Glutamatergic Calcium Responses in the Developing Lateral Superior Olive: Receptor Types and Their Specific Activation by Synaptic Activity Patterns

F. Aura Ene, Paul H. M. Kullmann, Deda C. Gillespie, and Karl Kandler

Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Submitted 13 March 2003; accepted in final form 2 July 2003

INTRODUCTION

One of the major functions of the auditory system is to determine the direction of incoming sound. The lateral superior olive (LSO) is the first station in the ascending auditory pathway that processes interaural intensity differences (Boudreau and Tsuchitani 1970; Caird and Klinke 1983; for review, see Oertel 1999). Neurons in this nucleus are excited by sound at the ipsilateral ear and are inhibited by sound at the contralateral ear. Excitation reaches the LSO via a glutamatergic projection from spherical bushy cells in the ipsilateral anteroventral cochlear nucleus (AVCN, reviewed in Thompson and Schofield 2000). Inhibition reaches the LSO via a glycinergic projection from the cochlear nucleus (AVCN, reviewed in Thompson and Schofield 1970; Caird and Klinke 1983; for review, see Oertel 1999).

Developmental regulation of LSO neurons and changes the axonal termination pattern of MNTB axons. These anatomical changes are accompanied by a number of physiological changes including abnormal inhibition, as well as excitatory, synaptic transmission (review by Sanes and Friauf 2000).

Despite the importance of synaptic activity for normal LSO development, the mechanisms by which synaptic activity exerts its effect on immature LSO neurons are still poorly understood. A number of studies in other neuronal systems have demonstrated the central role of calcium-dependent mechanisms in mediating the effects of activity on neuronal survival and development (Berridge 1998; Franklin and Johnson 1992; Spitzer et al. 2000). In the developing auditory system, the role of calcium-dependent mechanisms in activity-dependent neuronal survival is best understood in the cochlear nucleus of chickens. In this nucleus, glutamate released from 8th nerve terminals activates postsynaptic metabotropic glutamate receptors (mGluRs) that mobilize intracellular calcium. This activates protein kinases A and C, which in turn inhibit voltage-gated calcium channels, thereby preventing accumulation of toxic levels of 

$[Ca^{2+}]_{i}$ (Lachica et al. 1995; Zirpel and Rubel 1996; Zirpel et al. 1998). Recent evidence has indicated that calcium-dependent mechanisms are also crucial for the survival and maturation of neurons in the LSO (Lohmann et al. 1998), but the mechanisms by which synaptic activity increases $[Ca^{2+}]_{i}$ in the LSO are poorly understood. We have previously shown that glycineric/GABAergic synapses in the MNTB–LSO pathway increase $[Ca^{2+}]_{i}$ during the period when these synapses are depolarizing and are functionally eliminated (Kandler and Friauf 1995; Kim and Kandler 2003; Kullmann et al. 2002). However, the mechanisms by which glutamatergic afferents from the cochlear nucleus regulate postsynaptic $[Ca^{2+}]_{i}$ in the LSO are unknown. Developing LSO neurons express all major classes of ionotropic and metabotropic glutamate receptors (Caicedo and Ey-
balin 1999; Kandler and Friauf 1995; Kotak and Sanes 1996), indicating that glutamatergic synapses could elicit calcium responses by a variety of mechanisms such as calcium influx through Ca\(^{2+}\)-permeable AMPA, kainate, or N-methyl-D-aspartate (NMDA) receptors, depolarization-induced activation of voltage-gated calcium channels (VGCCs), or stimulation of metabotropic glutamate receptors (mGluRs) and mobilization of intracellular calcium.

In the present study, we have examined the contribution of GluRs to calcium responses in the developing LSO using Fura-2 calcium imaging in brain stem slices from neonatal mice. We found that all major types of GluRs can contribute to Fura-2 calcium imaging in brain stem slices from neonatal mice. We found that all major types of GluRs can contribute to synaptically elicited calcium responses and that activation of specific types of GluRs and their associated calcium entry pathways is dependent on the frequency of presynaptic stimulation. These results indicate that developing LSO neurons can rate-code spontaneous synaptic activity patterns from the cochlear nucleus by activating distinct calcium entry routes that may be linked to distinct second-messenger pathways and gene expression (West et al. 2002). Some of the results have previously been presented in abstract form (Negoita and Kandler 2002).

**METHODS**

**Animals and slice preparation**

Experiments were performed in brain stem slices of C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME) of both sexes aged between postnatal day 0 (P0: the date of birth) and P7. Experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Animals were anesthetized by hypothermia and decapitated, and the brains were removed and placed into cold (4–8°C) artificial cerebrospinal fluid (ACSF, composition in mM: 124 NaCl, 26 NaHCO\(_3\), 10 glucose, 5 KCl, 1.25 KH\(_2\)PO\(_4\), 1.3 MgSO\(_4\), 2 CaCl\(_2\), and 1 kynurenic acid, pH 7.4 when aerated with 95% O\(_2\)-5% CO\(_2\)). Transverse slices (200–300 \(\mu\)m thickness) were cut with a vibrotome (DTK-1500E, Ted Pella, Redding, CA), and slices in which the LSO and MNTB could be identified were selected for labeling.

**Fura-2 labeling**

Slices were first incubated in a concentrated Fura-2AM solution (100 \(\mu\)M; Molecular Probes, Eugene, OR) for 2–5 min followed by submersion in aerated ACSF containing 10 \(\mu\)M Fura-2AM, for 2–5 h at 30–32°C (Peterlin et al. 2000). All Fura-2AM staining solutions were prepared from 1 mM stock solution in DMSO. Previously, we showed that in the immature LSO, Fura-2AM preferentially labels neurons (Kullmann et al. 2002).

**Calcium imaging**

Individual slices were transferred into a recording chamber and were continuously superfused with oxygenated ACSF (30–32°C, perfusion rate 2–3 ml/min) that contained no kynurenic acid but 100 \(\mu\)M Trolox (Scheenen et al. 1996). Slices were imaged with an inverted epifluorescence microscope (Nikon Eclipse TE200) equipped with \(\times10\) and \(\times20\) air objectives (NA: 0.5 and 0.75 respectively). Ratiometric imaging was performed as previously described (Kullmann et al. 2002).

Fluorescence intensity was converted to intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) using the equation \([\text{Ca}^{2+}]_i = K_d\beta R - R_{\text{min}}) / (R_{\text{max}} - R)\), where \(R_{\text{min}}\) is the fluorescence ratio of Ca\(^{2+}\)-free Fura-2,

\(R_{\text{max}}\), is the ratio of Ca\(^{2+}\)-bound Fura-2, and \(\beta\) is the ratio of the fluorescence intensity of Ca\(^{2+}\)-free Fura-2 at 380 nm to the fluorescence intensity of Ca\(^{2+}\)-bound Fura-2 at 380 nm (Grynkiewicz et al. 1985). A \(K_d\) of 224 mM was used. \(R_{\text{min}}\) was determined in slices incubated in Ca\(^{2+}\)-free ACSF (Ca\(^{2+}\) was replaced by Mg\(^{2+}\)) with 2 mM EGTA and 4 \(\mu\)M Ca\(^{2+}\) ionophore Br-A23187 (Alomone Labs, Israel). \(R_{\text{max}}\) was measured in slices incubated in 10 mM CaCl\(_2\) (Kao 1994). Over the course of this study, the system was calibrated repeatedly, and the values for \(R_{\text{min}}\) ranged from 0.25 to 0.31, for \(R_{\text{max}}\) from 1.22 to 1.8, and \(\beta\) from 2.3 to 3.7.

**Electrical stimulation**

Afferent fibers from the AVCN to the LSO were stimulated using bipolar stainless steel electrodes (tip distance: 100–200 \(\mu\)FHC, Bowdoinham, ME). Electrodes were placed in the ipsilateral ventral acoustic stria (VAS), lateral to the LSO (Fig. 1A). Electrical stimuli consisted of constant current pulses (duration: 100 \(\mu\)s) with amplitudes between 50 and 300 \(\mu\)A (Master-8 and ISO-Flex; AMPI) delivered in single pulses or stimulus trains (10 pulses at 5, 10, 20, 50, and 100 Hz).

**Drug application**

Drugs were dissolved in ACSF from concentrated stock solutions and delivered via bath application or pressure application using patch electrodes (10 psi, 100- to 1,000-ms pulse duration; PV 820 picopump, WPI, Sarasota, FL).

![Ca\(^{2+}\)-responses in lateral superior olive (LSO) neurons elicited by electrical stimulation of the ipsilateral ventral acoustic stria (VAS). A: Brightfield image of a brain stem slice from a P3 mouse, illustrating the position of the stimulation electrode relative to the LSO. B: examples of Ca\(^{2+}\)-responses. Top: Ca\(^{2+}\) concentrations before (baseline) and after electrical stimulation of the VAS with a single stimulus, low-frequency (5 Hz), or high-frequency (50 Hz) stimulus train. Bottom: time course of Ca\(^{2+}\) responses from the cells delineated above. Arrowheads mark stimulation. C: average peak changes in [Ca\(^{2+}\)], elicited by single pulses, low-frequency and high-frequency stimulation. S, single stimulus; LF, low-frequency stimulation (10 pulses at 5–10 Hz); HF, high-frequency stimulation (10 pulses at 20–100 Hz).](http://jn.physiology.org/doi/10.220310.1101.org)
Drugs used in this study were as follows.

**AGONISTS OF GLURS.** N-methyl-d-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainic acid (KA), (S)-3,5-dihydroxyphenylglycine (DHPG), 4-carboxy-3-hydroxyphenylglycine (4C3HPG), l- (+) -2 -amino-4-phosphonobutyric acid (L-APV), and 1S, 3R -aminocyclopentane -trans -dicarboxylic acid (ACPD).

**ANTAGONISTS OF GLURS.** 6 -Cyano-7 -nitroquinoxaline-2,3-dione (CNQX), D -2 -amino-5 -phosphonopentanoic acid (D -APV), TTX (100 nM), and MCPG (1 mM; Alomone Labs, Jerusalem, Israel).

VGCCs BLOCKERS. Nicl₂ (nonspecific blocker of VGCCs at 2–5 mM (Gu et al., 1994), nifedipine (L-type channel inhibitor), α-conotoxin GVIA (N-type channel inhibitor), and α-agatoxin TK (P/Q-type channel inhibitor, Alomone Labs, Jerusalem, Israel).

Other drugs used were thapsigargin and tetrodotoxin (TTX, Alomone Labs).

Electrical stimulation was performed in the presence of bicuculline (10 μM) and strychnine (10 μM), and GluR agonists were applied in TTX (1 μM). For calcium-free ACSF, 2 mM EGTA was added and calcium was exchanged for equimolar concentrations of magnesium. If not stated otherwise, chemicals used were from Sigma (St. Louis, MO).

**Data analysis**

Image analysis was performed with the program Tillvision (T.I.L.L. Photonics). Images were low-pass filtered (Gaussian 3 × 3 kernel) and background subtraction was performed as described previously (Betz and Bewick 1993). Briefly, for each 380 image sequence, the lowest pixel value found in the sequence was subtracted from all pixels. For 340 images series, the histogram of pixel values for each image in a series was determined. The average of the mode values (peak of histogram) of images during baseline was calculated, and 80% of this value was subtracted from all pixels in the sequence.

Calcium concentrations were measured from the soma of cells in the focal plane. Cells with resting calcium levels >250 nM, with responses >1500 nM, and cells whose calcium concentration failed to return to baseline were excluded from analysis. Only changes in [Ca²⁺]i that exceeded 2 standard deviations of the baseline were regarded as responses.

**Electrical recordings**

For AMPA ionophoresis, visualized whole cell patch-clamp recordings were performed in voltage clamp (V_m = −65 mV) at room temperature. Recording electrodes had resistances of 2–3 MΩ and were filled with a solution containing (in mM): 110 d-glucionic acid, 110 CsOH-H₂O, 11 EGTA, 10 CsCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 0.3 Na-GTP, and 2 Mg-ATP-3.5H₂O. To isolate AMPAR-mediated currents, D-APV (50 μM), MCPG (1 mM), bicuculline (10 μM), strychnine (5 μM), and TTX (1 μM) were added to the ACSF. For electrical recordings only, desensitization of AMPARs was prevented with CTZ (50 μM), AMPA (10 mM in 0.15 M NaCl, pH 7.5) was ionophoretically applied close to the soma using pipettes with resistances ~70 MΩ. Retaining current was 20–50 nA (BVC-700, Dagan, Minneapolis, MN), and ejection current was 30–50 nA for 2s. Membrane currents were filtered at 1 kHz (Axopatch 1D, Axon Instruments), digitized (AD board, National Instrument, Austin, TX), and stored for off-line analysis. Data acquisition and analysis was performed using custom-written software running under the LabVIEW environment (National Instruments). For recordings of postsynaptic responses, d-glucionic acid and CsOH in the the pipette solution were substituted by 110 mM K-glucinate, and recordings were performed in current clamp, adjusting current injection to yield a V_m of approximately −60 mV. The ipsilateral pathway was stimulated using low- and high-intensity single stimuli and low frequency stimulus trains. The minimal current that elicited reliable EPSPs (failure rate <10%) was used for low intensity stimuli. An amplitude three times threshold was used for high-intensity stimulation. For low-frequency stimulus trains, 10 stimuli were applied at 10 Hz with low stimulus intensities.

For analysis, 5–10 excitatory postsynaptic potential (EPSP) traces were averaged, and peak amplitude and area of response were measured. The latter was determined by fitting a monoexponential curve to the decaying phase of responses, using the intersection of the fit with the V_m baseline as the endpoint of the response.

**Statistical analysis**

Statistical significance was analyzed using paired t-test, ANOVA followed by Student Newman-Keuls post hoc test, Fisher’s exact test and linear (Pearson) correlation test. If data were not normally distributed, the Mann-Whitney nonparametric statistical test was used. Throughout the text, values are expressed as means ± SE.

**RESULTS**

In this study we examined the contribution of ionotropic and metabotropic glutamate receptors to changes in [Ca²⁺]i, in immature LSO neurons. Responses were elicited by electrical stimulation of the ventral acoustic stria (VAS) or by bath application of specific GluR agonists. Our measurements focused on elongated cell bodies, which presumably represent principal cells (Helfert and Schwartz 1987; Ollo and Schwartz 1979; Rietzel and Friauf 1998). Marginal cells and very small round cells (diameter <5 μm) were excluded from further analysis. Results presented here include data from 801 neurons from 72 slices from animals aged between P0 and P7. The average resting Ca²⁺ concentration of neurons included in this study was 92 ± 2 nM (n = 647).

**Synaptically elicited calcium responses**

Electrical stimulation of the ipsilateral VAS, the major excitatory input from the AVCN to the LSO, consistently evoked Ca²⁺-responses in neurons throughout the LSO (Fig. 1). In accordance with previous electrophysiological studies (Caspar and Faingold 1989; Kandler and Friauf 1995; Kotak and Sanses 1995; Wu and Kelly 1992), VAS-elicited responses were glutamatergic because they were abolished by a mixture of the GluR antagonists CNQX (20 μM), APV (50–100 μM), and MCPG (1 mM; n = 45 cells, data not shown). The amplitudes of these Ca²⁺ responses increased with stimulation frequency (Fig. 1, B and C). Single electrical shocks elicited only small increases in [Ca²⁺]i (51 ± 7 nM, n = 54 cells), and responses returned to baseline within 2–5 s. Low-frequency stimulation (10 pulses at 5 and 10 Hz) elicited larger responses (138 ± 13 nM, n = 66 cells), and these responses were longer, returning to baseline within 5–15 s. High-frequency stimulation (10 pulses at 20, 50, and 100 Hz) elicited the largest responses (232 ± 19 nM, n = 66 cells) with the longest durations, returning slowly to the baseline within 3.5 to >15 s (limited by the duration of recordings).

The effects of stimulus frequency on response amplitude and duration could be due to frequency-dependent recruitment of the number of GluRs and/or the type of ionotropic and metabotropic GluRs. To address these possibilities, we investigated...
the recruitment of different GluR types as a function of stimulation frequency.

\textbf{Contribution of AMPA and kainate receptors}

To examine the contribution of AMPARs to the observed \( Ca^{2+} \) signals, the VAS was stimulated in the presence of the AMPA/kainate receptor antagonist CNQX (20 \( \mu M \)). Under these conditions, responses elicited by a single stimulus were nearly abolished (reduction: 92 \( \pm \) 2\%; peak amplitudes in control: 55 \( \pm \) 8 \( nM \), \( n = 40 \) cells; paired \( t \)-test, \( P < 0.01 \); Fig. 2) while responses to low-frequency trains were reduced by 73 \( \pm \) 3\% (control: 170 \( \pm \) 17 \( nM \), CNQX: 54 \( \pm \) 11 \( nM \), \( n = 43 \) cells; paired \( t \)-test, \( P < 0.01 \)). Responses elicited by high-frequency stimulation were the least affected by CNQX and were reduced by only 48 \( \pm \) 3\% (control: 277 \( \pm \) 27 \( nM \), CNQX: 148 \( \pm \) 27 \( nM \), \( n = 43 \) cells; paired \( t \)-test, \( P < 0.01 \)). These data indicate that the relative contribution of AMPA/kainate receptors to \( Ca^{2+} \)-responses decreases with increasing stimulus frequency (Fig. 2B).

Several nuclei in the mature and developing auditory brain stem express kainate receptors (Lachica et al. 1998; Lohrke and Friauf 2002; Petralia et al. 1994, 1996; review Petralia et al. 2000). To test whether these receptors contribute to an increase in \([Ca^{2+}]_i\), in LSO neurons, KA was applied in the presence of 25–50 \( \mu M \) GYKI 52466, an AMPAR antagonist (Lachica et al. 1998; Vizi et al. 1997). Under this condition, KA (5–25 \( \mu M \), 30 s) increased \([Ca^{2+}]_i\) (157 \( \pm \) 23 \( nM \), \( n = 37 \)) and CNQX (20 \( \mu M \)) reduced these responses to 28 \( \pm \) 5\% of control (\( n = 37 \) cells, P0 - P3; \( P < 0.01 \); paired \( t \)-test; Fig. 2, C and D).

AMPA/kainate receptors can increase \([Ca^{2+}]_i\), by two mechanisms: depolarization-induced activation of VGCCs or calcium influx through \( Ca^{2+} \)-permeable AMPA/kainate receptors. We thus compared AMPA/kainate receptor-mediated responses before and after blockade of VGCCs with Ni\(^{2+}\) (2–5 mM, G. Gu et al. 1994) (Fig. 3). Ni\(^{2+}\) blocked responses elicited by AMPA (1 \( \mu M \), 50 ms pressure application, \( n = 31 \) cells) or kainate in the presence of GYKI 52466 (\( n = 8 \) cells). Nickel (2 mM) had no effect on AMPA-elicited whole cell currents (\( P = 0.39 \), Student’s \( t \)-test; \( n = 4 \) cells; Fig. 3C). This indicates that AMPA and kainate receptors in neonatal LSO neurons are not \( Ca^{2+} \)-permeable, and that AMPA and kainate receptor-elicited \( Ca^{2+} \) responses are mediated via activation of VGCCs.

\textbf{Contribution of NMDA receptors}

The contribution of NMDA receptors (NMDARs) to synaptic \( Ca^{2+} \) responses was examined using the NMDAR antagonist APV (50–100 \( \mu M \); Fig. 4, A and B). APV had no effect on very small responses, indicating that for small responses, NMDARs do not contribute to somatic calcium changes (control: 8 \( \pm \) 2 \( nM \), APV: 10 \( \pm \) 4 \( nM \), \( n = 6 \) cells; paired \( t \)-test, \( P > 0.1 \)). For larger responses, however, APV reduced \( Ca^{2+} \) changes by \( \sim 50\% \), and this reduction was independent of the stimulus train frequency (Fig. 4, A and B). The absence of NMDA-mediated calcium influx at low response amplitudes suggests that NMDARs in LSO neurons are blocked by magnesium (Mayer et al. 1984; Nowak et al. 1984). In support of this, application of NMDA (50 \( \mu M \), 60 s) failed to elicit \( Ca^{2+} \) responses at physiological Mg\(^{2+}\) concentrations (1.3 mM; \( n = 83 \) cells, P1–P4; Fig. 4C) but elicited strong \( Ca^{2+} \) responses in Mg\(^{2+}\)-free ACSF (467 \( \pm \) 87 \( nM \), \( n = 83 \) cells) that were completely blocked by APV (50 \( \mu M \), \( n = 83 \)).

The \( \sim 50\% \) contribution of NMDARs to calcium responses was higher than expected based on previous electrophysiological studies indicating that ipsilaterally excitation to LSO neurons is mediated primarily or exclusively by non-NMDARs (Caspar and Faingold 1989; Kandler and Friauf 1995; Kotak and Sanes 1996; Wu and Kelly 1992). We thus investigated the contribution of NMDARs to ipsilaterally elicited EPSPs in our preparation. These experiments revealed that in neonatal mice (P3–P5), NMDARs contribute to EPSP amplitudes between 35 and 64\% and to response areas between 56 and 67\%, depending on stimulus conditions (Table 1, Fig. 4D). For high-intensity stimuli and stimulus trains, these values agree well with those we observed on calcium level, but for low-intensity responses, the obvious NMDAR contribution was higher for EPSPs. Taken together, these results demonstrate that in neonatal mice, NMDARs contribute considerably to ipsilaterally elicited calcium and voltage responses.

\textbf{Metabotropic glutamate receptors}

To examine whether and to what extent mGluRs contribute to synaptic \( Ca^{2+} \) responses, the VAS was stimulated while blocking ionotropic GluR receptors with APV (50–100 \( \mu M \)) and CNQX (20 \( \mu M \)). These antagonists abolished \( Ca^{2+} \) responses elicited by single stimuli in all cells and abolished responses to low-frequency trains in about half the cells (51%,...
agonist ACPD consistently increased \([\text{Ca}^{2+}]\), that is reversibly abolished by blocking voltage-gated calcium channels (VGCCs). On average, 2–5 mM Ni\(^{2+}\) reduces AMPA-elicited Ca\(^{2+}\)-responses to 2.2 ± 2.3% of control. \(*, P < 0.01,\) paired t-test. C: AMPARs are not affected by 2 mM Ni\(^{2+}\). Traces are single responses to AMPA iontophoresis. Bar indicates AMPA application. Graph illustrates average response from 4 cells.

23 of 45 cells. Responses to high-frequency trains were abolished in only 13% of cells (6 cells of the same 45 cells; Fisher’s exact test, \(P = 0.0002\)). Application of the mGluR antagonist MCPG (1 mM) significantly reduced responses to low-frequency stimuli that remained after APV/CNQX treatment (57% reduction, \(n = 39\) cells; paired t-test, \(P < 0.01\); Fig. 5A). In contrast, MCPG only slightly reduced APV/CNQX-resistant responses to low-frequency trains (from 16 ± 3 to 11 ± 2 nM, \(n = 22\) cells; Fig. 5B; paired t-test, \(P > 0.05\)). These results indicate that mGluRs in LSO neurons are recruited by high-frequency stimulus trains. For individual cells, there was no correlation between the response amplitude and the contribution of mGluRs (Pearson correlation coefficient \(r = 0.18\)), suggesting that stimulation frequency and not response amplitude determines mGluR activation.

Pharmacological analysis of mGluR-mediated responses

Previous electrophysiological studies demonstrated that stimulation of mGluRs can induce long-lasting membrane depolarizations of neonatal LSO neurons (Kotak and Sanes 1995). We thus performed an additional series of pharmacological experiments to investigate the mechanisms underlying mGluR-mediated calcium responses in more detail. Metabotropic glutamate receptors comprise a large family of receptors that are categorized into three groups (group I–III) based on their pharmacology, structure, and activation of intracellular pathways (Pin and Duvoisin 1995). We first determined which of these mGluRs increase \([\text{Ca}^{2+}]\) in LSO neurons using specific antagonists.

GROUP I mGluRs. Activation of group I mGluRs by the specific agonist DHPG (10–20 \(\mu M\)) (Ito et al. 1992) or the group II/III agonist ACPD consistently increased \([\text{Ca}^{2+}]\), (Fig. 6A). These responses had a distinctive profile consisting of a rapid, initial increase in \([\text{Ca}^{2+}]\) (peak response) followed by a long-lasting increase (plateau response). The amplitude of the plateau was ~35% of the amplitude of the peak response (average peak amplitude: 463 ± 26 nM, plateau amplitude: 162 ± 9 nM, \(n = 83\) cells).

GROUP II mGluRs. Activation of group II mGluRs by LCCG-I (5–10 \(\mu M\)) (Keele et al. 1999; Maiase et al. 1999) or by the group II-agonist/group I-antagonist 4C3HPG (200 \(\mu M\)) (Hayashi et al. 1994; Keele et al. 1999), elicited Ca\(^{2+}\)-responses with distinct peak and plateau components (Fig. 6B). Compared with group-I-elicited responses, peak responses of group II responses were significantly smaller (group II: 265 ± 28 nM, \(n = 45\) cells; group I: 463 ± 26 nM, \(n = 83\) cells, \(P < 0.01\); Fig. 4). These responses had a distinctive profile consisting of a rapid, initial increase in \([\text{Ca}^{2+}]\) (peak response) followed by a long-lasting increase (plateau response). The amplitude of the plateau was ~35% of the amplitude of the peak response (average peak amplitude: 463 ± 26 nM, plateau amplitude: 162 ± 9 nM, \(n = 83\) cells).

**FIG. 3.** AMPARs in neonatal LSO neurons of mice are Ca\(^{2+}\) impermeable. A: pressure application of AMPA (1 mM, 50 ms) induces a Ca\(^{2+}\)-response that is reversibly abolished by blocking voltage-gated calcium channels (VGCCs) with Ni\(^{2+}\) (5 mM). B: summary of data from 31 neurons between P0 and P4. On average, 2–5 mM Ni\(^{2+}\) reduces AMPA-elicited Ca\(^{2+}\)-responses to 2.2 ± 2.3% of control. \(*, P < 0.01,\) paired t-test. C: AMPARs are not affected by 2 mM Ni\(^{2+}\). Traces are single responses to AMPA iontophoresis. Bar indicates AMPA application. Graph illustrates average response from 4 cells.

**FIG. 4.** Contribution of N-methyl-D-aspartate receptors (NMDARs) to postsynaptic calcium and membrane potential responses. A: average peak amplitudes elicited by single, low-frequency, and high-frequency stimuli, before (C) and after (D) application of the NMDAR antagonist d-2-amino-5-phosphonopentanoic acid (APV, 50–100 \(\mu M\)). Small responses elicited by single stimuli (SS) are not affected by APV. Larger responses to single stimuli (SI) and responses to low- and high-frequency stimulus trains are reduced ~50%, \(*, P < 0.01,\) paired t-test. B: percent reduction of Ca\(^{2+}\)-responses by APV. *, significant difference from SS (ANOVA followed by Student-Newmann-Keuls, \(P < 0.01\)). C: NMDARs are blocked by Mg\(^{2+}\). Bath application of NMDA (50 \(\mu M, 60 \text{s}\)) in the presence of 1.3 mM Mg\(^{2+}\) fails to elicit a Ca\(^{2+}\)-response. In the absence of Mg\(^{2+}\) (0 mM), NMDA evokes a large Ca\(^{2+}\)-response, which is subsequently blocked by addition of APV (50 \(\mu M\)). D, top: traces of postsynaptic responses to a single stimulus at low intensity (\(S_{\text{low}}\)) and at high intensity (\(S_{\text{high}}\)), and to a train of low-frequency stimulus (LF) before (C) and after (D) APV. Traces are average of 5–10 consecutive responses. Bottom: percent reduction of EPSP amplitude and area by APV (100 \(\mu M\)), *, significant difference from control (paired t-test, \(P < 0.05,\) \(n = 5\) cells). Recordings are from P3–P5 mice.
t-test) while plateau responses had similar amplitudes (group II: 183 ± 17 nM, n = 45 cells; group I: 162 ± 9 nM, n = 83 cells; P > 0.1; t-test).

**GROUP III mGluRs.** The group III mGluR agonist L-AP4 (100–500 μM for 30–90 s) (Tanabe et al. 1993) never elicited Ca\(^{2+}\) responses (n = 42 cells; Fig. 6C). In the same slices, however, LSO neurons responded vigorously to KCl depolarizations (60 mV, 30 s), indicating that the absence of a response to L-AP4 was not due to decreased viability of these cells.

These results indicate that immature LSO neurons express group I and group II mGluRs, whose activation increases [Ca\(^{2+}\)]\(_i\), Group III mGluRs, however, are either not expressed in LSO neurons or are not coupled to intracellular calcium mobilization.

**Mechanisms of mGluR-elicted Ca\(^{2+}\) responses**

The best-characterized pathway by which mGluRs increase [Ca\(^{2+}\)]\(_i\) is through activation of PLC and the production of IP\(_3\), which releases calcium from internal stores (Conn and Pin 1997). To test the contribution of internal calcium stores to mGluR-elicted calcium responses in LSO neurons, calcium was depleted from the endoplasmic reticulum with thapsigargin (10 μM, 1- to 2-h incubation). This treatment completely blocked calcium responses after application of ACDP (100 μM; n = 33 cells, 2 slices; Fig. 6D), indicating that Ca\(^{2+}\) release from intracellular stores is necessary for mGluR-mediated responses.

To test the possible contribution of extracellular calcium, mGluRs were activated in Ca\(^{2+}\)-free ACSF. Under this condition, ACDP still increased [Ca\(^{2+}\)]\(_i\), but the initial peak was greatly reduced, and the plateau phase was completely abolished (peak reduced to 31 ± 8%; area reduced to 18 ± 5%, n = 9 cells, Fig. 6, E and F). Similar results were also observed with the group-I-specific agonist DHPG (peak reduced to 32 ± 2%, area reduced to 12 ± 1%, n = 82 cells) and the group-II-specific agonist 4C3HPG (peak reduced to 16 ± 2%, area reduced to 23 ± 3%, n = 19 cells). These results indicate that Ca\(^{2+}\) released from internal stores contributes only ~30% to the peak response, whereas extracellular Ca\(^{2+}\) contributes ~70% to the peak response and is the sole source of the plateau phase.

Because activation of mGluRs can induce membrane depolarizations (Crepel et al. 1994; Kotak and Sanes 1995), we also examined whether mGluRs activate VGCCs. To this end, we applied ACDP (20 μM) in the presence of specific antagonists to L-type (10 μM nifedipine), N-type (50–1,000 nM ω-conotoxin GVIA), and P/Q-type calcium channels (50 nM ω-agatoxin TK). None of these VGCC blockers significantly decreased the amplitudes of the peak or the plateau (Fig. 7) indicating that mGluR-activated influx of extracellular calcium does not involve VGCCs.

**DISCUSSION**

In the present study, we investigated the mechanisms by which glutamatergic inputs from the cochlear nucleus regulate [Ca\(^{2+}\)]\(_i\) in neonatal LSO neurons. Using Fura-2 calcium imaging in auditory brain stem slices, we found that synaptically elicited calcium responses are mediated by all major classes of ionotropic and metabotropic glutamate receptors and that activation of specific receptor types depends on response amplitude and stimulus frequency (Fig. 8). Pharmacological dissection of mGluR-mediated Ca\(^{2+}\) responses demonstrated that activation of both group I and group II mGluRs results in the release of calcium from internal stores, which is followed by a calcium-dependent activation of membrane calcium channels.

**Technical considerations**

In this study, calcium measurements were made exclusively from cell bodies due to the relatively high background staining.
in bulk-labeled slices and the thin diameter of LSO dendrites, both of which prevented the reliable detection of small fluorescent changes in dendritic segments.

Because in mature LSO neurons most glutamatergic inputs are located on dendrites, caution must be used in applying calcium measurements obtained from the soma to the dendrites. For example, due to the smaller intracellular volume of dendrites, the same amount of calcium in flux will produce a larger increase in \( \text{[Ca}^{2+}]_i \) in dendrites than in cell bodies. As seen in other neuronal types (Berridge 1998; Magee et al. 1998), VGCCs and intracellular calcium mobilization mechanism might be unequally distributed along single LSO neurons, resulting in locally distinct calcium responses. Thus while our approach allows us to draw basic conclusions about specific GluR activation, detailed dendritic calcium imaging data are necessary to understand the properties of synapse-specific calcium responses as well as synaptic interaction and integration from the standpoint of local calcium dynamics.

**AMPA/kainate receptor-mediated \( \text{Ca}^{2+} \) responses**

Single stimuli to the VAS elicited synaptic calcium responses that were primarily mediated by AMPARs. This agrees with previous electrophysiological studies in developing and adult animals that demonstrated that ipsilateral excitation to the LSO is primarily conveyed by non-NMDARs (Caspary and Faingold 1989; Kotak and Sanes 1996; Wu and Kelly 1992). Our results further demonstrate that these AMPAR-mediated \( \text{Ca}^{2+} \) responses result from activation of VGCCs rather than from \( \text{Ca}^{2+} \) influx though AMPARs. Thus AMPARs...
in neonatal mouse LSO neurons are Ca\textsuperscript{2+} impermeable and most likely contain GluR2 subunits (Hollmann et al. 1991; Verdoorn et al. 1991). This is consistent with previous studies that demonstrated the expression of GluR2 subunits during circuit maturation in the LSO of neonatal rats (Caicedo and Eybalin 1999; Caicedo et al. 1998) and the nucleus magnocellularis of embryonic chickens (Lawrence and Trussell 2000; Sugden et al. 2002). The calcium impermeability of GluR2-containing AMPARs reduces calcium entry during low synaptic activity. This could restrict activation of calcium-dependent intracellular pathways to periods of high synaptic activity, when, in addition to VGCCs, NMDARs, and mGluRs are also activated. Because GluR2 subunits play an important role in regulating activity-dependent synaptic plasticity (Jia et al. 1996) and internalization and stabilization of synaptic AMPARs (Borgdorff and Choquet 2002; Kim et al. 2001; Man et al. 2000), expression of GluR2 early in auditory brain stem development could play a role in synaptic maturation and organization.

**Calcium responses mediated by kainate receptors**

Kainate receptors are expressed in several nuclei of the mature and developing auditory brain stem (Lachica et al. 1998; Lohrke and Friauf 2002; Petralia et al. 1994, 1996; reviewed in Petralia et al. 2000), but their role in auditory processing or neuronal development is poorly understood. Preliminary results indicated that kainate receptors can elicit low-amplitude inward currents in developing LSO neurons (Vitten et al. 1999). Our results confirm and expand these previous studies by demonstrating that kainate receptors in the LSO are calcium impermeable and are thus most likely composed of the Q/R-edited forms of GluR5 and GluR6 subunits (Burnashev et al. 1995; Lee et al. 2001). It remains to be shown to what extent these kainate receptors participate in glutamatergic synaptic transmission (Frerking et al. 1998; Kidd and Isaac 1999) and, if they do, whether such participation is frequency-dependent or perhaps restricted to specific glutamatergic afferent pathways (DeVries and Schwartz 1999; Frerking and Nicoll 2000).

**NMDAR-mediated Ca\textsuperscript{2+} responses**

Previous studies indicated that LSO neurons express mRNA and protein of NMDARs (Caicedo and Eybalin 1999; Sato et al. 1999). Our results demonstrate that these NMDARs can be activated by synaptic inputs from cochlear nucleus fibers and that they contribute significantly to postsynaptic Ca\textsuperscript{2+} responses. Consistent with the expression of NR2A subunits (Caicedo and Eybalin 1999; Sato et al. 1999), we found that NMDARs in developing LSO neurons are magnesium sensitive, providing an explanation as to why NMDARs contributed to calcium responses only in larger responses, which most likely were accompanied by strong membrane depolarizations.

Once activated, NMDARs accounted for ~50% of the calcium influx and ~60% of the EPSP area. This contribution is higher than that reported in other studies, which indicated that ipsilateral excitation to LSO neurons is mediated primarily or exclusively by non-NMDARs (Caspar and Faingold 1989; Kandler and Friauf 1995; Kotak and Sanes 1996; Wu and Kelly 1992). However, because our animals were ~1 week younger than those used in previous studies, this difference most likely indicates that, as in many other brain regions, NMDAR-mediated responses are downregulated during maturation of the LSO. Because NMDAR-mediated calcium influx plays a central role in neuronal development, plasticity, and cell death in several systems (West et al. 2002; Wong and Ghosh 2002), the large contribution of NMDARs to calcium responses here suggests that NMDARs play an important part in the maturation of LSO neurons as well.

**Metabotropic glutamate receptor-mediated Ca\textsuperscript{2+} responses**

Based on subunit composition, pharmacology, and coupling to signal transduction pathways, mGluRs are classified into three categories, group I, II, and III (reviewed in Conn and Pin 1997). Group I mGluRs (mGluR1 and mGluR5) are coupled to G\textsubscript{i/o} type G proteins, which activate the PLC-IP\textsubscript{3} pathway and mobilize intracellular calcium. Group II mGluRs (mGluR2 and mGluR3) and group III mGluRs (mGluR4 and mGluR6–8) are associated with G\textsubscript{q/11}type proteins and generally are negatively coupled to adenylyl cyclase activity.
In the auditory brain stem, group I and II mGluRs are expressed in several auditory nuclei (Bilak and Moster 1998; Elezgarai et al. 1999, 2001; Jaarasma et al. 1998; Molitor and Manis 1997; Schwartz and Eager 1999; Takahashi et al. 1996; Zirpel et al. 2000; reviewed in Petralia et al. 2000). In the LSO, electrophysiological recordings have indicated expression of group II and/or III mGluRs on presynaptic glutamatergic terminals (Wu and Fu 1998) and group I mGluRs on postsynaptic LSO neurons (Kotak and Sanes 1995). Our results extend these findings by demonstrating that synaptic activation of postsynaptic group I and II mGluRs contributes to calcium responses in immature LSO neurons. These calcium responses were only observed with high-frequency stimulation, which is consistent with a perisynaptic location (Baude et al. 1993; Petralia et al. 1997; Takumi et al. 1999) and activation by glutamate spillover (Huang 1998; Isaacson 2000).

Stimulation of group I, as well as group II, mGluRs increased \([\text{Ca}^{2+}]_{i}\). The responses elicited by group II mGluR agonists were unexpected because group II mGluRs usually modulate adenylyl cyclase activity rather than increase IP3. It is unlikely that these responses were due to unspecific activation of group I mGluRs because [Ca\(^{2+}\)]\(_{i}\) also increased with 4C3HPG, which is not only an agonist for group II mGluRs but is also a potent antagonist for group I mGluRs (Hayashi et al. 1994). Although the exact intracellular pathways that underlie group II-elicited calcium responses remain unclear, the similarity of group II to group I responses suggests the involvement of IP3-dependent calcium mobilization. Very recently, group II mGluR-mediated activation of the PLC-IP3 pathway has been demonstrated in prefrontal cortex neurons (Otani et al. 2002), where group II mGluRs are critically involved in mediating activity-dependent synaptic plasticity.

Calcium responses elicited by mGluRs were characterized by a rapid, initial increase in [Ca\(^{2+}\)]\(_{i}\), that was followed by a lower-amplitude, prolonged calcium plateau. These responses were completely abolished if calcium stores were depleted by thapsigargin, indicating that calcium mobilization from intracellular stores is necessary for these responses. Recordings in zero extracellular calcium, however, indicated that mobilization of intracellular calcium contributed only a minor fraction (\(\sim 30\%\)) to the initial peak response. This is consistent with the idea that mobilization of intracellular calcium activates membrane calcium channels and causes an influx of extracellular calcium. VGCCs do not contribute to this calcium influx because responses were unaffected by specific antagonists of VGCCs. Potential candidates for this channel are members of the transient receptor potential channels, some of which are activated by mobilization of intracellular calcium (Clapham et al. 2001). Further experiments to characterize this calcium channel in LSO neurons are important in light of its large contribution to mGluR-mediated calcium responses during the period when the survival of LSO neurons depends on calcium (Lohmann et al. 1998) and synaptic maturation depends on glutamatergic inputs (Kotak and Sanes 1997).

**Stimulus-dependent recruitment of different classes of glutamate receptors**

Prior to hearing onset, spontaneous activity is present in the avian and mammalian central auditory pathway (Glowatzki and Fuchs 2000; Guummer and Mark 1994; Jones and Perez 2001; Kotak and Sanes 1995; Kros et al. 1998; Lippe 1994; Romand and Ehret 1990). Accumulating evidence indicates that this spontaneous activity plays a critical role in neuronal survival (Lachica et al. 1995; Zirpel and Rubel 1996) and in the normal physiological and anatomical development of central auditory connections (Gabriele et al. 2000; Kitzes et al. 1995; Russell and Moore 1995; reviewed in Sanes and Friauf 2000). Spontaneous activity in the immature auditory system is rhythmic and oscillatory, consisting of bursts of activity that are separated by periods of low-frequency events (Jones and Perez 2001; Kotak and Sanes 1995; Lippe 1994), a pattern that is reminiscent of the activity patterns in other developing systems (reviewed in Zhang et al. 2000). With the exception of the visual system, in which burst-like synthetic activity in the form of retinal waves is thought to play an instructive role in the formation of eye-specific layers in the lateralgeniculate nucleus (Stellwagen and Shatz 2002), the exact function of the burst-like pattern of spontaneous activity has remained largely elusive. Our results demonstrate that different temporal patterns of synaptic activity in the LSO activate distinct calcium-entry pathways (Fig. 8) and, most likely, distinct cellular pathways and gene expression (West et al. 2002). Although the exact nature of these intracellular events remains to be shown, our results are consistent with the idea that different patterns ofafferent activity serve distinct developmental roles, highlighting the importance of the temporal pattern of spontaneous activity in the immature auditory system.

We thank N. Allman for excellent technical support. We are grateful to E. Aizenman for comments on an earlier version of the manuscript and to N. K. Baba for valuable advice. We also appreciate the constructive suggestions made by anonymous reviewers.

**DISCLOSURES**

This work was supported by the National Institute on Deafness and Other Communication Disorders (DC-04199), the Alfred P. Sloan foundation, a Presidential Early Career Award (K. Kandler), a predoctoral fellowship from the Center for Neural Basis of Cognition (F. A. Ene), and T32 NS07391 (D. C. Gillespie).

**REFERENCES**


Caicedo A, Kungel M, Pujol R, and Friauf E. Glutamate-induced Co2+ uptake in rat auditory brain stem neurons reveals developmental changes in...


