AMPA Receptor-Mediated, Calcium-Dependent CREB Phosphorylation in a Subpopulation of Auditory Neurons Surviving Activity Deprivation

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Although dependence on afferent synaptic activity has been shown for central neurons in every sensory system, the mechanisms of afferent maintenance of target sensory neurons are not understood. Neurons in the cochlear nucleus (CN) require afferent activity for maintenance and survival. One of the earliest changes seen after activity deprivation is an increase in intracellular calcium that leads to the death of 30% of the neuronal population. Sixty minutes after deafferentation, the surviving neurons show increased phosphorylation of the transcription factor calcium/cAMP response element-binding protein (CREB). CREB phosphorylation in activity-deprived CN neurons is dependent on increased intracellular calcium resulting from influx through

AMPA receptors and is mediated by calcium/calmodulin-dependent kinases and protein kinase A. We conclude that in CN neurons, the deafferentation-induced increase in calcium activates at least two kinase pathways that phosphorylate CREB in surviving neurons. We hypothesize that this phosphorylation results in the transcription of genes containing the calcium/cAMP response element within their promoter regions, and these genes code for proteins that allow the neurons to compensate for their hypercalcemic, activity-deprived state.

Key words: chick nucleus magnocellularis; activity-dependent; deafferentation; calcium homeostasis; propidium iodide; neuronal survival; cell death; mouse AVCN

Afferent regulation of target tissues is a common feature of the developing nervous system. Experimental manipulations of afferent activity have been shown to alter structure, function, and neuronal survival of developing sensory systems (Hubel and Wiesel, 1965; Dubin et al., 1986; Stryker and Harris, 1986; Brunjes, 1994). In the auditory system, elimination of afferent input alters the metabolism, morphology, and survival of target neurons (for review, see Rubel, 1978; Moore, 1992; Friauf and Lohmann, 1999; Parks, 1999). Despite the pervasiveness of these cellular interactions, little is known about the intercellular and intracellular signals responsible for these effects.

Neurons in the cochlear nucleus [nucleus magnocellularis (NM)] of embryonic and early posthatch chicks require afferent input from the eighth cranial nerve for maintenance and survival. Elimination of this input results in the death of 20–40% of the NM neurons, whereas the surviving neurons display profound changes in morphology, metabolism, and physiology (for review, see Rubel et al., 1990). Neurons in the neonatal mouse anteroventral cochlear nucleus (AVCN, the mammalian homolog of the avian NM) also show dependence on afferent activity from the eighth nerve (Trune, 1982) (Mostafapour et al., 2000). One of the earliest changes seen in NM after deafferentation is an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) (Zirpel et al., 1995b). This increase in $[Ca^{2+}]_i$ can be prevented by activation of metabotropic glutamate receptors (mGluRs) (Zirpel and Rubel, 1996) linked to protein kinase A (PKA) and protein kinase C (PKC) signal transduction pathways (Lachica et al., 1995; Zirpel et al., 1995a, 1998). Blockade of mGluRs during eighth nerve activity results in a rapid and large increase in $[Ca^{2+}]_i$ (Zirpel and Rubel, 1996) caused, in part, by continued activation of highly Ca^{2+} -permeable AMPA receptors (Otis et al., 1995; Ravindranathan et

al., 2000). Although this deafferentation-induced increase in $[Ca^{2+}]_i$ and the mechanisms preventing it are well characterized, the source of the Ca^{2+} and its effects on NM neurons are unknown.

Activation of the transcription factor calcium/cAMP response element-binding protein (CREB) is dependent on phosphorylation by PKA, Ca^{2+} /calmodulin-dependent kinases (CaMKs), or ribosomal S6 kinases (RSKs) (Walton and Dragunow, 2000). One of the most well characterized signals for CREB phosphorylation and activation is an increase in $[Ca^{2+}]_i$ (Sheng et al., 1990; Deisseroth et al., 1996; Hardingham et al., 1997; S. C. Hu et al., 1999; Rajadhyaksha et al., 1999). CREB phosphorylation and activation have been shown to be important for the survival of neurons in a number of different conditions, including hypercalcemia (Walton et al., 1999; Walton and Dragunow, 2000). Thus CREB phosphorylation is a likely candidate for one of the mechanisms underlying the changes seen in cochlear nucleus neurons after deafferentation. We hypothesize that the $[Ca^{2+}]_i$ increase in NM neurons after deafferentation activates kinase pathways that phosphorylate CREB, resulting in the transcription of genes that enable the neurons to survive the deafferentation-induced hypercalcemic state.

Using an antibody that recognizes specifically the phosphorylated form of CREB (Ginty et al., 1993), we examined CREB phosphorylation in cochlear nucleus neurons receiving or after termination of synaptic activation via eighth nerve activity. We find that after cessation of afferent activity, cochlear nucleus neurons show a transient phosphorylation (peaking at 50 min) of CREB in a percentage of neurons equal to the percentage of surviving neurons. Using dynamic calcium imaging, we show that treatments that block AMPA receptor activation, or prevent increases in $[Ca^{2+}]_i$, also block the phosphorylation of CREB. CREB phosphorylation is attenuated, but not completely blocked, by inhibition of staurosporine-sensitive kinases (e.g., PKA, PKC, and RSKs), CaMKs (with KN-62), or PKA [with Rp-adenosine 3',5'-cyclic monophosphate triethylamine (Rp-cAMPS)], but not by inhibition of mitogen-activated protein kinase kinase (MAPKK; with PD98059). Inhibition of both staurosporine-sensitive and CaM kinases eliminates CREB phosphorylation. Constitutive activation of PKA (with Sp-cAMPS) increases the number of neurons showing CREB phosphorylation. The number of neurons

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showing CREB phosphorylation is negatively correlated with the number of dead or dying neurons. These results suggest that after deafferentation, AMPA receptors are activated by ambient glutamate, causing an increase in $[Ca^{2+}]_i$ that activates more than one calcium-dependent kinase pathway. These kinases then phosphorylate CREB in a subpopulation of neurons, consistent with the hypothesis that CREB-mediated transcription allows these neurons to survive in their activity-deprived, hypercalcemic environment.

MATERIALS AND METHODS

Cochlea removals

White Leghorn chicks, 4–7 d posthatch (P4–P7), were anesthetized with halothane. Right cochleae were removed as described by Born and Rubel (1985). The auricle was removed, and the tympanic membrane was perforated with fine forceps. The columella was extracted, and forceps were inserted through the oval window. The basilar papilla was removed and visually examined to ensure complete deafferentation. If the basilar papilla was not 75% intact, or if the lagena was not visible, the surgery was deemed a failure, and the animal was not used for experimentation. After a successful cochlea ablation, the middle ear was packed with Gelfoam. The chick was allowed to survive the appropriate amount of time, anesthetized with ketamine and pentobarbital, and perfused transcardially with 0.9% saline containing 1000 IU heparin and 2 mg/ml $NaNO_2$ followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brainstem was dissected free and placed in 4% paraformaldehyde for 3 hr and then stored in 30% sucrose in 0.1 M phosphate buffer at 4°C overnight. Tissue was then embedded and frozen in optimal cutting temperature (OCT) compound (VWR Scientific, Salt Lake City, UT). By the use of a cryostat, 18- μ m-thick coronal sections were cut through the brainstem at the level of the auditory nuclei and placed on microscope slides. Care was taken to acquire sections that were symmetric, thus providing a within-animal control (deafferented vs intact cochlear nuclei).

Mouse pups aged 5 d (P5) were anesthetized with halothane. Right cochleae were removed as described by Mostafapour et al. (2000). The oval window was exposed by dissecting away the pinna and aspirating the soft tissue filling the middle ear at this age. The tip of a flame-polished Pasteur pipette was then inserted through the oval window, and the cochlea was aspirated with gentle suction. The middle ear was packed with Gelfoam, and the incision was closed with cyanoacrylate adhesive. The pup was allowed to survive for 60 min, anesthetized with cooling, and decapitated. The entire cranium was immersed in 4% paraformaldehyde overnight. The brain was dissected free from the cranium, placed in 4% paraformaldehyde for 1 hr, rinsed with PBS (137 mM NaCl, 2.7 mM KCl, 5 mM Na_2HPO_4 , and 1.7 mM KH_2PO_4 , pH 7.4), rinsed with distilled water, and placed in 70% ethanol overnight. Brains were then dehydrated and embedded in paraffin. Ten micrometer sections containing the cochlear nuclei bilaterally were obtained using a microtome and mounted on microscope slides. Sections on slides were deparaffinized and rehydrated.

Phospho-CREB immunohistochemistry

Chick tissue. Tissue sections were blocked for 20 min with 1% normal goat serum and 0.4% saponin in PBS. Sections were then incubated overnight at 4°C with anti-phospho-CREB (rabbit polyclonal; 1:1000; Upstate Biotechnology, Lake Placid, NY) or anti-CREB (rabbit polyclonal; 1:1000; Upstate Biotechnology) diluted in blocking buffer, pH 7.3–7.4. Primary antibody incubation was continued for an additional 1–3 hr at room temperature, followed by three 10 min washes in PBS. Alexa 594 secondary antibody (1:800 in 0.4% normal goat serum in PBS; Molecular Probes, Eugene, OR) was applied for 90 min at room temperature, followed by three 10 min washes with PBS. Slides were coverslipped with Fluorsave (Calbiochem, La Jolla, CA) and stored in the dark at 4°C until analyzed. Controls for each run were processed identically except instead of a primary antibody incubation, they were incubated in blocking solution.

Mouse tissue. Antigen retrieval was performed on the sections of mouse tissue. Slides were placed in a Coplin jar containing 10 mM citric acid solution, pH 6, and microwaved on high power until the solution boiled. Evaporated fluid was replaced with distilled H_2O , and the boiling procedure was repeated two more times. After the final boiling, slides were allowed to cool and rinsed in distilled H_2O . Immunohistochemistry was performed identically as described above for the chick tissue. No primary controls were included in each run.

Image acquisition. By the use of confocal laser-scanning microscopy (Bio-Rad, Hercules, CA), labeled sections were “optically sectioned.” The optical section corresponding to the 50th percentile of the entire section thickness was chosen for analysis. Because quantitative fluorescence analysis was not performed, acquisition was optimized for image quality. Images were acquired via a Fluor 40 $\times$ oil immersion objective (1.3 numerical aperture; Nikon). Image resolution was set at 1024 $\times$ 1024 pixels, and all acquisitions were Kalman filtered (factor of four). Images were converted to TIFF format using NIH Image (public domain software developed at the United States National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image>).

Data analysis. Cells were counted as either positive or negative on the basis of a clearly identifiable nucleus, labeled or not labeled, respectively.

Cells with ambiguous labeling or an unidentifiable nucleus were excluded from analysis. For each image, all positive- and negative-labeled cells were counted, and the positive-labeled cells were expressed as the percentage of total cells. Neurons and glia were counted separately. Multiple sections were counted for each animal, averaged, and treated as $n = 1$. Images were prepared for presentation using Photoshop (Adobe Systems, San Jose, CA). ANOVA and two-tailed t tests were performed using Statview (SAS Institute, Cary, NC).

In vitro experiments

Slice preparation. Live *in vitro* tissue slices (300 μ m) were acquired as described previously (Zirpel et al., 1995a,b, 1998; Zirpel and Rubel, 1996) and maintained in oxygenated artificial CSF (ACSF; 130 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 , and 10 mM D-glucose, pH 7.4). For pharmacology experiments, a drug was added to ACSF and was present throughout the dissection and slicing procedure. At the end of each experiment, slices were immersed in 4% paraformaldehyde for 1 hr, cryoprotected, rinsed, embedded in OCT, and sectioned for immunohistochemistry as described above.

Electrophysiology. Methods for stimulating and recording from *in vitro* chick brainstem slices have been described previously (Jackson et al., 1985; Hyson and Rubel, 1989; Zirpel and Rubel, 1996; Zirpel et al., 1998). A concentric bipolar-stimulating electrode (FHC, Bowdoinham, ME) was placed on the eighth nerve dorsal and lateral to NM. Stimulation pulses (50–100 μ sec duration; 60–80 V) were delivered at a rate of 0.1–1.0 Hz. Responses were recorded with an ACSF-filled microelectrode (~ 1 M Ω) attached to an intracellular amplifier and monitored on an oscilloscope.

Calcium imaging. Tissue slices were incubated in oxygenated ACSF containing 8 μ M fura-2 AM, 1.7% anhydrous dimethylsulfoxide, and 0.03% pluronic for 30 min at room temperature. Slices were placed in the imaging chamber and bathed in normal, oxygenated ACSF for ~ 5 min before data acquisition. The ratiometric fluorescence-imaging techniques used in this study were similar to those described previously (Zirpel et al., 1995a,b, 1998, 2000; Zirpel and Rubel, 1996). Paired 350/380 excitation images were acquired approximately every 3–5 sec or at 1 min intervals, and ratios were determined on a pixel-by-pixel basis. Intracellular calcium concentration was estimated according to the method of Grynkiewicz et al. (1985) using NIH Image. Five-point external calibrations were performed on the imaging system routinely, and the K_d for fura was calculated with each calibration. The range of K_d values was 75–172 nM. Up to 10 neurons were analyzed for any given experiment. However, each slice was considered $n = 1$, and the data from all neurons in an experiment were averaged. Cells were not included in the analysis if the baseline $[Ca^{2+}]_i$ was >250 nM, because this concentration is indicative of a dying cell (Zirpel et al., 1998). Cells were neither added to nor subtracted from the analysis of an experiment except when a complete loss of fluorescence was observed, indicating cell death (Johnson et al., 1994).

Values of $[Ca^{2+}]_i$ were plotted as a function of time using EXCEL (Microsoft, Redmond, WA) and Cricket Graph (Cricket Software, Malvern, PA). Data are presented as the means ± 1 SEM unless otherwise indicated.

Propidium iodide labeling. For selected experiments, 1.5 μ M propidium iodide (PI) was added to the superfusate at the 30 min time point. Thirty minutes later, an image was captured, *in situ*, through a 600 nm long-pass filter using 510 nm excitation. PI-labeled neurons were counted and expressed as a percentage of the sum of PI-labeled and fura-2-labeled neurons.

Western immunoblots

Westerns were performed as described previously (Zirpel et al., 2000). Brainstem areas containing the nucleus magnocellularis from P2 to P7 chicks were dissected out in ACSF or in ACSF containing a selected drug at room temperature for a 60 min total deafferentation time. Nuclei magnocellularis were then dissected from the brainstem and homogenized in lysis buffer [50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, and protease inhibitors (7 μ g/ml chymostatin, 7 μ g/ml leupeptin, 7 μ g/ml antipain, 7 μ g/ml pepstatin, 5 μ g/ml aprotinin, and 350 μ g/ml PMSF)]. One brainstem was dissected in ACSF and homogenized immediately in lysis buffer. The samples were sonicated (3–5 sec) and centrifuged at 14000 rpm for 40 min at 4°C. The protein concentration of the samples was determined using a BCA assay (Bio-Rad). Twenty-five and fifty micrograms of protein were resolved via 10% SDS-PAGE (100 V for 2.5 hr at room temperature) for blots to be probed with anti-CREB and anti-phospho-CREB, respectively. Gels were transferred onto Immobilon-P membranes (Millipore, Bedford, MA) at 30 V overnight at 4°C. Blots were probed with anti-CREB or anti-phospho-CREB polyclonal antibodies diluted 1:1000. Blots were rinsed in buffer B (0.5 M NaCl, 0.02 M Tris base, and 0.1% Tween 20, pH 7.5) and incubated with donkey anti-rabbit Ig, horseradish peroxidase-linked whole antibody (Amersham), diluted 1:5000 at room temperature for 1.5 hr. Blots were rinsed in buffer B followed by buffer A (0.5 M NaCl and 0.02 M Tris base, pH 7.5). Bound secondary antibodies were developed by enhanced chemiluminescence using ECL Western Blotting Detection Reagents (Pharmacia, Piscataway, NJ). Blots were scanned into a computer, and the relative levels of CREB or phosphorylated-CREB (p-CREB) were determined by densitometry using NIH Image. To insure consistent measurements, a box of defined size was

Figure 1. CREB phosphorylation after deafferentation in a subset of neurons. *A*, Chick cochlear nucleus immunostained for p-CREB 60 min after an *in vivo*, unilateral cochlea removal. Note that both neuronal and glial (arrowheads) nuclei show staining. *B*, Chick cochlear nucleus contralateral to cochlea removal (i.e., intact) immunostained for p-CREB. *C*, Percentage of p-CREB+ cells in chick cochlear nucleus neurons and glia as a function of time after an *in vivo* cochlea ablation and *in vitro* slice preparations. Error bars represent the SEM. For *in vivo* cochlea ablation, n = a minimum of 3 animals per data point. For *in vitro* data, n = a minimum of 5 slices per data point. *D*, Mouse (P5) AVCN immunostained for p-CREB 60 min after unilateral cochlea ablation. *a*, AVCN ipsilateral to cochlea ablation. *b*, AVCN contralateral to cochlea ablation (i.e., intact). Scale bar: *A*, *B*, *D*, 30 μ m.

used to measure the optical density of each band in a given lane at the same position in the y dimension on each blot.

RESULTS

Activity deprivation results in CREB phosphorylation

To determine the state of CREB phosphorylation in neurons receiving, and neurons deprived of, normal synaptic activity, we examined p-CREB labeling in the cochlear nuclei of chicks that had undergone unilateral cochlea ablation. Removal of the cochlea eliminates all excitatory input to the ipsilateral cochlear nucleus while leaving intact the normal afferent activity in the contralateral cochlear nucleus. Figure 1*A* shows an example of p-CREB labeling in NM 60 min after cochlea removal. Glial nuclei (arrowheads) are easily distinguished from neuronal nuclei, and it is readily apparent that the labeling is localized to the nuclei of both glia and neurons. Cells were counted as either labeled or not; no attempt was made to quantify varying degrees of p-CREB labeling. p-CREB labeling was never seen in glia or neurons contralateral to deafferentation (i.e., on the side receiving normal eighth nerve input; Fig. 1*B*). p-CREB labeling was first seen 30 min after cochlea ablation when $72 \pm 12\%$ of the glia labeled positive for p-CREB (Fig. 1*C*, $\circ$). The percentage of p-CREB-labeled (p-CREB+) glia remained elevated at 50 min ($70 \pm 11\%$), fell to $20 \pm 8\%$ at 70 min, and was absent at 90 min. Neuronal p-CREB labeling was first observed 50 min

after cochlea ablation when $74 \pm 14\%$ of the neurons were p-CREB+ (Fig. 1*C*, $\blacksquare$). The percentage of p-CREB+ neurons remained high at 60 min ($70 \pm 10\%$), fell to $43 \pm 8\%$ at 70 min, and was absent by 90 min. To facilitate pharmacological manipulation, activity deprivation-induced CREB phosphorylation was also demonstrated using *in vitro* slice preparations. The eighth nerve was unilaterally stimulated (field potentials were recorded to ensure synaptic activation; data not shown) for defined periods of time after which the slices were processed for anti-p-CREB immunohistochemistry. As with the *in vivo* cochlea removal, this allows for a within-slice control in which one NM receives normal input from the ipsilateral eighth nerve and the contralateral NM is activity deprived. The timing and pattern of CREB phosphorylation in neurons from *in vitro* slices mirrored that seen with *in vivo* cochlea removal; p-CREB+ neurons were first seen at 50 min and were absent at 90 min (Fig. 1*C*, $\triangle$). The percentage of p-CREB+ neurons observed in the *in vitro* slice preparations was slightly lower than that seen with cochlea ablation ($53 \pm 11\%$ vs $74 \pm 14\%$ at 50 min and $52 \pm 12\%$ vs $70 \pm 10\%$ at 60 min) perhaps because, in part, of damage caused by the dissection and slicing procedure. p-CREB labeling was never observed in glia or neurons in the NM ipsilateral to nerve stimulation. The remainder of the experiments in this report were conducted using *in vitro* slice preparations, and thus, control or no stimulation refers to the 60 min *in vitro* percentage of

Figure 2. Deafferentation-induced CREB phosphorylation is Ca^{2+} dependent. *A*, Effects of ionomycin on $[\text{Ca}^{2+}]_i$ and CREB phosphorylation. *a*, Fura-2-measured mean $[\text{Ca}^{2+}]_i$ increase in chick NM neurons in the absence of activity in normal ACSF (■; 38 neurons; $n = 4$ slices) and in ACSF containing $5 \mu\text{M}$ ionomycin (○; 47 neurons; $n = 5$ slices). Error bars represent the SEM and are $<20 \text{ nM}$ where not visible. *b*, Percentage of p-CREB+ NM neurons in the presence (◆) and absence (△) of $5 \mu\text{M}$ ionomycin as a function of time in the absence of activity *in vitro*. Error bars represent the SEM ($n = 4$ for the 30 min ionomycin data point, 5 for the 60 min data point, and 3 for all others). The *No Stimulation* data are the same as those presented in Figure 1C. *B*, Effects of $[\text{Ca}^{2+}]_i$ on CREB phosphorylation. *a*, Fura-2-measured mean $[\text{Ca}^{2+}]_i$ of NM neurons in the absence of activity in normal ACSF (■; 38 neurons; $n = 4$ slices; same data shown in *Aa*), in ACSF containing 1 mM ACPD (△; 39 neurons; $n = 4$ slices), in ACSF containing $1 \mu\text{M}$ nifedipine (○; 37 neurons; $n = 4$ slices), and in normal ACSF after incubation in $5 \mu\text{M}$ BAPTA AM ($K_d = 160 \text{ nM}$; ◇; 29 neurons; $n = 3$ slices). Error bars represent the SEM and are $<20 \text{ nM}$ where not visible. *b*, Percentage of p-CREB+ NM neurons after 60 min in the four conditions shown in *Ba*. Error bars represent the SEM. Control, $n = 19$; BAPTA, $n = 3$; ACPD, $n = 3$; and nifedipine, $n = 3$. *, $p < 0.05$.

p-CREB+ neurons shown in Figure 1C. All chick cochlear nucleus neurons show diffuse nuclear and cytoplasmic labeling for CREB (data not shown).

To determine whether CREB phosphorylation occurred in mice as well as chicks after deafferentation, four mouse pups underwent unilateral cochlea ablation. Sixty minutes after cochlea ablation, a subpopulation of AVCN neurons showed p-CREB labeling (Fig. 1*Da*). No p-CREB+ cells were observed in the AVCN contralateral to cochlea removal (Fig. 1*Db*). The percentage of p-CREB+ AVCN neurons ipsilateral to cochlea ablation ($53 \pm 13\%$) is similar to the mean number of AVCN neurons reported by Trune (1982) (66%) and by Mostafapour et al. (2000) (40%) to survive cochlea ablation. Similarly, the percentage of chick NM neurons that show CREB phosphorylation 60 min after cochlea ablation ($70 \pm 10\%$) is equivalent to the percentage of neurons that survive deafferentation (70%) (Born and Rubel, 1985). All mouse cochlear nucleus neurons show diffuse nuclear and cytoplasmic labeling for CREB (data not shown).

Activity deprivation-induced CREB phosphorylation is calcium dependent

After cessation of eighth nerve input, NM neurons show a gradual $[\text{Ca}^{2+}]_i$ increase from a resting level of $100 \pm 13 \text{ nM}$ to $242 \pm 18 \text{ nM}$ at 90 min (Fig. 2*Aa*, ■) (see also Zirpel and Rubel, 1996). Adding $5 \mu\text{M}$ ionomycin, a Ca^{2+} ionophore, to the superfusate of *in vitro* slices containing NM causes the $[\text{Ca}^{2+}]_i$ increase to occur sooner (Fig. 2*Aa*, ○) but does not alter the level reached ($267 \pm 25 \text{ nM}$) at 90 min. In parallel with this more rapid ionomycin-induced $[\text{Ca}^{2+}]_i$ increase is a temporal phase advance of peak p-CREB labeling in NM neurons (Fig. 2*Ab*). Thirty minutes after deafferentation $55 \pm 12\%$ of the ionomycin-treated neurons are p-CREB+ (compared with 0% of the control neurons in normal ACSF), and at 60 min this percentage has dropped to $11 \pm 12\%$ versus $52 \pm 12\%$ of the control neurons. These results suggest that CREB phosphorylation is mediated by increased $[\text{Ca}^{2+}]_i$.

If increased calcium is indeed the signal for CREB phosphorylation, preventing the $[\text{Ca}^{2+}]_i$ increase should prevent CREB phosphorylation. Adding $5 \mu\text{M}$ BAPTA AM, a Ca^{2+} chelator, to the fura-2 loading solution not only prevents the deafferentation-induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 2*Ba*, ◇) but also causes a lower resting $[\text{Ca}^{2+}]_i$ in NM neurons ($63 \pm 25\%$ vs $100 \pm 13\%$). Sixty minutes after deafferentation, only $4.8 \pm 4.8\%$ of the BAPTA-treated NM neurons show p-CREB labeling (Fig. 2*Bb*). A one-factor ANOVA revealed a very highly significant effect of experimental treatment on the percentage of p-CREB+ neurons [$F_{(3,65)} = 12.64$; $p < 0.0001$]. *Post hoc* comparisons with Scheffé's method showed several significant differences between groups, including a lower percentage of p-CREB+ neurons in the BAPTA treatment group than in controls ($p = 0.03$).

Reducing $[\text{Ca}^{2+}]_i$ by a second, independent mechanism shows a similar effect on CREB phosphorylation. 1-Amino-1,3-cyclopentanedicarboxylic acid (ACPD) prevents the increase in $[\text{Ca}^{2+}]_i$ in NM neurons after deafferentation by activating metabotropic glutamate receptors, which play a pivotal role in NM neuron $[\text{Ca}^{2+}]_i$ homeostasis (Zirpel and Rubel, 1996; Zirpel et al., 1998). ACPD (1 mM) added to the superfusate of slices prevented the $[\text{Ca}^{2+}]_i$ increase (Fig. 2*Ba*, △) and reduced the percentage of p-CREB+ neurons at 60 min to $6.2 \pm 2.9\%$ (Fig. 2*Bb*; $p = 0.04$).

The L-type voltage-gated calcium channel antagonist nifedipine ($1 \mu\text{M}$) had no effect on the increase in $[\text{Ca}^{2+}]_i$ in the absence of activity (Fig. 2*Ba*, ○) or on the percentage of p-CREB+ NM neurons ($44 \pm 4\%$; $p = 0.043$) 60 min after activity deprivation (Fig. 2*Bb*).

Altering the temporal characteristics of the $[\text{Ca}^{2+}]_i$ increase in NM neurons after deafferentation also alters the temporal characteristics of p-CREB labeling. Furthermore, preventing the $[\text{Ca}^{2+}]_i$ increase via two different mechanisms virtually eliminates p-CREB labeling. Therefore, we conclude that the phosphorylation of CREB in NM neurons after deafferentation is a calcium-dependent process.

Figure 3. The $[Ca^{2+}]_i$ increase and CREB phosphorylation depend on AMPA receptor activation. *A*, Fura-2-measured mean $[Ca^{2+}]_i$ of NM neurons in the absence of activity in normal ACSF (■; 38 neurons; $n = 4$ slices; same data shown in Fig. 2*Aa*) and in ACSF containing 30 μ M CNQX (△; 37 neurons; $n = 5$ slices), 50 μ M GYKI 52466 (GYKI; ●; 33 neurons; $n = 4$ slices), or 100 μ M CTZ (◇; 37 neurons; $n = 5$ slices) is shown. Error bars represent the SEM. *B*, AMPA receptor blockade inhibits CREB phosphorylation. The percentage of p-CREB+ NM neurons 60 min after deafferentation in the conditions described in *A* and after incubation in ACSF containing 200 μ M APV is shown. Error bars represent the SEM. CNQX, $n = 13$; GYKI 52466, $n = 4$; CTZ, $n = 4$; APV, $n = 3$. *, $p < 0.05$. No stim, No stimulation.

AMPA receptors mediate the increase in $[Ca^{2+}]_i$ and CREB phosphorylation

AMPA receptors expressed by NM neurons show extremely rapid kinetics (Raman and Trussell, 1992; Raman et al., 1994) that allows these neurons to encode faithfully temporal aspects of acoustic stimuli (Trussell, 1999). The AMPA receptor subunit composition that allows rapid kinetics also imparts Ca^{2+} permeability to the receptor complex (Geiger et al., 1995). Thus NM neurons express AMPA receptors that are highly permeable to Ca^{2+} (Otis et al., 1995; Ravindranathan et al., 2000).

Residual neurotransmitter in the synaptic cleft in the absence of activity is a well established phenomenon (Barbour et al., 1994; Timmerman and Westerink, 1997), and whole-cell patch-clamped NM neurons in an *in vitro* slice show miniature EPSCs in the absence of eighth nerve stimulation (L. Zirpel, unpublished observations). It is therefore plausible that after an *in ovo* cochlea removal and *in vitro*, there is spontaneous leak of glutamate from the eighth nerve terminals that activates AMPA receptors enough to cause the gradual increase in $[Ca^{2+}]_i$ seen in NM neurons.

To test this hypothesis of AMPA receptor-mediated Ca^{2+} influx, 30 μ M CNQX, a AMPA/kainate receptor antagonist, or 50 μ M GYKI 52466, a more specific AMPA receptor antagonist (Donevan and Rogawski, 1993), was added to the superfusate of slices while $[Ca^{2+}]_i$ was monitored. Figure 3*A* shows that both CNQX (△) and GYKI 52466 (●) prevented the $[Ca^{2+}]_i$ increase in NM neurons in the absence of activity. Cyclothiazide (CTZ) is a benzothiadiazide that inhibits the rapid desensitization of AMPA receptors (Partin et al., 1993; Wong and Mayer, 1993). Addition of 100 μ M CTZ to the superfusate of slices had no effect on the $[Ca^{2+}]_i$ increase in NM neurons (Fig. 3*A*, ◇). NMDA receptors have been shown to contribute little to synaptic transmission (Zhang and Trussell, 1994) or calcium regulation (Zirpel et al., 1995a) in NM neurons in late embryonic or early posthatch chicks. In agreement with this idea, application of the NMDA receptor

Figure 4. Western immunoblots confirm immunohistochemistry. *A*, Immunoblot analysis of CREB levels using 25 μ g of protein from isolated NM receiving normal stimulation (Stim), in ACSF for 60 min with no stimulation (No stim), and in ACSF containing 30 μ M CNQX for 60 min (CNQX). Rat cerebellum (Rat cb.; 25 μ g) was used as a positive control. *B*, Immunoblot analysis of phosphorylated-CREB levels using 50 μ g of isolated NM tissue from the identical stimulation, no stimulation, and CNQX groups described for the CREB Western immunoblot in *A*. NM tissue in ACSF containing BAPTA AM for 60 min (BAPTA) was also included. Rat cerebellum (25 μ g) was included as a positive control.

antagonist ($\pm$)-2-amino-5-phosphonopentanoic acid (APV; 200 μ M) had no effect on the increase in $[Ca^{2+}]_i$ in the absence of activity (data not shown).

CNQX and GYKI 52466 both significantly reduced the number of p-CREB+ NM neurons 60 min after deafferentation (Fig. 3*B*; $p < 0.0001$ and $p = 0.012$, respectively). CTZ showed a trend of increasing the number of p-CREB+ NM neurons (Fig. 3*B*), but this effect was not statistically significant ($p = 0.16$). APV had no effect on the percentage of p-CREB+ NM neurons (Fig. 3*B*; $p = 0.71$). The results of these experiments indicate that the deafferentation-induced $[Ca^{2+}]_i$ increase in NM neurons and the underlying CREB phosphorylation require activation of AMPA receptors.

To confirm the specificity of the antibodies on chick tissue, Western immunoblots were performed using antibodies against CREB and phospho-CREB. Figure 4*A* shows that the anti-CREB antibody recognizes an appropriately sized protein (43 kDa) in both rat cerebellum (positive control) and chick tissue. Chick tissue consisted of isolated NM from the following three conditions: normal stimulation (no p-CREB via immunohistochemistry), no stimulation for 60 min (high p-CREB levels via immunohistochemistry), and no stimulation in the presence of 30 μ M CNQX for 60 min (very low p-CREB levels via immunohistochemistry). This antibody recognizes a 30 kDa protein, which may be a CRE-modulating protein, as well as CREB in different states of phosphorylation (Ginty et al., 1993). Thus, only the lower of the three bands near 43 kDa was quantified using NIH Image. The results of the CREB Western show that there are no significant differences in CREB levels among the chick groups. It can also be seen that CNQX treatment decreases the intensity of the upper bands that represent phosphorylated-CREB. For the p-CREB Western the stimulation, no stimulation, and CNQX groups were identical to those used for the CREB Western. NM tissue treated with BAPTA for 60 min in the absence of activity was also included. Consistent with our immunohistochemistry results, the p-CREB Western shows significant bands at the appropriate size for the no stimulation or control tissue, and p-CREB levels are attenuated by BAPTA and CNQX treatment. The p-CREB antibody also recognizes a 38 kDa protein that may be phosphorylated activating transcription factor-1 (Ginty et al., 1993).

Figure 5. Kinase activity affects CREB phosphorylation. Percentage of p-CREB+ NM neurons after 60 min of deafferentation in normal ACSF ($n = 19$; same control data shown in Figs. 2*Bb*, 3*B*) and in ACSF containing 1 μ M staurosporine ($n = 5$), 1 μ M KN-62 ($n = 3$), the combination of 1 μ M KN-62 and 1 μ M staurosporine (KN-62 + Stauro; $n = 3$), 100 μ M Rp-cAMPS ($n = 4$), PD98059 ($n = 9$; pooled data from 1, 10, and 50 μ M; see Results), and 100 μ M Sp-cAMPS ($n = 3$). *, $p < 0.05$.

Inhibition of CaM kinases and staurosporine-sensitive kinases prevents CREB phosphorylation

CREB can be phosphorylated by a number of kinases, including PKA, PKC, CaM kinases, and RSKs (Walton and Dragunow, 2000). To determine whether these particular kinases were involved in CREB phosphorylation in NM neurons after deafferentation, we pharmacologically inhibited kinases in slices and assayed for CREB phosphorylation after 60 min of deafferentation. At micromolar concentrations, staurosporine is a nonspecific kinase inhibitor (Shapiro et al., 1996) that inhibits, among others, PKA, PKC, and RSKs but not CaM kinases (Hidaka and Kobayashi, 1992). Application of 1 μ M staurosporine to slices for 60 min of deafferentation reduced the percentage of p-CREB+ NM neurons by 51% (Fig. 5; from $52 \pm 6\%$ of total neurons to $25 \pm 2\%$). Similarly, inhibition of CaM kinases with 1 μ M KN-62 resulted in a 65% reduction in the percentage of p-CREB+ NM neurons (Fig. 5; from 52 to $18 \pm 3\%$). Inhibition of PKA with 100 μ M Rp-cAMPS reduced the number of p-CREB+ neurons by 61% (Fig. 5; from 52 to 20%; $p = 0.0007$). The combination of 1 μ M staurosporine and 1 μ M KN-62 eliminated p-CREB labeling in NM neurons (Fig. 5; $8 \pm 3\%$). This value is significantly different from the control ($p = 0.036$) but not from 0 (one-sample t test, $t = 1.76$; $p = 0.22$). MAPKK inhibition eliminates downstream activation of RSKs and thus CREB phosphorylation (Impey et al., 1998a). Application of the MAPKK inhibitor PD98059 (Alessi et al., 1995) at 1, 10, or 50 μ M ($n = 3$ for each concentration) had no effect on the number of p-CREB+ NM neurons after 60 min of activity deprivation (Fig. 5; $p = 0.1339$ on nine slices from all three concentrations). Activation of PKA (with 100 μ M Sp-cAMPS) in the absence of activity attenuates the increase in $[Ca^{2+}]_i$ (Zirpel et al., 1998) but increases the percentage of p-CREB+ NM neurons (Fig. 5; $p = 0.0466$). None of the kinase inhibitors used had any effect on the increase in $[Ca^{2+}]_i$ (data not shown) (but see Zirpel et al., 1998).

Preliminary experiments ($n = 3$ for each treatment; data not shown) further support the involvement of PKA in the deafferentation-induced CREB phosphorylation. Stimulating PKA directly with 1 mM 8-bromo-cAMP or indirectly with the adenylate cyclase activator forskolin (50 μ M) for 30 min [when CREB phosphorylation is not normally observed (Fig. 1*C*)] results in p-CREB+ labeling in 30–50% of the NM neurons. Conversely, stimulating PKC with phorbol 12-myristate 13-acetate (PMA; 100 nM) or application of the inactive dideoxyforskolin (50 μ M) or 4 α -PMA did not result in any p-CREB+ NM neurons 30 min after activity deprivation.

Thus, at least two different kinases, a minimum of one CaM kinase and PKA but not PKC or MAPKK/RSK, converge to phosphorylate CREB in response to the AMPA receptor-mediated, deafferentation-induced increase in $[Ca^{2+}]_i$.

p-CREB labeling is correlated with neuronal survival

To determine whether p-CREB labeling occurs in a random subpopulation of NM neurons or in the subpopulation of NM neurons that survive deafferentation, slices in various treatment groups were labeled with PI 30 min before fixation for p-CREB immunohistochemistry as described by Zirpel et al. (1998). PI is a fluorescent dye that binds to DNA and, when used in live tissue, functions as an exclusion dye that labels dead or dying cells (Jiang et al., 1993; Wilde et al., 1994; Behl et al., 1995; Kuroda et al., 1995). However PI is not fixable when used in live tissue, and this precludes double-label experiments with immunohistochemistry. Figure 6 shows that treatments that prevent CREB phosphorylation in activity-deprived NM neurons without preventing the $[Ca^{2+}]_i$ increase result in a higher number of PI-labeled neurons. However, activation of PKA with Sp-cAMPS attenuates the increase in $[Ca^{2+}]_i$ (Zirpel et al., 1998), increases the number of p-CREB+ NM neurons (Fig. 5), and decreases the number of PI-labeled neurons (Fig. 6, *top*, *bottom*). There is a strong and significant negative correlation between the proportions of PI+ and p-CREB+ NM neurons across the five experimental treatments ($r^2 = 0.943$; $p < 0.01$). These results indicate that CREB phosphorylation occurs in the NM neurons that will survive the deafferentation-induced hypercalcemia.

DISCUSSION

Afferent regulation of target neurons is a well established phenomenon in the developing nervous system. Although this has been demonstrated in all sensory systems, little is known about the mechanisms underlying this regulation or the converse of neuronal degeneration, or survival, in the absence of afferent activity. In this study we have shown that a subpopulation of auditory neurons that survive deafferentation shows CREB phosphorylation in response to the AMPA receptor-mediated increase in $[Ca^{2+}]_i$. This CREB phosphorylation is the result of the activity of at least two Ca^{2+} -dependent kinase pathways. This study suggests that in conditions of activity deprivation, neurons are able to implement active mechanisms that allow them to cope with and survive in their new environment.

This study and others have shown that CREB phosphorylation is Ca^{2+} dependent (Sheng et al., 1990; Deisseroth et al., 1996; Hardingham et al., 1997; S. C. Hu et al., 1999). Whereas other studies have demonstrated CREB phosphorylation in response to increased levels of synaptic activity (Deisseroth et al., 1996; Impey et al., 1998b; Lin et al., 1998; Sgambato et al., 1998; Moon et al., 1999; Sakaguchi et al., 1999), we show here an increase in CREB phosphorylation in response to *activity deprivation*. However, even in the absence of eighth nerve synaptic activity, AMPA receptor activity is required for the increase in $[Ca^{2+}]_i$ that drives CREB phosphorylation in cochlear nucleus neurons. Some studies have suggested that Ca^{2+} influx through L-type calcium channels and/or NMDA receptor channels is required for CREB phosphorylation (Hardingham et al., 1999; Rajadhyaksha et al., 1999; Mermelstein et al., 2000). This does not seem to be the case in cochlear nucleus neurons because specific inhibition of AMPA receptors, which leaves available NMDA receptors for neuron depolarization and Ca^{2+} influx, completely prevents the $[Ca^{2+}]_i$ increase and subsequent CREB phosphorylation. In addition, inhibiting L-type Ca^{2+} channels or NMDA receptors with nifedipine or APV, respectively, has no effect on the deafferentation-induced increase in $[Ca^{2+}]_i$ in NM neurons or the number of p-CREB+ neurons. Perkinson et al. (1999) report that in primary cultures of mouse striatal neurons, Ca^{2+} -permeable AMPA receptors cause an increase in $[Ca^{2+}]_i$ with no contribution from voltage-gated Ca^{2+} channels or NMDA receptors. This $[Ca^{2+}]_i$ increase stimulates

Figure 6. CREB phosphorylation is inversely correlated with propidium iodide labeling. *Top*, PI labeling in NM after 60 min of activity deprivation in the presence of the PKA activator Sp-cAMPS. *Middle*, PI labeling after 60 min of activity deprivation in the presence of the nonspecific kinase inhibitor staurosporine. NM neurons show clearly labeled nuclei and diffusely labeled cytoplasm. Smaller, punctate labeling is glial nuclei. Scale bar: A, B, 30 μ m. *Bottom*, Percentage of p-CREB+ NM neurons as a function of the percentage of propidium iodide-labeled neurons after 60 min of deafferentation in (from left to right on x-axis) ACSF containing 100 μ M Sp-cAMPS, normal ACSF, and ACSF containing 1 μ M staurosporine, 1 μ M KN-62, or the combination of 1 μ M staurosporine and 1 μ M KN-62. Error bars represent the SEM ($n = 6$ for all PI-labeling points and 19 for control p-CREB, 5 for staurosporine p-CREB, and 3 for the KN-62, staurosporine + KN-62, and Sp-cAMPS p-CREB groups).

a mitogen-activated protein kinase cascade resulting in CREB phosphorylation.

Residual glutamate in synaptic clefts has been reported to be in the low micromolar range (Sarantis et al., 1993; Jay et al., 1999). This concentration of glutamate has also been reported to stimulate NMDA receptors but not AMPA/kainate receptors (Sarantis et al., 1993). Because of the unique morphological characteristics of the eighth nerve calyx synapses (Parks, 1981; Carr and Boudreau, 1991), which cover two-thirds of the NM neuron's surface, it is plausible to hypothesize that glutamate may reach higher concentrations—high enough to activate the Ca^{2+} -permeable AMPA receptors on these neurons. The NM neuron AMPA receptors have an EC_{50} of glutamate (Raman and Trussell, 1992; Raman et al., 1994) that is an order of magnitude lower than that of the mGluRs in NM (Zirpel et al., 1994, 1995a). Thus, in the absence of activity, the residual glutamate within the eighth nerve synaptic cleft may activate the AMPA receptors, but not the mGluRs. Because the mGluRs mediate the calcium-clearing and homeostatic mechanisms of NM neurons (Zirpel and Rubel, 1996; Zirpel et al., 1998, 2000; Kato and Rubel, 1999), after deafferentation an important Ca^{2+} regulatory mechanism is disabled, and the Ca^{2+} influx through AMPA receptors becomes cumulative and causes a gradual $[\text{Ca}^{2+}]_i$ increase. Also consistent with AMPA receptor involvement is the report by Solum et al. (1997) showing that AMPA receptor antagonists prevent both normal developmental neuronal death and deafferentation-induced death in the auditory nuclei of chick embryos.

CREB can be phosphorylated on Ser133 by PKA, PKC, CaM kinases, and RSKs (Walton and Dragunow, 2000). There is considerable interaction among these kinase pathways and the MAPK pathway, resulting in complex phosphorylation mechanisms that converge on CREB (Roberson et al., 1999). In murine F9 cells, basic FGF induced CREB phosphorylation via stimulation of the PKA and MAPK pathways (Hansen et al., 1999). Similarly, Impey et al. (1998a) demonstrated in pheochromocytoma-12 (PC-12) cells and hippocampal neurons that RSK phosphorylation of CREB depends on PKA activation and that CaMKIV is not essential for CREB phosphorylation. It is not surprising, then, that our results indicate that at least two different kinase pathways are capable of phosphorylating CREB in NM neurons after deafferentation. The protein kinase A and C pathways play a significant role in NM neuron calcium homeostasis during normal eighth nerve stimulation (Zirpel et al., 1998) and are therefore prime candidates for involvement in Ca^{2+} -dependent CREB phosphorylation. However, our results indicate that CaM kinases and PKA are the primary CREB-phosphorylating kinases active in NM neurons after activity deprivation. That PD98059 had no effect on the number of p-CREB+ neurons suggests that RSKs are not involved and hints that PKC also may not be directly involved because it is thought that PKC mediates CREB phosphorylation via the MAPK/RSK pathway.

CREB phosphorylation has been shown to be critical for neuronal survival in a variety of cell types and conditions. CREB phosphorylation protects PC-12 cells from okadaic acid-induced apoptosis (Walton et al., 1999) and seems to protect hippocampal dentate gyrus neurons from ischemia (B. R. Hu et al., 1999; Walton and Dragunow, 2000). Some of the survival-promoting effects of NGF also seem to be mediated by CREB phosphorylation (Finkbeiner et al., 1997; Riccio et al., 1999; for review, see Finkbeiner, 2000). After deafferentation, 30% of chick cochlear nucleus neurons die (Born and Rubel, 1985). Our results suggest that CREB phosphorylation occurs in the remaining 70% of the activity-deprived neurons and promotes their survival in their new hypercalcemic environment. All chick cochlear nucleus neurons express CREB, and all show increased $[\text{Ca}^{2+}]_i$ after deafferentation. Why, then, do only 70% show CREB phosphorylation and survive? It seems that the deafferentation-induced rise in $[\text{Ca}^{2+}]_i$ serves as a general signal to which 30% of the neurons respond by dying (perhaps via Ca^{2+} -mediated apoptosis) and 70% respond by phosphorylating CREB and implementing compensatory mechanisms.

In agreement with this hypothesis, there appears to be an $[Ca^{2+}]_i$ threshold above which NM neurons begin to die (Zirpel et al., 1998); manipulations that prevent the $[Ca^{2+}]_i$ increase also prevent increased neuronal death. In the present study, we show that manipulations (namely, kinase inhibition) that prevent CREB phosphorylation in the presence of the $[Ca^{2+}]_i$ increase result in increased neuronal death. Hence, in the absence of activity, increased $[Ca^{2+}]_i$ serves as a signal for neuronal death or CREB phosphorylation: those neurons unable to phosphorylate or prevented from phosphorylating CREB die.

Our working hypothesis, based on the current study and consistent with the existing data, follows. Ambient glutamate in the eighth nerve synaptic cleft activates Ca^{2+} -permeable AMPA receptors on the postsynaptic NM neurons. This allows an influx of Ca^{2+} that causes a gradual increase in $[Ca^{2+}]_i$ because of the absence of mGluR-mediated mechanisms for clearing Ca^{2+} . This increased $[Ca^{2+}]_i$ activates Ca^{2+} -sensitive adenylate cyclases (AC). The cAMP generated by AC activates PKA. PKA then directly phosphorylates CREB. Increased Ca^{2+} would also interact with CaM, translocate to the nucleus, and activate a CaMK, most likely CaMKIV (Deisseroth et al., 1996; Chawla et al., 1998). CaMK would then phosphorylate CREB. In this scenario, staurosporine would inhibit PKA, leaving CaMK active to phosphorylate CREB. Our results showed a 51% decrease in CREB phosphorylation with staurosporine (Fig. 5). Alternatively, in this scenario, inhibition of CaM kinase with KN-62 would leave active PKA to phosphorylate CREB. Our results showed a 65% decrease in CREB phosphorylation with KN-62 and a complete elimination of CREB phosphorylation in the presence of both KN-62 and staurosporine. Inhibition of PKA with Rp-cAMPS showed a reduction in CREB phosphorylation similar in magnitude to that seen with staurosporine, suggesting that the majority of staurosporine effects are mediated by inhibition of PKA. After being phosphorylated by these pathways, CREB interacts with CREB binding protein and initiates the transcription of genes containing the Ca^{2+} /cAMP response element within their promoter region. These gene products may be proteins that allow the neuron to compensate for the deafferentation-induced hypercalcemia and absence of synaptic activity. One gene of particular interest, which is known to be regulated by CREB transcription, is *Bcl-2* (Wilson et al., 1996). BCL-2 is well known to serve an antiapoptotic role, and recent studies have shown that BCL-2 message expression is upregulated within hours of NM deafferentation (Wilkinson and Hyson, 2000). Future experiments using the genetics of a mouse model of deafferentation-induced CREB phosphorylation will help elucidate which genes are transcribed and the mechanisms that allow neurons to survive activity deprivation.

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