Glutamatergic and GABAergic Agonists Increase $[Ca^{2+}]_i$ in Avian Cochlear Nucleus Neurons

E. A. Lachica, B. Maya Kato, W. R. Lippe, Edwin W Rubel

Virginia Merrill Bloedel Hearing Research Center, Department of Otolaryngology-Head and Neck Surgery, University of Washington School of Medicine, Box 357923, Seattle, Washington 98195

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ABSTRACT: **Neurons of the avian cochlear nucleus, nucleus magnocellularis (NM), are stimulated by glutamate, released from the auditory nerve, and GABA, released from both interneurons surrounding NM and from cells located in the superior olivary nucleus. In this study, the Ca^{2+} indicator dye Fura-2 was used to measure Ca^{2+} responses in NM stimulated by glutamate- and GABA-receptor agonists using a chicken brainstem slice preparation. Glutamatergically stimulated Ca^{2+} responses were evoked by kainic acid (KA), α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid (AMPA), and *N*-methyl-D-aspartate (NMDA). KA- and AMPA-stimulated changes in $[Ca^{2+}]_i$ were also produced in NM neurons stimulated in the presence of nifedipine, an L-type Ca^{2+} channel blocker, suggesting that KA- and AMPA-stimulated changes in $[Ca^{2+}]_i$ were carried by Ca^{2+}-permeable receptor channels. Significantly smaller changes in $[Ca^{2+}]_i$ were produced by NMDA. When neurons were stimulated in an alkaline (pH 7.8) superfusate, NMDA responses were potentiated. KA- and AMPA-stimulated responses were not affected by pH. Several agents known to stimulate metabotropic receptors in other systems were tested on NM neurons bathed in a Ca^{2+} free-EGTA–buffered media, including L-cysteine sulfinic acid (L-CSA), *trans*-azetidine dicarboxylic acid (t-ADA), *trans*-aminocyclopentanedicarboxylic acid (*t*-ACPD), and homobromoibotenic acid (HBI). The only agent to reliably and dose-dependently increase $[Ca^{2+}]_i$ was HBI, an analog of ibotenate. GABA also stimulated increases in $[Ca^{2+}]_i$ in NM neurons. GABA-stimulated responses were reduced by agents that block voltage-operated channels and by agents that inhibit Ca^{2+} release from intracellular stores. Whereas GABA-A receptor agonist produced increases in $[Ca^{2+}]_i$ GABA-B and GABA-C receptor agonists had no effect. There appear to be several ways for $[Ca^{2+}]_i$ to increase in NM neurons. Presumably, each route represents a means by which Ca^{2+} can alter cellular processes.** © 1998 John Wiley & Sons, Inc. J Neurobiol 37: 321–337, 1998

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Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) stimulate a number of intracellular events. In neuronal systems, Ca^{2+} has been shown to play a role in chemical transmission, phosphorylation of receptor proteins, gene expression, and modulation of cytoplasmic messengers. Ca^{2+} is also known to play an important role in the cell death process. In the sensory deprived avian cochlear nucleus, nucleus magnocellularis (NM), elevations in $[Ca^{2+}]_i$ precede the cellular changes that are characteristic of neurons that will die (Zirpel et al., 1996; Lachica et al., 1996). Ca^{2+} may trigger a wide variety of changes in gene expression and may be involved in producing the phenotypic changes displayed by sensory-deprived NM neurons. We have not yet determined how or why $[Ca^{2+}]_i$ increases in deafferented neurons. Understanding how $[Ca^{2+}]_i$ is normally regulated in these neurons represents a logical first step toward answering this question.

Nucleus magnocellularis is composed of a single population of large, adendritic neurons that receive a

Correspondence to: E. W Rubel

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single source of excitatory input from the auditory nerve (Boord, 1969; Parks and Rubel, 1978; Jhaveri and Morest, 1982a,b). Two GABAergic inputs exist: one from interneurons in the region of NM (von Bartheld et al., 1989) and one from the superior olivary nucleus (Lachica et al., 1994). Auditory nerve terminals release glutamate from large endbulbs that envelop NM neurons, covering over 60% of the somatic surface (Parks and Rubel, 1978; Parks, 1981; Zhou and Parks, 1992; Raman and Trussell, 1992). The local circuit interneurons and olivary neurons release GABA via bouton-shaped endings that form axosomatic connections (Code et al., 1989).

The effects of glutamate on $[Ca^{2+}]_i$ in other neurons are well known. Changes in $[Ca^{2+}]_i$ generally result from stimulation of NMDA- or non-NMDA–type receptors. Non-NMDA receptors are divided into two types based upon their sensitivity to α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid (AMPA) and kainic acid (KA). Using video-enhanced ratiometric fluorometry, we show that NMDA and non-NMDA receptor agonists increase $[Ca^{2+}]_i$ in NM neurons. We demonstrate that NMDA-stimulated Ca^{2+} responses are pH dependent. We also show that changes in $[Ca^{2+}]_i$ stimulated by AMPA and KA are carried by Ca^{2+}-permeable non-NMDA receptor channels.

Glutamate can also stimulate a change in $[Ca^{2+}]_i$ via a metabotropic glutamate receptor (mGluR) coupled to the enzyme phospholipase C (Sugiyama et al., 1989; Aramori and Nakanishi, 1992; Zirpel et al., 1994). In NM neurons, homobromoibotenic acid (HBI) reliably and dose dependently liberated Ca^{2+} from stores. Although the receptor target for the selective action of HBI in NM neurons is not known, the results strongly suggest that this agent acts on a phospholipase C-linked metabotropic glutamate receptor.

The effects of GABA on neuronal $[Ca^{2+}]_i$ are not as well studied (however, see Obrietan and van den Pol, 1995; Parramon et al., 1995; Obrietan and van den Pol, 1996). We show that a novel GABA receptor stimulates an increase in $[Ca^{2+}]_i$. A small fraction of this change in $[Ca^{2+}]_i$ (ΔCa^{2+}) appears to be carried by dihydropyridine-sensitive, high voltage–operated Ca^{2+} channels (VOCCs). The remainder is due to Ca^{2+} released from intracellular stores, indicating that the GABA receptor expressed by NM neurons may be G protein linked.

In avians, nucleus magnocellularis neurons are responsible for detecting the microsecond disparities that mark the arrival of incoming sounds. Like all sensory neurons, NM neurons transmit information forward to higher-order sensory nuclei. In the case of NM, however, the anatomy and connectivity are such that a premium is placed on the rapid and faithful transmission of signals. Presumably, little postprocessing is completed at this level. Consequently, the NM system serves as an excellent model for studies that are designed to understand how sensory signals affect neurons at the membrane receptor and subcellular level. In this study, which examines the calcium changes that occur in NM as a result of glutamatergic and GABAergic transmission, our results show that there are multiple routes of entry available to Ca^{2+} in NM neurons, and therefore, several ways in which Ca^{2+} can alter cellular physiology.

MATERIALS AND METHODS

A detailed account of the dissection, fluorometry, and perfusion system has been described previously (Lachica et al., 1995). Briefly, brainstem slices (300 μm thick) containing the rostral portion of NM were acquired from 17- to 18-day-old white Leghorn chicken embryos, staged using Hamilton and Hamburger (1951) criteria. These sections were transferred immediately into a warm (40°C), oxygenated salt solution containing 5 μM Fura-2 AM (Molecular Probes), 0.1% dimethylsulfoxide (DMSO), and 0.02% pluronic acid. After 20–25 min, slices were placed in a Leiden-style microscope-stage chamber and superfused with an artificial cerebrospinal fluid (aCSF) composed of 125 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 8 mM Hepes, 8 mM $NaHCO_3$, 10 mM glucose, and 3.5 mM $CaCl_2$ (pH 7.4). The fluorescent emissions of neurons excited by 340- and 380-nm wavelengths [provided by a Xenon source (Ushio, Inc.)] were collected by a ×40, 1.4 NA (Nikon) fluorite objective, passed through a 510-nm emission filter, and recorded by an intensified charge-coupled device (Hamamatsu). Analog images were digitized (Matrox), and a ratio of the fluorescent intensity, reported as nanomoles of Ca^{2+} concentration (Grynkiewicz et al., 1985), was computed using software designed by Universal Imaging Corp. (West Chester, PA). All of the data were collected at room temperature. The duration of individual experiments typically ranged from 10 to 15 min.

It is important to emphasize that these experiments were conducted on an acute slice preparation. We have assumed that the drug effects described in this article were not mediated by glial cells or other elements acting on NM neurons. These assumptions are supported by results from physiological studies on dissociated neurons (conducted by other investigators) that used the same drugs as those in this study and reported findings consistent with the observations that we describe in the present report.

Ionotropic Glutamate Receptor Agonists: Non-NMDA Receptors

Nucleus magnocellularis neurons were stimulated with different concentrations of the glutamate agonists KA, AMPA, and NMDA, and dose–response curves were generated. A

single slice was stimulated with a single concentration of only one agonist. Each experiment was reproduced at least four times. Agonists were delivered in a superfusate containing 10 μm nifedipine, a Ca^{2+} channel antagonist, to ensure that voltage operated calcium channels (VOCCs) did not contribute to changes in cell Ca^{2+} (ΔCa^{2+}). Nifedipine, a dihydropyridine that blocks L-type calcium channels (Nowycky et al., 1985; Carbone and Swandulla, 1989), reduces KCl-stimulated Ca^{2+} responses in NM neurons when used at concentrations of 1–10 μM by 90% (Lachica et al., 1995).

Changes in cell $[Ca^{2+}]_i$ were produced by 15- to 25-s superfusion of KA, a 40- to 60-s superfusion of AMPA, or a 3-min-long superfusion of NMDA.

Kainic acid– and AMPA-stimulated changes in $[Ca^{2+}]_i$ were challenged by the following agents: $CdCl_2$, an inorganic compound that nonspecifically blocks voltage operated Ca^{2+} channels; N-methyl-D-glucamine (NMG), a nonpermeable cation which prevents neuronal depolarization (by superfusing neurons in an Na^+/K^+ free aCSF); 8-(diethylamino)octyl-3,4,5-triethoxybenzoate (TMB8), an inhibitor of intracellular Ca^{2+} release (see, e.g., Chiou and Hong, 1995; Griffiths et al., 1994); cyclothiazide (CTZ), a benzothiadiazide that inhibits AMPA receptor desensitization (Desai et al., 1995; Wong and Mayer, 1993; Yamada and Tang, 1993); and GYKI 52466, a non-NMDA receptor antagonist that inhibits AMPA responses (Donevan and Rogawski, 1993; Wilding and Huettner, 1995).

Ionotropic Glutamate Receptor Agonists: NMDA Receptors

Neurons were superfused with an Mg^{2+}-free, 10-μM glycine-supplemented aCSF for 1 min before they were stimulated with 1 m*M* NMDA. NMDA-stimulated $[Ca^{2+}]_i$ responses were then challenged by the following agents: phorbol myristate acetate (PMA), used to stimulate protein kinase C activity; the membrane permeable analog of cyclic adenosine monophosphate, 8-bromo-cyclic AMP, and the adenylate cyclase activator, forskolin, used to stimulate protein kinase A activity; the isoquinolinsulfonimdes, H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) and H8 (*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride), used to block, respectively, C and A kinase activity. The effects of the metabotropic glutamate receptor (mGluR) agonist *trans*-aminocyclopentanedicarboxylic acid (*t*-ACPD), and the mGluR antagonist methyl-4-carboxyphenylglycine (MCPG) were also examined.

Finally, KA- and NMDA-stimulated changes were examined in perfusates that were 0.4 pH units greater or less than normal (pH 7.4). In these experiments, KA- or NMDA-stimulated increases in $[Ca^{2+}]_i$ were evoked, then allowed to buffer in normal aCSF. After basal calcium levels were restored, slices were superfused for a minimum of 5 min in the pH-modified perfusate, then restimulated.

Metabotropic Glutamate Receptor Agonists: Intracellular Stores

Ca^{2+} efflux from intracellular stores was stimulated in NM neurons superfused in aCSF lacking Ca^{2+} and buffered with 1 m*M* EGTA (Sigma, St. Louis, MO). Several agents were examined (see Results for details). We are confident that changes in calcium caused by an agent were attributable to release from stores because each of these agents was delivered in a Ca^{2+}-free, EGTA-buffered vehicle. We also examined the effects of the following agents to ensure that the $\Delta[Ca^{2+}]_i$ stimulated by a particular mGluR-agonist was due to the liberation of stored Ca^{2+}: nifedipine, TMB8, thapsigargin, and MCPG.

GABA

The effects of GABA were tested on NM neurons superfused in normal aCSF and Ca^{2+}-free aCSF. The effects of the GABA-A receptor agonist muscimol were tested on NM neurons superfused in normal aCSF only; the GABA-B and GABA-C agonists baclofen and *cis*-4-aminocrotonic acid, respectively, were tested on NM neurons superfused in normal and Ca^{2+}-free aCSF. The effects of the following agents on GABA-stimulated responses were also examined: BAY K 8644, a dihydropyridine agonist that potentiates changes in $[Ca^{2+}]_i$ carried by L-type voltage operated channels; TMB8, the Ca^{2+} antagonist; heparin, an antagonist of inositol trisphosphate receptors (IP3Rs); and CNQX, an antagonist of ionotropic glutamate receptors.

Statistics

Statistical analyses were performed using one- or two-way analyses of variance (ANOVA). Post hoc analyses were assessed using the Scheffé test. Student *t* test was used when appropriate.

In Results, changes in $[Ca^{2+}]_i$ are reported as the means $\pm$ standard error of the mean (S.E.M.). The total number of neurons examined is reported, followed by the total number of slices that were examined. For statistical comparisons, a conservative approach was used; we computed the mean value of all neurons in a slice and used that value as a single observation.

Materials

L-Cysteine sulfinic acid (L-CSA), (*t*-ACPD), and homobromoibotenic acid (HBI) were purchased from Tocris (St. Louis, MO). The remaining pharmaceuticals were purchased from Research Biochemical (Natick, MA) unless otherwise specified. All other chemicals were of analytical grade.

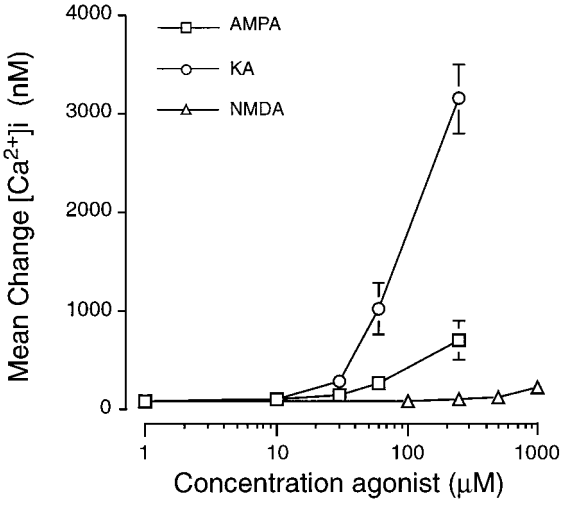

Figure 1 AMPA, KA, and NMDA increase $[Ca^{2+}]_i$ in NM neurons. Dose–response curves plot the mean change in $[Ca^{2+}]_i$ produced by increasing concentrations of three glutamate receptor agonists. Responses were measured in aCSF containing 10 μM nifedipine to block any potential contribution of voltage-operated Ca^{2+} channels. Where absent, error bars are smaller than the symbol size. Error bars = S.E.M.

RESULTS

In the sections that follow, we show that changes in $[Ca^{2+}]_i$ are produced in NM neurons stimulated with the non-NMDA receptor agonists AMPA and KA, and with NMDA. We also demonstrate that changes in $[Ca^{2+}]_i$ can be stimulated with a metabotropic glutamate receptor agonist believed to activate phospholipase C. Finally, we show that GABA-stimulated changes in $[Ca^{2+}]_i$ are the result of influx of Ca^{2+} through voltage operated channels and from Ca^{2+} released from intracellular stores.

Changes in $[Ca^{2+}]_i$ Are Evoked by Glutamate Receptor Agonists

Figure 1 plots the mean maximal change in $[Ca^{2+}]_i$ (±S.E.M.) in NM neurons stimulated by different concentrations of AMPA, KA, and NMDA. In NM, non-NMDA receptors are permeable to Ca^{2+} (Otis et al., 1995). For this reason, the Ca^{2+} responses plotted in Figure 1 were measured in neurons treated with 10 μM nifedipine to block the contributions of voltage-operated Ca^{2+} channels. Each data point is based on neuronal responses from at least four different brainstem slices (an average of 12 neurons per slice). In separate experiments, Ca^{2+} responses to AMPA and KA were also tested in $CdCl_2$-supplemented and NMG-modified aCSF (data not shown), which blocks the contributions of all VOCCs. Robust responses were stimulated by these agents under each of these conditions. The amplitude of KA- and AMPA-stimulated responses measured in NM neurons superfused in normal media did not differ from KA-/AMPA-stimulated responses in Na^+/K^+-free media.

KA, AMPA, and NMDA increased $[Ca^{2+}]_i$. Figure 1 shows that the responses evoked by KA and AMPA at any given concentration were always greater than the changes produced by NMDA. NMDA-stimulated increases in $[Ca^{2+}]_i$ were not observed in roughly 70% of the neurons examined at the highest concentration (1.0 m*M* NMDA). Ca^{2+} responses were reliably produced by brief pulses (10–15 s) of KA and AMPA applied in micromolar concentrations, whereas prolonged exposures (roughly 3 min) of millimolar concentrations of NMDA were required to produce a Ca^{2+} response. KA always produced a greater $\Delta[Ca^{2+}]_i$ than the equivalent concentration of AMPA, and it took the neurons longer to buffer KA-stimulated increases in $[Ca^{2+}]_i$ even though AMPA was applied for a longer period of time. These results show that $[Ca^{2+}]_i$ in NM neurons can be increased by agents that stimulate non-NMDA and NMDA receptor channels.

AMPA- and KA-Stimulated Increases in $[Ca^{2+}]_i$

Nucleus magnocellularis neurons possess Ca^{2+}-permeable AMPA-preferring receptors (Raman and Trussell, 1992; Zhang and Trussell, 1994). These receptors are rapidly desensitizing (Raman and Trussell, 1992, 1995), which could explain why AMPA-stimulated responses were consistently smaller than the KA-stimulated responses. This possibility was examined by studying AMPA-stimulated $[Ca^{2+}]_i$ responses in the presence of cyclothiazide (CTZ), an agent that blocks glutamate receptor desensitization (Partin et al., 1993). A total of 59 neurons ($n = 5$ slices) were studied. The $\Delta[Ca^{2+}]_i$ evoked by AMPA before and after the neurons were superfused with 50 μM CTZ was not significantly different. Before CTZ treatment, 50 μM AMPA produced a mean $\Delta[Ca^{2+}]_i$ of 243 ± 30 n*M;* in the presence of CTZ, 50 μM AMPA caused a mean $\Delta[Ca^{2+}]_i$ of 292 ± 52 n*M.* This concentration of CTZ has been shown to reverse AMPA receptor desensitization in NM neurons (Trussell et al., 1993). KA-stimulated Ca^{2+} responses were also not affected by CTZ treatment. These results suggest that receptor desensitization does not contribute to the difference in the $\Delta[Ca^{2+}]_i$ stimulated by KA versus AMPA under the conditions of this study.

Another possible explanation for the different

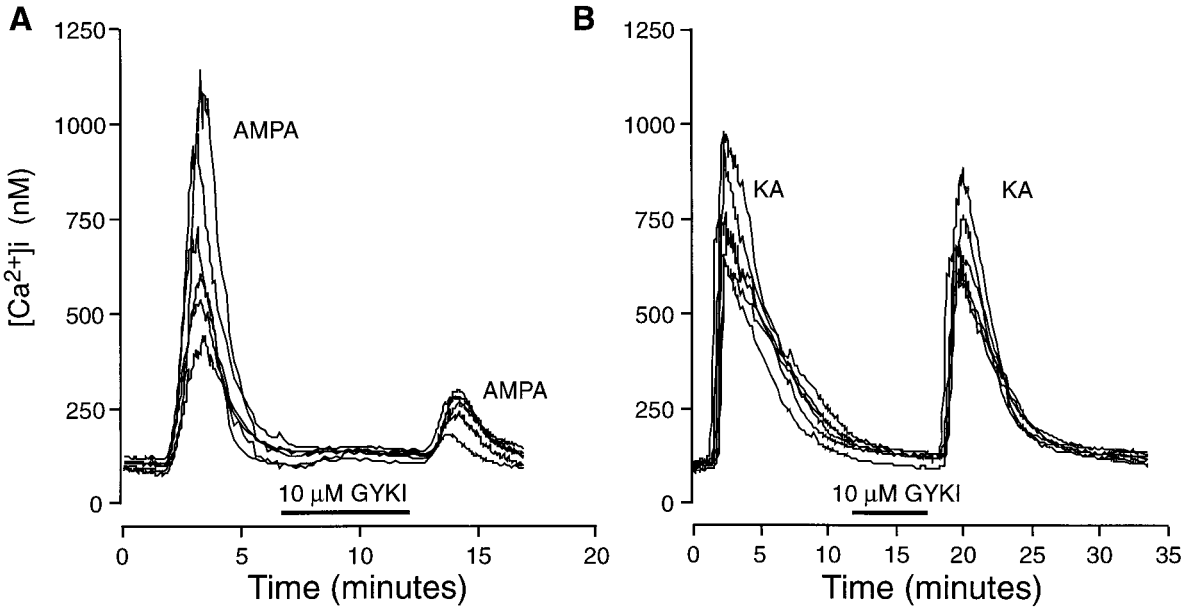

Figure 2 Ca^{2+} response stimulated by AMPA and KA are differentially affected by GYKI 52466. (A) Ca^{2+} responses of six NM neurons stimulated with 50 μM AMPA before and after they were treated with 10 μM GYKI 52466 for 10 min. (B) Ca^{2+} responses of six neurons (from a different slice preparation) stimulated with 25 μM KA before and after they were treated with 10 μM of GYKI 52466. Both experiments were conducted in Na^+/K^+-free aCSF to eliminate the contribution of VOCCs to the agonist-stimulated Ca^{2+} response. Na^+ and K^+ were replaced by the impermeable cation NMG.

magnitude of responses to AMPA versus KA is that AMPA and KA stimulate different receptors in NM. GYKI 52466, an agent that has been shown preferentially to attenuate AMPA-stimulated currents (e.g., Lerma et al., 1993), was used to test this possibility. For these experiments, NM neurons superfused in Na^{2+}-free media (to eliminate the $\Delta[Ca^{2+}]_i$ contributed by voltage-operated channels), were initially stimulated with either AMPA or KA, exposed to 10 μM GYKI for 5 min, then restimulated. As seen in Figure 2, GYKI dramatically reduced AMPA-stimulated changes in $[Ca^{2+}]_i$, while KA-stimulated Ca^{2+} responses were unaffected. The responses of 42 KA-stimulated neurons ($n = 4$) were compared to 56 AMPA-stimulated neurons ($n = 4$ slices). Statistical analyses show that AMPA-stimulated responses were significantly reduced ($t = 5.314$; $p < .001$). On average, 10 μM GYKI reduced the AMPA-stimulated responses by approximately 66% (Fig. 3).

NMDA-Stimulated ΔCa^{2+} Are Sensitive to $[H^+]_o$

NMDA receptors express several modulatory sites that are capable of reducing NMDA receptor sensitivity [see McBain and Mayer (1994) for review]. Thus, the weak NMDA response seen in NM neurons could be explained by (a) a small number of receptors, or (b) negatively modulated receptors. Several agents that have been shown to alter NMDA receptor sensitivity were examined. In each case, neurons were initially stimulated with 1 mM NMDA for 3 min, exposed to a test agent, then restimulated with 1 mM NMDA. Table 1 summarizes these results.

The effects of some of these agents were inconsistent. The A-kinase–blocking agent H8, for example, significantly reduced the Ca^{2+} response to NMDA (100 μM; 64 neurons; $n = 5$ slices), whereas A-kinase accumulating forskolin had no effect (100 μM; 75 neurons; $n = 4$ slices). Similarly, the C-kinase activator PMA significantly reduced the response to NMDA (0.5 μM; 55 neurons; $n = 5$ slices), but the C-kinase blocker H7 did not have a reliable effect (100 μM; 55 neurons; $n = 4$ slices). In other systems, these agents have been shown to have very specific, reproducible, predictable effects on NMDA receptor responses.

NMDA responses were also unaffected by spermidine (10 and 100 μM; 57 neurons; $n = 4$ slices), a polyamine that potentiates NMDA currents, by KN-62 (10 and 100 μM; 63 neurons; $n = 5$ slices), an inhibitor of Ca^{2+}/calmodulin-dependent protein ki-

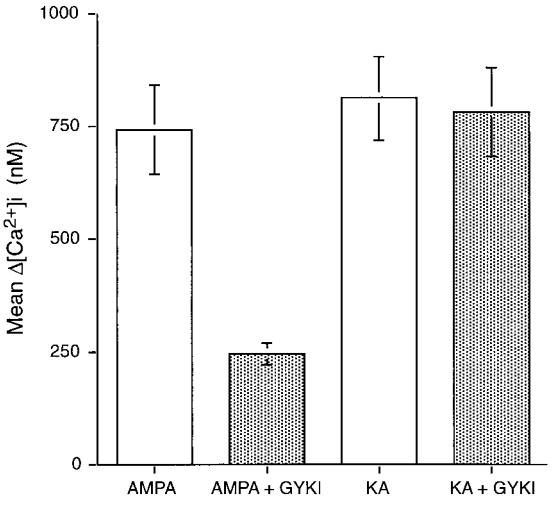

Figure 3 GYKI 52466 reduces AMPA-stimulated Ca^{2+} responses in NM neurons. Effects of GYKI 52466 on Ca^{2+} responses to 50 μM AMPA and 25 μM KA. White bars labeled "AMPA" and "KA" represent the mean change in Ca^{2+} stimulated by these agents in untreated NM neurons. Shaded bars labeled "AMPA + GYKI" and "KA + GYKI" represent the mean response stimulated by these agents in neurons treated for 5 min with 10 μM GYKI 52466. AMPA-stimulated responses were significantly attenuated by GYKI; KA responses were not affected. Error bars = S.E.M.

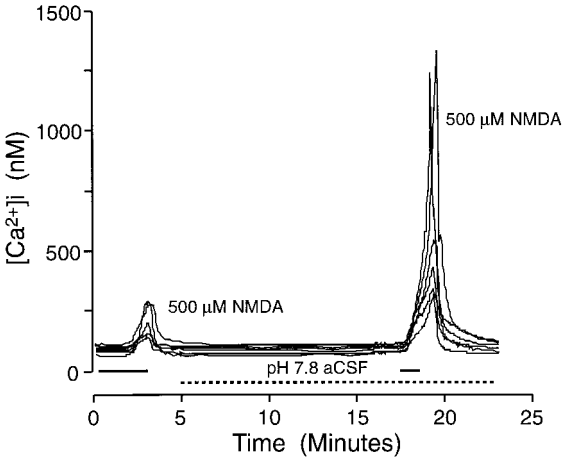

Figure 4 NMDA-stimulated Ca^{2+} response are pH sensitive. NMDA-stimulated Ca^{2+} responses are shown for six NM neurons perfused in normal aCSF and pH-modified aCSF. Black bars mark the duration of agonist stimulation. Note the shorter period of stimulation during the superfusion of pH-modified media. Dashed bar marks the duration of treatment superfusion.

nase, and glycine (200 μM; 62 neurons; $n = 4$ slices). Each of these agents has been shown to reliably alter NMDA responses in other neuronal systems.

NMDA-stimulated Ca^{2+} responses were extremely sensitive to pH. In particular, the $\Delta[Ca^{2+}]_i$ was significantly potentiated when NMDA receptors were stimulated in an alkaline environment. An example of this effect is seen in Figure 4, which shows the responses of neurons stimulated with 500 μM NMDA in normal (pH 7.4) and alkaline (pH 7.8) media. In normal media, 100 μM NMDA (60 neurons; $n = 5$ slices) produced a very small $\Delta[Ca^{2+}]_i$, 8.4 $\pm$ 4 n*M*. The mean $\Delta[Ca^{2+}]_i$ of 68 neurons ($n = 6$ slices) stimulated with 100 μM NMDA in alkaline aCSF was 371 $\pm$ 71 n*M*. Acidification of the media did not affect the NMDA response; the mean response measured in 43 neurons ($n = 4$ slices) stimulated with 100 μM NMDA in pH 7.0 media was 12 $\pm$ 7 n*M*. As shown in Figure 5(B), the magnitude of the potentiation in alkaline media was greater for progressively higher concentrations of NMDA.

We determined that the effect of pH is not common to all glutamate receptors, by examining KA-stimulated responses in normal, alkaline, and acidic media. Figure 5(A) compares the responses to 250 μM NMDA and 25 μM KA in normal and alkaline aCSF. Figure 5(B,C) shows the mean $\Delta[Ca^{2+}]_i$ in alkaline and acidic media to varying concentrations of NMDA and KA, respectively. The results of these experiments indicate that KA-stimulated responses are relatively insensitive to the ranges of pH studied here. A two-way ANOVA comparing the Ca^{2+} responses to NMDA and KA in different pH conditions (pH 7.8, 7.4, and 7.0) was performed. The main effect of pH and the interaction term were significant ($ps < .01$). Pairwise comparisons revealed that the significant interaction effect was due to the effect of alkaline versus normal pH on NMDA-stimulated responses.

Table 1 Effects of Modulatory Agents on NMDA-Stimulated Ca^{2+} Responses

Agent	Concentration (μM)	Mean Difference (n*M*)	S.D. (n*M*)
H8*	100	212	115
H7	100	62	53
Forskolin	100	20	60
PMA†	0.50	277	96
KN-62	100	72	58
Spermidine	100	35	53
Glycine	200	76	56

The maximal Ca^{2+} (ΔCa^{2+}_{max}) response to 1 m*M* NMDA was measured and compared to the ΔCa^{2+}_{max} evoked by NMDA after the neurons were exposed to the agents listed. The concentration of the agent and mean difference in maximal Ca^{2+} response (and standard deviation) are shown.

* $p < .05$ (two-tailed *t* test).

† $p < .01$ (two-tailed *t* test).

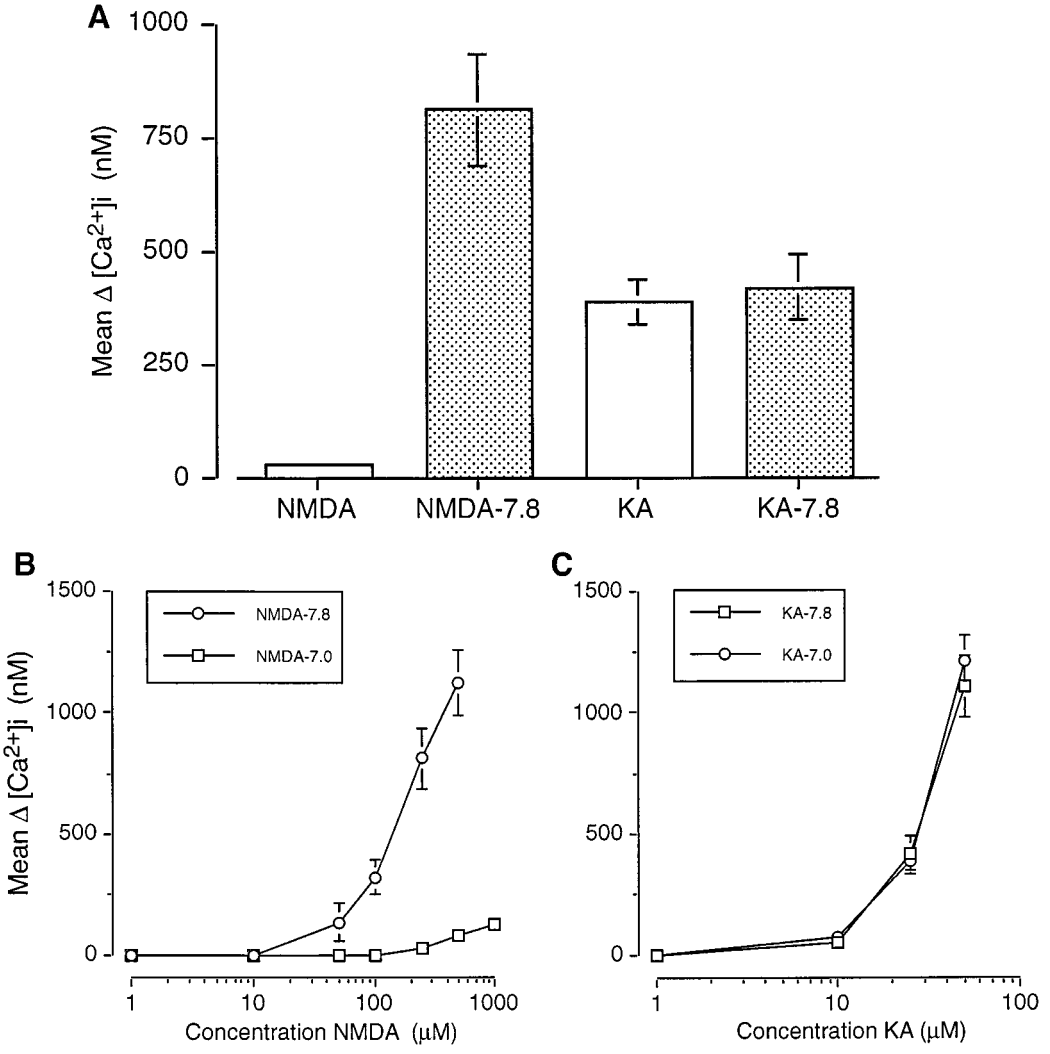

Figure 5 NMDA receptors in NM are pH sensitive. (A) Effects of different pH values on NMDA- and KA-stimulated responses. White bars labeled "NMDA" and "KA" represent the mean change in $[Ca^{2+}]_i$ stimulated by these agents in normal aCSF. Shaded bars labelled "NMDA-pH" and "KA-pH" represent the mean change in $[Ca^{2+}]_i$ stimulated by these agents in pH 7.8 aCSF. Test concentrations of NMDA and KA were 250 and 25 μM, respectively. (B,C) Dose–response effects of NMDA and KA on Ca^{2+} responses produced in alkaline and acidic aCSF. Four different slices were used to generate each data point. Each slice was exposed to one concentration of a single agent in a single type of environment (i.e., pH 7.0 vs. 7.8). Note that the values calculated for the acidic conditions did not differ from normal condition (pH 7.4). Error bars = S.E.M. Where not shown, error bars are smaller than the symbol.

Changes in $[Ca^{2+}]_i$ Stimulated from Intracellular Ca^{2+} Stores

Figure 6 plots the mean $\Delta[Ca^{2+}]_i$ measured in NM neurons stimulated with a variety of mGluR-activating agents in 0 Ca^{2+} aCSF buffered with EGTA. Each data point is based upon measurements from four different slices (on average, 12 neurons per slice). Individual slices were exposed to a single concentration of a single agent.

Ca^{2+} responses were rarely evoked by submillimolar concentrations of glutamate; 5–10 mM glutamate usually stimulated a rise in $[Ca^{2+}]_i$, but even these responses were not always reliable. Reliable responses were never produced by L-CSA or *t*-ADA. L-CSA has been shown to stimulate phospholipase D (Boss et al., 1994) and cyclic AMP activity independent of Go activation (Boss and Boaten, 1995). *t*-ADA (Favaron et al., 1993; Kozikowski et al., 1993) is believed to stimulate a re-

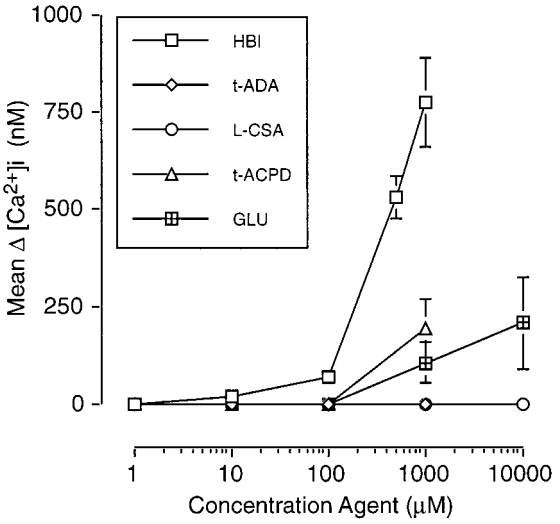

Figure 6 HBI dose dependently stimulates Ca^{2+} efflux from intracellular stores. Dose–response curves show the effects of five agents on $[Ca^{2+}]_i$ in NM neurons superfused in 0 Ca^{2+} aCSF. Homobromoibotenic acid (HBI) was the only agent that reliably and dose dependently increased $[Ca^{2+}]_i$ in NM neurons. t-ADA = *trans*-azetidine-dicarboxylic acid; L-CSA = L-cysteine sulfinic acid; t-ACPD = *trans*-aminocyclopentanedicarboxylic acid; GLU = glutamate. Error bars = S.E.M. Where not shown, error bars are smaller than the symbol.

ceptor exclusively linked to the activation of phospholipase C and the liberation of Ca^{2+} from inositol trisphosphate (IP_3) sensitive stores. *t*-ACPD evoked a $\Delta[Ca^{2+}]_i$ only at the highest concentration tested. It is important to note, however, that 1 m*M t*-ACPD did not always elevate $[Ca^{2+}]_i$, and in some cases $[Ca^{2+}]_i$ was reduced from baseline. The only agent tested that reliably and dose dependently stimulated Ca^{2+} efflux was homobromoibotenic acid (HBI), with an EC_{50} of approximately 250 μM.

Homobromoibotenic acid–stimulated changes in $[Ca^{2+}]_i$ were significantly reduced by TMB-8, (100 μM; 61 neurons; four slices; $t = 5.313$; $p < .001$) and thapsigargin (100 n*M;* 54 neurons; four slices; $t = 4.037$; $p < .001$). Nifedipine (10 μM), CNQX (50 μM), and APV (50 μM) did not reduce HBI-stimulated Ca^{2+} responses (57 neurons; $n = 4$ slices). MCPG (1 m*M;* 63 neurons; $n = 5$ slices) and L-AP3 (1 m*M;* 41 neurons; $n = 3$ slices), agents that have been shown to inhibit the actions of mGluRs, also failed to reduce HBI-stimulated Ca^{2+} responses. These data are summarized in Table 2.

Changes in $[Ca^{2+}]_i$ Stimulated by GABA

A total of 62 neurons ($n = 4$ slices) were used to study changes in $[Ca^{2+}]_i$ stimulated by GABA. As seen in Figure 7, GABA-stimulated responses consisted of a brief Ca^{2+} spike followed by a small, sustained component. GABA-stimulated responses were not dose dependent over the range of concentrations examined (10 μM to 5 m*M*). Instead, they were all or none. A concentration of 500 μM GABA reliably increased $[Ca^{2+}]_i$ in NM neurons superfused in normal and 0 Ca^{2+} aCSF [Fig. 7(A)]. Because of the complex nature of the GABA-stimulated Ca^{2+} response, comparative statistical analyses based on the mean maximal change in $[Ca^{2+}]_i$ were not performed. Instead, analyses were conducted on the total area demarcated by the waveform. Specifically, we enclosed the response area with a line segment whose beginning and end points were defined by the rise in $[Ca^{2+}]_i$ to GABA and the return to prestimulus $[Ca^{2+}]_i$, respectively.

The magnitude of the $\Delta[Ca^{2+}]_i$ produced by GABA in Ca^{2+}-free aCSF was not significantly different from the Ca^{2+} response seen in neurons superfused in normal aCSF. These data suggest that a large portion of the GABA-stimulated response originated from intracellular stores. To test this hypothesis, 55 neurons ($n = 4$ slices) were exposed to 100 μM TMB8 and then stimulated with 500 μM GABA in Ca^{2+}-free aCSF buffered with 1 m*M* EGTA. TMB8 reduced GABA-stimulated responses by nearly 60%. The effects of heparin (100 U/mL), an IP_3R-antagonist, were similarly tested on 31 neurons ($n = 3$ slices). Heparin reduced GABA-stimulated responses by 72%. CNQX, an ionotropic glutamate receptor antagonist that was used as a positive control, did not affect GABA-stimulated responses.

These experiments suggest that the GABA receptor expressed by NM neurons is capable of activating a second-messenger system that liberates Ca^{2+} from

Table 2 Antagonist Effects on HBI-Stimulated Ca^{2+} Responses

Agent	Concentration (μM)	Mean Difference (n*M*)	S.D. (n*M*)
TMB8*	100	453	21
Thapsigargin*	0.10	343	33
Nifedipine	10	23	12
CNQX	50	14	8
APV	50	21	33
MCPG	1000	84	80
L-AP3	1000	51	37

The maximal Ca^{2+} (ΔCa^{2+}_{max}) response to 500 μM HBI was measured and compared to the ΔCa^{2+}_{max} evoked by HBI after the neurons were exposed to the agents listed. The concentration of the agent and mean difference in maximal Ca^{2+} response (and standard deviation) are shown.

* $p < .001$ (two-tailed *t* tests).

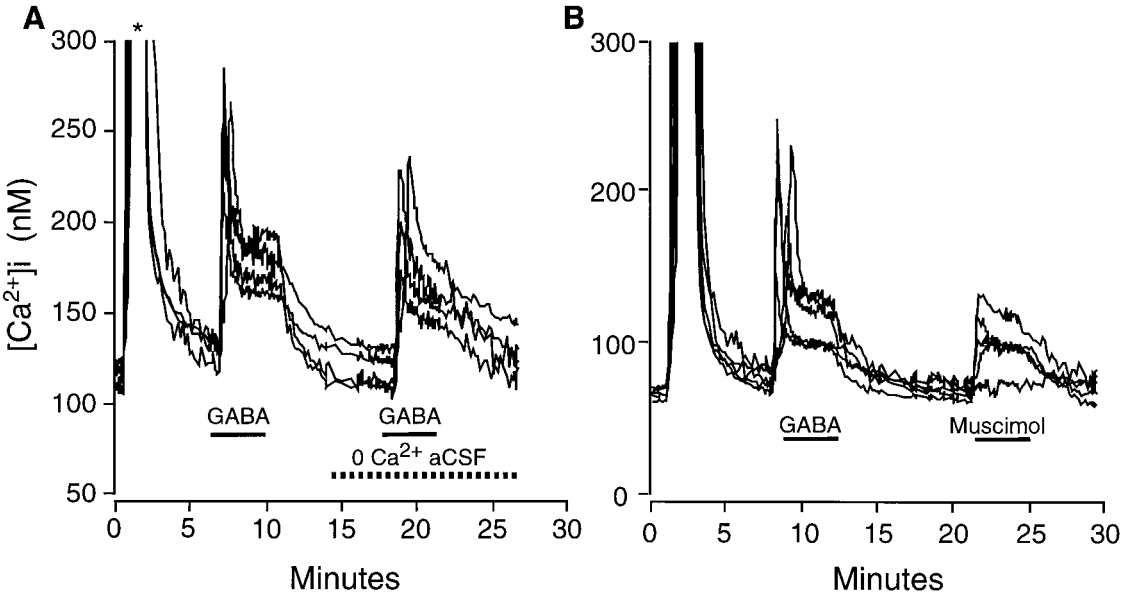

Figure 7 GABA increases $[Ca^{2+}]_i$ in NM neurons. (A) Effects of 500 μM GABA on the $[Ca^{2+}]_i$ of six NM neurons stimulated in normal aCSF and 0 Ca^{2+} aCSF. (B) Ca^{2+} responses generated in six NM neurons stimulated first by 500 μM GABA, and then by 100 μM muscimol, a GABA-A receptor agonist. Note that the large early Ca^{2+} transient seen in GABA-stimulated neurons is not produced by muscimol. In addition, note that muscimol does not produce an increase in $[Ca^{2+}]_i$ in every NM neuron. This was a consistent finding. Black bar marks the duration of agent stimulation. Dashed line marks the duration of the 0 Ca^{2+} aCSF superfusion.

IP_3-sensitive stores. Thus, it could be argued that the GABA receptor is G-protein linked and should be sensitive to the GABA-B receptor agonist, baclofen. A total of 138 neurons ($n = 12$ slices) were exposed to 10, 100, or 1000 μM baclofen in 0 Ca^{2+} aCSF. Calcium responses were never seen in baclofen-stimulated neurons. The GABA-C receptor agonist *cis*-aminocrotonic acid (100 and 1000 μM) also failed to stimulate an increase in $[Ca^{2+}]_i$.

Figure 7(B) shows the Ca^{2+} responses of NM neurons stimulated by GABA and the GABA-A receptor agonist muscimol (46 neurons; $n = 4$ slices). As with GABA-stimulated Ca^{2+} responses, muscimol-stimulated responses were all or none. One hundred micromoles of muscimol reliably produced an increase in $[Ca^{2+}]_i$ that did not differ in magnitude from responses stimulated by 1000 μM muscimol. Muscimol-stimulated Ca^{2+} responses were, on average, 73% smaller than GABA-stimulated Ca^{2+} responses. This difference appears to reflect the absence of the large, early Ca^{2+} transient that is observed when neurons are stimulated by GABA.

Finally, the effects of the dihydropyridine agonist BAY K 8644 were tested on neurons stimulated with muscimol (29 neurons; $n = 3$ slices) and GABA (57 neurons; $n = 5$ slices). Changes in $[Ca^{2+}]_i$ stimulated by either agent were augmented significantly by 10 μM BAY K 8644. In the presence of BAY K 8644, GABA-stimulated responses were 55% larger than normal; muscimol-stimulated responses were 39% larger. These results indicate that a small component of the GABA-stimulated Ca^{2+} response could be attributable to an L-type VOCC.

DISCUSSION

In this study, videofluorometric techniques were used to show that ionotropic and metabotropic glutamate receptor agonists and GABA are capable of increasing $[Ca^{2+}]_i$ of NM neurons. The main results are summarized in Figure 8. First, large increases in $[Ca^{2+}]_i$ were reliably produced by the ionotropic glutamate receptor agonists AMPA and kainate, which stimulate a Ca^{2+}-permeable ionophore. Studies using GYKI 53466 indicate that AMPA-preferring and kainate-preferring receptors may be expressed by NM neurons. Second, large changes in $[Ca^{2+}]_i$ were produced by NMDA, but only when NM neurons were stimulated in an alkaline medium. Thus, the NMDA receptors of NM neurons appear to possess a proton-sensitive modulatory site. It could be argued that neurons stimulated in alkaline media are slightly depolarized,

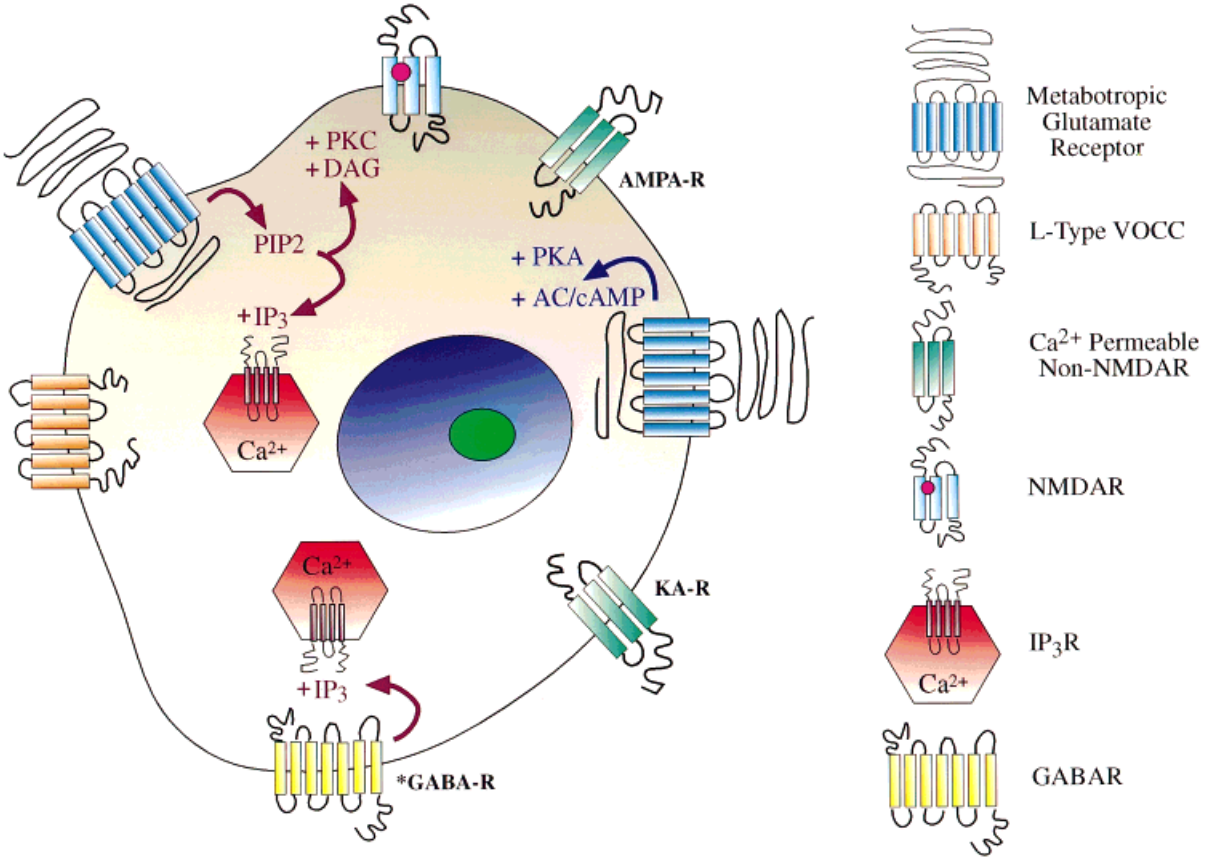

Figure 8 Schematic diagram of an individual NM neuron showing receptors that can be activated to stimulate an increase in $[Ca^{2+}]_i$. NM neurons possess two Ca^{2+}-permeable non-NMDA receptors (non-NMDAR, represented by the green tetramer), one sensitive to AMPA (AMPA-R) and one sensitive to kainic acid (KA-R). They also possess pH-sensitive NMDA receptors (NMDAR). We previously determined (Lachica et al., 1995) that NM neurons possess a metabotropic glutamate receptor (represented by the gray heptamers) that increases cyclic AMP activity and attenuates Ca^{2+} increases carried by L-type voltage operated channels (represented by the red hexamers). NM neurons also possess a metabotropic receptor linked to phospholipase C that liberates Ca^{2+} from inositol trisphosphate (IP_3)-sensitive stores (represented by the red hexagon). A GABAergic receptor (GABA-R) also stimulates an increase in $[Ca^{2+}]_i$. This receptor is illustrated as a heptamer because the majority of the GABA-stimulated Ca^{2+} response originates from intracellular stores, suggesting a G-protein–dependent process.

which could account for their increased sensitivity to NMDA. However, KA-stimulated response were not affected by changes in pH. Furthermore, NMDA-stimulated changes produced in neurons that were pharmacologically depolarized by 10 m*M* KCl did not differ from NMDA-stimulated Ca^{2+} responses seen in non–KCl-treated neurons (data not shown). Third, changes in $[Ca^{2+}]_i$ linked to cytoplasmic organelles were produced by HBI, a metabotropic glutamate receptor agonist. Finally, GABA produced an increase in $[Ca^{2+}]_i$ that was sensitive to agents that affect Ca^{2+} stores and high-voltage-operated channels. Thus, in NM neurons, $[Ca^{2+}]_i$ can increase in several ways: (a) via voltage-operated channels, (b) via Ca^{2+}-permeable non-NMDA receptor channels and NMDA receptor channels, (c) via second-messenger–linked intracellular stores, and (d) by GABA. Each main result is discussed below.

Ca^{2+}-Permeable Non-NMDA Receptor Channels in NM

Physiological and pharmacological studies (Otis et al., 1995; Zhou et al., 1995) and the fluorometric analyses of the present report indicate that non-NMDA receptor channels in NM are Ca^{2+} permeable. Ca^{2+}-permeable non-NMDA receptor channels are not unusual. Many neuronal types have been shown to

express them, including striatal neurons (Murphy et al., 1987; Murphy and Miller, 1989), hippocampal neurons (Iino et al., 1990), retinal bipolar cells (Gilbertson et al., 1991), retinal ganglion cells (Leinders-Zuffal et al., 1994), cerebellar Purkinje cells (Pruss et al., 1991), cerebellar granule cells (Hack and Balazs, 1995), and spiral ganglion cells of the auditory nerve (Simmons et al., 1994). They appear to be the abundant form of non-NMDA receptor in the immature CNS (Pellegrini-Giampietro et al., 1992).

In the intact brain, it is likely that increases in $[Ca^{2+}]_i$ carried by Ca^{2+}-permeable receptor channels are much more localized than the changes we produced in this study by bath application of agonists. It is possible that elevations in $[Ca^{2+}]_i$ carried by these iGluRs regulate a process(es) different from those mediated by Ca^{2+} changes carried by VOCCs (Lachica et al., 1995). For example, these iGluRs could be involved in phosphorylative processes that alter glutamate receptor physiology [see, e.g., Tan et al. (1994) and Yakel et al. (1995)].

AMPA- and KA-Preferring Receptors in NM

Converging lines of evidence indicate that AMPA and KA stimulate distinct receptor channels in a variety of neuronal systems. The differential effects of GYKI 52466 on AMPA- and KA-stimulated Ca^{2+} responses in the present study support the hypothesis that at least two distinct ionotropic receptor types may be expressed in NM. KA-sensitive/AMPA-insensitive receptors are not unique to NM neurons. KA-preferring receptors are expressed by neurons in the spinal cord, cerebellum, striatum, and hippocampus, and by glial cells (Somogyi et al., 1990; Herb et al., 1992; Lerma et al., 1993; Chen et al., 1995; Hack and Balazs, 1995; Renard et al., 1995).

The task of differentiating AMPA from KA receptors has been impeded by the absence of adequate pharmacological tools. In fact, pharmacological differences had not been identified until recently (e.g., Verdoorn et al., 1994; Nielsen et al., 1995; Shimizu-Sasamata et al., 1996). A growing number of investigators appear to agree that AMPA and KA receptors can be distinguished on the basis of a benzodiazepine recognition site (Donvean and Rogawski, 1993; Zorumski et al., 1993; Lerma et al., 1993; Paternain et al., 1995) that is sensitive to GYKI 52466.

Evidence for a KA-AMPA receptor distinction is the kainate receptor-encoding cDNAs (Seeburg, 1993). It should be noted, however, that the physiological responses produced by homomeric assemblies of kainate receptors GluR5 and GluR6, and heteromeric assemblies of kainate receptors GluR7, KA1, and KA2 (Herb et al., 1992), are not consistent with the physiological profile of glutamate receptors that have been described in NM (Raman and Trussell, 1992, 1995; Zhang and Trussell, 1994). In fact, physiological studies indicate that KA-sensitive receptors are not expressed by NM neurons. Considering the differential effects of GYKI on KA- and AMPA-stimulated Ca^{2+} responses, a reevaluation of NM neuron non-NMDA receptor expression may be warranted.

H^+-Sensitive NMDA Receptors Channels in NM

In NM neurons, NMDA stimulates significant increases in $[Ca^{2+}]_i$, but only in an alkaline environment. NMDA receptors possess multiple modulatory sites [see McBain and Mayer (1994) for review] and are uniquely sensitive to $[H^+]_o$ (Tang et al., 1990; Traynelis and Cull-Candy, 1991; Vyklicky et al., 1990).

Until recently (Zhang et al., 1995), the expression of NMDA receptors by NM neurons in the pre- and post-hatchling chick was controversial. NMDA responses are robust at early stages of development but decline significantly during the last week of embryonic development (Zhou and Parks, 1992). The decline of NMDA responses appears to coincide exactly with the morphological development of the auditory nerve end- bulb [see Rubel and Parks (1988) for review]. The end- bulb is a synaptic specialization that covers over 60% of the somatic surface (Parks, 1981). We suspect that the endbulb limits the diffusion of glutamate from the synaptic cleft, and thereby produces a pool of glutamate. In addition to this sink of glutamate, a sink of lactic acid, which builds up as a consequence of vesicular transmission (Duffy et al., 1975; Chapman et al., 1977; Mutch and Hansen, 1984), and a sink of arachidonic acid, released as a by-product of glutamatergic transmission (Pellerin and Wolfe, 1991; Dumuis et al., 1993; Stella et al., 1995), could acidify the synaptic cleft (Krishtal et al., 1987; Chesler and Kaila, 1992) and mask NMDA receptor responses in late embryonic and hatchling chickens.

The acidification of the cleft (or the inhibition of the pH-sensitive modulatory site) could account for the inconsistent results that we observed in our examination of second-messenger–stimulating agents on NMDA receptor responses. Now that we know that this modulatory site exists, it may be useful to reexamine how changes in A- and C-kinase activity alter NMDA receptor functions in NM neurons.

NMDA receptors have been shown to play an important role in CNS development and plasticity. Their contributions to developmental or modulatory events are thought to be associated with changes in the expression of NMDA receptor (NR) subunits, which implies the generation of a new receptor protein. Another explanation is that NR sensitivity is altered as a consequence of acid/base shifts associated with changes in synaptic architecture. Extreme shifts in pH are not required. In fact, the variations in pH required to alter NR responses occur during the normal course of synaptic transmission (Chesler, 1990). It is also important to note that the H^+-modulatory site of the NMDA receptor is located on the NR1 subunit, not on the modulatory NR2 subunits (Traynelis et al., 1995). Thus, the proton modulatory site of the NMDA receptor represents an efficient means of producing a rapid change in receptor physiology in an unstable environment.

Intracellular Ca^{2+} Stores in NM

Homobromoibotenic acid reliably and dose dependently increased $[Ca^{2+}]_i$ in NM neurons superfused in Ca^{2+} free, EGTA-buffered media. HBI-stimulated $[Ca^{2+}]_i$ increases were reduced by TMB8 and thapsigargin, agents known to inhibit Ca^{2+} mobilization from intracellular stores. They are not affected by ryanodine, an agent that inhibits Ca^{2+} release from organelles bearing Ca^{2+}-sensitive channels [see Kato et al. (1996)] or by the ionotropic glutamate receptor agonist CNQX. These results indicate that HBI liberates Ca^{2+} from intracellular stores associated with the endoplasmic reticulum.

Large changes in $[Ca^{2+}]_i$ were not reliably produced by glutamate or *t*-ACPD, which are normally used to stimulate IP_3-dependent Ca^{2+} release from stores. Both agents stimulate PI hydrolysis in NM neurons (Zirpel et al., 1994). In addition, *t*-ADA, another mGluR agonist that is believed to preferentially stimulate phospholipase C (Favaron et al., 1993), did not produce a rise in $[Ca^{2+}]_i$ in NM neurons. We have not yet determined whether HBI stimulates PI hydrolysis in NM neurons as it does in other systems (Chung et al., 1994; Thomsen et al., 1994).

Glutamate and *t*-ACPD stimulate numerous enzymes, and this could explain why changes in $[Ca^{2+}]_i$ are not stimulated by these agents in NM neurons (Zirpel et al., 1995). In addition to phospholipase C, glutamate and *t*-ACPD (in other systems) stimulate phospholipase D (Boss and Conn, 1992; Holler et al., 1993; Sarri et al., 1995), adenylate cyclase (Schoepp and Johnson, 1993; Winder and Conn, 1993; Wang and Johnson, 1995), and possibly cyclic GMP phosphodiesterase (Okada, 1992; Thoreson and Miller, 1994). The effectors regulated by any of these enzymes are capable of inhibiting Ca^{2+} responses associated with intracellular stores by phosphorylating the IP_3 receptor, which expresses several modulatory sites (Supattapone et al., 1988; Ferris et al., 1991; Shimizu et al., 1993).

GABA-Stimulated Ca^{2+} Responses in NM

The effects of TMB8 and heparin indicate that GABA liberates Ca^{2+} from intracellular stores in NM neurons. This result is consistent with the idea that G-protein–coupled GABA-B receptors are expressed by NM neurons. However, Ca^{2+} responses were not produced by the GABA-B receptor agonist baclofen.

The physiological and pharmacological profile of the GABA receptor expressed by NM neurons is beginning to be described in hatchling and embryonic chickens (Hyson et al., 1995; Brenowitz and Trussell, 1997; Lu et al., 1997). The receptor can be activated by muscimol, which we also show stimulates a rise in $[Ca^{2+}]_i$; yet it lacks the same features typical of a chloride-linked GABA-A receptor channel. For example, receptor activation produces a depolarization, rather than a hyperpolarization, which could account for the portion of the GABA-stimulated Ca^{2+} response sensitive to BAY K 8644. The effects of BAY K 8644 on GABA-stimulated responses are of interest as they indicate that GABA will activate L-type Ca^{2+} channels. Interestingly, GABA appears to produce small changes in membrane voltage, enough to activate outward K^+ conductances (Reyes et al., 1994; Hyson et al., 1995).

α-Aminobutyric acid receptors that are bicuculline/baclofen insensitive, called GABA-C receptors (Zhang et al., 1995), have been described (Qian and Dowling, 1995; Pan and Lipton, 1995). We are inclined to argue that NM neurons do not express GABA-C type receptors. Functionally speaking, activation of GABA-C receptors inhibits Ca^{2+} current (Pan and Lipton, 1995). More importantly, GABA-C receptors are sensitive to *cis*-4-aminocrotonic acid (Matthews et al., 1994), an agent that did not stimulate a Ca^{2+} response in NM. It would appear that NM neurons possess a unique baclofen/bicuculine/CACA-insensitive GABA receptor. GABA receptors of NM possess the binding characteristics of type I GABA-A receptors but lack the a_1-receptor subunit that normally defines type I receptors (Hyson and Sadler, 1997).

If the GABA receptor is G-protein linked, then GABA represents an alternative source of second-

messenger stimulation in NM neurons that are no longer receiving auditory nerve signals. Thus, GABA may provide important cellular signals that help attenuate degenerative changes in NM neurons that are no longer receiving auditory nerve stimulation. The trophic effects of GABA are well known, particularly in the developing nervous system (Yamashita and Fukuda, 1993; Ben-Ari et al., 1994; Gaiarsa et al., 1995).

α-Aminobutyric acid–stimulated changes in $[Ca^{2+}]_i$ carried by L-type voltage-operated Ca^{2+} channels have been reported elsewhere (Wang et al., 1994; Berninger et al., 1995; Leinekugel et al., 1995; Obrietan and van den Pol, 1995). Other studies have shown that GABA can trigger Ca^{2+} release from stores (Nilsson et al., 1993; Parramon et al., 1995). However, a specific link with IP_3-sensitive stores or PI hydrolysis has not been made.

Neuroprotective Mechanisms at the Glutamatergic Synapse

Nucleus magnocellularis neurons tonically discharge at rates of 50–100 Hz (Sullivan and Konishi, 1984; Warchol and Dallos, 1990). It could be argued that they continuously fire at rates that would be toxic to most neurons. With all the possible routes of entry and efflux available to Ca^{2+}, NM neurons must possess an extremely efficient means of managing glutamatergically stimulated Ca^{2+} loads.

Nucleus magnocellularis neurons appear to have multiple mechanisms to prevent Ca^{2+} from increasing to toxic levels. We have previously shown that the activation of cAMP reduces voltage-dependent increases in cell Ca^{2+} (Lachica et al., 1995). More recent studies show that a protein kinase C–activating mGluR attenuates increases in $[Ca^{2+}]_i$ that are released from IP_3-sensitive stores (Kato and Rubel, 1998). Glutamate also appears to reduce Ca^{2+} liberated from ryanodine-sensitive stores (Kato et al., 1996). Observations made in the present report indicate that $\Delta[Ca^{2+}]_i$ carried by NMDA receptors can be depressed *in vitro* by the $[H^+]_o$. Oddly enough, NM neurons appear to be so dependent upon glutamatergically associated mechanisms to buffer $[Ca^{2+}]_i$ changes that $[Ca^{2+}]_i$ increases rapidly and dramatically in the absence of glutamatergic stimulation (Zirpel et al., 1995; Zirpel and Rubel, 1996). This observation suggests that glutamatergically activated second messengers may work synergistically with conventional $[Ca^{2+}]_i$ buffering systems, i.e., pumps, exchangers, and sequestering organelles. This topic merits further investigation. Surely, changes in neurotransmitter-stimulated second-messenger function need to be appraised in injured and developing neurons to completely understand how changes in $[Ca^{2+}]_i$ affect neuronal form, physiology, and function.

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