JOURNAL OF NEUROPHYSIOLOGY
Vol. 76, No. 1, July 1996. *Printed in U.S.A.*

RAPID PUBLICATION

Glutamate Modulates Intracellular Ca^{2+} Stores in Brain Stem Auditory Neurons

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SUMMARY AND CONCLUSIONS

1. Fura-2 imaging was used to measure the effects of glutamate on caffeine-sensitive Ca^{2+} stores in neurons of the avian cochlear nucleus, n. magnocellularis (NM).

2. On average, 100-mM caffeine stimulated a 250-nM increase in intracellular calcium ion concentration {$[Ca^{2+}]i$} in Ca^{2+}-free media; 1-mM glutamate significantly attenuated caffeine-stimulated Ca^{2+} responses.

3. The metabotropic glutamate receptor agonist, ACPD, also inhibited the caffeine-stimulated rise in $[Ca^{2+}]i$.

4. Glutamate has an important role in regulating Ca^{2+} stores in NM neurons. Glutamate-deprivation (viz. cochlear removal) results in a rise in $[Ca^{2+}]i$ that may, in part, be the result of release from Ca^{2+} stores. We hypothesize that Ca^{2+}-induced Ca^{2+} release stores (CICRs) may be involved in deprivation-induced cell death.

INTRODUCTION

Neurons in the avian cochlear nucleus, n. magnocellularis (NM), are tonically stimulated by glutamate released from auditory nerve axons. In young chicks, removing or pharmacologically silencing this input triggers a series of intracellular events, resulting in the death of 30% of the neurons and atrophy of the remaining population (Rubel et al. 1990). One of the earliest changes is an elevation in intracellular calcium ion concentration ($[Ca^{2+}]i$) (Zirpel et al. 1995). Increases in $[Ca^{2+}]i$ contribute to cell death in other systems (Choi 1995; Trump and Berezesky 1995), and we hypothesize that the changes in $[Ca^{2+}]i$ observed in afferent-deprived NM neurons are Ca^{2+}-linked (Lachica et al. 1996).

How $[Ca^{2+}]i$ increases in afferent-deprived NM neurons is unknown. Presumably, normal regulatory mechanisms become compromised without orthodromic activation of membrane receptors. The role of Ca^{2+}-induced Ca^{2+} release stores (CICRs) (Endo et al. 1970) is of interest because these stores augment increases in $[Ca^{2+}]i$ in a variety of other systems (Friel and Tsien 1992; Reber et al. 1993; Thayer et al. 1988). It is important, therefore, to determine if CICRs exist in NM neurons and to ascertain whether glutamate modulates CICRs.

METHODS

Tissue preparation

The methods used in the present study have been described in detail (Lachica et al. 1995). Coronal brain stem slices (350 μM) containing NM were obtained from 18-day–old White Leghorn chicken embryos. Slices were incubated in oxygenated artificial cerebral spinal fluid (aCSF) containing 5-μM fura-2-AM (Molecular Probes, Eugene, OR) at 40°C for 25 min, then rinsed with aCSF for 10 min before beginning the experiment.

Microfluorometry

The $[Ca^{2+}]i$ of NM cells was measured by using fluorometric videomicroscopy. Fluorescent emissions were acquired at 3-s intervals, ratiometrically compared, and converted to nM $[Ca^{2+}]i$ by using Universal Imaging Corp. (West Chester, PA) software. Changes in $[Ca^{2+}]i$ are reported as the mean ± SE.

Pharmaceuticals

aCSF and Ca^{2+}-free aCSF were freshly made each day. aCSF was prepared as described previously (Lachica et al. 1995). The Ca^{2+}-free aCSF was composed of 125-mM NaCl, 5-mM KCl, 1.25-mM KH_2PO_4, 4.4-mM $MgCl_2$, 26-mM sodium bicarbonate, 10-mM dextrose and 1-mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). The following drugs were delivered in Ca^{2+}-free aCSF: 100-mM caffeine; 10-μM, 100-μM, and 1-mM glutamate; 10-μM, 100-μM, and 1-mM ± *trans* 1-amino-1,3 cyclopentanedicarboxylic acid (ACPD); 1-mM (±)-a-methyl-4-carboxyphenylglycine (MCPG); 100-μM ryanodine; 50-μM kainic acid (KA). Drugs were purchased from Research Biochemicals, (Natick, MA), except glutamate, which was acquired from Sigma. In every case, neurons were pharmacologically depolarized with KCl to test cell viability. There is a 30-s delay between initiation of stimulus delivery and the time the test chamber was completely saturated with the test solution. This delay is not corrected for in the figures.

RESULTS

NM neurons possess CICRs

To determine if CICRs are present in NM neurons, cells were superfused with caffeine, which liberates Ca^{2+} from CICRs in other systems (Konishi and Kurihara 1987; Mekhail-Ishak et al. 1987). Figure 1*A* plots the $\Delta[Ca^{2+}]i$ of seven NM neurons superfused with 60-mM KCl in Ca^{2+}-free aCSF, 100-mM caffeine in Ca^{2+}-free aCSF, and 60-mM KCl in normal aCSF (Ca^{2+}-containing). A $\Delta[Ca^{2+}]i$ was not seen in cells pharmacologically depolarized with KCl in Ca^{2+}-free media. Superfusion with caffeine (65 neurons, n = 4 slices) caused a mean $\Delta[Ca^{2+}]i$ of 254 ± 23 nM. This rise in $[Ca^{2+}]i$ was presumably the result of Ca^{2+} efflux from CICRs, because caffeine was delivered in Ca^{2+}-free medium, and because the caffeine-stimulated responses were eliminated by ryanodine (Fig. 2*B*), a CICR antagonist (Feher and Lipford 1985). Finally, KCl in aCSF evoked a

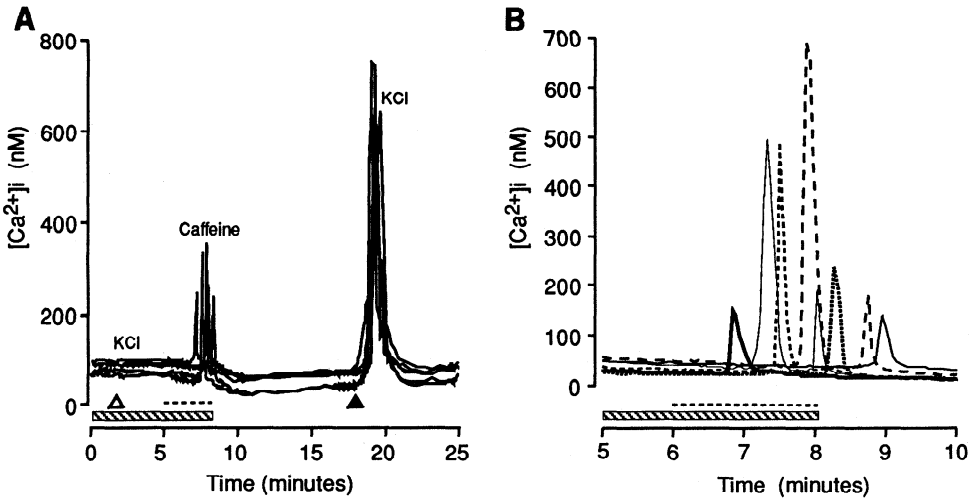

FIG. 1. Changes in intracellular calcium ion concentration ($[Ca^{2+}]i$) caused by KCl and caffeine. *A*: responses of 7 n. magnocellularis (NM) neurons to 60-mM KCl applied in Ca^{2+} free artificial cerebral spinal fluid (aCSF, △), 100-mM caffeine, and 60-mM KCl in normal aCSF (▲). Caffeine stimulates a rise in $[Ca^{2+}]i$ that is smaller in magnitude and decreases to subresting levels after the Ca^{2+} spike. *B*: caffeine-stimulated Ca^{2+} transients of 7 neurons in the same slice are asynchronous. ▩, Ca^{2+}-free aCSF superfusion; – – –, duration of caffeine stimulus.

large rise in $[Ca^{2+}]i$ which was largely the result of Ca^{2+} influx through voltage-gated channels (Lachica et al. 1995).

Caffeine-stimulated Ca^{2+} responses differ from those evoked by pharmacological depolarization with KCl (Fig. 1*A*). They are much smaller in magnitude than the $\Delta[Ca^{2+}]i$ caused by KCl. With our bath application method, caffeine-stimulated increases in $[Ca^{2+}]i$ develop after a latency of ~1 to 3 min; whereas, KCl evoked transients consistently occur within 30 s. In addition, caffeine-stimulated release of Ca^{2+} from CICRs varies in time and amplitude between neurons within a slice (Fig. 1*B*). After the Ca^{2+} rise evoked by caffeine, $[Ca^{2+}]i$ falls to less than basal levels (the post-caffeine "undershoot"; Usachev et al. 1993). The average post-caffeine $[Ca^{2+}]i$ was 61 ± 6 nM (93 neurons, $n = 7$ slices). This value is significantly different ($P < 0.0001$) than basal $[Ca^{2+}]i$ of the same NM neurons bathed in aCSF (91 ± 4 nM) or Ca^{2+}-free aCSF (92 ± 5 nM).

Ca^{2+} from CICRs contributes to the $[Ca^{2+}]i$ with membrane depolarization

CICRs potentiate voltage-dependent changes in $[Ca^{2+}]i$ (Friel and Tsein 1992; Thayer et al. 1988). To determine whether or not this occurs in NM neurons, we superfused 40 neurons ($n = 4$ slices) with 100-μM ryanodine between sequential KCl stimuli. Ryanodine significantly reduced voltage-dependent $\Delta[Ca^{2+}]i$ from an average of 1222 ± 372 nM to 624 ± 235 nM, an average of 49% ($P < 0.05$).

Glutamate modulates Ca^{2+} release from CICRs via an mGluR

Figure 2*A* shows the effect of glutamate, ACPD, and KA on the $\Delta[Ca^{2+}]i$ attributable to CICRs. Slices were depolarized with KCl, then perfused with one of these agents for 5 min in Ca^{2+}-free media, and then stimulated with caffeine. 1-mM glutamate (67 neurons, $n = 6$ slices) significantly reduced the mean caffeine response from 254 ± 23 nM to 58 ± 18 nM ($P < 0.001$) (Fig. 2, *A*, *C* and *D*). The suppressive effect of glutamate on caffeine-stimulated increases in $[Ca^{2+}]i$ was dose-dependent (Fig 2*D*); 100-μM glutamate (54 neurons, $n = 5$ slices) significantly attenuated the $\Delta[Ca^{2+}]i$ from CICRs to 149 ± 34 nM ($P = 0.03$).

The mGluR agonist, ACPD, also inhibited CICR responses (Figs. 2*A* and 3*B*). The mean $\Delta[Ca^{2+}]i$ stimulated by caffeine after neurons were exposed to 100-μM ACPD (41 neurons, $n = 4$ slices) was 128 ± 64 nM ($P < 0.05$). 10-μM and 1-mM ACPD in Ca^{2+} free aCSF did not cause a significant change in Ca^{2+} release from CICRs. As a control, 42 neurons ($n = 3$ slices) were exposed to 50-μM KA, an ionotropic glutamate receptor (iGluR) agonist, in Ca^{2+} free aCSF. The mean increase in $[Ca^{2+}]i$ to caffeine after KA treatment (265 ± 102 nM) was not significantly different from the response to caffeine alone ($P = 0.462$) (Fig. 2*A*).

The suppressive effect of glutamate was reversed by the mGluR antagonist, MCPG (46 neurons, $n = 4$ slices). The average $\Delta[Ca^{2+}]i$ evoked by caffeine after the co-application of 1-mM MCPG and 1-mM glutamate was 181 ± 30 nM (Fig 2*B*). This response was statistically greater than the $\Delta[Ca^{2+}]i$ seen in caffeine-stimulated neurons exposed to 1-mM glutamate alone 58 ± 18 nM ($P < 0.01$).

In our study, glutamate and KA did not stimulate a rise in $[Ca^{2+}]i$ as a result of Ca^{2+} influx through ionotropic receptors because they were delivered in Ca^{2+}-free aCSF. This finding is consistent with previous studies that have examined the effects of glutamate and its agonists in NM neurons (Kato et al. 1996; Lachica et al. 1995).

DISCUSSION

Our interest in studying the regulation of Ca^{2+} stores in NM neurons comes as the result of two observations: *1*) there is rapid cell death and atrophy of NM neurons after afferent deprivation in neonatal chicks (Rubel et al. 1990); and *2*) $[Ca^{2+}]i$ increases rapidly in NM neurons deprived of afferent signals (Zirpel et al. 1995). Changes in $[Ca^{2+}]i$ are believed to play an important role in cell death in neuronal and non-neuronal systems (Choi 1995; Orrenius and Nicotera 1993; Trump and Berezesky 1995). Therefore, it is likely that increases in $[Ca^{2+}]i$ after afferent deprivation

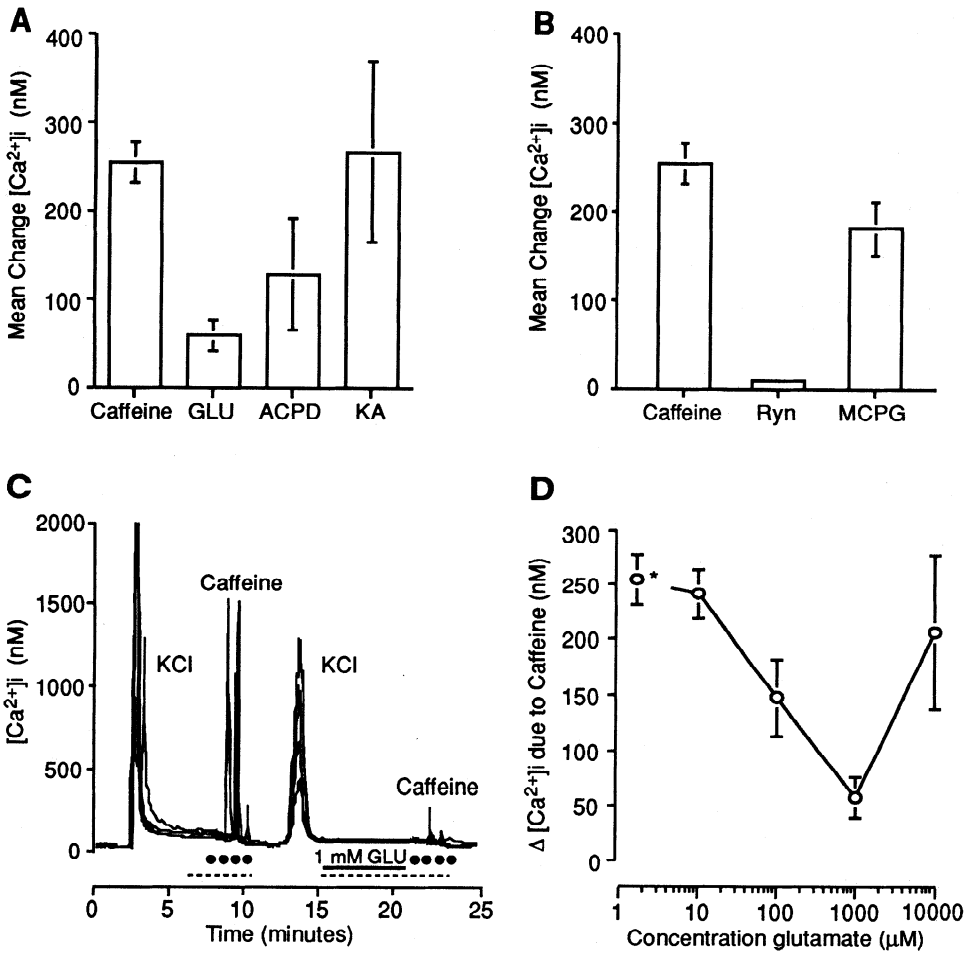

FIG. 2. Glutamate modulates Ca^{2+}-induced Ca^{2+} release stores (CICRs). *A*: mean ± SE $\Delta[Ca^{2+}]i$ produced by 100-mM caffeine alone or after 5-min superfusion of 1-mM glutamate (GLU), 100-μM metabotropic glutamate receptor agonist (ACPD), or 50-μM kainic acid (KA). *B*: mean ± SE $\Delta[Ca^{2+}]i$ produced by 100-μM ryanodine (Ryn) and 1-mM a-methyl-4-carboxyphenylglycine (MCPG) (co-applied with 1-mM glutamate) on $\Delta[Ca^{2+}]i$ attributable to CICRs. Normal response to 100-mM caffeine is shown for comparison. *C*: 1-mM glutamate suppresses caffeine-stimulated $\Delta[Ca^{2+}]i$. – – –, Ca^{2+}-free aCSF superfusion; · · ·, caffeine stimulus; ——, glutamate superfusion. *D*: dose-response effect of glutamate on caffeine-stimulated Ca^{2+} responses; * represents caffeine response in the absence of glutamate. All drugs were delivered in Ca^{2+}-free aCSF.

contribute to events resulting in NM neuron atrophy and death.

$[Ca^{2+}]i$ may increase via influx through plasma membrane channels, efflux from intracellular stores or from inadequate Ca^{2+} buffering. Because deafferented NM neurons are no longer electrically active (Born et al. 1991), it seems likely that the rise in $[Ca^{2+}]i$ is the result of Ca^{2+} release from Ca^{2+} storing organelles, rather than Ca^{2+} influx through ligand operated channels in the cell membrane. This idea stimulated our interest in identifying the pharmacological profile of Ca^{2+} stores and their regulation in NM neurons.

In this study, we demonstrated that CICRs are present in

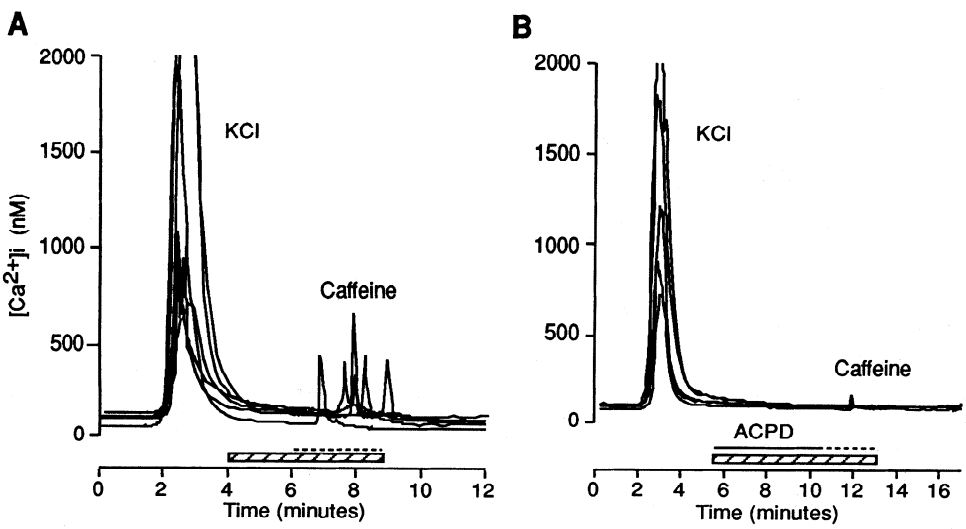

FIG 3. ACPD modulates CICR responses. *A*: CICR responses in control neurons. *B*: 100-μM ACPD suppresses CICR responses. – – –, Caffeine superfusion; ——, ACPD superfusion.

NM neurons. More importantly, we found that glutamate negatively modulates CICRs, reducing the $\Delta[Ca^{2+}]i$ resulting from caffeine-induced release from CICRs by 40%. Therefore, it could be argued that in the normal afferented state, glutamate serves to prevent the potentially deleterious release of Ca^{2+} from CICRs.

The existence of such a mechanism would be valuable to NM neurons because they are tonically stimulated by glutamate. Spontaneous rates of NM activity are ~100 Hz (Warchol and Dallos 1990). Furthermore, the auditory nerve envelops the neurons with a giant cup-shaped ending that limits the diffusion of glutamate. As a result, it is possible that as the concentration of neurotransmitter in the synaptic cleft approaches 1 mM, the predicted concentration at glutamatergic synapses (Clements et al. 1992). Interestingly, caffeine-stimulated responses were significantly reduced by 1-mM glutamate. It is also important to note that this concentration of glutamate has been shown to be maximally efficacious in stimulating mGluRs1–7 (Abe et al. 1992; Aramori and Nakanishi 1992; Nakajima et al. 1993; Okamoto et al. 1994; Tanabe et al. 1992, 1993).

We were surprised to find that higher concentrations of glutamate failed to suppress Ca^{2+} release from CICRs. This may be due to receptor desensitization (e.g., Aronica et al. 1993; Catania et al. 1991; Herrero et al. 1994) or the activation of other second messenger systems (Bockaert et al. 1993; Nakanishi 1992; Schoepp and Conn 1993; Schoepp et al. 1995) by the higher glutamate concentrations. To date, mGluRs have been shown to regulate phosphoinositide hydrolysis, cAMP, arachidonic acid, cGMP, and phospholipase D activity (see Boss et al. 1994; Glaum and Miller 1993a,b; Schoepp and Conn 1993; Winder et al. 1993). Unfortunately, the precise concentrations at which glutamate activates mGluRs coupled to different second messenger systems is not known.

Previously, we reported that the mGluR agonist ACPD reduces voltage-dependent Ca^{2+} responses (Lachica et al. 1995). Present experiments with ACPD and MCPG demonstrate that the glutamatergic effect on CICRs is also mGluR-dependent: 100-μM ACPD reliably inhibited increases in Ca^{2+} due to CICRs, while MCPG, used at its most efficacious concentration (Hayashi et al. 1994), attenuated glutamate's suppressive effect. The iGluR agonist, KA, had no effect on Ca^{2+} release from CICRs. These data suggest that metabotropic effects may act broadly to reduce stimulation-induced increases in cell calcium, independent of its source. To test this hypothesis it will be necessary to identify the second messenger that mimics glutamatergic inhibition of Ca^{2+}. In the case of the mGluR-mediated reductions in voltage-dependent Ca^{2+} fluxes, cAMP appears to play a significant role (Lachica et al., 1995). Preliminary studies indicate that cAMP effectors are not involved in the mGluR-mediated inhibition of caffeine-stimulated $\Delta[Ca^{2+}]i$.

The authors thank Drs. William R. Lippe and Lance Zirpel for helpful discussion and scholarship.

This research was supported by National Institute of Deafness and Other Communications Disorders Grants DC-00520 and DC-00018.

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Received 23 January 1996; accepted in final form 10 April 1996.

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