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Eighth Nerve Activity Regulates Intracellular Calcium Concentration of Avian Cochlear Nucleus Neurons via a Metabotropic Glutamate Receptor

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SUMMARY AND CONCLUSIONS

1. Neurons in the cochlear nucleus, nucleus magnocellularis (NM), of embryonic and neonatal chicks are dependent on eighth nerve activity for their maintenance and survival. Removing this input results in the death of 20–40% of the NM neurons and profound changes in the morphology and metabolism of surviving neurons.

2. One of the first changes in NM neurons after an in vivo cochlea removal is an increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Increased $[Ca^{2+}]_i$ has been implicated in a number of neuropathologic conditions.

3. In this study, we orthodromically and antidromically stimulated NM neurons in an in vitro brain stem slice preparation and monitored NM field potentials while simultaneously assessing the $[Ca^{2+}]_i$ of NM neurons using fura-2.

4. During continuous orthodromic stimulation, $[Ca^{2+}]_i$ of NM neurons remained constant at 80 nM. In the absence of stimulation, NM neuron $[Ca^{2+}]_i$ increased steadily to 230 nM by 90 min. Antidromic and contralateral stimulation produced a $[Ca^{2+}]_i$ increase in NM neurons that was similar in magnitude but slightly more rapid than that observed in the absence of stimulation.

5. Addition of the metabotropic glutamate receptor (mGluR) antagonists (R,S)-α-methyl-4-carboxyphenylglycine or 2-amino-3-phosphonopropionic acid to the superfusate during continued orthodromic stimulation resulted in a dose-dependent, rapid, and dramatic increase in NM neuron $[Ca^{2+}]_i$ without affecting the postsynaptic field potentials recorded from NM.

6. The ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione and 2-amino-5-phosphonovalerate eliminated NM field potentials during continued orthodromic stimulation but did not result in an increase in $[Ca^{2+}]_i$.

7. Continuous superfusion of *trans*-(±)-aminocyclopentane dicarboxylate, but not glutamate, prevented the increase in $[Ca^{2+}]_i$ in the absence of stimulation.

8. These results suggest that NM neurons rely on eighth nerve activity-dependent activation of a mGluR to maintain physiological $[Ca^{2+}]_i$. Removal of this mGluR activation results in an increase in $[Ca^{2+}]_i$ that may contribute to the early stages of degeneration and eventual death of these neurons.

INTRODUCTION

Afferent regulation of target tissues or structures is a common feature of the developing nervous system. Experimental manipulations of afferent activity have been shown to alter structure and function of developing sensory systems (Brunjes 1994; Dubin et al. 1986; Hubel and Wiesel 1965, 1970; Movshon and Kiorpes 1990; Rhoades et al. 1990; Stryker and Harris 1986; Wiesel and Hubel 1963a,b; Woolsey 1990) as well as the neuromuscular junction (Lømo and Rosenthal 1972). Despite the pervasiveness of these cellular interactions, little is known about the inter- and intracellular signals responsible for these effects.

In the auditory system, elimination of afferent input alters the metabolism, morphology, and survival of target neurons (Levi-Montalcini 1949; Moore 1992; Parks 1981; Rubel 1978; Woolf et al. 1983). In the embryonic and neonatal chick, neurons of the cochlear nucleus, nucleus magnocellularis (NM), are critically dependent on eighth nerve activity for maintenance and survival (reviewed in Rubel et al. 1990). Eliminating this input, either by cochlea ablation or perilymphatic injection of the sodium channel blocker tetrodotoxin, results in the death of 20–40% of NM neurons. The surviving neurons display a number of changes in their metabolic activity and morphology. Using an in vitro slice preparation, Hyson and Rubel (1989) showed that activity-dependent regulation of NM neurons required the release of a "trophic substance" from the eighth nerve terminals.

A likely signaling molecule for this intercellular trophic regulation is the neurotransmitter at the eighth nerve-NM synapse, glutamate (Jackson et al. 1985; Martin 1985; Nemeth et al. 1983). In addition to ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors (Raman and Trussell 1992; Zhou and Parks 1992a,b), NM neurons express one or more subtypes of metabotropic glutamate receptor (mGluR) that stimulate phospholipase C (PLC) and, possibly, adenylate cyclase (AC) signal transduction cascades (reviewed in Lachica et al. 1995b). mGluR activation of the PLC cascade in NM neurons generates IP_3 (Zirpel et al. 1994), which subsequently liberates Ca^{2+} from internal stores (Zirpel et al. 1995b) and presumably activates protein kinase C (PKC) (Nishizuka 1984, 1986). mGluR activation of the AC cascade in NM neurons inhibits Ca^{2+} influx through L-type voltage-operated calcium channels (Lachica et al. 1995a), presumably through adenosine 3′,5′-cyclic monophosphate (cAMP) activation of protein kinase A (PKA).

Increases in intracellular calcium concentrations ($[Ca^{2+}]_i$) have been implicated in a number of conditions that are pathological to neurons (Choi 1987, 1988; Kennedy 1989; Siesjö 1988) including sensory deprivation (Feldman et al. 1990). Alternatively, neuronal survival in culture can be *enhanced* by elevating $[Ca^{2+}]_i$ (Franklin et al. 1995;

Larmet et al. 1992). In developing neurons, $[Ca^{2+}]_i$ must be maintained within a narrow range for optimal development and survival (Kater et al. 1988; Koike et al. 1989). Perturbation of this $[Ca^{2+}]_i$ homeostasis by altering influx or intracellular release mechanisms results in an increase in neuronal death (Larmet et al. 1992). These studies suggest that strict regulation of $[Ca^{2+}]_i$ is critical for neuronal survival.

NM neurons show a large increase in $[Ca^{2+}]_i$ within 1 h after cochlea ablation (Zirpel et al. 1995a), suggesting that a rise in $[Ca^{2+}]_i$ may be involved in the signaling pathway resulting in transneuronal atrophy and cell death in this system. The hypothesis that deprivation-induced transneuronal changes are triggered by a chronic increase in $[Ca^{2+}]_i$ was tested by employing simultaneous nerve stimulation, electrophysiological recording of NM field potentials, and fura-2 calcium imaging to determine how eighth nerve activity affects NM neuron $[Ca^{2+}]_i$. This approach takes advantage of Hyson and Rubel (1989, 1995)'s paradigm, in which it was shown that orthodromic, but not antidromic, stimulation prevents the early changes associated with cell atrophy and death. Because the mGluR(s) expressed by NM neurons are capable of directly and indirectly regulating $[Ca^{2+}]_i$, it is plausible that stimulation of one or more mGluRs is necessary to maintain normal $[Ca^{2+}]_i$ homeostasis and prevent a pathological increase in $[Ca^{2+}]_i$ in activity-deprived NM neurons. This hypothesis was tested using agonists and antagonists of ionotropic and metabotropic glutamate receptors while monitoring NM neuron $[Ca^{2+}]_i$ in the presence and absence of stimulation.

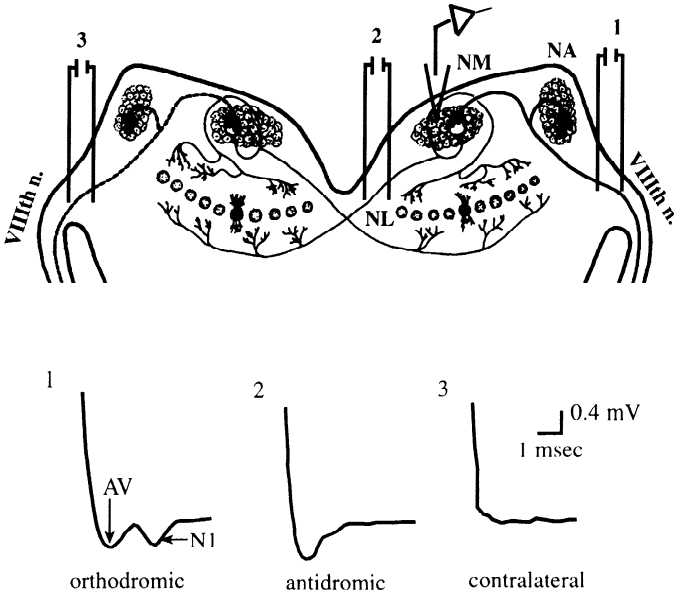


FIG. 1. Schematic diagram of chick brain stem auditory system showing experimental paradigms used in these experiments. Nucleus magnocellularis (NM) neurons were stimulated orthodromically by placing a bipolar stimulating electrode on the ipsilateral VIIIth nerve (1). Antidromic stimulation was achieved by placing stimulating electrode on contralaterally projecting axons of NM neurons that reside in crossed dorsal cochlear tract (2). Contralateral stimulation was performed by placing stimulating electrode on contralateral VIIIth nerve (3). Ipsi- and contralateral are relative to right NM in which fura-2 imaging was conducted and from which field potentials were recorded. NM response to orthodromic stimulation is biphasic, consisting of afferent volley (AV) followed by postsynaptic neuronal response (N1). Antidromic stimulation elicits a monophasic response consisting of only the electrical response of NM neurons. Contralateral stimulation elicits no field potential from NM.

METHODS

Tissue preparation

Brain stem slices (300 μm thick) were obtained from 17- to 18-day-old chick embryos as described previously (Zirpel et al. 1995b). A thick coronal section containing the entire brain stem was obtained from the decapitated embryo. The cerebellum and optic tectum were dissected free, and the remaining brain stem tissue was immersed in a 4% agarose solution. When the agarose solidified, 300-μm-thick coronal sections containing bilateral NM were acquired using a tissue slicer. All manipulations were performed in oxygenated artificial cerebrospinal fluid (ACSF). The slices then were allowed to equilibrate to room temperature in oxygenated ACSF for 45 min, at which time, they were placed in an oxygenated ACSF solution containing 5–10 μM fura-2 AM (Molecular Probes, Eugene, OR), 1.7% anhydrous dimethylsulfoxide (DMSO; Aldrich Chemical, Milwaukee, WI), and 0.03% Pluronic (Molecular Probes) for 30 min. Slices then were placed in a teflon imaging chamber (Meridian Instrument, Kent, WA) and continuously superfused with oxygenated ACSF at a rate of 3 ml/min. All experiments were performed at room temperature.

Electrophysiology

Techniques for stimulating and recording in chick brain stem slices in vitro have been described previously (Hyson and Rubel 1989). The stimulus-recording paradigms employed in this study are diagrammed in Fig. 1. Twisted teflon-coated silver wires (Medwire, Mt. Vernon, NY) comprised the bipolar stimulating electrode. All stimulation consisted of 1- to 4-mA, 20-μs pulses delivered at a rate of 5 Hz. Recording electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) to resistances of 1–5 MΩ and filled with ACSF.

ORTHODROMIC STIMULATION. To achieve orthodromic stimulation of NM, the stimulating electrode was placed on the eighth nerve (VIIIth n.) root lateral to NM. The VIIIth nerve is the sole excitatory input to NM (Born et al. 1991) and is a glutamatergic synapse (Jackson et al. 1985; Martin 1985; Nemeth et al. 1983; Raman and Trussell 1992; Zhou and Parks 1992a,b).

ANTIDROMIC STIMULATION. Antidromic stimulation of NM was achieved by placing the stimulating electrode on the crossed dorsal cochlear tract, medial to NM. The crossed dorsal cochlear tract is composed of NM axons projecting contralaterally to the third order auditory nucleus, nucleus laminaris.

CONTRALATERAL STIMULATION. The configuration for contralateral stimulation was identical to that for orthodromic stimulation except that the stimulating electrode was placed on the VIIIth nerve stump contralateral to fura-2 imaging of NM.

NO STIMULATION. Electrodes were placed as for orthodromic stimulation but no stimulus was delivered.

RECORDING. Field potentials were monitored in all stimulated slices by placing a recording electrode in NM. Responses were amplified (WPI M-707), displayed on an oscilloscope (Tektronix, Beaverton, OR) and recorded photographically.

Fura-2 calcium imaging

Fluorometric imaging techniques using this preparation have been described previously (Zirpel et al. 1995b). Slices containing fura-2-loaded NM neurons were placed in the imaging chamber

on the stage of a Nikon Diaphot inverted microscope and alternately excited with 340- and 380-nm wavelengths of light. Fura-2 fluorescence emission was obtained at 510 nm by an intensified CCD (Hamamatsu, Japan). Paired 340/380 fluorescence ratio images were acquired every 10, 30, or 180 s. NM neurons are identified easily (see Fig. 2) and were chosen randomly for analysis only if the ratio image was >50 pixels. Cells were outlined for analysis using Image-1/Fluor software (Universal Imaging, West Chester, PA). Ratios (340/380) were converted to intracellular calcium concentrations ($[Ca^{2+}]_i$) by the Image-1/Fluor software using a high-low calibration technique (Grynkiewicz et al. 1985). The Kd of fura-2 was assumed to be 224 nM (Grynkiewicz et al. 1985). Neurons with initial $[Ca^{2+}]_i$ of ≥250 nM were assumed to have been injured in the tissue preparation and were excluded from the data acquisition process (see Fig. 5). The majority of slices contained no cells with $[Ca^{2+}]_i$ this high, and few slices contained more than four cells with high $[Ca^{2+}]_i$: average = 3%. Cells chosen for analysis were monitored continuously for the duration of the experiment; no cells were added, and regions were removed only after complete loss of fluorescent signal. Complete loss of fluorescence only occurred in ~1 of every 40 cells imaged.

Data analysis

$[Ca^{2+}]_i$ levels for neurons within a given slice were averaged at 5-min intervals. For statistical analyses, a conservative approach was used. The mean $[Ca^{2+}]_i$ for all cells analyzed in a given slice at a particular time point was used as a single observation. Thus *n*s for each statistical comparison were the number of slices in a treatment group, except where otherwise noted. Data are presented as means ± SE. The total number of neurons is presented followed by *n* = number of slices. Data were plotted using Excel (Microsoft, Redmond, WA) and Cricket Graph III (Cricket Software, Malvern, PA). Two-way analysis of variance and *t*-tests were performed using Statview (Abacus Concepts, Berkeley, CA) and SAS/STAT (SAS Institute, Cary, NC).

Drugs, chemicals, and media

Slices were continuously superfused at a rate of ~3 ml/min with oxygenated ACSF consisting of (in mM) 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 glucose. Calcium-free ACSF was obtained by substituting $MgCl_2$ for $CaCl_2$ and buffering with 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

(R,S)-α-methyl-4-carboxyphenylglycine (MCPG), *trans*-(±)-aminocyclopentane dicarboxylate (ACPD), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 2-amino-5-phosphonovalerate (APV) were obtained from Research Biochemicals (Natick, MA). Glutamate and 2-amino-3-phosphonopropionic acid (AP3) were obtained from Sigma Chemical (St. Louis, MO). All pharmaceuticals were dissolved in ACSF, with the exception of CNQX, which was dissolved in anhydrous DMSO and then brought to a final concentration in ACSF. All solutions were prepared within 24 h of use. All other reagents were of analytical grade.

RESULTS

Electrophysiology

The field potentials in orthodromically stimulated NM are biphasic (Fig. 1): the initial negative deflection immediately after the stimulus artifact is the afferent volley produced by the presynaptic terminals (Hackett et al. 1982; Jackson et al. 1985); the second negative deflection is the postsynaptic NM response (Hackett et al. 1982; Zhou and Parks 1992a). Antidromic stimulation of NM produces a monophasic response consisting of a NM electrical response immediately after the stimulus artifact (Fig. 1). Stimulus levels were adjusted to produce field potentials that were 90% of the maximum amplitude recorded. The response amplitude to antidromic stimulation did not differ significantly from the response amplitude to orthodromic stimulation. The stimulation rate for all experiments was 5 Hz. Field potential amplitudes ranged between 0.1 and 1.2 mV and did not vary by >30% (most field potentials increased slightly) for ≥120 min in all experiments in which they were monitored, with the exception of ionotropic receptor antagonist experiments (see *Effects of AP3, CNQX, and APV on NM neuron* $[Ca^{2+}]_i$ *during VIIIth nerve stimulation*).

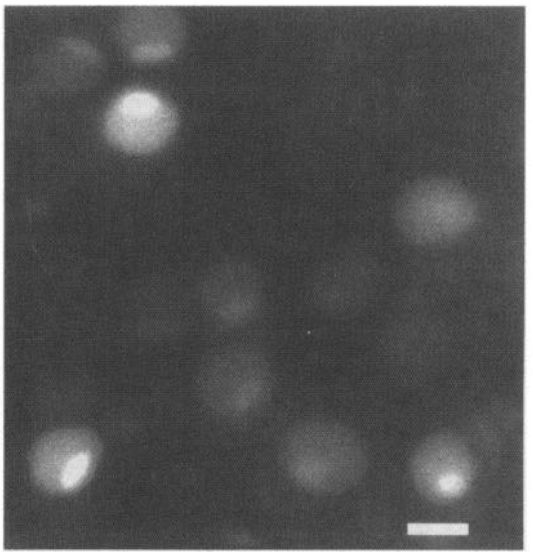


FIG. 2. NM neurons labeled with fura-2. Note uniform fura-2 labeling of cytoplasm of NM neurons. Scale bar = 20 μm.

Fura-2 imaging

NM neurons are spherical, adendritic cells with a diameter of ~30 μm. Figure 2 shows NM neurons labeled with fura-2. Fura-2 labeling of NM neurons was uniform throughout the cytoplasm with an occasional intensely labeled nucleus. The average $[Ca^{2+}]_i$ of NM neurons before the initiation of stimulation was 96 ± 2 nM (674 neurons, *n* = 69 slices randomly selected from experimental population).

VIIIth nerve stimulation

If VIIIth nerve activity regulates $[Ca^{2+}]_i$ of NM neurons, $[Ca^{2+}]_i$ levels would be expected to remain stable during orthodromic stimulation of NM but change in the absence of activity. Figure 3 shows the effects of different stimulus paradigms on NM neuron $[Ca^{2+}]_i$. By the time data acquisition is initiated, NM neurons have been deprived of afferent activity for ~80 min. [Initiation of orthodromic stimulation of NM neurons via the VIIIth nerve appeared to decrease the $[Ca^{2+}]_i$ of this group of NM neurons over the first 30 min from 86 ± 8 to 78 ± 15 nM. This change was not statistically significant (2-tailed *t*-test, $P = 0.55$).] Orthodromically stimulated NM neurons maintain a $[Ca^{2+}]_i$ level of 78 ± 2 nM for the duration of the experiments: 90 min

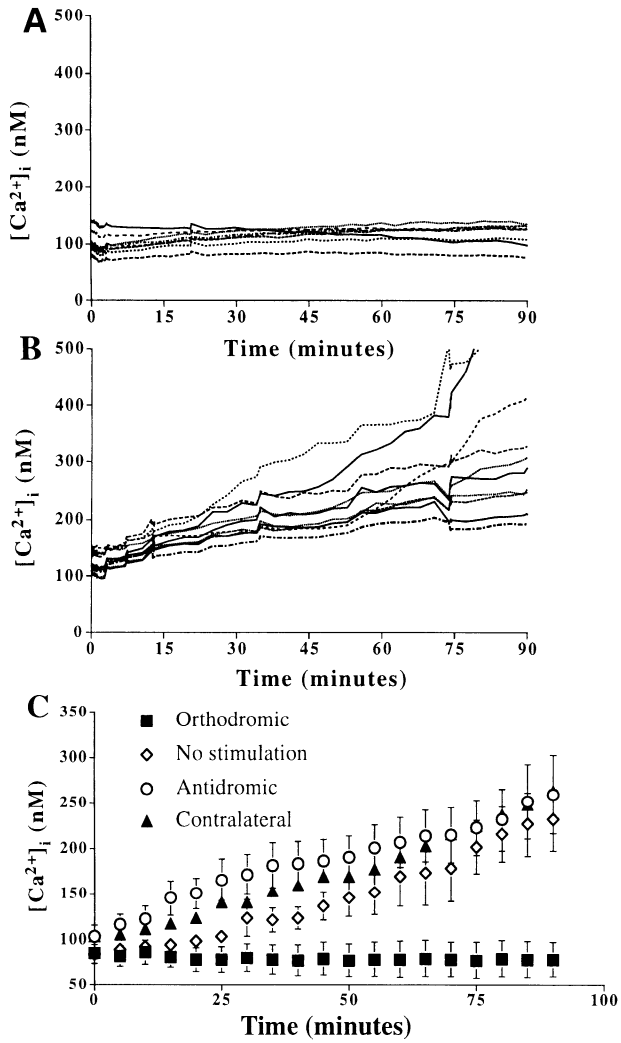


FIG. 3. Orthodromic stimulation maintains low intracellular calcium concentrations in NM neurons. *A*: $[Ca^{2+}]_i$ levels in 10 NM neurons receiving continuous 5-Hz orthodromic stimulation. Note that $[Ca^{2+}]_i$ levels are stable for the duration of 90-min experiment. *B*: $[Ca^{2+}]_i$ levels in 10 NM neurons receiving 5-Hz antidromic stimulation. Note that $[Ca^{2+}]_i$ increases steadily during course of 90-min experiment. *C*: average NM $[Ca^{2+}]_i$ levels in response to different stimulus paradigms: filled squares, 5 Hz orthodromic (35 neurons, $n = 4$ slices); open diamonds, no stimulation (51 neurons, $n = 4$ slices); open circles, 5 Hz antidromic (46 neurons, $n = 4$ slices); filled triangles, 5 Hz contralateral (43 neurons, $n = 4$ slices), which served as a control group. Error bars are standard error of mean and are <10 nM where not seen.

(Fig. 3, *A* and *C*, filled squares). In other experiments, we have found that orthodromically stimulated NM neurons can maintain this level of $[Ca^{2+}]_i$ for ≥3 h.

In the absence of stimulation, the average $[Ca^{2+}]_i$ of NM neurons increased gradually from 85 ± 10 to 232 ± 34 nM by 90 min (Fig. 3*C*, open diamonds). Antidromic stimulation of NM neurons resulted in an increase in average $[Ca^{2+}]_i$ from 104 ± 12 to 260 ± 43 nM over 90 min (Fig. 3, *B* and *C*, open circles). NM neurons in slices in which the contralateral eighth nerve was stimulated, showed an increase in average $[Ca^{2+}]_i$ that is similar in magnitude to the increase observed in unstimulated NM neurons, reaching 264 ± 50 nM at 90 min (Fig. 3*C*, filled triangles). This final concentration is comparable with that attained by both unstimulated and antidromically stimulated NM neurons. Two-way analysis of variance on all experimental groups showed a significant effect of time [$F(18,216) = 32.60$, $P = 0.0001$], treatment [$F(3,12) = 4.07$, $P = 0.033$], and a significant interaction [$F(54,216) = 4.30$, $P < 0.0001$]. It is evident from Fig. 3*C* that the orthodromic stimulation group is significantly different than the other three groups. Another two-way analysis of variance was performed on the remaining groups; antidromic, contralateral and no stimulation. This analysis revealed a significant effect of time [$F(18,162) = 35.37$, $P = 0.0001$] but not of treatment [$F(2,9) = 0.65$, $P = 0.547$]. Furthermore, the interaction was not significant [$F(36,162) = 0.37$, $P = 0.9996$], indicating that the effect of time did not differ reliably between groups. Overall, these results indicate that orthodromic stimulation of NM neurons maintains $[Ca^{2+}]_i$ at low levels: <100 nM.

It is interesting to note that the $[Ca^{2+}]_i$ of NM neurons in all three groups not receiving orthodromic stimulation reached equivalent levels by 90 min. Inspection of Fig. 3*C* also shows that the $[Ca^{2+}]_i$ of antidromically stimulated NM neurons increases more rapidly than in unstimulated NM neurons. A post hoc two-way analysis of variance was performed on the unstimulated and antidromically stimulated groups over the first 25 min of Ca^{2+} measurements. This analysis revealed a significant effect of treatment [$F(1,6) = 6.63$, $P = 0.043$], time [$F(5,7) = 9.427$, $P < 0.0001$], and a significant interaction [$F(5,66) = 3.36$, $P = 0.016$]. This rapid increase in $[Ca^{2+}]_i$ in antidromically stimulated NM neurons is probably due to direct electrical activation of voltage-gated Ca^{2+} channels without the inhibitory effect of mGluR activation (Lachica et al. 1995a) that would be present during orthodromic stimulation.

Effects of MCPG on the actions of NM neuron mGluRs

Previous studies have shown that bath application of glutamate has three mGluR-mediated effects on NM neurons: inhibition of Ca^{2+} influx through L-type channels in response to depolarization with KCl (Lachica et al. 1995a); release of Ca^{2+} from intracellular stores (Zirpel et al. 1995b); and inhibition of Ca^{2+} influx through a kainate-sensitive receptor channel (E. A. Lachica, R. Rübsamen, and E. W Rubel, unpublished results). At a concentration of 1 mM, the phenylglycine derivative MCPG is an antagonist for the mGluR1 and mGluR2 subtypes of glutamate receptors (Hayashi et al. 1994). Figure 4 shows the results of bath application of glutamate and of coapplying 1 mM MCPG with glutamate, on the Ca^{2+} response of NM neurons to depolarization with KCl. Figure 4, *A* and *B*, shows that MCPG (1 mM) inhibits the ability of submillimolar concentrations of glutamate to attenuate the NM neuron Ca^{2+} response to depolarization with 60 mM KCl. An application of 60 mM KCl produced a 148 ± 25% increase in average NM neuron $[Ca^{2+}]_i$. After a 60-s application of 500 μM glutamate, a second application of 60 mM KCl elicited only a 35 ± 5% increase in average $[Ca^{2+}]_i$ of NM neurons (Fig. 4*A*, *inset*); a significant 76% reduction in response amplitude ($P < 0.001$, Fisher's PLSD, 28 neurons, $n = 4$ slices). MCPG inhibited this glutamate effect; subsequent to a 1-min application of 1 mM MCPG followed by a

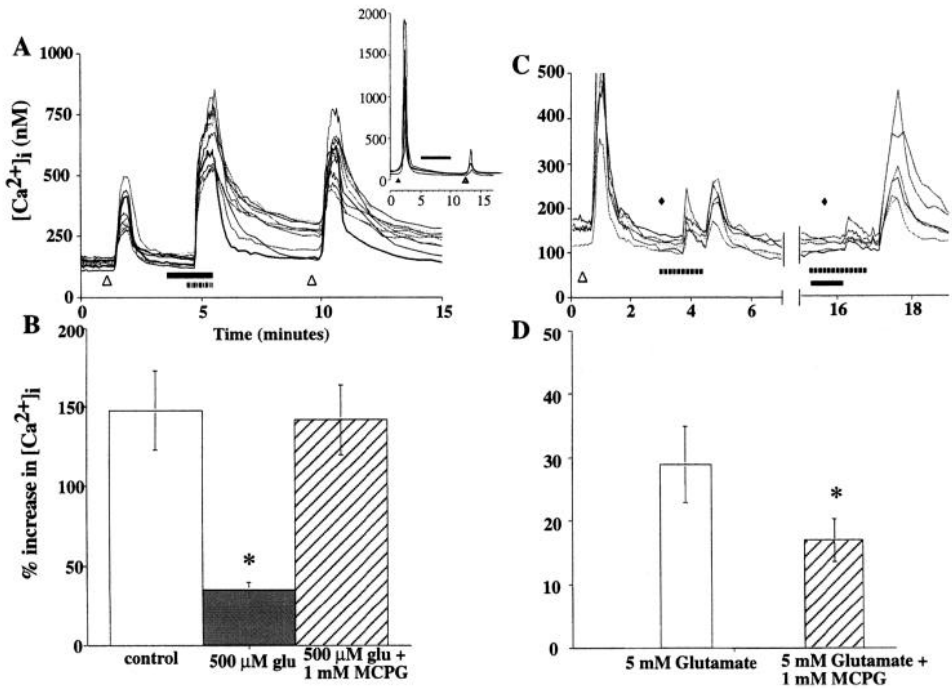


FIG. 4. (R,S)-α-methyl-4-carboxyphenylglycine (MCPG) inhibits glutamate-stimulated attenuation of Ca^{2+} influx (*A* and *C*) and release of Ca^{2+} from internal stores (*B* and *D*). *A*: traces showing $[Ca^{2+}]_i$ levels in 11 NM neurons depolarized with a 30-s application of 60 mM KCl (△) before and after coapplication of 500 μM glutamate (– – –) and 1 mM MCPG (——). Tissue was superfused for 1 min with 1 mM MCPG before coapplication of 1 mM MCPG and 500 μM glutamate. Note that in presence of 1 mM MCPG, 500 μM glutamate elicits a large increase in $[Ca^{2+}]_i$ and *does not* attenuate subsequent reponse to 60 mM KCl. *Inset* shows that a 30-s application of 60 mM KCl (small ▲) increases $[Ca^{2+}]_i$ and that application of 500 μM glutamate (solid bar) alone does not elicit an increase in $[Ca^{2+}]_i$, but attenuates subsequent response to depolarization with 60 mM KCl (large ▲). *B*: histogram showing mean responses, as percent increase above basal $[Ca^{2+}]_i$ levels (± SE) of NM neurons to a 30-s depolarization with 60 mM KCl after 3 different treatments: control, 500 μM glutamate, or 500 μM glutamate and 1 mM MCPG after a 1-min pretreatment with 1 mM MCPG. MCPG antagonizes ability of glutamate to *inhibit* response of NM neurons to depolarization. [$*P < 0.0001$, Fisher's PLSD (28 neurons, $n = 4$ slices)]. *C*: traces from 6 NM neurons showing that a 1-min application of 1 mM MCPG (——) attenuates ability of a 1-min co-application of 1 mM MCPG and 5 mM glutamate (♦) to increase $[Ca^{2+}]_i$ of NM neurons superfused with calcium-free ACSF (– – –). Note that initial response to 60 mM KCl is truncated. Also note that rebound response upon returning slice to normal ACSF is potentiated after coapplication of glutamate and MCPG. *D*: histogram showing mean ± SE $[Ca^{2+}]_i$ increase elicited by 5 mM glutamate in calcium-free ACSF in both absence and presence of 1 mM MCPG. Note that MCPG attenuates, but does not abolish, ability of glutamate to release Ca^{2+} from internal stores ($*P < 0.05$, paired *t*-test, $n = 23$ neurons).

1-min coapplication of 500 μM glutamate and 1 mM MCPG, application of 60 mM KCl elicited a 142 ± 21% increase in average NM neuron $[Ca^{2+}]_i$, an increase not significantly different from the response to an initial 60-mM KCl application.

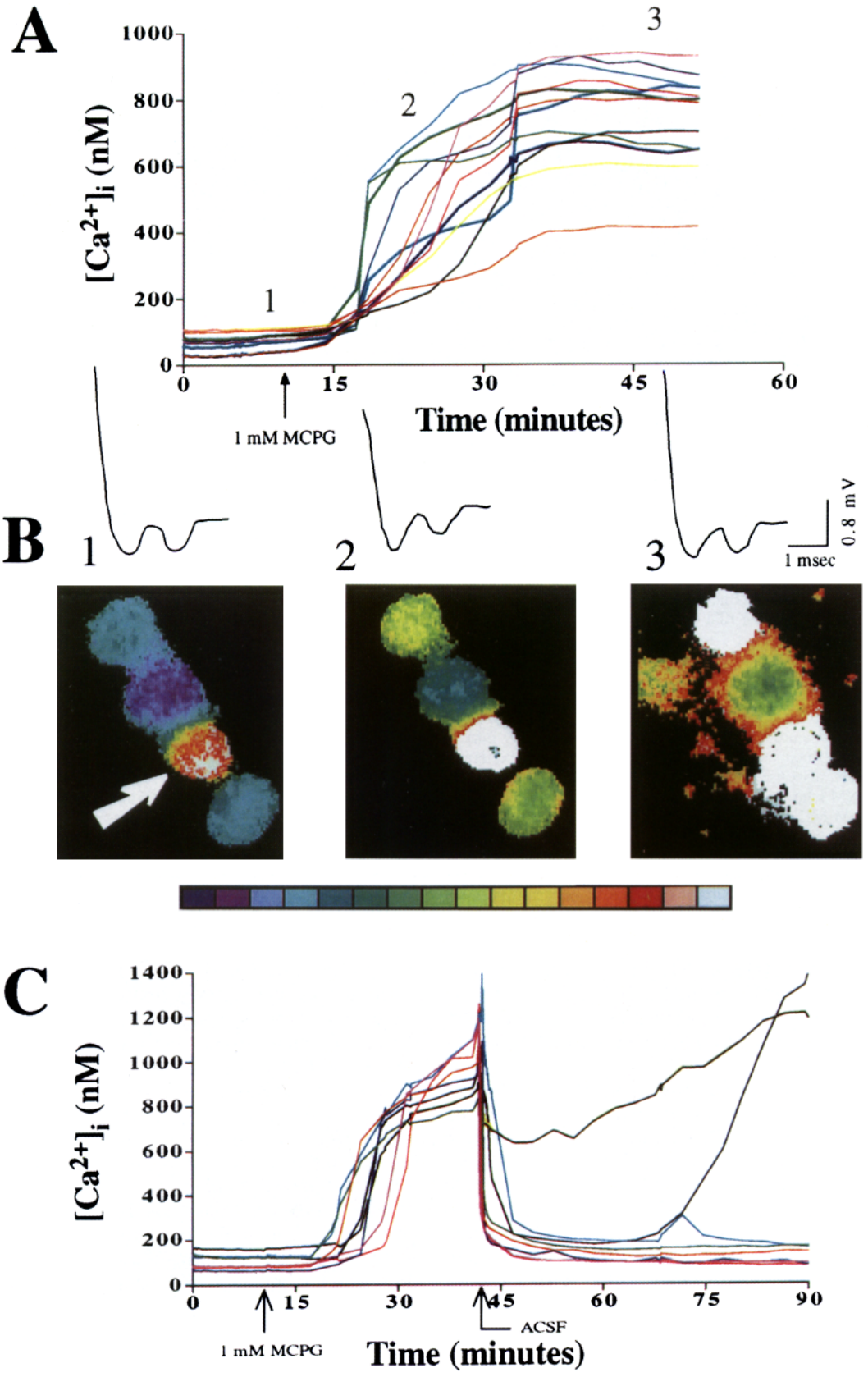
NM neurons do *not* normally show a robust Ca^{2+} response to 500 μM glutamate (Fig. 4*A*, *inset*) (Lachica et al. 1995a, but see also Zirpel et al. 1995b). However, it is interesting to note that, in the presence of MCPG, NM neurons consistently show a large increase in $[Ca^{2+}]_i$ in response to 500 μM glutamate (e.g., Fig. 4*A*). This is perhaps due to MCPG's block of the mGluR inhibition of Ca^{2+} influx through both L-type channels (Lachica et al. 1995a) and non-*N*-methyl-D-aspartate ionotropic glutamate receptor channels (E. A. Lachica, R. Rübsamen, and E. W Rubel, unpublished data).

Figure 4, *C* and *D*, shows that MCPG also attenuates, but does not abolish, the ability of glutamate (5 mM) to release Ca^{2+} from intracellular stores. A 60-s application of 5 mM glutamate in calcium-free ACSF elicited a 29 ± 12% increase in average NM neuron $[Ca^{2+}]_i$. Pretreatment and coapplication of 1 mM MCPG significantly attenuates this response such that 5 mM glutamate elicited only a 17 ± 6% increase in $[Ca^{2+}]_i$ (paired *t*-test, $n = 23$ neurons, $P = 0.002$). Previous studies have shown that responses to repeated glutamate applications in calcium-free ACSF show no attenuation (Zirpel et al. 1995b).

The results of these experiments suggest that MCPG has a powerful antagonistic effect on the mGluR(s) expressed by NM neurons, activation of which normally results in release of stored calcium and attenuation of Ca^{2+} influx through L-type Ca^{2+} channels. In addition, these results suggest that if a mGluR linked to these particular effects is responsible for preventing the $[Ca^{2+}]_i$ increase in orthodromically stimulated NM neurons, this effect should be blocked by MCPG.

Effects of MCPG on NM neuron $[Ca^{2+}]_i$ during VIIIth nerve stimulation

Figures 5 and 6 show the results of experiments performed to determine the role of mGluRs in the regulation of $[Ca^{2+}]_i$


A
1000
800
600
400
200
0
[Ca2+]i (nM)
1
2
3
0
15
30
45
60
Time (minutes)
1 mM MCPG
B
1
2
3
0.8 mV
1 msec
C
1400
1200
1000
800
600
400
200
0
[Ca2+]i (nM)
0
15
30
45
60
75
90
1 mM MCPG
ACSF
Time (minutes)

by VIIIth nerve activity. In these experiments, slices were stimulated initially orthodromically to ensure orthodromic regulation of $[Ca^{2+}]_i$ and then subjected to one of three treatments: termination of stimulation; continued orthodromic stimulation; or addition of 1 mM MCPG with continued orthodromic stimulation. In the first group, NM was stimulated orthodromically for 30 min, at which time the stimulus was terminated (Fig. 6*A*, ○). The subsequent increase in NM neuron $[Ca^{2+}]_i$ was nearly identical to that observed in unstimulated slices (Fig. 3, ◇), reaching a mean concentration of 237 ± 20 nM by 90 min after stimulus termination (43 neurons, $n = 4$). The second group is identical to that shown in Fig. 3 in which continued orthodromic stimulation maintains $[Ca^{2+}]_i$ at ~80 nM (Fig. 6*A*, ■).

In the third group, NM was stimulated orthodromically for 10 min to verify stable $[Ca^{2+}]_i$ levels, and then 1 mM MCPG was added to the superfusate and orthodromic stimulation continued for an additional 60 min. The increase in $[Ca^{2+}]_i$ that followed was very rapid and large (Figs. 5, *A* and *B*, and 6*A*, ◆), reaching a concentration of 416 ± 86 nM 20 min after application of MCPG (65 neurons, $n = 6$ slices). NM neuron $[Ca^{2+}]_i$ continued to increase for another 35 min, reaching a mean concentration of 556 ± 178 nM. It is important to note that the addition of MCPG did not alter the latency or amplitude of postsynaptic field potentials recorded from NM. Representative potentials recorded during the experiment are shown in Fig. 5, and the data are summarized in Table 1.

To determine the reversibility of the effect of MCPG, two experiments (27 neurons) were performed in which 1 mM MCPG was removed from the superfusate after being present for 30 min. Immediately after the removal of MCPG, $[Ca^{2+}]_i$ levels in most NM neurons fell from the elevated levels to levels approaching 100 nM within 10 min (Fig. 5*C*). However, the $[Ca^{2+}]_i$ of a small number of the neurons (5/27) did not fully recover after removal of MCPG, and remained >400 nM.

The specificity of MCPG is thought to be dose dependent with 1 mM antagonizing mGluR1 and mGluR2 subtypes of mGluRs and the actions at lower concentrations becoming subtype specific (Hayashi 1994). MCPG (100 μM) did not affect the $[Ca^{2+}]_i$ of NM neurons receiving continuous orthodromic stimulation (Fig. 6*B*, ●; 49 neurons, $n = 4$ slices). MCPG (10 μM) did not affect the $[Ca^{2+}]_i$ of orthodromically stimulated NM neurons for 65 min (Fig. 6*B*, □; 42 neurons, $n = 4$ slices). However, from 70 min until the termination of the experiments at 90 min, NM neurons receiving orthodromic stimulation in the presence of 10 μM MCPG showed $[Ca^{2+}]_i$ levels that were elevated (to ~250 nM), compared with orthodromically stimulated NM neurons. This may indicate an effect on a specific subtype of mGluR at this concentration when compared with nonspecific mGluR antagonism at 1 mM (Hayashi et al. 1994).

Finally, the effects of bath application of MCPG alone on NM neuron $[Ca^{2+}]_i$ was tested in four slices for each of the above three concentrations. MCPG did not affect $[Ca^{2+}]_i$ levels or dynamics of neurons in unstimulated slices.

The results of these experiments support the hypothesis that orthodromic activity regulates $[Ca^{2+}]_i$ in NM neurons via a mGluR and that the effect of blocking this receptor with MCPG is reversible, at least for the majority of NM neurons.

Effects of AP3, CNQX, and APV on NM neuron $[Ca^{2+}]_i$ during VIIIth nerve stimulation

AP3 is a putative antagonist at several subtypes of mGluRs (Abe et al. 1992; Houamed et al. 1991; Schoepp and Johnson 1989). However, in NM neurons, 1 mM AP3 shows no affect on glutamate-stimulated phosphatidylinositol (PI) metabolism (Zirpel et al. 1994), glutamate-stimulated release of Ca^{2+} from intracellular stores (Zirpel et al. 1995b), nor mGluR-mediated inhibition of Ca^{2+} influx through L-type channels (E. A. Lachica, personal communication). Figure 6*C* shows results of application of AP3 to orthodromically stimulated NM neurons. AP3 (1 mM) applied to NM neurons receiving orthodromic stimulation resulted in a $[Ca^{2+}]_i$ increase that reached 350 ± 45 nM by 90 min after application (Fig. 6*C*, +; 43 neurons, $n = 4$ slices; $P < 0.05$ at 80-, 85-, and 90-min time points compared with orthodromic stimulation group). AP3 (100 μM) had no significant effect on the $[Ca^{2+}]_i$ of orthodromically stimulated NM neurons (Fig. 6*C*, ◇; 34 neurons, $n = 4$ slices). Neither 1 mM nor 100 μM AP3 had any effect on the magnitude nor latency of NM field potentials (Table 1) nor on the magnitude and dynamics of the $[Ca^{2+}]_i$ increase observed in unstimulated slices (data not shown; $n = 3$ slices for each concentration).

Submillilmolar concentrations of the ionotropic glutamate receptor antagonists APV and CNQX block eighth nerve synaptic transmission to NM neurons (Zhou and Parks 1992a,b). CNQX and APV have no effect on glutamate-stimulated PI metabolism (Zirpel et al. 1994) or release of Ca^{2+} from intracellular stores (Zirpel et al. 1995b). Application of 30 μM CNQX and 200 μM APV completely abolished the NM field potential within 60 s of application (Table 1), but did not affect the $[Ca^{2+}]_i$ of orthodromically stimulated NM neurons (Fig. 6*C*, ▶; 45 neurons, $n = 4$ slices); $[Ca^{2+}]_i$ remained at ~80 nM despite complete loss of the postsynaptic response.

The results of these of experiments further suggest that activation of a mGluR is necessary for the regulation of NM neuron $[Ca^{2+}]_i$ by orthodromic activity.

FIG. 5. Orthodromic stimulation increases NM neuron $[Ca^{2+}]_i$ in presence of MCPG. *A*: traces from 12 NM neurons receiving 5 Hz orthodromic stimulation. MCPG (1 mM) was added to superfusate at time indicated (↑). Numbers indicate times at which field potentials and images in *B* were acquired. *B*: MCPG does not affect field potential of NM but results in a large increase in $[Ca^{2+}]_i$. Numbers indicate corresponding times in *A* at which field potential and accompanying ratio images were acquired. Ratio images show 3 of 12 NM neurons in *A*. Note NM neuron with high $[Ca^{2+}]_i$ at beginning of experiment (→, *B1*). This cell presumably was damaged in dissection process and excluded from analysis. Fluorescence ratios (340/380) are pseudocolored and correspond to $[Ca^{2+}]_i$ as indicated by color scale, which represents nanomolar $[Ca^{2+}]_i$ ranging from 10.5 (dark purple) to 1051 (white). *C*: traces from 7 NM neurons receiving 5 Hz orthodromic stimulation. MCPG (1 mM) was added and subsequently washed out as indicated (↑). Note rapid increase in $[Ca^{2+}]_i$ when MCPG was added, and subsequent decrease in $[Ca^{2+}]_i$ upon removal of MCPG and that not all NM neurons returned to baseline levels after this very large increase in $[Ca^{2+}]_i$.

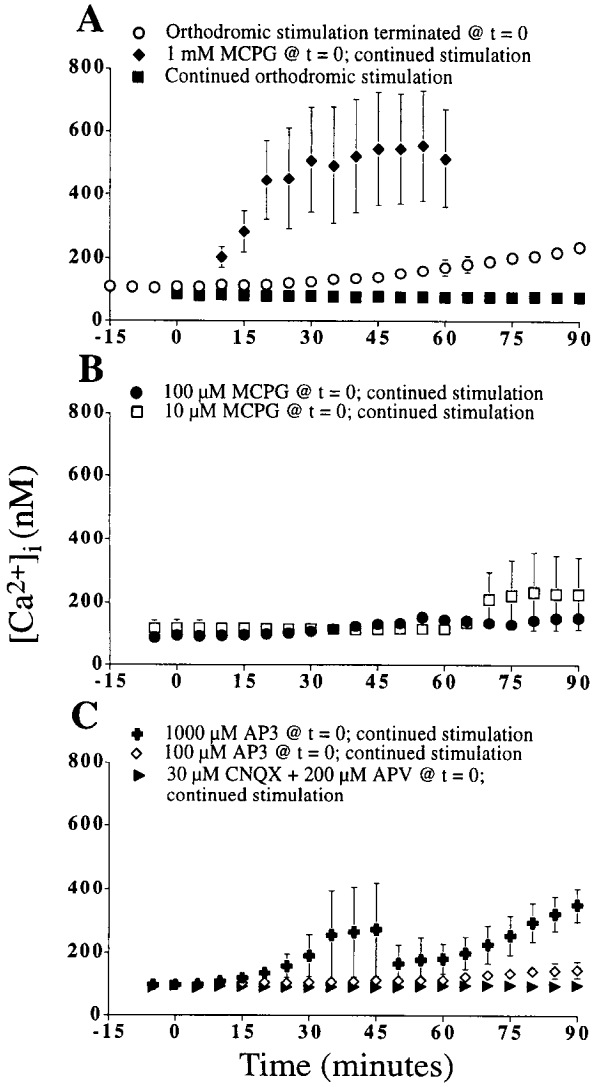


FIG. 6. Metabotropic and ionotopic receptor antagonists have different effects on $[Ca^{2+}]_i$ in stimulated NM neurons. *A*: 1 mM MCPG inhibits ability of orthodromic stimulation to suppress $[Ca^{2+}]_i$ of NM neurons. Mean $[Ca^{2+}]_i$ levels are plotted at 5-min intervals for 3 conditions: 5-Hz orthodromic stimulation (■, 35 neurons, $n = 4$ slices); 5-Hz orthodromic stimulation terminated at $t = 0$ (○, 43 neurons, $n = 4$ slices); and continued 5-Hz orthodromic stimulation with the addition of 1 mM MCPG at $t = 0$ (♦, 65 neurons, $n = 6$ slices). Error bars represent SE and where not seen are <10 nM. *B*: effect of MCPG is dose dependent. This graph shows plots of mean $[Ca^{2+}]_i$ of NM neurons receiving 5-Hz orthodromic stimulation with addition of 100 μM MCPG (●; 49 neurons, $n = 4$ slices) or 10 μM MCPG (□; 42 neurons, $n = 4$ slices) at $t = 0$. Error bars represent SE and where not seen are <10 nM. *C*: effects of 2-amino-3-phosphonopropionic acid (AP3) and ionotropic receptor antagonists on NM neuron $[Ca^{2+}]_i$ during orthodromic stimulation. Mean NM neuron $[Ca^{2+}]_i$ is plotted at 5-min intervals for 3 conditions during continued 5-Hz orthodromic stimulation: addition of 1,000 μM AP3 at $t = 0$ (+, 43 neurons, $n = 4$ slices); addition of 100 μM AP3 at $t = 0$ (◇, 34 neurons, $n = 4$ slices); and addition of 30 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 200 μM 2-amino-5-phosphonovalerate (APV) at $t = 0$ (▶, 45 neurons, $n = 4$ slices). Error bars represent SE and where not seen are <10 nM.

Effects of agonist superfusion on NM neuron $[Ca^{2+}]_i$

To determine whether mGluR activation is sufficient to maintain low NM neuron $[Ca^{2+}]_i$, slices were superfused with either *trans*-(±)-ACPD or glutamate in the absence of stimulation. These results are shown in Fig. 7. *Trans*-(±)-ACPD is a specific agonist of mGluRs and has dose-dependent effects on NM neuron $[Ca^{2+}]_i$. At millimolar concentrations, *trans*-(±)-ACPD causes release of $[Ca^{2+}]_i$ from intracellular stores (Zirpel et al. 1995b), whereas micromolar concentrations attenuate influx of Ca^{2+} through voltage-gated Ca^{2+} channels in NM neurons (Lachica et al. 1995a). Superfusion of 100 μM *trans*-(±)-ACPD did not elicit release of Ca^{2+} from intracellular stores, but did delay the $[Ca^{2+}]_i$ increase of NM neurons in unstimulated slices (Fig. 7, ○; 30 neurons, $n = 3$ slices). The $[Ca^{2+}]_i$ of NM neurons in the presence of 100 μM ACPD did not become significantly elevated until 75 min (compared with 30 min in unstimulated slices), but then increased rapidly to 218 ± 33 nM at 90 min. NM neurons in unstimulated slices superfused with 500 μM *trans*-(±)-ACPD showed no increase in mean $[Ca^{2+}]_i$ over the course of 90-min experiments (Fig. 7, ◆; 46 neurons, n = 4 slices). However, NM neurons in two of the four slices superfused with 500 μM *trans*-(±)-ACPD showed a transient increase in $[Ca^{2+}]_i$ representative of release from intracellular stores (seen as an increase at the 5-min time point in Fig. 7). The $[Ca^{2+}]_i$ of these NM neurons then gradually returned to baseline levels within 15 min and remained there for the remainder of the experiments.

TABLE 1. *Effects of glutamate receptor antagonists on N1 amplitude of NM field potentials during 5-Hz stimulation*

Preamplitude, mV	Treatment, μM	Postamplitude, mV	Post/Pre Ratio
0.55 ± 0.12	1,000 MCPG	0.57 ± 0.13	1.04
0.16 ± 0.02	100 MCPG	0.19 ± 0.03	1.19
0.31 ± 0.04	10 MCPG	0.31 ± 0.04	1.00
0.49 ± 0.12	1,000 AP3	0.52 ± 0.13	1.06
0.30 ± 0.08	100 AP3	0.34 ± 0.07	1.13
0.28 ± 0.05	30 CNQX + 200 APV	0.0 ± 0.003	0.00

Amplitudes are expressed as means ± SE for $n = 4$ slices for all groups except 1,000 μM MCPG where $n = 6$ slices. NM, nucleus magnocellularis; MCPG, (R,S)-α-methyl-4-carboxyphenylglycine; AP3, 2-amino-3-phosphonopropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; APV, 2-amino-5-phosphonovalerate.

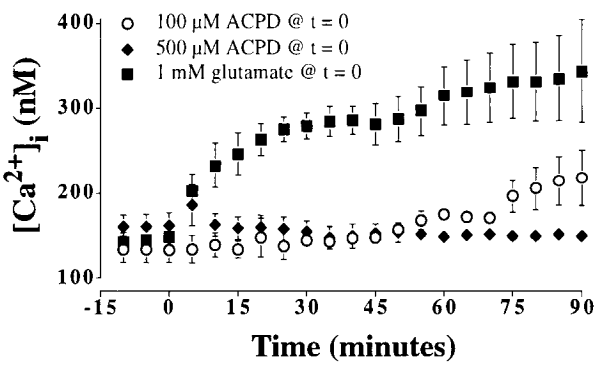


FIG. 7. Superfusion of 500 μM *trans*-(±)-ACPD prevents the $[Ca^{2+}]_i$ increase in unstimulated NM neurons. Mean $[Ca^{2+}]_i$ levels are plotted at 5-min intervals for unstimulated slices in following 3 conditions: superfusion of 100 μM *trans*-(±)-ACPD (○, 30 neurons, $n = 3$ slices); superfusion of 500 μM *trans*-(±)-ACPD (◆, 46 neurons, $n = 4$ slices); and superfusion of 1 mM glutamate (■, 36 neurons, $n = 4$ slices). Error bars represent SE and are <10 nM where not seen. Note that initial $[Ca^{2+}]_i$ of these experimental groups is slightly higher than 100 nM.

Glutamate elicits release of Ca^{2+} from intracellular stores at millimolar concentrations (Zirpel et al. 1995b), but also activates ionotropic receptors resulting in membrane depolarization and Ca^{2+} influx through agonist-activated cation channels and voltage-activated Ca^{2+} channels (Raman and Trussell 1992; Zhou and Parks 1992a,b; E. A. Lachica, R. Rübsamen, and E. W. Rubel, unpublished data). Superfusion of 1 mM glutamate resulted in a rapid increase in $[Ca^{2+}]_i$ of NM neurons in unstimulated slices (Fig. 7, ■; 36 neurons, $n = 4$ slices). NM neuron $[Ca^{2+}]_i$ continued to increase reaching a level of 344 ± 60 nM 90 min after the initiation of superfusion. This $[Ca^{2+}]_i$ increase in the presence of glutamate indicates that in addition to activation of ionotropic glutamate receptors, which allow Ca^{2+} influx and also depolarize the neurons and allow influx through voltage-gated calcium channels, this concentration of glutamate is either not sufficient to activate the mGluR-mediated inhibition of influx, or, if so, the activation of the ionotropic receptors overwhelms this particular effect.

Analysis of variance of the superfusion experimental groups (100 μM ACPD, 500 μM ACPD, and 1 mM glutamate), the no-stimulation group, and the orthodromic stimulation group revealed a significant effect of superfusion treatment [$F(4,98) = 69.35$, $P < 0.0001$]. Post hoc analysis (Scheffe's F) showed that the 100 μM *trans*-(±)-ACPD group did not differ significantly from the no-stimulation group ($P = 0.233$); the 500 μM *trans*-(±)-ACPD group did not differ from the orthodromic stimulation group ($P = 0.947$) but did differ from the 100 μM *trans*-(±)-ACPD group ($P < 0.0001$); the 1 mM glutamate superfusion group differed from all other groups ($P < 0.0001$).

These results show that superfusion of 500 μM *trans*-(±)-ACPD, but not 100 μM *trans*-(±)-ACPD nor 1 mM glutamate, can maintain NM neuron $[Ca^{2+}]_i$ at levels comparable with those maintained by orthodromic stimulation.

DISCUSSION

In this study, we have shown that orthodromic stimulation maintains $[Ca^{2+}]_i$ levels of NM neurons at ~80 nM in an in vitro slice preparation. Deprivation of stimulation, antidromic stimulation, and contralateral eighth nerve stimulation all result in gradual, large increases in $[Ca^{2+}]_i$ of NM neurons. Application of the metabotropic glutamate receptor antagonists MCPG or AP3 blocks the ability of orthodromic stimulation to maintain $[Ca^{2+}]_i$, resulting in a rapid (in the case of MCPG) and large increase in $[Ca^{2+}]_i$ without affecting the gross electrical responses of NM neurons. Conversely, application of the ionotropic glutamate receptor antagonists CNQX and APV completely abolishes the electrical response of NM neurons but does not affect the ability of orthodromic stimulation to maintain NM neuron $[Ca^{2+}]_i$. In addition, we have shown that MCPG antagonizes glutamate-stimulated inhibition of L-type Ca^{2+} channels and Ca^{2+} release from intracellular stores in NM neurons. Superfusion of 500 μM *trans*-(±)-ACPD maintains NM neuron $[Ca^{2+}]_i$ at concentrations comparable with those maintained by orthodromic stimulation. In the remainder of this report, we will discuss these results in the context of: intracellular calcium concentrations and neuronal survival; the role of metabotropic glutamate receptors in other systems; and the functional significance of this mGluR-mediated regulation of $[Ca^{2+}]_i$ in terms of afferent regulation of NM neurons.

$[Ca^{2+}]_i$ and cell viability

Strict regulation of $[Ca^{2+}]_i$ is critical for neuronal survival and development (Carafoli 1987; Franklin and Johnson 1992; Kennedy 1989; Koike et al. 1989; Siesjö 1988). Increased $[Ca^{2+}]_i$ has been implicated in neuronal death and degeneration in a number of conditions including hypoxia, ischemia, seizures, excitotoxicity (reviewed in Choi 1992), and apoptosis (Wyllie et al. 1984). In this study, we demonstrate that orthodromic stimulation of NM neurons maintains NM neuron $[Ca^{2+}]_i$ at low levels, ~80 nM. This concentration is well within the range considered normal for healthy neurons (Siesjö 1988).

NM neurons in vivo that are deprived of VIIIth nerve activity show a number of rapid changes (reviewed in Rubel et al. 1990) that are consistent with known actions of elevated $[Ca^{2+}]_i$, including decreased stability of cytoskeletal elements, increases in mitochondria (Hyde and Durham 1994), degradation of polyribosomes (Rubel et al. 1991), decreased protein synthesis (Steward and Rubel 1985; Born and Rubel 1988), and decreases in ribosomal RNA (Y10B) antigenicity (Garden et al. 1994). NM neurons in an in vitro brain stem slice deprived of eighth nerve activity similarly show decreased protein synthesis (Hyson and Rubel 1989) and decreased Y10B labeling (Hyson and Rubel 1995). Although orthodromic stimulation prevents these changes, comparable levels of postsynaptic electrical activity achieved by antidromic stimulation are insufficient to prevent the downregulation of protein synthesis and ribosomal RNA antigenicity. In fact, antidromically stimulated NM neurons show a greater inhibition of protein synthesis than do activity-deprived NM neurons (Hyson and Rubel 1989). Parallel results are presented in this study in which $[Ca^{2+}]_i$ is the dependent variable. These results are consistent with the proposal that a rise in $[Ca^{2+}]_i$ triggers some of the early metabolic events that eventually will lead to deprivation-induced changes in NM neurons. We show that NM neurons deprived of VIIIth nerve activity, and NM neurons that are stimulated antidromically, show gradual increases in $[Ca^{2+}]_i$ that approach 300 nM by 90 min; a time at which changes in protein synthesis and Y10B antigenicity are apparent (Born and Rubel 1988; Hyson and Rubel 1989, 1995; Steward and Rubel 1985). $[Ca^{2+}]_i$ is a well-known regulator of protein synthesis (Brostrom and Brostrom 1990; Kimball and Jefferson 1992; Preston and Berlin 1992). Though no specific concentration of $[Ca^{2+}]_i$ has been characterized as pathologic or lethal, increases on the order of hundreds of nanomolar can induce subsequent neuronal degeneration and death (Larmet et al. 1992; Michaels and Rothman 1990). It is also interesting to note that the $[Ca^{2+}]_i$ of antidromically stimulated NM neurons increased more rapidly than in NM neurons deprived of stimulation (Fig. 3).

Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are G-protein-linked receptors that regulate intracellular second messenger systems including PI metabolism (Sugiyama et al.

1987) and adenylate cyclase activity (Cartmell et al. 1992; Schoepp et al. 1992; Winder and Conn 1992). The effects of mGluR activation include modulation of electrophysiological properties (McBain et al. 1994; Zheng and Gallagher 1992), modulation of ionotropic glutamate receptor currents (Bleakman et al. 1992; Glaum and Miller 1993; Greengard et al. 1991; Kinney and Slater 1993), modulation of L-type Ca^{2-} channels (Chavis et al. 1994, 1995; Lester and Jahr 1990; Sahara and Westbrook 1993; Sayer et al. 1992; Swartz and Bean 1992; Zeilhofer et al. 1993), modulation of *c-fos* transcription (Sheng et al. 1990), regulation of mGluR mRNA (Bessho et al. 1993), and regulation of astrocyte proliferation (Condorelli et al. 1989). mGluR activation also has been shown to attenuate excitotoxicity (Koh et al. 1991; Pizzi et al. 1993), reduce the degenerative effects of hypoxia (Opitz and Reymann 1993) and ischemia (Chiamulera et al. 1992) and function synergistically with nerve growth factor to prevent cell death in cultured Purkinje cells (Mount et al. 1993). In NM neurons, mGluR activation results in increased PI metabolism (Zirpel et al. 1994), release of Ca^{2+} from internal stores (Zirpel et al. 1995b), and inhibition of Ca^{2-} influx through L-type channels (Lachica et al. 1995a) and through ionotropic glutamate receptor channels (E. A. Lachica, R. Rübsamen, and E. W Rubel, unpublished results), presumably through an increase in cAMP levels.

In this study, we show that the phenylglycine derivative MCPG, a nonspecific mGluR antagonist (Hayashi et al. 1994), antagonizes the ability of glutamate to inhibit L-type Ca^{2+} channels and partially antagonizes the ability of glutamate to liberate Ca^{2+} from internal stores; two mGluR effects that may be important to $[Ca^{2+}]_i$ regulation during synaptic activation. Additionally, MCPG prevents orthodromic stimulation from maintaining NM neuron $[Ca^{2+}]_i$ at low levels. The mGluR antagonist AP3 also prevents orthodromic stimulation from maintaining NM neuron $[Ca^{2+}]_i$ but does not affect the ability of glutamate to release Ca^{2+} from internal stores (Zirpel et al. 1995b). Neither MCPG nor AP3 influenced the magnitude of the postsynaptic field potentials recorded in NM at any of the concentrations tested. The ionotropic glutamate receptor antagonists CNQX and APV do not affect the ability of orthodromic stimulation to maintain NM neuron $[Ca^{2+}]_i$ at low levels although they completely blocked the postsynaptic response. These results suggest that the ability of VIIIth nerve activity to regulate NM neuron $[Ca^{2+}]_i$ is, in large part, mediated by a mGluR. The fact that high (1,000 μM) and low (10 μM) concentrations of MCPG have very different temporal effects on and affect the magnitude of $[Ca^{2+}]_i$ changes differently may suggest that two subtypes of mGluR are involved in eighth nerve activity regulation of NM neuron $[Ca^{2+}]_i$. MCPG has been used at this concentration range (500–1,000 μM) to successfully antagonize mGluR actions on the following: voltage-gated Ca^{2+} channels of cortical neurons (1 mM) (Choi and Lovinger 1996); presynaptic depression at corticostriatal synapses (500 μM) (Lovinger and McCool 1995); phosphoinositide metabolism in cortical astrocytes (1 mM) (Miller et al. 1994); phosphoinositide metabolism in *Xenopus* oocytes (1 mM) (Saugstad et al. 1995); inhibition of I_{AHP} in hippocampal CA1 neurons (500 μM) (Manzoni et al. 1994); prevention of β-amyloid peptide-induced apoptosis (1 mM) (Copani et al. 1995a) and neuronal apoptosis in culture (3 mM) (Copani et al. 1995b). The time course of these MCPG antagonist effects ranged from seconds (Choi and Lovinger 1996; Saugstad et al. 1995) to minutes (Kemp et al. 1994; Lovinger and McCool 1995; Manzoni et al. 1994) and showed reversibility within the same time courses (Choi and Lovinger 1996; Lovinger and McCool 1995; Manzoni et al. 1994). Conversely, in the presence of the ionotropic receptor antagonists, NM neuron $[Ca^{2+}]_i$ remains low due to the continued activation of the mGluR(s) despite the lack of electrical activation.

The extremely rapid and large increase in $[Ca^{2+}]_i$ of NM neurons undergoing continuous orthodromic stimulation in the presence of high concentrations of MCPG is not unexpected if one remembers that the ionotropic response of NM neurons to VIIIth nerve activity is unaffected by MCPG. Thus release of glutamate from the eighth nerve stimulates an influx of Ca^{2+} through both the ionotropic receptor channels and the voltage gated calcium channels, thus contributing to the rapid and large increase in $[Ca^{2+}]_i$.

We have not made a systematic attempt to identify the subtype of mGluR mediating this action on NM neurons. To do so would require an extensive sampling of the myriad of compounds that appear to have inconsistent and nonspecific effects depending on the cell type examined. While this work is being performed, it is beyond the scope and intent of the present report.

Functional significance

Sensory deprivation is well characterized as having dramatic effects on the survival, morphology, metabolism and physiology of target neurons in the developing CNS (Brunjes 1994; Dubin et al. 1986; Globus 1975; Hubel and Wiesel 1970; Moore 1992; Wiesel and Hubel 1963a,b; Woolf et al. 1983). Deprivation of synaptic activity also has been shown to have dramatic effects on second messenger systems, including PI metabolism (Nicoletti et al. 1987) and PKC activity (Elkabes et al. 1993). Despite the abundance of studies characterizing the long-term effects of sensory deprivation on CNS neurons, little is known about the intracellular cascade of events underlying these phenomena. A long-term goal of our work has been to describe the cellular events that may result in these long-lasting changes in CNS pathways and structures. Based on this work, we propose a mechanism for the afferent regulation of NM neurons by VIIIth nerve activity. Although the following proposal is speculative at this time, it fits the data from previous and current studies from this laboratory and provides a framework for future studies on this system as well as more complex systems.

$[Ca^{2+}]_i$ can increase in three ways: influx through voltage gated Ca^{2+} channels, influx through ligand-gated channels, and release from internal stores. PKA and PKC can modulate all three of these pathways at a number of points in the cascade of events, including L-type channels, ionotropic receptors, and IP_3 receptors on internal Ca^{2+} stores (Bleakman et al. 1992; Ferris et al. 1991; Swartz 1993; Swartz et al. 1993; Taylor and Marshall 1992; Wang et al. 1991). In addition, PKC activation has been shown to increase neuronal (Wakade et al. 1988) and lymphocyte (Lucas et al. 1994) survival in vitro. In NM neurons, mGluR activation

inhibits Ca^{2+} influx through L-type channels (Lachica et al. 1995a), modulates release of Ca^{2+} from internal stores (Kato et al. 1996; Zirpel et al. 1995b) and inhibits Ca^{2+} influx through ionotropic glutamate receptor channels (E. A. Lachica, R. Rübsamen, and E. W. Rubel, unpublished observations). This makes the NM mGluR an ideal candidate for playing a major role in the regulation NM neuron $[Ca^{2+}]_i$. In the absence of orthodromic stimulation, $[Ca^{2+}]_i$ of NM neurons rises significantly on a time course that parallels the well-characterized cytosolic and metabolic changes observed to precede cellular atrophy and death after activity deprivation. Because MCPG and AP3 eliminate the ability of orthodromic stimulation to maintain low $[Ca^{2+}]_i$ levels, it is reasonable to propose that VIIIth nerve activity maintains NM neurons by regulating $[Ca^{2+}]_i$ via a metabotropic glutamate receptor that activates both the PKC and PKA signal transduction pathways. When this regulation is prevented, a rise in $[Ca^{2+}]_i$ may trigger a variety of apoptotic pathways as well as compensatory events, such as mitochondrial proliferation, which may serve to buffer the elevated $[Ca^{2+}]_i$.

Although it initially may appear paradoxical that activation of a mGluR that releases Ca^{2+} from intracellular stores ultimately results in prevention of an increase in $[Ca^{2+}]_i$, it makes sense when one remembers that $[Ca^{2+}]_i$ homeostasis is a dynamic, multicomponent phenomenon (Carafoli 1987). Included among the myriad mechanisms of $[Ca^{2+}]_i$ homeostasis are the IP_3/PKC (Nahorski 1988) and cAMP/PKA (Ghosh and Greenberg 1995) signal transduction pathways that we have shown to be present in NM neurons (reviewed in Lachica et al. 1995b). NM neurons show ongoing spontaneous activity rates of 100 Hz and phase locking to auditory nerve input $\leq$9,000 Hz (Warchol and Dallos 1990). This rate of activation of mGluRs, in addition to ionotropic receptor activation, creates ideal conditions for dynamic $[Ca^{2+}]_i$ homeostasis with mechanisms for increasing $[Ca^{2+}]_i$ (influx through iGluR channels, voltage-gated Ca^{2+} channels and release from intracellular stores) and mechanisms for keeping those increases in check [inhibition of influx through voltage-gated Ca^{2+} channels (Lachica et al. 1995a) and iGluR channels (E. A. Lachica, R. Rübsamen, and E. W. Rubel, unpublished data) and inhibition of release from intracellular stores (Kato et al. 1996)] balancing out to maintain physiological levels of $[Ca^{2+}]_i$. Upon loss of synaptic activity, this balance is offset in the direction of an increase in $[Ca^{2+}]_i$ through influx, release from stores, decreased pumping efficiency of Ca^{2+}-ATPases and may be facilitated by Ca^{2+}-induced Ca^{2+} release and influx through a Ca^{2+}-activated Ca^{2+} current, both of which appear to be present in NM neurons (Kato et al. 1996; Zirpel et al. 1995a).

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