I_Ba²⁺ (n = 3 cells). B: in the presence of nimodipine (long gray bar on the top of the graph), FPL 64176 no longer enhanced the HVA I_Ba²⁺ (n = 3 cells). Note the reduction of HVA I_Ba²⁺ by nimodipine in the beginning of the recording. C: effects of successive application of ω-CTx-GVIA (1 and 3 µM) on HVA I_Ba²⁺. Application of ω-CTx-GVIA (1 µM) largely inhibited the HVA I_Ba²⁺ and increasing the concentration of the toxin by threefold did not further block the HVA I_Ba²⁺ (n = 3 cells). D: effects of successive application of ω-Aga-IVA (100 and 300 nM) on HVA I_Ba²⁺. Application of ω-Aga-IVA (100 nM) inhibited the HVA I_Ba²⁺ and a threefold concentration increase did not result in more inhibition (n = 5 cells).
ment of $I_{\text{Ba}^{2+}}$ by FPL 64176 (2 μM) was abolished (Fig. 3B, n = 3 cells), indicating that L-type channel $I_{\text{Ba}^{2+}}$ of NM neurons could be completely blocked by nimodipine (10 μM). To test the effectiveness of ω-CTx-GVIA (1 μM) and ω-Aga-IVA (100 nM) on N- and P/Q-type Ca$^{2+}$ channels, respectively, we tripled the concentration of each blocker successively for 5 min after the original concentration was applied for 5 min. Neither blocker at their tripled concentration produced additional effectiveness (Fig. 3, B, n = 3 cells), indicating that L-type channel $I_{\text{Ba}^{2+}}$ of NM neurons could be completely blocked by nimodipine (10 μM).

To test the effectiveness of -CTx-GVIA (1 μM) and -Aga-IVA (100 nM) on N- and P/Q-type Ca$^{2+}$ channels, respectively, we tripled the concentration of each blocker successively for 5 min after the original concentration was applied for 5 min. Neither blocker at their tripled concentration produced additional effectiveness (Fig. 3, B, n = 3 cells), indicating that N- and P/Q-type HVA currents were maximally blocked under our experimental conditions. Therefore we feel confident that the concentrations of each blocker used in the experiments presented in the following text is appropriate to assess the type(s) of HVA VGCCs modulated by mGluR activation.

**Activation of mGluRs inhibits HVA $I_{\text{Ba}^{2+}}$ of NM neurons**

Figure 4A shows an example in which glutamate (100 μM, in the presence of iGluR antagonists DNQX and AP-5) inhibited HVA $I_{\text{Ba}^{2+}}$ of a NM neuron. The inhibitory effect developed fully at ~1 min after glutamate application and decreased over time in the presence of continued glutamate, possibly due to desensitization of mGluRs (Aronica et al. 1993; Catania et al. 1991; Kammermeier and Ikeda 2002).

Figure 4B shows the relationship between inhibition of HVA $I_{\text{Ba}^{2+}}$ and glutamate concentration. Glutamate (1 μM) had no effect on HVA $I_{\text{Ba}^{2+}}$ (n = 6 cells). Glutamate at concentrations of 10, 100, and 500 μM, inhibited the HVA $I_{\text{Ba}^{2+}}$ by 6.8 ± 1.5% (n = 4 cells), 16.3 ± 3.1% (n = 18 cells), and 15.9 ± 4.9% (n = 5 cells), respectively. One-way ANOVA showed a
significant effect of glutamate concentration on the percent inhibition of HVA \(I_{Ba^{2+}}\); \(F(3,29) = 3.685, P < 0.05\). Post hoc Fisher’s test showed significant differences between the treatment of 1 and 100 \(\mu M\) (\(P < 0.01\)) and 1 and 500 \(\mu M\) (\(P < 0.05\)). The response appeared to be saturated at 100 \(\mu M\) because glutamate (500 \(\mu M\)) produced no further inhibition of HVA \(I_{Ba^{2+}}\). Results of inhibition of HVA \(I_{Ba^{2+}}\) by higher glutamate concentration (1 \(mM\)) are not reported because this concentration of glutamate depolarized NM cells and decreased the input resistance, even in the presence of DNQX and AP-5 (\(n = 6\) cells). The inhibition appeared to be voltage dependent (Fig. 4C, \(n = 7\) cells), with little or no inhibition occurring at potentials more negative than –25 mV. Figure 4D shows that a depolarizing prepulse (+80 mV, duration of 50 ms) relieved the inhibition. Glutamate (100 \(\mu M\)) inhibited the current by 18.5 ± 2.5 and 8.8 ± 2.11% before and after the prepulse, respectively (\(P < 0.05, n = 5\) cells). These results are consistent with previous reports on G-protein-coupled neurotransmitter receptors (e.g., Bean 1989; Choi and Lovinger 1996; Sahara and Westbrook 1993; Swartz and Bean 1992). 1S,3R-ACPD, a nonspecific mGluR agonist, mimicked the inhibitory effect of glutamate on HVA \(I_{Ba^{2+}}\) (Fig. 4E). 1S,3R-ACPD (200 \(\mu M\)) inhibited HVA \(I_{Ba^{2+}}\) by an average of 12.4 ± 3.2% (\(n = 11\) cells).

The inhibition of HVA \(I_{Ba^{2+}}\) by glutamate was not blocked by iGluR antagonists; DNQX (50 \(\mu M\)) and AP-5 (100 \(\mu M\)) were present in the recording solutions in all experiments, further supporting the conclusion that mGluRs mediated this inhibition. MCPG is reported to be a competitive antagonist of group I and II mGluRs, but not group III (Hayashi et al. 1994). Somewhat surprisingly, MCPG (1 mM) failed to block the inhibitory effect of glutamate (100 \(\mu M\)) on HVA \(I_{Ba^{2+}}\) (data not shown). Similar findings have been reported in other neuron types. For example, in neurons of the medial nucleus of the trapezoid body (MNTB), 1S,3S-ACPD (20–50 \(\mu M\)), another nonspecific mGluR agonist, reduces the probability of transmitter release and the effect is not antagonized by MCPG at the concentration of 0.5–1 mM (Barnes-Davies and Forsythe 1995). Similarly, in cortical neurons, MCPG (1 mM) only partially blocks 1S,3R-ACPD (50 \(\mu M\))-mediated inhibition of HVA Ca\(^{2+}\) currents (Sayer 1998).

We assessed the contribution of each mGluR group using specific group agonists. Figure 4F shows an example in which group I agonist 3,5-DHPG (200 \(\mu M\)), group II agonist DCG-IV (10 \(\mu M\)), and group III agonist t-AP4 (1 \(\mu M\)) were applied sequentially during the recording of HVA \(I_{Ba^{2+}}\) of a NM neuron. The order of the application of the agonists was varied in different cells. The concentrations of 3,5-DHPG, DCG-IV, and t-AP4 are 3, 33, and 10 times higher than their EC\(_{50}\) obtained from cell lines, respectively, so that all members in each mGluR group can be activated regardless of their potency, and the agonists are reported to have minimal cross-talk (reviewed in Cartmell and Schoepp 2000; Conn and Pin 1997). As seen in Fig. 4F, each mGluR group agonist inhibited the HVA \(I_{Ba^{2+}}\) of the sample NM neuron. The slow kinetics of HVA \(I_{Ba^{2+}}\) shown in the inset is not typical of our recording and was likely due to residual inhibition from earlier application of groups I and II agonists. The kinetics of the currents prior to any drug application was faster than the traces shown in the inset to Fig. 4F (data not shown). Other investigators have also reported that inhibition of Ca\(^{2+}\) channels, particularly N-type channels, by neurotransmitter receptors via a voltage- and G-protein-dependent mechanism, distorts channel activation kinetics (e.g., Choi and Lovinger 1996; Garcia-Ferreiro et al. 2001; Lambert and Wilson 1996). The average percent inhibition of HVA \(I_{Ba^{2+}}\) by group I–III mGluR agonists was 16.2 ± 3.0, 16.5 ± 4.0, and 41.2 ± 9%, respectively (Fig. 4G; \(n = 9, 8, \) and 8 cells, respectively, for group I–III mGluR agonists). One-way ANOVA showed significant difference in the effect of group I–III mGluR agonists on HVA \(I_{Ba^{2+}}\) (\(F(2,22) = 6.059, P < 0.01\)). Post hoc Fisher’s test showed significant difference between the treatment of 3.5-DHPG and t-AP4 (\(P < 0.01\)), and DCG-IV and t-AP4 (\(P < 0.01\)) but not between 3.5-DHPG and DCG-IV (\(P > 0.5\)). Because t-AP4 (1 \(\mu M\)) produced the largest inhibition, we further examined the effects on HVA \(I_{Ba^{2+}}\) of t-AP4 at two smaller doses (1 and 100 \(\mu M\)). t-AP4 at a concentration of 100 \(\mu M\) inhibited the HVA \(I_{Ba^{2+}}\) by 11.2 ± 3.1% (\(n = 6\) cells), while t-AP4 at a concentration of 1 \(\mu M\) produced little changes (1.9 ± 1.3%) of the current (\(n = 5\) cells). However, glutamate (100 \(\mu M\))-mediated inhibition of the HVA \(I_{Ba^{2+}}\) was marginally blocked by CPPG (5 \(\mu M\)), a group III mGluR antagonist. The percent inhibition was 14.9 ± 3.4 and 12.3 ± 2.4% in the absence and presence of CPPG (\(n = 4\) cells, data not shown), suggesting that nonspecific effects of t-AP4 especially at high concentration (1 \(mM\)) might be present.

Which type(s) of HVA \(I_{Ba^{2+}}\) are inhibited?

Glutamate-mediated inhibition of HVA \(I_{Ba^{2+}}\) was examined in the absence and presence of \(\omega\)-CTx-GVIA (1 \(\mu M\), \(n = 5\) cells). In Fig. 5, we report the total HVA \(I_{Ba^{2+}}\) that is inhibited by glutamate (100 \(\mu M\)) and the percent inhibition when the
N-type $I_{Ba^{2+}}$, the most dominant component, is blocked by ω-CTx-GVIA (1 μM). Figure 5A shows an example of these experiments. After ω-CTx-GVIA (1 μM) was applied for 5 min to block N-type Ca$^{2+}$ channels (and in the continued presence of ω-CTx-GVIA), the second application of glutamate (100 μM) produced less inhibition of HVA $I_{Ba^{2+}}$ compared with the first application of the agonist. Note that three stable current traces (duration of 1 min) were obtained prior to the second glutamate application, indicating that blocking of N-type channels reached a relatively stable condition. Figure 5B shows two groups of individual current traces before, during, and after glutamate application; taken at the times indicated by a-f in Fig. 5A. Traces a–c were obtained in the absence of ω-CTx-GVIA and traces d–f in the presence of ω-CTx-GVIA. The average percent inhibition of total HVA $I_{Ba^{2+}}$ (100 μM) produced less inhibition of HVA $I_{Ba^{2+}}$, that was inhibited by application of glutamate (100 μM) in the presence of ω-CTx-GVIA was presumably non-N-type HVA $I_{Ba^{2+}}$. The average results ($n = 5$ cells) are shown in Fig. 5C. The average percent inhibition of the total HVA $I_{Ba^{2+}}$ was 21.1 ± 4.3% and that contributed by non-N-type HVA $I_{Ba^{2+}}$ was 8.0 ± 1.8% (relative to the total HVA $I_{Ba^{2+}}$ under control condition, measured as the average of predrug and washout conditions to account for rundown and in this case incomplete recovery of glutamate’s effects, see METHODS). The percent inhibition of N-type $I_{Ba^{2+}}$ is therefore estimated to be 13.1%. Both N-type and non-N-type $I_{Ba^{2+}}$ were inhibited by mGluR activation.

1S,3R-ACPD did not modify $I_{Na^{+}}$ or $I_{K^{+}}$ nor the firing properties of NM neurons

Figure 6A shows that 1S,3R-ACPD (200 μM) did not alter $I_{Na^{+}}$ recorded by using a 100 ms depolarization pulse stepping from −60 to −30 mV ($n = 5$ cells, 1.12 ± 0.15 and 1.10 ± 0.13 nA for control and 1S,3R-ACPD, respectively; paired t-test $P > 0.05$). In addition, the total $I_{K^{+}}$, recorded by using a 100 ms depolarization pulse stepping from −60 to +10 mV, was not affected by 1S,3R-ACPD (200 μM) either (Fig. 6B, $n = 5$ cells, 7.12 ± 0.62 and 7.20 ± 0.57 nA for control and 1S,3R-ACPD, respectively; paired t-test $P > 0.05$). Consistent with the lack of modulation on $I_{Na^{+}}$ and $I_{K^{+}}$, 1S,3R-ACPD did not change the firing properties of NM neurons in response to current injections including threshold current (the minimal

![Image](http://jn.physiology.org/DownloadedFrom/10.22033.4/04/13/2017)
current needed to elicit an AP), action potential (AP) threshold, AP height (the difference between the AP threshold and the peak), AP half-width (duration at half-amplitude), rectification (ratio of input resistance depolarized to rest over input resistance hyperpolarized to rest), and the peak values of the depolarization voltage sag (Fig. 6C and Table 1). A small but significant depolarization of the resting membrane potential was observed when 1S,3R-ACPD (200 μM) was bath-applied (Table 1). Also a small and consistent decrease in the absolute amplitude of the voltage sag was observed in three cells (the 4th cell did not show a sag). The voltage sag is likely due to activation of hyperpolarization-activated cationic channels (Ih), which are also found in other auditory nuclei (Bal and Oertel 2000; Banks et al. 1993; Cuttle et al. 2001).

**DISCUSSION**

We report three main findings: both LVA and HVA \( I_{Ba^{2+}} \) were observed in NM neurons and four pharmacological types of HVA VGCCs were identified, N-type being the most dominant; activation of mGluRs reliably inhibited both N- and non-N-type channel HVA \( I_{Ba^{2+}} \) in NM neurons and agonists of all three groups of mGluRs produced some inhibition of HVA \( I_{Ba^{2+}} \); and voltage-gated Na\(^+\) and K\(^+\) currents and firing properties of NM neurons were not subject to modulation by mGluRs. Therefore activation of mGluRs appears to limit Ca\(^{2+}\) influx through VGCCs, participating in regulation of Ca\(^{2+}\) homeostasis of NM neurons.

**N- not L-type channel HVA \( I_{Ba^{2+}} \) dominates in NM neurons**

The general characteristics of voltage dependence and kinetics of LVA and HVA \( I_{Ba^{2+}} \) is similar to what was reported by Koyano et al. (1996). However, the relative abundance of Ca\(^{2+}\) channel types we observed differed from their report. Our pharmacological analyses indicated that the N-type channels contributed over 50% to the total HVA \( I_{Ba^{2+}} \). Koyano et al. (1996), on the other hand, reported predominantly L-type HVA VGCC, based on the fact that nifedipine (20 μM), an L-type channel antagonist, almost completely blocked the HVA \( I_{Ba^{2+}} \). Technical difficulties related to reliable recording of Ca\(^{2+}\) currents are probably responsible for the discrepancy. It is well known that Ca\(^{2+}\) channel currents are more susceptible to rundown than other ionic channel currents (Byerly and Yazejian 1986; Hao et al. 1999). The nearly complete blockade of HVA \( I_{Ba^{2+}} \) by nifedipine reported by Koyano and colleagues may have resulted from the additive effects of L-channel blockade and rundown of Ca\(^{2+}\) conductances. Using the same internal and external solutions as Koyano et al. (1996), we found that rundown of the HVA \( I_{Ba^{2+}} \) was considerably faster than that recorded using our solutions (n = 8 cells, data not shown). Under our recording conditions, bath application of another L-type Ca\(^{2+}\) channel blocker (nimodipine, 10 μM) blocked 17.9 ± 4.2% (Fig. 2) of the HVA \( I_{Ba^{2+}} \), and bath application of nifedipine (20 μM) for 5 min inhibited only 31.8 ± 4.7% of the current (n = 4 cells, data not shown).

Our finding that N- not L-type Ca\(^{2+}\) channels dominate in NM neurons also contradicts with an earlier report from our laboratory in which Ca\(^{2+}\) imaging was used to show that Ca\(^{2+}\) transients in response to high concentration of KCl were largely blocked by L-type channel blocker, nifedipine (Lachica et al. 1995). Although the reason for this discrepancy is unclear, given the difference in inactivation of L-type (τ > 500 ms) versus N-, P/Q-, and R-type (τ ~ 50 – 80 ms) channels (Hille 2001), the difference between the current study and Lachica et al. (1995) may be due to the difference in the time course of the depolarization used to activate VGCCs. We applied depolarizing voltage pulses with duration of 100 ms during which little inactivation of the HVA \( I_{Ba^{2+}} \) was seen, while Lachica et al. (1995) used bath application of KCl to depolarize NM neurons, during which N-, P/Q- and R-type channels may be inactivated at an early time so that the Ca\(^{2+}\) transients are largely due to influx through less inactivating L-type channels and blocking L-type channels largely eliminated the remaining Ca\(^{2+}\) transients.

On the presynaptic terminals from the auditory nerve onto NM neurons, N-type Ca\(^{2+}\) channels seem to predominate and P/Q-type channels may be present, while L- and R-type channels appear to be absent (Sivaramakrishnan and Laurent 1995). In contrast, somatic P/Q-type channel currents in NM neurons seem to be the smallest components (12% of the total HVA \( I_{Ba^{2+}} \) and both L- and R-type channels appear to be abundant. In bushy cells of anteroventral cochlear nucleus (AVCN), the mammalian homologue of NM, multiple HVA Ca\(^{2+}\) channels are found as well with about equal proportion (~30%) of L-, N-, and R-type channels and minimal P/Q-type channels (Doughty et al. 1998). In our experiments, blocking N-type channels did not result in noticeable changes in parameters of current injection-induced action potentials (AP) of NM neurons, including AP threshold, latency, height, maximal rising slope, or width (n = 5 cells, data not shown). This suggests that N-type Ca\(^{2+}\) channel currents don’t participate in the generation of APs in NM neurons. N-type channels are also found at the soma of other neuronal types (reviewed in Stefani et al. 1996). Hence, it does not seem that auditory neurons are unique in the somatic expression of N-type Ca\(^{2+}\) channels for their particular functions. The distinct functions of multiple VGCCs in the CNS neurons are not clear except their pharmacological sensitivity (Hille 2001).

**Involvement of different mGluRs in the modulation of VGCCs**

There are at least eight mammalian cloned mGluRs divided into three groups according to their sequence similarity, phar-
maciological properties, and signaling pathways (reviewed in Bruno et al. 2001). Inhibition of HVA VGCCs by activation of mGluRs has now been reported in many other neuronal types and the relative involvement of different mGluRs in the modulation of VGCCs appears to vary widely between different neurons (reviewed in Catterall 2000; Stefani et al. 1996).

Results of the current study indicate the involvement of all three groups of mGluRs in modulation of VGCCs of NM neurons, with group III appearing predominant. This is an uncommon situation in which all three groups of mGluRs seem to exist on the postsynaptic neurons and all target to VGCCs. In many other systems, group I and II mGluRs are found to be located on postsynaptic cells, modulating a variety of cellular processes; and Group II and III mGluRs on presynaptic terminals, functioning as either autoreceptors modulating glutamatergic transmission or heteroreceptors modulating nonglutamatergic transmission (Cartmell and Schoepf 2000). However, we remain cautious with respect to the relative abundance of the mGluR groups on NM neurons since results obtained by experiments using mGluR antagonists (MCPG and CPPG) were inconclusive. Genetic and histochemical analyses are needed to determine which group(s) of mGluR RNA are expressed and the sites of protein expression in NM neurons.

Lack of modulation of firing properties of NM neurons by mGluRs

The amount of depolarization-induced Ca2+ influx through VGCCs is presumably proportional to action potential (AP) parameters such as spike frequency and AP width. These parameters are known to be modulated by mGluRs in some other systems. For example, neuronal excitability, measured by the number of spikes in response to prolonged depolarizing current injection, is enhanced by activation of group I mGluRs in hippocampal CA1 neurons (Ireland and Abraham 2002). Also in CA1 neurons, mGluR agonist 1S,3R-ACPD broadens the width of APs elicited by current injection (Hu and Storm 1991). By increasing neuronal excitability, mGluRs reduce firing adaptation of hippocampal CA1 neurons (Desai et al. 1992; Pacelli and Kelso 1991) or CA3 neurons (Charpak et al. 1990). Modulation of $I_{K+}$ or $I_{Na+}$ by mGluRs is presumably the underlying mechanism for these phenomena (reviewed in Anwyl 1999; Cantrell and Catterall 2001).

We found no modulation of $I_{K+}$ or $I_{Na+}$ of NM neurons by stimulation of mGluRs, consistent with the observation that the firing properties (threshold current, AP threshold, AP height, AP half-width, and rectification) of NM neurons are not altered by activation of mGluRs. However, depolarization of the resting membrane potential (RMP) might be partially correlated with the activation of $I_{h}$ channels (shown as a reduction in the absolute amplitude of the voltage sag in Fig. 6). $I_{h}$ channels can influence RMP (Bal and Oertel 2000; Maccarreri et al. 1993) and are enhanced by increased levels of cAMP (Banks et al. 1993; Cuttle et al. 2001). Therefore activation of mGluRs by 1S,3R-ACPD in NM neurons may increase cAMP level (Lachica et al. 1995) and then activate $I_{h}$ channels, resulting in membrane depolarization. Although 1S,3R-ACPD caused a depolarization of the RMP, it is unlikely that Ca2+ flux through HVA VGCCs in NM neurons will be influenced because the change in RMP is very small (1.3 mV) and the changed RMP is below the HVA VGCC activation potential (Fig. 1) so that the inactivation of the channels, if any, would be little. Therefore inhibition of HVA VGCCs by activation of mGluRs in NM neurons could induce a net reduction of Ca2+ influx.

Functional significance

Intracellular signaling cascades activated by mGluRs result in a large number of important modulatory functions (reviewed in Anwyl 1999; Cartmell and Schoepf 2000; De Blasi et al. 2001). Protection of neurons from damage due to excitotoxicity has been previously reported (reviewed in Bruno et al. 2001) and the protective effect can be dependent on inhibition of Ca2+ influx through VGCCs (Colwell and Levine 1999; Kimura et al. 1999). These studies highlight the fact that because of the nature of their coding functions and their synaptic input, neurons face a variety of challenges with respect to Ca2+ homeostasis and Ca2+ signaling. This has been emphasized with respect to motoneurons by Keller and associates (Lips and Keller 1999; Vanselow and Keller 2000).

NM neurons and their mammalian homologs in the anteroventral cochlear nucleus (AVCN) fire spontaneously at very high rates (≥100 Hz) and are driven at rates upward of 250 Hz (Born et al. 1991; Koppl 1997; Rubel and Parks 1975; Rubasmen et al. 1995; Stopp and Whitfield 1961; Sullivan and Komish 1984; Warchol and Dallos 1990). These chronically high activity levels in combination with the constraints of using the Ca2+ permeable AMPA receptor (Parks 2000), places an unusually high demand on cochlear nucleus neurons with respect to Ca2+ homeostasis. In several previous reports, we have stressed that activation of mGluRs is one of the strategies to maintain Ca2+ homeostasis in NM neurons (Rubel and Fritzsch 2002; Rubel et al. 2004). The global inhibitory effect on Ca2+ conductance by mGluR activation, shown in the current study, may limit depolarization-induced Ca2+ entry into NM neurons, participating in regulation of Ca2+ homeostasis of these neurons. On the other hand, it is important to note that this mechanism inhibited ~20% of HVA $I_{Ba+}$ under the conditions of our study, and this mechanism presumably works in concert with a host of other processes that appear hypertrophied in these neurons and their mammalian counterparts. For example, previous studies from our laboratory suggest that Ca2+ release from intracellular stores is modulated by mGluRs (Kato and Rubel 1999; Kato et al. 1996). In addition, NM neurons and AVCN neurons express unusually high levels of the major Ca2+ binding proteins, calbindin, calretinin, and parvalbumin (Braun 1990; Caicedo et al. 1996; Korada and Schwartz 2000; Kubke et al. 1999; Parks et al. 1997; Vater and Braun 1994). It will be of considerable interest to determine the differential contributions of these and other Ca2+ regulating mechanisms to insuring survival of both young and adult neurons.

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