Glutamate Regulates IP_3-Type and CICR Stores in the Avian Cochlear Nucleus

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Kato, B. Maya and Edwin W Rubel. Glutamate regulates IP_3-type and CICR stores in the avian cochlear nucleus. *J. Neurophysiol.* 81: 1587–1596, 1999. Neurons of the avian cochlear nucleus, nucleus magnocellularis (NM), are activated by glutamate released from auditory nerve terminals. If this stimulation is removed, the intracellular calcium ion concentration ($[Ca^{2+}]_i$) of NM neurons rises and rapid atrophic changes ensue. We have been investigating mechanisms that regulate $[Ca^{2+}]_i$ in these neurons based on the hypothesis that loss of Ca^{2+} homeostasis causes the cascade of cellular changes that results in neuronal atrophy and death. In the present study, video-enhanced fluorometry was used to monitor changes in $[Ca^{2+}]_i$ stimulated by agents that mobilize Ca^{2+} from intracellular stores and to study the modulation of these responses by glutamate. Homobromoibotenic acid (HBI) was used to stimulate inositol trisphosphate (IP_3)-sensitive stores, and caffeine was used to mobilize Ca^{2+} from Ca^{2+}-induced Ca^{2+} release (CICR) stores. We provide data indicating that Ca^{2+} responses attributable to IP_3- and CICR-sensitive stores are inhibited by glutamate, acting via a metabotropic glutamate receptor (mGluR). We also show that activation of C-kinase by a phorbol ester will reduce HBI-stimulated calcium responses. Although the protein kinase A accumulator, Sp-cAMPs, did not have an effect on HBI-induced responses. CICR-stimulated responses were not consistently attenuated by either the phorbol ester or the Sp-cAMPs. We have previously shown that glutamate attenuates voltage-dependent changes in $[Ca^{2+}]_i$. Coupled with the present findings, this suggests that in these neurons mGluRs serve to limit fluctuations in intracellular Ca^{2+} rather than increase $[Ca^{2+}]_i$. This system may play a role in protecting highly active neurons from calcium toxicity resulting in apoptosis.

INTRODUCTION

The viability of brain stem neurons in the cochlear nucleus of young animals is dependent on tonic stimulation by the auditory nerve (see Lachica et al. 1996; Rubel et al. 1990 for reviews). In the absence of auditory nerve stimulation, immature cochlear nucleus neurons undergo a number of cytological changes that culminate in cellular atrophy or death. Using the avian cochlear nucleus, nucleus magnocellularis (NM), we have studied the early events that precede cell atrophy and death when afferent stimulation is eliminated or transiently blocked. Our goal is to identify the intracellular signals that are activated by afferent stimulation and that regulate the survival of NM neurons.

One of the earliest events seen in unstimulated NM neurons is a rise in intracellular calcium ion concentration ($[Ca^{2+}]_i$) (Zirpel and Rubel 1996). We believe that the deregulation of $[Ca^{2+}]_i$ stimulates cellular events that are characteristic of dying neurons. Thus it is possible that $[Ca^{2+}]_i$ imbalance may trigger a cascade of cell death events such as those seen in excitotoxicity (Randall and Thayer 1992).

We have not determined what mechanisms are responsible for causing the elevated $[Ca^{2+}]_i$ seen in afferent-deprived NM neurons. In neurons, $[Ca^{2+}]_i$ can increase in at least two ways: influx across plasmalemma channels or efflux from the second-messenger regulated Ca^{2+} storing organelles. Because deafferented NM neurons are no longer electrically active (Born et al. 1991), contributions to the elevated $[Ca^{2+}]_i$ by ligand- or voltage-operated channels seem unlikely. Numerous studies indicate that Ca^{2+} released from intracellular Ca^{2+}-storing organelles plays a major role in neuronal pathologies caused by elevated $[Ca^{2+}]_i$ (e.g., Frandsen and Shousboe 1992, 1993). In addition, cytopathologic changes that are associated with glutamate-induced excitotoxicity (presumed to be caused by supernormal $[Ca^{2+}]_i$) can be corrected partially in neurons that have been treated with agents that preferentially block Ca^{2+} release from intracellular stores (see e.g., Yoon et al. 1996; Zhang et al. 1993).

These observations led to the present study, which describes the pharmacological regulation of intracellular Ca^{2+} stores in NM neurons. Ca^{2+} liberation from organelles is a ligand-gated event. Two distinct receptor channels have been identified: one is sensitive to inositol trisphosphate (IP_3), a metabolite of phosphatidylinositol (PI) hydrolysis, and the other is sensitive to Ca^{2+}. Anatomically distinct IP_3 receptor (IP_3R) channels and Ca^{2+}-sensitive Ca^{2+} channels (called CICRs, ''Ca^{2+}-induced Ca^{2+} release'') have been identified. Receptor-binding studies indicate that the IP_3R and CICR are unique proteins (Supattapone et al. 1988). Furthermore the organelles that they are attached to occupy spatially distinct sites in a neuron, indicating that the IP_3R and CICR are functionally unique (Feng et al. 1992; Kijima et al. 1993; Sharp et al. 1993; Shoshan-Barmatz et al. 1991; Walton et al. 1991).

Glutamate is the excitatory neurotransmitter released from auditory nerve endings onto NM neurons. Previously we demonstrated that glutamate significantly can reduce voltage-dependent increases in $[Ca^{2+}]_i$ (Lachica et al. 1995). Further studies showed that glutamate also attenuates elevations in $[Ca^{2+}]_i$ that are attributed to CICR stores (Kato et al. 1996). One of the specific aims of the present report was to determine whether glutamate modulates Ca^{2+} increases stimulated by IP_3R-activating agents. In addition, we wanted to begin investigating the intracellular pathways involved in glutamate reg-

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ulation of intracellular stores. We hypothesize that the elevated $[Ca^{2+}]_i$ seen in sensory-deprived NM neurons is partly due to the absence of glutamate-dependent inhibition of release from intracellular Ca^{2+} stores.

Using video-enhanced fluorometric techniques, the present study shows that IP_3- and Ca^{2+}-sensitive intracellular stores are present in NM neurons. We demonstrate that glutamate activation of metabotropic receptors reduces changes in $[Ca^{2+}]_i$ due to activation of IP_3R- and CICR-regulated stores. Interestingly, changes in $[Ca^{2+}]_i$ due to IP_3R and CICR stores appear to be regulated by second-messenger systems that do not attenuate the Ca^{2+} increases carried by voltage-operated channels.

METHODS

Tissue preparation

White leghorn chicken embryos, aged 17–18 days, were removed from their shells and decapitated. The brain stems were rapidly dissected out and blocked above and below the level of the cochlear nucleus. Specimens were placed into 5% low-melting-point agarose dissolved in artificial cerebrospinal fluid (ACSF) for 3 min. The ACSF was composed of (in mM) 125 NaCl, 5 KCl, 1.25 KH_2PO_4, 1.3 $MgCl_2$, 26 $NaHCO_3$, 10 dextrose, and 3.1 $CaCl_2$. When the agarose solidified, a square block containing the brain stem was cut out and glued to the stage of a vibratome. The entire block then was immersed in cold ACSF.

Coronal brain stem slices (300 μM) through NM were made. Slices were loaded with 5 μM FURA-2 acetoxymethyl ester (FURA-2), dissolved in a 0.1% dimethylsulfoxide, 0.025% pluronic acid, ACSF solution. The tissue slice approximately mid way through NM was incubated in the oxygenated FURA-2/ACSF solution at 40°C for 25 min. The slice then was rinsed in fresh ACSF and prepared for imaging. Imaging experiments were conducted at room temperature.

Drug application

FURA-2-loaded brain stem slices were placed in a Leiden-style chamber on an inverted microscope stage and anchored to the floor of the chamber with a stainless steel net. Pharmacological agents were applied to the slices in bath application via a stainless steel capillary inlet tube (1-mm diam) positioned on top of the net. A second capillary tube connected to suction rested on the floor of the chamber, establishing laminar flow of the superfusate at a rate of ~3 ml/min. Specimens were superfused continuously with oxygenated solution throughout the experiment. Media reservoirs containing the solutions were attached to the capillary tube delivering the superfusate by a length of plastic tubing ~30 cm long. As a result, there was a delay between the time the solutions were changed at the level of the reservoir and the time that the solution in the test chamber containing the specimen was completely changed. This delay was ~30 s and is not corrected for in the figures.

Pharmaceuticals

Fresh solutions of ACSF and Ca^{2+}-free ACSF were made daily. The Ca^{2+}-free ACSF was identical in composition to the normal ACSF with the exception that it lacked $CaCl_2$ and contained 1 mM EGTA and 4.4 mM $MgCl_2$. At the beginning of each experiment, neurons typically were depolarized pharmacologically with 60 mM KCl in ACSF to assess cell viability. All other drugs were delivered in Ca^{2+}-free ACSF. These agents can be grouped into six general categories as follows: drugs that stimulate Ca^{2+} release from intracellular stores: homo-bromo-ibotenate (HBI) and caffeine; agents that block Ca^{2+} release from stores: TMB-8 hydrochloride (TMB8) and ryanodine; glutamate and its receptor agonists: *trans*-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD), *trans*-azetidine dicarboxylic acid (t-tADA), L(+)-2-amino-3-phosphonopropionic acid (AP3), L(+)-cysteine sulfinic acid (L-CSA), kainic acid (KA); glutamate receptor antagonists: (±)-*a*-methyl-4-carboxyphenylglycine (MCPG); protein kinase activators: Sp-cAMPS triethylamine (Sp-cAMPS) and phorbol 12-myristate 13-acetate (PMA); and protein kinase inhibitors: H7 dihydrochloride (H7) and H8 dihydrochloride (H8).

Microfluorometry

NM neurons were excited alternately by 340 and 380 nm wavelengths from a xenon source (Ushio). Excitation wavelengths were obtained using interference filters from Chroma Technology (Brattleboro, VT). Emitted light was passed through a ×40 fluor oil-immersion objective (Nikon) attached to a Nikon Diaphot inverted microscope, through a 480-nm long-pass exit filter, and finally into an image intensifier coupled to a CCD camera (Hamamatsu, Japan). Cells were exposed to UV light, attenuated to 3% its normal intensity by neutral density filters, during data-collection periods only. Exposure time for each wavelength was 500–750 ms controlled via a computer-controlled shutter and filter wheel (Sutter Instruments, Novato, CA). Paired images were captured every 3 s. Data were obtained by comparing the intensity of fluorescent emission to 340- and 380-nm excitation wavelengths. The difference in emitted fluorescence was expressed as a ratio (F340:F380) that was compared with a standard curve for free Ca^{2+} constructed from solutions of known Ca^{2+} and FURA-2 concentrations. As a result, ratios of fluorescent intensity were translated directly to Ca^{2+} concentrations using software designed by Universal Imaging (West Chester, PA). The K_d of hydrolyzed FURA-2 for Ca^{2+} was assumed to be 224 nM (Grynkiewicz et al. 1985). Numerical values reported are an average of >50 adjacent pixels for each cell. All results are presented as mean change in $[Ca^{2+}]_i \pm 1$ SE. The total number of neurons and number of slices that were tested in each experiment are indicated parenthetically. Typically 10–13 neurons were studied in each slice. For statistical comparisons, a conservative approach of computing the mean value of all neurons in a slice, and using that value as a single observation was used.

Materials

FURA-2 was obtained from Molecular Probes (Eugene, OR), glutamate was purchased from Sigma, and L-CSA and HBI were acquired from Tocris-Cookson (St. Louis, MO). All other pharmaceuticals were bought from Research Biochemicals (Natick, MA) and the remaining reagents were of analytic grade.

Statistics statistical analyses were performed using Student's *t*-test or one-way ANOVA, as required. Post hoc analyses were computed using Scheffe's test.

RESULTS

NM neurons possess two types of intracellular Ca^{2+} stores: IP_3-sensitive stores and CICR stores

At least two pharmacologically distinct types of intracellular stores have been described: IP_3-sensitive stores and Ca^{2+}-sensitive CICR stores. In this study, HBI, a metabotropic glutamate receptor agonist linked to the phospholipase C (PLC) signal transduction cascade, was used to stimulate calcium release from IP_3-sensitive stores, and caffeine was used to stimulate CICR stores.

Figure 1 shows the changes in $[Ca^{2+}]_i$ seen in six NM neurons stimulated with 500 μM HBI followed by 100 mM caffeine. The doses used have been shown to reliably stimulate large increases in $[Ca^{2+}]_i$ in NM neurons (Kato et al. 1996; Lachica et al. 1998). Before stimulation, the cells were given a

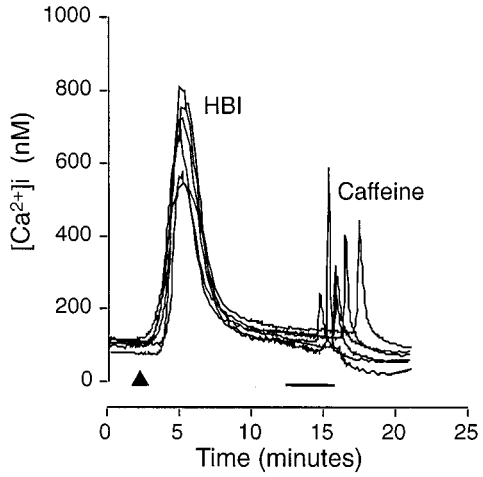

FIG. 1. Homobromoibotenic acid (HBI) and caffeine stimulate increases in $[Ca^{2+}]_i$. Ca^{2+} responses of neurons stimulated with 500 μM HBI and 100 μM caffeine. Neurons were exposed to HBI for 1 min (▲ and caffeine for 3 min. (black bar). Both responses were generated in Ca^{2+}-free artificial cerebrospinal fluid (ACSF) + 1 mM EGTA.

3-min washout period in Ca^{2+}-free ACSF with 1 mM EGTA. HBI and caffeine were delivered in Ca^{2+}-free ACSF. Therefore elevations in $[Ca^{2+}]_i$ elicited by these agents can be attributed to Ca^{2+} mobilized from intracellular stores and are not due to calcium influx through the plasma membrane.

On the average (72 neurons, $n = 6$ slices), 500 μM HBI increased $[Ca^{2+}]_i$ by 530 ± 55 nM. The mean $\Delta[Ca^{2+}]_i$ (75 neurons, $n = 6$ slices) produced by caffeine was 331 ± 51 nM. Both of these values differ significantly from fluctuations in basal $[Ca^{2+}]_i$ levels in these neurons, which varies ~100 nM. We observed that HBI-and caffeine-stimulated Ca^{2+} responses differed in their spatial and temporal characteristics. Elevations in $[Ca^{2+}]_i$ evoked by HBI developed slowly to a maximum and were buffered over a protracted period of time. These HBI-stimulated increases in $[Ca^{2+}]_i$ developed and decayed synchronously in response to the presentation and withdrawal of the HBI stimulus. In contrast, the caffeine-stimulated Ca^{2+} responses occurred asynchronously between NM neurons in a single brain stem slice. These Ca^{2+} transients rose rapidly and were buffered quickly in the presence of continued caffeine superfusion. The caffeine-stimulated Ca^{2+} responses usually were distinguished by a ''postcaffeine undershoot''—a period immediately after the caffeine-stimulated $\Delta[Ca^{2+}]_i$ where Ca^{2+} levels sink below resting levels (Kato et al. 1996; Usachev et al. 1993). The HBI-treated neurons never demonstrated this undershoot.

The effects of two agents that block Ca^{2+} release from intracellular stores, TMB8 and ryanodine, were tested on HBI- and caffeine-stimulated cells. HBI-stimulated Ca^{2+} increases (Fig. 2) were attenuated significantly ($[Ca^{2+}]_i = 85 \pm 7$ nM; $P < 0.001$) when neurons were pretreated with 100 μM TMB8 for 5 min (17 neurons, $n = 3$ slices). However, exposure to 100 μM ryanodine did not significantly affect HBI-stimulated Ca^{2+} responses ($[Ca^{2+}]_i = 400 \pm 47$ nM; 42 neurons = 4 slices). Another set of cells was stimulated with caffeine after exposure to 100 μM concentrations of either ryanodine or TMB8. Although both agents significantly attenuated caffeine-stimulated Ca^{2+} responses, the actions of ryanodine were greater than those of TMB8. The average $\Delta[Ca^{2+}]_i$ seen in ryanodine-treated, caffeine-stimulated neurons was 57 ± 16 nM (71 neurons, $n = 6$ slices). This is much less than the $\Delta[Ca^{2+}]_i$ to caffeine alone ($P < 0.0001$). The average $\Delta[Ca^{2+}]_i$ seen in TMB8-treated caffeine-stimulated neurons was 103 ± 27 nM, and it is significantly smaller than in slices treated with caffeine alone ($t = 8.4$; $P < 0.01$).

Glutamate modulates Ca^{2+} release from intracellular stores

In vivo, Ca^{2+} mobilized from IP_3-sensitive or CICR stores should be linked to the release of glutamate from the auditory nerve terminal. However, in NM, glutamate does not appear to stimulate a $\Delta[Ca^{2+}]_i$ at the concentrations that have been shown to reliably stimulate increases $[Ca^{2+}]_i$ in other neurons (Lachica et al. 1995, 1998). Millimolar concentrations are required to raise $[Ca^{2+}]_i$ in NM neurons, and even at these concentrations, glutamate-stimulated elevations in $[Ca^{2+}]_i$ due to the influx or efflux of Ca^{2+} are variable (Lachica et al. 1998; Zirpel et al. 1995b). We previously demonstrated that glutamate significantly attenuates increases in $[Ca^{2+}]_i$ in KCl-stimulated NM neurons (Lachica et al. 1995), and it is possible that glutamate has a similar effect on Ca^{2+} mobilized from intracellular stores. The remainder of this report is directed at evaluating this glutamate effect on IP_3R stores and further analyzing its effect on CICR-stores (see Kato et al. 1996).

Figure 3*A* shows that large increases in $[Ca^{2+}]_i$ were elicited repeatedly in NM neurons by multiple exposures to 500 μM HBI. Thus IP_3R stores are not labile and the release of Ca^{2+} from IP_3R stores is not dependent on the store being ''reloaded'' with Ca^{2+}. When 1 mM glutamate is applied to the slice, the HBI-stimulated $[Ca^{2+}]_i$ transients are reduced markedly (Fig. 3*B*) This effect lasts for several minutes. Figure 4 shows that the suppressive effect of glutamate on Ca^{2+} responses from intracellular stores is dose dependent. At a concentration of 1.0 mM, glutamate maximally reduced HBI-stimulated

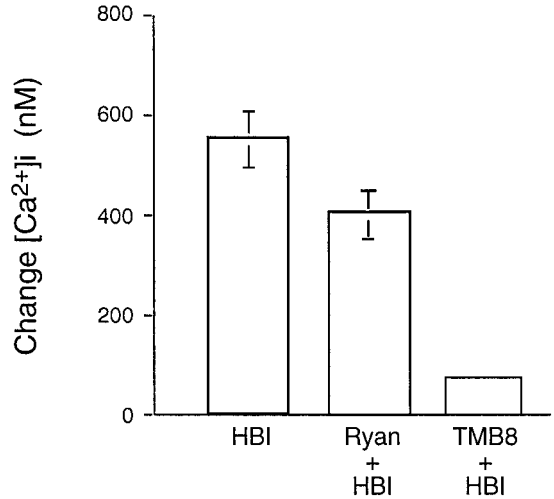

FIG. 2. Effects of ryanodine and TMB-8 hydrochloride (TMB8) on Ca^{2+} efflux from HBI-sensitive stores. Neurons were exposed to either 100 μM ryanodine or 100 μM TMB8 for 5 min in 0 Ca^{2+} ACSF + EGTA before they were stimulated with 500 μM HBI (1 min). HBI stimulation was made in the continued presence of either ryanodine or TMB8. Bar marked HBI represents Ca^{2+} responses measured in neurons that were not exposed to test agents. Bar marked Ryan + HBI represents the mean change in $[Ca^{2+}]_i$ measured in HBI-stimulated neurons exposed to ryanodine. Bar marked TMB8 + HBI represents the mean change in $[Ca^{2+}]_i$ measured in HBI-stimulated neurons exposed to TMB8. Error bars = 1 SE.

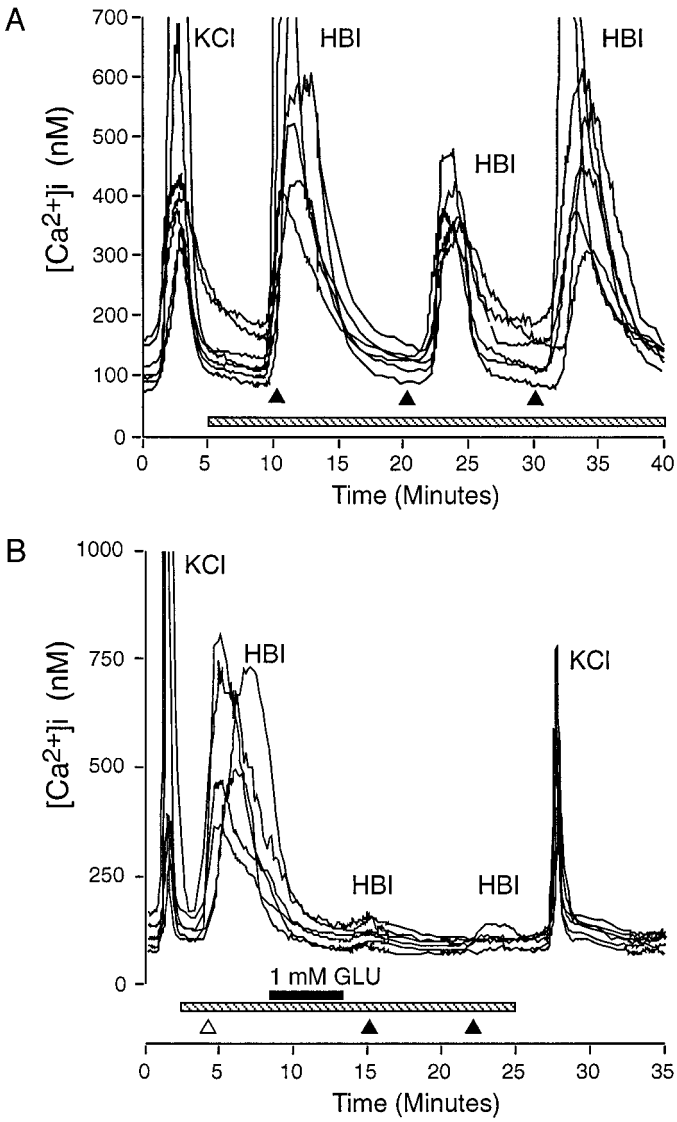

FIG. 3. Ca^{2+} responses of HBI in normal and glutamate-treated neurons. *A*: HBI can stimulate sequential increases in $[Ca^{2+}]_i$. First $\Delta[Ca^{2+}]_i$ was created by a 30 s pulse of 60 mM KCl. Subsequent transients were generated by 500 μM HBI presented for 1 min in 0 Ca^{2+} ACSF + EGTA (▲). ▨, period and duration of 0 Ca^{2+} superfusate. *B*: glutamate suppresses HBI-stimulated Ca^{2+} responses. First Ca^{2+} transient was created by a 30-s pulse of 60 mM KCl. After this $\Delta[Ca^{2+}]_i$ was buffered, the superfusate was switched to 0 Ca^{2+} ACSF + EGTA, wherein neurons were stimulated with 500 μM HBI for 1 min (△). After HBI-stimulated $\Delta[Ca^{2+}]_i$ was buffered, neurons were exposed to 1 mM glutamate (GLU) for 5 min (black bar), then stimulated again with 500 μM HBI (▲). Final Ca^{2+} transient was generated by 60 mM KCl to show that Ca^{2+} increases still could be generated in these neurons.

Ca^{2+} changes by 460 nM (Fig. 4); a half-maximal effect was produced by 100 μM glutamate. Glutamate also attenuates caffeine-stimulated changes in $[Ca^{2+}]_i$ (see Kato et al. 1996, Fig. 2*C*). This effect is also dose dependent. The caffeine-stimulated $\Delta[Ca^{2+}]_i$ was reduced most efficaciously by 100 μM glutamate. Interestingly, 1 and 10 mM glutamate did not suppress the caffeine response to the same degree as 100 μM glutamate. In fact, 10 mM glutamate did not have a statistically significant effect on caffeine-stimulated calcium responses (see also Kato et al. 1996, Fig. 2*D*).

Glutamate effects on Ca^{2+} responses produced by HBI and caffeine were mimicked by the mGluR agonist t-ACPD. The t-ACPD reduction of HBI-stimulated transients were also dose dependent (Fig. 4). Maximal reduction of the HBI response was elicited by 100 μM t-ACPD. The $\Delta[Ca^{2+}]_i$ seen in HBI-stimulated neurons (54 neurons; $n = 4$ slices) exposed to 100 μM t-ACPD was only 57 ± 11 nM. This same concentration of t-ACPD was also the most effective in reducing caffeine-stimulated transients. The $\Delta[Ca^{2+}]_i$ of caffeine-stimulated neurons (41 neurons; $n = 4$ slices) treated with 100 μM t-ACPD was 108 ± 19 nM. These dose-dependent effects of glutamate and t-ACPD on Ca^{2+} stores may be due to receptor downregulation, receptor desensitization, or to other second-messenger activation. Again, it is important to note that higher concentrations of t-ACPD did not have an inhibitory effect on HBI- or caffeine-stimulated responses. For example, 1 mM t-ACPD potentiated the $\Delta[Ca^{2+}]_i$ stimulated by HBI. Caffeine-stimulated responses neither were potentiated nor inhibited by 1 mM t-ACPD. The fact that the higher concentrations of glutamate and t-ACPD do not inhibit HBI- and caffeine-stimulated responses is important. These findings suggest that the suppressive effect of glutamate and t-ACPD is not due to competitive binding at the level of the mGluR.

To determine whether the suppressive effect of glutamate on intracellular Ca^{2+} stores was modulated specifically by a metabotropic receptor, the effect of the ionotropic receptor agonist, kainic acid (KA; 50 μM), was tested on a separate set of neurons stimulated with HBI (37 neurons; $n = 3$ slices) or caffeine (45 neurons; $n = 4$ slices). This dose of kainate used has been shown to effectively stimulate the KA/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor without being toxic to the cell (Lachica et al. 1998). The HBI- and caffeine-stimulated Ca^{2+} responses of the KA-treated neurons did not differ from normal (data not shown).

The mGluR antagonist, MCPG, partially reversed glutamate effects on IP_3R and CICR stores (Fig. 5, *A* and *B*). These results are shown in Fig. 5, *A* and *B*, respectively. Fifty-two neurons ($n = 4$) were stimulated with 500 μM HBI immediately after the coapplication of 1 mM MCPG and 1 mM glutamate. The mean $\Delta[Ca^{2+}]_i$ measured in these neurons was

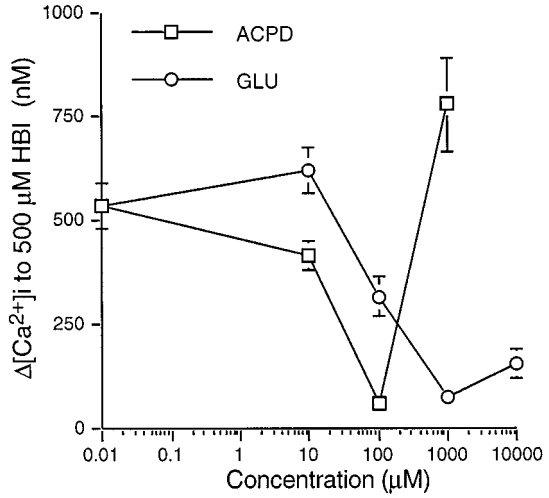

FIG. 4. Effects of glutamate and *trans*-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD) on HBI are dose dependent. *A*: dose-response curves plot $\Delta[Ca^{2+}]_i$ to 500 μM HBI in neurons superfused for 5 min with ascending concentrations of glutamate (GLU; ○) or t-ACPD (ACPD, □). These data represent means (±1 SE) from 4 different slices (avg. 12 neurons/slice) for each data point.

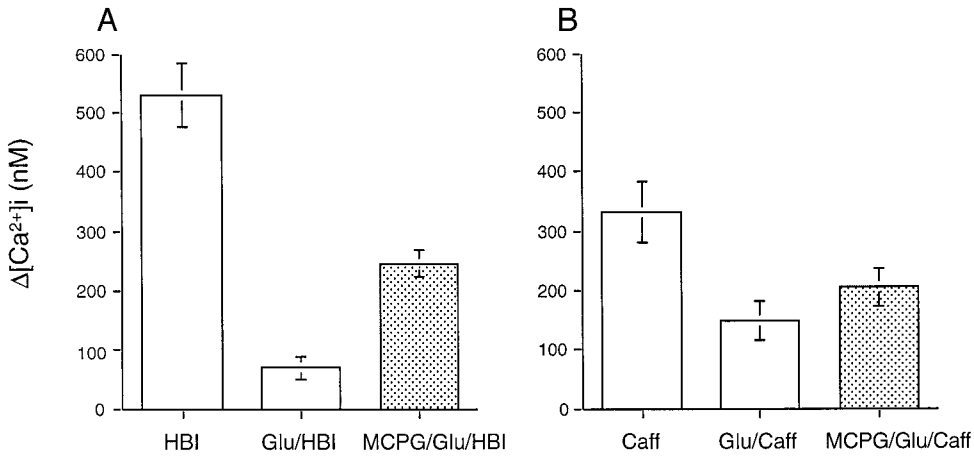

FIG. 5. Effects of (±)-*a*-methyl-4-carboxyphenylglycine (MCPG) on the glutamatergic inhibition of HBI- and caffeine-stimulated calcium responses. *A*: bar marked Glu represents mean $\Delta[Ca^{2+}]_i$ to 500 μM HBI in control neurons; bar marked Glu/HBI represents the mean $\Delta[Ca^{2+}]_i$ to 500 μM HBI in neurons treated with 1 mM glutamate; bar marked MCPG/Glu/HBI shows the mean $\Delta[Ca^{2+}]_i$ of neurons treated with 1 mM MCPG and 1 mM glutamate before stimulation with 500 μM HBI. *B*: mean $\Delta[Ca^{2+}]_i$ in neurons stimulated with 100 mM caffeine. Bar marked Caff shows response to caffeine alone; bar marked Glu/Caff represents caffeine response in neurons pretreated with 1 mM glutamate; bar marked MCPG/Glu/Caff demonstrates caffeine response in neurons pretreated with 1 mM MCPG and 1 mM glutamate.

246 ± 22 nM. Although this value was much smaller than the response to HBI in untreated neurons (Scheffe's test, $P < 0.0001$), it was significantly greater than the rise in $\Delta[Ca^{2+}]_i$ stimulated by HBI in neurons exposed only to glutamate ($P < 0.01$). Therefore it could be argued that the mGluR antagonist, MCPG, partially blocks the suppressive effect of glutamate. The effects of MCPG on glutamate inhibition of caffeine responses were tested on a separate group of cells (43 neurons; $n = 4$ slices). The mean $\Delta[Ca^{2+}]_i$ measured in these neurons was 205 ± 31 nM. Although this value was not statistically different from the response to caffeine seen in untreated neurons, it also did not differ significantly from the responses of caffeine-stimulated cells that were exposed only to glutamate.

Effects of kinase inhibitors and activators on HBI-stimulated responses

Metabotropic glutamate receptors act mainly by modulating the effectors of one of two pathways: the phospholipid/Ca^{2+} signaling pathway or the adenylate cyclase/cyclic AMP transduction system. Preliminary attempts have been made to determine the signaling pathways underlying mGluR regulation of calcium-storing organelles. We examined the Ca^{2+} responses of HBI-stimulated neurons after they were exposed to the following conditions: coapplication of 1 mM glutamate and 100 μM H7, an inhibitor of protein kinase C or coapplication of glutamate and 100 μM H8, an inhibitor of protein kinase A. The doses of H7 and H8 have been proven to be effective in inhibiting protein kinases (e.g., Chik et al. 1995; Glaum and Miller 1995). In addition, we examined responses to HBI in the presence of 1 μM PMA, a C-kinase-activating phorbol ester and in the presence of 1 mM sp-cAMP, an analogue of A-kinase-activating cAMP. These agents were tested in the absence of glutamate stimulation. The results of these experiments are summarized in Fig. 6.

HBI-stimulated $\Delta[Ca^{2+}]_i$ was examined in 60 neurons ($n = 4$ slices) exposed to glutamate and H7 and in 66 neurons ($n = 4$ slices) exposed to glutamate and H8 (Fig. 6*A*). The mean $\Delta[Ca^{2+}]_i$ seen in Glu-H7-treated neurons was 313 ± 36 nM, and the mean $\Delta[Ca^{2+}]_i$ seen in Glu-H8 treated neurons was 42 ± 5 nM. It is important to note that the responses of Glu-H7-treated slices were greater than the $\Delta[Ca^{2+}]_i$ of HBI-stimulated neurons exposed solely to glutamate ($P < 0.0001$). In contrast, the Ca^{2+} responses of Glu-H8-treated neurons did not differ significantly from the responses of neurons treated with glutamate alone. These data indicate that H7 partially reversed the effects of glutamate. An alternative explanation is that HBI-stimulated Ca^{2+} responses may be attenuated by an increase in C kinase activity. As a control, additional slices were tested with H7 or H8 alone (without glutamate) and were not found to alter resting $[Ca^{2+}]_i$ (data not shown).

The actions of PMA and Sp-cAMPs on HBI-stimulated $\Delta[Ca^{2+}]_i$ were also very different (Fig. 6*B*). PMA reduced HBI-stimulated responses by ~90% to 48 ± 6 nM (59 neurons; $n = 4$ slices). Sp-cAMPS, however, did not significantly alter HBI-stimulated $\Delta[Ca^{2+}]_i$. On the average, HBI evoked a 455 ± 51 nM $\Delta[Ca^{2+}]_i$ in Sp-cAMPs treated neurons (54 neurons; $n = 4$ slices).

Effects of kinase inhibitors and activators on caffeine-stimulated responses

We performed a parallel set of experiments to assess the effects of the protein kinase inhibitors and activators on caffeine-stimulated $\Delta[Ca^{2+}]_i$. Caffeine-stimulated responses were examined in 77 neurons ($n = 4$ slices) treated with H7 alone and 67 neurons ($n = 4$ slices) treated with H8 alone. The $\Delta[Ca^{2+}]_i$ in H7-treated neurons was 135 ± 19 nM and 151 ± 14 nM in H8-treated cells. These values did not differ from one another nor did they differ from the ΔCa^{2+} measured in caffeine-stimulated neurons treated only with glutamate. These results suggest that both H7 and H8, acting by themselves, attenuate caffeine-stimulated Ca^{2+} responses as effectively as glutamate.

Fifty-two neurons ($n = 4$ slices) were exposed to glutamate and H7 and 68 neurons ($n = 4$ slices) were exposed to glutamate and H8 before stimulation with caffeine (Fig. 7*A*). The $\Delta[Ca^{2+}]_i$ measured in Glu-H7-treated neurons was 163 ± 28 nM. This value is 50% smaller than that measured in the cells treated only with caffeine and is not significantly different from the $\Delta[Ca^{2+}]_i$ in caffeine-stimulated neurons exposed to glutamate or H7 alone. The $\Delta[Ca^{2+}]_i$ measured in Glu-H8-treated neurons was 27 ± 7 nM. This is significantly smaller than the caffeine-stimulated $\Delta[Ca^{2+}]_i$ in cells treated with glutamate ($P < 0.0001$), demonstrating that H8 potentiates the inhibitory effects of glutamate on CICR stores. This suggests that glutamate and H8 may act through different mechanisms to suppress Ca^{2+} release from CICRs.

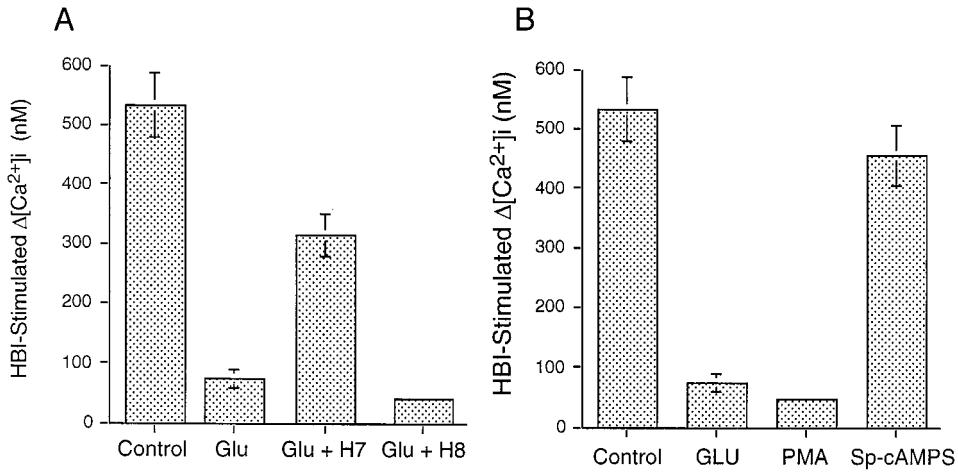

FIG. 6. Effects of the kinase inhibitors and activators on HBI-stimulated responses. *A*: effects of H7 and H8 on glutamate inhibition of HBI-stimulated Ca^{2+} responses are shown here. Bar marked Control represents mean $\Delta[Ca^{2+}]_i$ measured in neurons stimulated for 1 min with 500 μM HBI. Bar marked Glu represents mean $\Delta[Ca^{2+}]_i$ measured in neurons exposed to 1 mM glutamate for 5 min before being stimulated by 500 μM HBI. Bar marked Glu + H7 represents mean $\Delta[Ca^{2+}]_i$ measured in neurons exposed to a cocktail of 100 μM H7 and 1 mM glutamate before they were stimulated with 500 μM HBI. Bar marked Glu + H8 represents mean $\Delta[Ca^{2+}]_i$ measured in neurons exposed to a cocktail of 100 μM H8 and 1 mM glutamate before they were stimulated with 500 μM HBI. *B*: effects of phorbol 12-myristate 13-acetate (PMA) and Sp-cAMPS on HBI-stimulated Ca^{2+} responses. Bar marked Control represents mean $\Delta[Ca^{2+}]_i$ measured in neurons stimulated with 500 μM HBI for 1 min. Bar marked Glu represents mean $\Delta[Ca^{2+}]_i$ measured in neurons exposed to 1 mM glutamate for 5 min before they were stimulated with HBI. Bar marked PMA represents mean $\Delta[Ca^{2+}]_i$ measured in neurons exposed to 1 μM PMA for 5 min before they were HBI-stimulated. Bar marked Sp-cAMPS represents mean $\Delta[Ca^{2+}]_i$ measured in neurons exposed to Sp-cAMPS for 5 min before they were stimulated with HBI. Error bars = 1 SE.

The Ca^{2+} responses of 61 caffeine-stimulated neurons ($n = 4$ slices) that were exposed to 1 μM PMA were compared with Ca^{2+} responses of 63 caffeine-stimulated neurons ($n = 4$ slices) exposed to 1 mM Sp-cAMPS. The actions of PMA and Sp-cAMPS on caffeine-stimulated Ca^{2+} responses were nearly identical (Fig. 7*B*). Both agents suppressed caffeine-stimulated responses. The mean caffeine-stimulated $\Delta[Ca^{2+}]_i$ seen in PMA-treated neurons was 122 $\pm$ 17 nM. The $\Delta[Ca^{2+}]_i$ in neurons treated with Sp-cAMPS and caffeine was 128 $\pm$ 12 nM.

Effects of other mGluR-stimulating agents

mGluRs can be activated by a number of agents. We evaluated the effects of L-cysteine sulfinic acid (L-CSA), L-2-amino-3-phosphonopropionic acid (L-AP3), and *trans*-azetidine dicarboxylic acid (t-ADA) on HBI and caffeine-stimulated Ca^{2+} responses. L-CSA has been shown to stimulate phospholipase D and cyclic AMP activities (Boss and Boaten 1995; Boss et al. 1994). L-AP3 has been shown to block and stimulate mGluRs (Irving et al. 1990; Lonart et al. 1992; Manev et al. 1993; Saugstad et al. 1995). t-ADA is believed to be a selective agonist of a mGluR coupled to phosphoinositide hydrolysis. Each agent was tested at concentrations of 1, 100, and 1,000 μM. HBI- and caffeine-stimulated Ca^{2+} responses were not inhibited by any of these agents.

DISCUSSION

It is well known that tight regulation of intracellular free calcium levels is essential to cellular survival. Neurons and other excitable cells are challenged particularly in this respect because their responses to extrinsic stimuli result in calcium

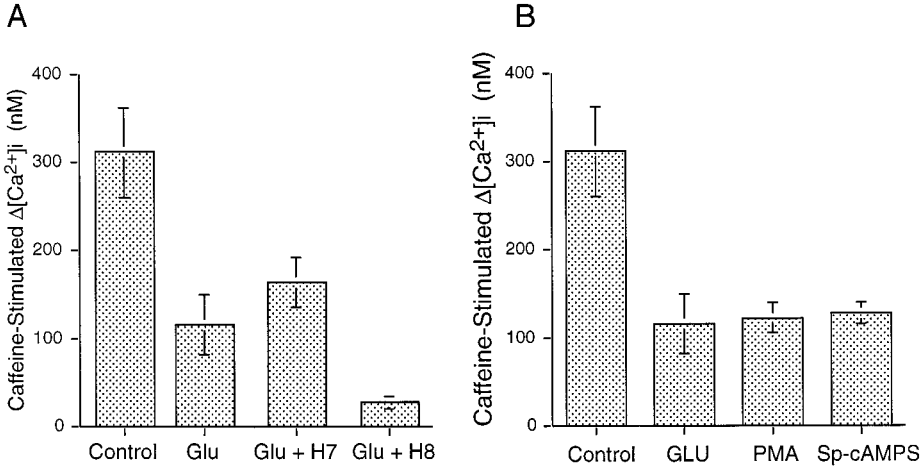

FIG. 7. Effects of kinase inhibitors and activators on caffeine-stimulated $\Delta[Ca^{2+}]_i$. *A*: effects of H7 and H8 on glutamate inhibition of caffeine-stimulated Ca^{2+} responses are shown here (conventions as in Fig. 6*A*). *B*: effects of PMA and Sp-cAMPS on caffeine-stimulated Ca^{2+} responses (see Fig. 6*B* for explanation). Error bars = 1 SE.

influxes due to activation of membrane channels and calcium or release from internal stores. Fluctuation in levels of intracellular calcium resulting from short periods of high levels of stimulation usually are bound, sequestered, or efficiently extruded from the cell. In very active neurons, however, calcium levels could rise rapidly to toxic levels without cellular machinery limiting its accumulation. Auditory system neurons of birds and mammals are known for their high levels of intrinsic activity. For example, many eighth nerve neurons have spontaneous firing rates of 50–100 spikes/s in the absence of acoustic stimulation (Liberman 1978) and rates may rise to levels four to five times that level during an acoustic stimulus. Nucleus magnocellularis neurons also show very high rates of ongoing activity, averaging ~100 spikes/s in the absence of stimulation and 300–400 spikes/s during stimulation (Rubel and Parks 1975; Stopp and Whitfield 1961; Warchol and Dallos 1990). Furthermore we have shown previously that in the absence of eighth nerve activity, intracellular-free calcium levels in NM neurons rise three- to fourfold within 3 h (Zirpel and Rubel 1996; Zirpel et al. 1995a). This time period corresponds to deprivation-induced degenerative events that result in cell death and atrophy (Kelley et al. 1997; Rubel et al. 1990). Consequently, we recognized that NM neurons, and presumably other highly active neurons, need specialized mechanisms for limiting calcium accumulation and that these mechanisms should respond to synaptic stimulation. In a previous study, we showed that a pathway involving one or more metabotropic glutamate acting through A kinase inhibits calcium entry through low-voltage-activated channels (Lachica et al. 1995). This study investigates intracellular Ca^{2+} storing organelles in NM neurons and their regulation by glutamate. It describes the mechanisms underlying glutamate regulation of IP_3R-type Ca^{2+}-storing organelles. In addition, this study further analyzes glutamate modulation of CICR-type Ca^{2+}-storing organelles (see Kato et al. 1996), focusing on the intracellular pathways involved in this regulation.

NM neurons possess both IP_3R- and CICR-type Ca^{2+}-storing organelles. Changes in $[Ca^{2+}]_i$ linked to the activation of IP_3Rs and CICRs differ in a number of ways. Ca^{2+} released from IP_3R stores is reduced by TMB8 but not by ryanodine. CICR stores are sensitive to the effects of both of these agents but are particularly sensitive to ryanodine. The temporal characteristics of Ca^{2+} responses attributable to IP_3Rs and CICRs are also different. Within a sample of neurons treated with HBI, $[Ca^{2+}]_i$ increased and decreased in conjunction with the presence and withdrawal of HBI. On the other hand, it was difficult to predict exactly when a given NM neuron would respond to caffeine. HBI-stimulated Ca^{2+} transients were buffered over a protracted period of time, whereas caffeine-stimulated transients were buffered rapidly and the $[Ca^{2+}]_i$ transiently sank slightly below prestimulus basal $[Ca^{2+}]_i$.

Calcium increases stimulated by HBI and caffeine were both inhibited by glutamate, the neurotransmitter released at the auditory nerve ending, and by t-ACPD, a mGluR agonist (Favaron et al. 1993). The inhibitory effect of glutamate and t-ACPD on HBI- and caffeine-stimulated responses were long lasting, reversible and dose dependent. These results suggest that stimulation of one or more mGluRs triggers intracellular events that attenuate calcium increases originating from calcium-storing organelles. t-ACPD usually activates an IP_3-dependent increase in cell calcium by stimulating the hydrolysis of PIP_2 (Palmer et al. 1988). However, at the concentrations used to inhibit IP_3R and CICR responses, t-ACPD does not cause a $\Delta[Ca^{2+}]_i$ in NM neurons (Lachica et al. 1998). This finding indicates that t-ACPD does not exclusively stimulate phospholipase C activity in NM neurons. The identity of the effectors that are mGluR modulated in NM neurons is not yet known.

It could be argued that the glutamate effect on HBI-stimulated transients is due to receptor desensitization, given that these two agents may act on the same family of binding sites. There are several observations that suggest that this cannot explain the results presented. First, HBI repeatedly stimulates calcium increases (Fig. 3*A*). Second, glutamate and t-ACPD have similar inhibitory effects on caffeine-stimulated responses (which are not linked to a membrane-affiliated receptor). Third, the dose-dependent effects of glutamate and t-ACPD are not linear—higher concentrations of glutamate and t-ACPD were less efficacious than lower concentrations (Fig. 4). Finally, previous studies have shown that HBI stimulates phosphoinositide hydrolysis by binding to mGluRs other than those sensitive to ACPD (Chung et al. 1994; Littman et al. 1995; Thomsen et al. 1994).

We recently have conducted and presented the results of preliminary experiments using caged IP^3 to directly stimulate calcium release from IP_3-sensitive stores (Kato and Rubel 1998). Brain stem slices were incubated in caged IP_3 and calcium fluxes were studied with Oregon Green. All experiments were performed in calcium-free media, buffered with 1 mM EGTA. In most neurons, photolysis of the cage resulted in large increases in the intensity of the fluorophore, indicating an elevation in $[Ca^{2+}]_i$ elicited by release of IP_3. In the presence of 1 mM glutamate, these calcium elevations were suppressed dramatically. These preliminary data support the results presented above and provide further support for the concept that the glutamate-stimulated cascade directly inhibits calcium release from IP^3-sensitive stores.

Protein kinase C inhibits IP_3 responses in NM

Preliminary studies examining the roles of A and C kinase pathways in the inhibition of the IP_3 response have led to a relatively clear picture. Ca^{2+} responses attributable to IP_3Rs were reduced by PMA, a C kinase activator. They were unaffected by Sp-cAMPS, a cyclic AMP analogue that activates cyclic AMP-dependent protein kinase I and II. The role of C kinase is strengthened by experiments using the kinase inhibitor, H7. Glutamate reduced HBI-stimulated responses by 86%; when coapplied with H7, responses were reduced by only 41%. Because H7 did not alter HBI responses by itself, it can be concluded tentatively that the glutamate inhibitory effects on IP_3Rs are reversed partially when C kinase activity is attenuated.

The effects of protein kinase C (PKC) on HBI-stimulated responses is of interest because PKC is activated by diacylglycerol (DAG), the second major metabolite of PIP_2 hydrolysis. PMA reduces the HBI response by 90%. Conventionally, C kinase activation is thought to result from a collaborative interaction between DAG and Ca^{2+} released from IP_3R stores. The collaboration is synergistic but is not obligatory. In NM neurons, the DAG branch of the cascade may be particularly sensitive. This may explain why IP_3-regulated Ca^{2+} transients are so rarely observed in glutamate-stimulated NM neurons, as

suggested by the large concentrations of glutamate required to elicit an increase in $[Ca^{2+}]_i$ in calcium-free superfusate (Zirpel et al. 1995b).

The inhibitory effects of PKC on IP_3-regulated Ca^{2+} signaling have been observed previously in a number of cell types, including neurons (Shimizu et al. 1993). PKC can alter $[Ca^{2+}]_i$ in several ways. Because the modulatory domain of the IP_3 receptor is a substrate for several protein kinases (Supattapone et al. 1988), including PKC (Ferris et al. 1991), PKC may attenuate Ca^{2+} efflux via receptor phosphorylation. PKC also may act farther down stream in the cascade by promoting Ca^{2+} sequestration (Nishi et al. 1994; Shivan and Alexander 1995; Werth et al. 1996). PKC also acts up stream, preventing PIP_2 hydrolysis (Luo et al. 1995; Shimizu et al. 1993).

A reduction in the rate of IP_3 production appears to be the least likely explanation for the glutamatergic inhibition of Ca^{2+} signaling. Previous studies of NM neurons demonstrated that concentrations of glutamate and t-ACPD that do not themselves affect $\Delta[Ca^{2+}]_i$ stimulate an increase in the levels of PI metabolites (Zirpel et al. 1994, 1995b).

Kinase modulation of CICR responses in NM neurons

Clearly, additional pharmacological studies are needed to understand the regulation of CICR stores in NM neurons. Our preliminary experiments yielded a confusing picture. Every agent used in this study that modulated kinase activity inhibited CICR-transients by ~60%, which is equivalent to the amount of reduction affected by glutamate and t-ACPD. The paradoxical combinatory effects of glutamate and H8 suggest that CICRs in NM are subject to modulation by multiple mechanisms.

It is difficult to draw any conclusions from these data, as these results are incompatible with existing information on the CICRs in neurons. For example, in other neurons, caffeine-stimulated responses have been potentiated by PKA phosphorylation (Yoshida et al. 1992). In NM, the A kinase activator Sp-cAMPS inhibited caffeine responses. The inhibitory effects of PMA on CICRs were equally surprising and may be explained by Ca^{2+} sequestration, not receptor modulation, because the brain CICR is thought to be a poor substrate for PKC phosphorylation (Guerrini et al. 1995; Takasago et al. 1991; Witcher et al. 1992; Yoshida et al. 1992).

Complicating matters further is the fact that caffeine, independent of its Ca^{2+}-liberating abilities, reduces phosphodiesterase activity (Sawynok and Yaksh 1993). Thus all of the manipulations could be confounded by this effect. Ultimately, our understanding of the signaling pathways responsible for the CICR regulation may be dependent on the discovery of agents that exclusively stimulate the Ca^{2+} release from CICRs without directly activating other second-messenger activity.

Glutamate attenuates any increase $[Ca^{2+}]_i$ via multiple mGLuRs

We previously have shown that glutamate inhibits $[Ca^{2+}]_i$ increases stimulated by KCl and KA. It is important to note that the $[Ca^{2+}]_i$ increases produced by KCl and KA are carried by different channels. Voltage-dependent changes in $[Ca^{2+}]_i$ are carried mainly by dihydropyridine sensitive voltage-operated channels (Lachica et al. 1995). Changes in $[Ca^{2+}]_i$ stimulated by KA are carried by a Ca^{2+} permeable non-*N*-methyl-D-aspartate (non-NMDA) receptor channels (Lachica et al. 1996). Interestingly, the increases in $[Ca^{2+}]_i$ carried voltage operated calcium channels or Ca^{2+} permeable non-NMDA receptor channels are reduced by agents that stimulate protein kinase A activity. Phorbol esters, which inhibit $[Ca^{2+}]_i$ increases from stores, do not affect KCl- or KA-stimulated responses. Two conclusions may be drawn from these results when they are paired with the observations described in the present report. First, NM neurons must possess at least two distinct mGluRs, one that activates adenylate cyclase and a second that activates phospholipase C. Second, NM neurons use somewhat separate, parallel pathways to inhibit increases in Ca^{2+} originating from the exterior and the interior of the cell. Currently, mGluRs have been linked directly to the PLC, adenylate cyclase, and phospholipase D (PLD) transduction systems. L-CSA, an agent that potentiates PLD and cAMP activities (Boss and Boaten 1995; Boss et al. 1994), did not inhibit Ca^{2+} responses stimulated by HBI or caffeine. We have not determined whether L-CSA alters transmembrane-related $\Delta[Ca^{2+}]_i$.

Ca^{2+} stores may contribute to supranormal $[Ca^{2+}]_i$ levels in dying neurons

The present study was stimulated by the observation that $[Ca^{2+}]_i$ rapidly increases in NM neurons deprived of excitatory afferent activity (Zirpel et al. 1995a) and that this effect is prevented by orthodromic stimulation of the auditory nerve (Zirpel and Rubel 1996). We previously have demonstrated that manipulations that prevent this increase also prevent the early events associated with deprivation-induced cell death and neuronal atrophy (Hyson and Rubel 1989, 1995). We hypothesize that changes in $[Ca^{2+}]_i$ are an obligatory step in these and other deprivation-induced neuronal changes (Hartlage-Rübsamen and Rubel 1996; Hyson and Rubel 1989; Lachica et al. 1995; Zirpel and Rubel 1996).

We do not know why $[Ca^{2+}]_i$ increases in deafferented neurons. A number of possible explanations exist. $[Ca^{2+}]_i$ may increase as a result of deregulation of intracellular Ca^{2+} stores. This may be due to the loss of inhibition of Ca^{2+} release from these stores that normally is effected by glutamate. In deafferented NM neurons, Ca^{2+} stores still may be activated via the projections from the superior olivary nuclei, which contain GABAergic neurons (Lachica et al. 1994). GABA depolarizes NM neurons (Hyson et al. 1995) and stimulates increases in $[Ca^{2+}]_i$ (Lachica et al. 1998). In NM, as in other neurons, the GABA-stimulated $\Delta[Ca^{2+}]_i$ is sensitive to dihydropyridines and agents that block Ca^{2+} release from intracellular stores (Berninger et al. 1995; Ito et al. 1995; Nilsson et al. 1993; Parramon et al. 1995; Spergel et al. 1995) In fact, a large component of the GABA-stimulated rise in Ca^{2+} can be eliminated by the IP_3R antagonist, heparin (Lachica et al. 1998). Thus in deafferented neurons, GABA-linked IP_3R-stimulated Ca^{2+} changes could increase to levels that typically are kept in check by a mGluR. The IP_3-associated Ca^{2+} increase could be potentiated further by CICR stores, which also would be ''glutamate-liberated'' in deafferented neurons, resulting in a supernormal level of $[Ca^{2+}]_i$ that triggers cytopathologic events.

The central role assigned to the Ca^{2+} storing organelles in the scenario just described is not unusual. It has become apparent that pathological increases in cell Ca^{2+} are not always caused by Ca^{2+} influx. Ca^{2+} mobilization from stores triggers apoptosis in HL-60 promyelocytic leukemia cells, and MCF-7 estradiol-receptor sensitive breast tumor cells (Vandewalle et al. 1995; Zhu and Loh 1995). Agents that specifically block Ca^{2+} release from stores have been shown to rescue or prevent glutamate toxicity in cortical neurons (Frandsen and Schousboe 1992, 1993) and ameliorate the effects of ischemia and other toxic agents in hippocampal neurons (Yoon et al. 1996; Zhang et al. 1993). Clearly the role of intracellular stores in the regulation of $[Ca^{2+}]_i$ and their contribution to the cytopathology exhibited by deafferented NM neurons requires further investigation.

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