Mitochondrial Regulation of Calcium in the Avian Cochlear Nucleus

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Mostafapour, Sam P., Edward A. Lachica, and Edwin W Rubel. Mitochondrial regulation of calcium in the avian choclear nucleus. *J. Neurophysiol.* 78: 1928–1934, 1997. The role of mitochondria and the endoplasmic reticulum in buffering $[Ca^{2+}]_i$ in response to imposed calcium loads in neurons of the chick cochlear nucleus, nucleus magnocellularis (NM), was examined. Intracellular calcium concentrations were measured using fluorometric videomicroscopy. After depolarization with 125 mM KCl, NM neurons demonstrate an increase in $[Ca^{2+}]_i$ that returns to near-basal levels within 6 min. Addition of the protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) dissipated the mitochondrial membrane potential, as evidenced by increased fluorescence when cells were loaded with rhodamine-123. Two micromolar CCCP had minimal effect on baseline $[Ca^{2+}]_i$. However, 2 or 10 μM CCCP interfered with the ability of NM cells to buffer $[Ca^{2+}]_i$ in response to KCl depolarization without significantly affecting peak $[Ca^{2+}]_i$. Oligomycin also interfered with postdepolarization regulation of $[Ca^{2+}]_i$, but blocked late (7–8 min postdepolarization) increases in $[Ca^{2+}]_i$ caused by CCCP. Thapsigargin had no effect on baseline, peak, or postdepolarization $[Ca^{2+}]_i$ in NM cells. These results suggest that normal mitochondrial membrane potential and ATP synthesis play an important role in buffering $[Ca^{2+}]_i$ in response to imposed calcium loads in NM neurons. Furthermore, the endoplasmic reticulum does not appear to play a significant role in either of these processes. Thus increases in mitochondrial number and function noted in NM cells after deafferentation may represent an adaptive response to an increased cytosolic calcium load.

INTRODUCTION

Neurons of the avian cochlear nucleus, nucleus magnocellularis (NM), are stimulated tonically by glutamate released from auditory nerve endings. Ipsilateral cochlear removal or action potential blockade triggers a series of intracellular events leading to the death of 20–40% of these neurons (Born and Rubel 1985; Lachica et al. 1996; Rubel et al. 1990). During this process, these cells undergo characteristic metabolic and morphological changes similar to apoptosis (Garden et al. 1994; Hartlage-Rübsamen and Rubel 1996). One of the earliest changes noted is an increase in intracellular calcium ($[Ca^{2+}]_i$) (Zirpel et al. 1995), a pattern observed during cell death in other systems (Choi 1995; Trump and Berezesky 1995). The mechanisms involved in this rise in $[Ca^{2+}]_i$ have not yet been determined. A number of possibilities exist, one of which is the failure of Ca^{2+} sequestration by organelles or extrusion of Ca^{2+} from the cell.

The organellar compartmentation of Ca^{2+} has been found to play a role in regulating $[Ca^{2+}]_i$ in a number of neuronal and nonneuronal systems including the NM (Herrington et al. 1996; Kato et al. 1996; Kiedrowski and Costa 1995; Schinder et al. 1996; Thastrup et al. 1990). In this regard, much recent work has focused on the sarco-endoplasmic reticular complex and the mitochondrion. For example, inhibition of the sarco-endoplasmic reticulum Ca^{2+} (SERCA) pumps has been shown to perturb Ca^{2+} homeostasis in nonneuronal cell lines (Thastrup et al. 1990). Mitochondria also possess Ca^{2+} transport systems (Gunter and Gunter 1994) and have been found to play a role in calcium regulation in some types of neurons (Kiedrowski and Costa 1995; Schinder et al. 1996; Werth and Thayer 1994). Deafferentation of NM causes an increase in mitochondrial number and an increase in oxidative metabolism (Durham and Rubel 1985; Hyde and Durham 1990, 1994b). Furthermore, inhibitors of mitochondrial function specifically potentiate cell death in the avian cochlear nucleus after deafferentation (Garden et al. 1994; Hartlage-Rübsamen and Rubel 1996; Hyde and Durham 1994a). How mitochondrial inhibition results in increased cell death after deafferentation in the NM is unknown.

Recent evidence suggests that neuronal mitochondria rapidly sequester Ca^{2+} when cells are exposed to high calcium loads (Herrington et al. 1996). Furthermore, changes in mitochondrial potential ($\Delta\Psi_m$) have been noted in excitatory neurotoxicity and apoptosis in other cell types (Schinder et al. 1996; White and Reynolds 1996; Zamzami et al. 1995). We sought to determine the relative roles of mitochondria and the endoplasmic reticulum in the regulation of $[Ca^{2+}]_i$ in NM neurons in the basal state and after imposed Ca^{2+} loads.

METHODS

Tissue preparation

The methods used for tissue preparation in the present study have been described in detail elsewhere (Lachica et al. 1995). Briefly, coronal brain stem slices (300 μm) containing NM were obtained from 18-day-old White Leghorn chicken embryos. Slices were incubated in oxygenated artificial cerebral spinal fluid (ACSF) containing either 5 μM fura-2-AM (Molecular Probes, Eugene, OR) at 40°C for 25 min or 0.5 μg/ml rhodamine-123 at 40°C for 15 min. Slices then were rinsed in ACSF and placed in the perfusion chamber for microscopic analysis. The time from animal death to the end of experimental measurements was <60 min. Glucose (10 mM) was present in the medium at all times.

Microfluorometry

The $[Ca^{2+}]_i$ of NM cells loaded with fura-2 was measured using fluorometric videomicroscopy. Fluorescent images were alternately acquired using excitation wavelengths at 340 and 380 nm at 3- or 10-s intervals. Each image then was compared radiometrically and converted to nanomolar $[Ca^{2+}]_i$ by using Universal Imaging (West Chester, PA) software. Changes in $[Ca^{2+}]_i$ are reported as

means ± SE. Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) of cells loaded with rhodamine-123 were measured as changes in the brightness of fluorescent emission (arbitrary units based on 256 gray levels) at 3- or 10-s intervals.

Pharmaceuticals

ACSF was prepared as described previously (Lachica et al. 1995) and was made fresh each day. Briefly, the ACSF was composed of (in mM) 125 NaCl, 5 KCl, 1.25 KH_2PO_4, 1.3 $MgCl_2$, 26 $NaHCO_3$, 10 dextrose, and 3.1 $CaCl_2$. High potassium ACSF was composed of (in mM) 5 NaCl, 125 KCl, 1.25 KH_2PO_4, 1.3 $MgCl_2$, 26 $NaHCO_3$, 10 dextrose, and 3.1 $CaCl_2$. Carbonylcyanide m-chlorophenylhydrazone (CCCP), thapsigargin, and oligomycin were prepared as stock solutions in 99.8% anhydrous dimethyl sulfoxide. All pharmaceuticals were obtained from Sigma (St. Louis, MO) with the exception of thapsigargin (Calbiochem, La Jolla, CA). All solutions were changed by bath application. Thus, there is a 30-s delay between initiation of stimulus delivery and the time the test chamber is saturated completely with the given test solution. This delay is not corrected for in the figures.

Statistical analysis

Each brain stem slice represents a single experiment, and only one slice was examined per animal. For each brain stem slice, the mean NM neuronal $[Ca^{2+}]_i$ was calculated at each time point, and these data were taken to represent a single experiment. All results are expressed as means ± SE, and represent at least three separate experiments. Statistical comparisons were performed using an unpaired Student's *t*-test.

RESULTS

CCCP depolarized mitochondria in NM neurons

The mitochondrial matrix normally is negative with respect to the cell cytoplasm, with the mitochondrial membrane potential ($\Delta\Psi_m$) across the inner membrane ranging from −90 to −160 mV (Gunter and Gunter 1994; Lemasters et al. 1995). To confirm that the protonophore CCCP depolarized mitochondria in NM neurons, cells were loaded with rhodamine-123 and examined using videomicroscopic fluoroscopy. The lipophilic dye rhodamine-123 is also cationic and is accumulated in the negatively charged mitochondrial matrix where it is quenched partially (Johnson et al. 1980, 1981). When the mitochondria depolarize, the dye redistributes to the cytoplasm where it is dequenched. This is observed as an increase in the fluorescence of the cell under microfluoroscopic analysis (Johnson et al. 1980, 1981).

As shown in Fig. 1*A*, the addition of CCCP for 1 min caused an immediate, reversible change in $\Delta\Psi_m$. Continued perfusion with CCCP eventually caused saturation of the image due to continued depolarization and leakage of rhodamine-123 into the cytoplasm (data not shown). The addition of oligomycin, an inhibitor of mitochondrial ATP-synthase, had minimal effect on $\Delta\Psi_m$ (Fig. 1*B*).

CCCP causes disruption of $[Ca^{2+}]_i$ buffering after KCl depolarization

CCCP inhibits uptake of Ca^{2+} into mitochondria and causes release of pooled Ca^{2+} (Herrington et al. 1996). We therefore sought to determine if mitochondria sequester Ca^{2+} in the basal state and after the cell is exposed to an imposed

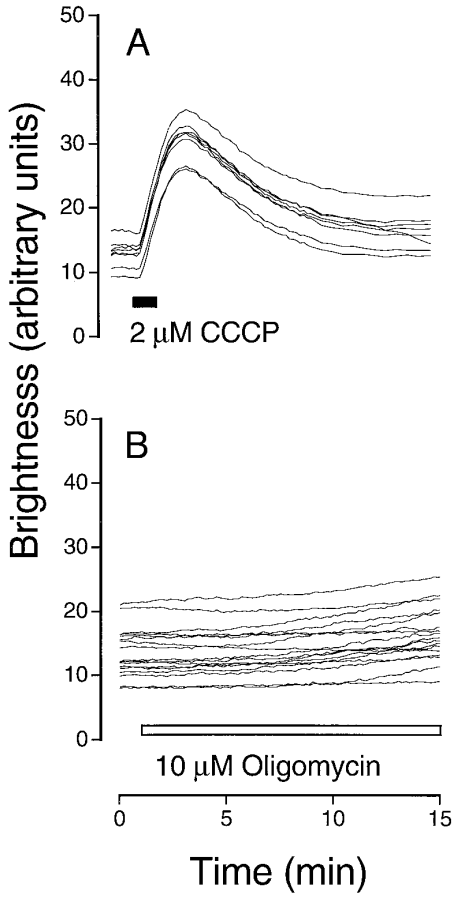

FIG. 1. carbonylcyanide m-chlorophenylhydrazone (CCCP)-induced depolarization of mitochondria in nucleus magnocellularis (NM) cells. Cells were loaded with rhodamine-123 as described in METHODS and superfused with artificial cerebrospinal fluid (ACSF). *A*: 2 micromolar CCCP was added to the superfusate for 1 min (■). *B*: 10 micromolar oligomycin was added to the superfusate for the time indicated (□). Data shown represents a single experiment, each tracing represents a single cell. These conditions were replicated on at least 3 different slices.

Ca^{2+} load. As Fig. 2*A* demonstrates, 2 μM CCCP did not affect basal $[Ca^{2+}]_i$ in NM neurons.

When NM neurons are exposed to 125 mM KCl for 20 s, $[Ca^{2+}]_i$ rises rapidly to 0.5–5 μM and typically returns to basal levels in 5–6 min, as shown in Figs. 2*B* and 3*B*. A number of mechanisms may be responsible for this clearance of Ca^{2+} from the cytosol, including plasma membrane extrusion and organellar sequestration (Gunter 1994; Trump and Berezesky 1995). Thus it is possible that while cytosolic Ca^{2+} concentrations have returned to baseline within 5 min, mitochondria or endoplasmic reticulum may retain large pools of Ca^{2+}, presumably for later extrusion from the cell. To determine if such a pool exists in the mitochondria of NM neurons after a large imposed Ca^{2+} load, 2 μM CCCP was added to the superfusate 5 min after KCl depolarization. As shown in Fig. 2*C*, no releasable Ca^{2+} pool was detected when CCCP was added at this time. However, addition of 2 μM CCCP 1 min before depolarization did interfere with postdepolarization recovery of $[Ca^{2+}]_i$, as shown in Fig. 2*D*. In this case, the majority of cells continue to demonstrate a rapid initial removal of cytosolic Ca^{2+} after KCl depolarization. However, in the continued presence of CCCP, cyto-

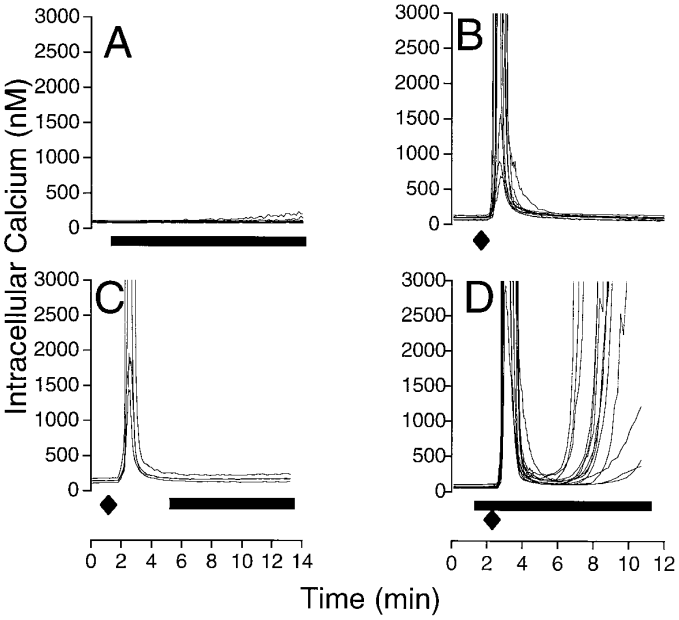

FIG. 2. Effect of 2 μM CCCP on basal and stimulated $[Ca^{2+}]_i$ in NM neurons. Cells were loaded with fura-2 as described in METHODS. *A*: cells were superfused with ACSF ± 2 μM CCCP (■) as indicated. *B*: typical responses of NM cells to depolarization with 125 mM KCl times 20 s (♦). *C*: 2 micromolar CCCP (■) was perfused for the time indicated, starting 5 min after depolarization (♦). *D*: 2 micromolar CCCP (■) was added 1 min before depolarization (♦). Each graph represents a single experiment, each tracing represents a single cell. These conditions were replicated on at least 3 different slices.

solic $[Ca^{2+}]$ subsequently showed a large secondary increase.

These data are summarized in Figs. 5 and 6. As shown in Fig. 5, 2 μM CCCP did not affect basal $[Ca^{2+}]_i$. Interestingly, 10 μM CCCP appeared to increase basal $[Ca^{2+}]_i$ (Fig. 5) after 7–8 min of treatment, though this was not statistically significant when examined by unpaired Student's *t*-test. Addition of 2 or 10 μM CCCP 1 min before depolarization did not affect peak $[Ca^{2+}]_i$ (4,253 ± 334 nM and 3,980 ± 413, respectively, Fig. 6). However, CCCP at either dose caused a significant secondary rise in $[Ca^{2+}]_i$ compared with untreated cells (Fig. 6). Ten micomolar CCCP also appeared to cause a greater disturbance in postdepolarization $[Ca^{2+}]_i$ than 2 μM CCCP, though this difference was not statistically significant by unpaired student's *t*-test ($P = 0.15–0.35$). These data suggest maintenance of $\Delta\Psi_m$ is critical for normal $[Ca^{2+}]_i$ buffering mechanisms.

SERCA pump inhibition does not affect $[Ca^{2+}]_i$

The endoplasmic reticulum serves as a store of Ca^{2+} in a number of systems, and inhibition of SERCA pumps has been shown to disrupt Ca^{2+} homeostasis (Thastrup et al. 1990; Trump and Berezesky 1995). To test whether the endoplasmic reticulum serves either of these roles in NM neurons, cells were treated with thapsigargin, a specific inhibitor of the family of SERCA pumps (Lytton et al. 1991; Thastrup et al. 1990). As shown in Fig. 3*A*, 1 μM thapsigargin, a concentration that effectively blocks all SERCA pumps (Lytton et al. 1991; Thastrup et al. 1990), had no effect on baseline $[Ca^{2+}]_i$. No mobilization of thapsigargin-sensitive pools of Ca^{2+} was noted with the addition of 1 μM thapsigargin 5 min after depolarization (Fig. 3*C*). Furthermore, addition of 1 μM thapsigargin 1 min before depolarization did not affect the buffering of $[Ca^{2+}]_i$ when compared with control (Figs. 3, *B* and *D*).

To confirm that thapsigargin was effective in this preparation, we performed the following experiment: Thapsigargin (1 μM) was added to the preparation with the subsequent application of caffeine (100 mM) to release calcium from internal stores. The caffeine then was washed out in continued presence of thapsigarin. In all cases, a second application of 100 mM caffeine caused no further release from internal stores, demonstrating the effectiveness of thapsigargin in blocking reuptake (data not shown).

The effect of thapsigargin on basal and postdepolarization regulation of $[Ca^{2+}]_i$ in NM neurons is summarized in Figs. 5 and 6. Pretreatment of cells with 1 μM thapsigargin 1 min before depolarization had no effect on peak $[Ca^{2+}]_i$ (4,072 ± 539 nM). In addition, $[Ca^{2+}]_i$ was unaffected at all time points thereafter (260 ± 67 nM and 120 ± 20 nM at 2 and 6 min postdepolarization, respectively). These data suggest that the endoplasmic reticulum does not play a significant role in regulating $[Ca^{2+}]_i$ in the basal state or after imposed Ca^{2+} loads.

Inhibition of ATP-synthase disrupts $[Ca^{2+}]_i$ *homeostasis*

Disruption of $\Delta\Psi_m$ with CCCP blocks oxidative phosphorylation, and, in this state, glycolysis provides the primary means of ATP synthesis. When depolarized with CCCP, the mitochondrial ATP synthase may reverse in a

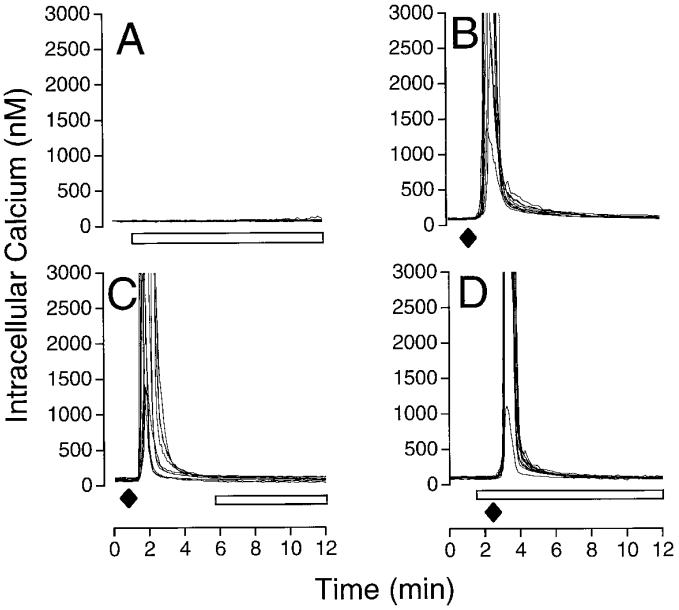

FIG. 3. Effect of 1 μM thapsigargin on basal and stimulated $[Ca^{2+}]_i$ in NM neurons. Cells were loaded with fura-2 as described in METHODS. *A*: cells were superfused with ACSF ± 1 μM thapsigargin (□) as indicated. *B*: typical responses of NM cells to depolarization with 125 mM KCl times 20 s (♦). *C*: 1 micromolar thapsigargin (□) was perfused for the time indicated, starting 5 min after depolarization (♦). *D*: 1 micromolar thapsigargin (□) was added 1 min before depolarization (♦). Each graph represents a single experiment, each tracing represents a single cell. These conditions were replicated on at least 3 different slices.

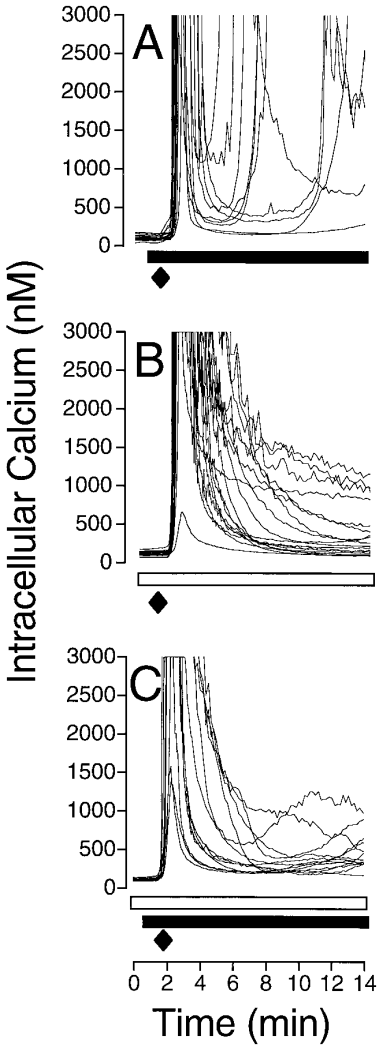

FIG. 4. Effect of 10 μM CCCP on basal and stimulated $[Ca^{2+}]_i$ in NM neurons. Cells were loaded with fura-2 as described in METHODS. All cells were superfused in aCSF ± drugs as indicated. *A*: 10 micromolar CCCP (■) was added 1 min before depolarization (♦). *B*: 10 micromolar oligomycin (□) was added 5 min before depolarization with 125 mM KCl (♦). *C*: 10 micromolar oligomycin (□) was added 5 min before depolarization with 125 mM KCl (♦) and 10 μM CCCP (■) was added 1 min before depolarization. Each graph represents a single experiment, each tracing represents a single cell. These conditions were replicated on at least 3 different slices.

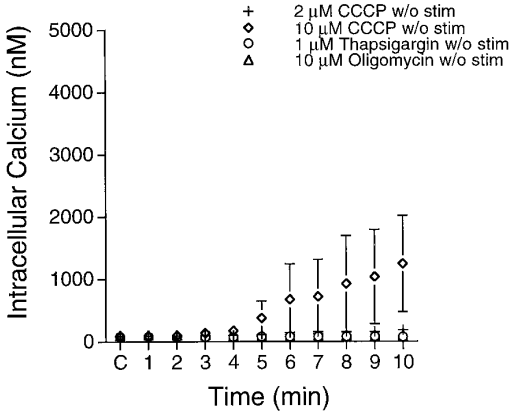

FIG. 5. Time course of $[Ca^{2+}]_i$ in NM neurons without depolarization. Cells were examined in the basal state (C) and after treatment with the indicated drugs. Abscissa indicates time of exposure to drug. No cells were depolarized during these experiments. Data shown represent the means ± SE of 3–15 experiments consisting of 4–24 cells per experiment.

futile cycle that attempts to restore $\Delta\Psi_m$, further depleting ATP produced via glycolysis (Budd and Nicholls 1996a,b). Thus treatment with CCCP alone causes both mitochondrial depolarization and rapid depletion of ATP. Recent work has indicated that mitochondrial ATP synthesis is important in regulating $[Ca^{2+}]_i$ (Budd and Nicholls 1996a,b). We next sought to investigate the importance of ATP-dependent processes in regulating $[Ca^{2+}]_i$ in NM neurons. To separate the mechanisms of mitochondrial Ca^{2+} transport via the inner membrane uniporter (dependent on $\Delta\Psi_m$) and ATP-dependent mechanisms that may be present in the plasma membrane, we used an inhibitor of ATP synthase, oligomycin. Inhibition of ATP synthase with oligomycin thus should allow reduction of cellular ATP without affecting $\Delta\Psi_m$. Because oligomycin blocks reversal of the ATP synthase, any acceleration of ATP depletion produced by CCCP should be prevented (Werth and Thayer 1994).

Cells treated with 10 μM CCCP demonstrate a rapid initial removal of cytosolic Ca^{2+} after KCl depolarization (Fig. 4*A*). These cells subsequently show a large secondary increase in $[Ca^{2+}]_i$ in the continued presence of 10 μM CCCP (Fig. 4*A*). Cells treated with 10 μM oligomycin demonstrated a delayed decrease in $[Ca^{2+}]_i$ after depolarization (Fig. 4*B*). Cells treated with both 10 μM oligomycin and 10 μM CCCP demonstrate a delayed decrease in $[Ca^{2+}]_i$ after depolarization without a secondary increase in $[Ca^{2+}]_i$ despite the continued presence of CCCP (Fig. 4*C*).

These data are summarized in Fig. 7. Treatment with 10 μM oligomycin had no effect on basal $[Ca^{2+}]_i$ (Fig. 5), but disrupted early postdepolarization buffering of $[Ca^{2+}]_i$. Intracellular calcium concentrations in oligomycin-treated cells were 1,964 ± 532 nM at 2 min postdepolarization ($P < 0.01$ vs. control, Fig. 7). Ten micromolar CCCP also caused an increase in $[Ca^{2+}]_i$ after depolarization. Intracellu-

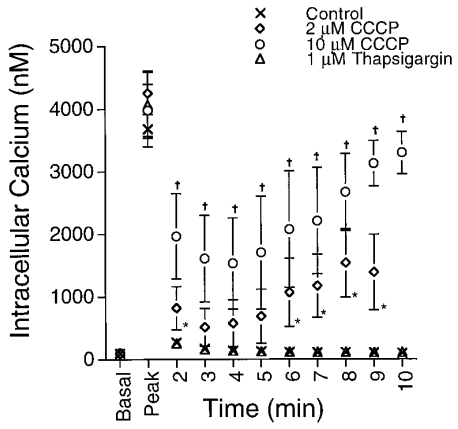

FIG. 6. Effect of mitochondrial or sarco-endoplasmic reticulum Ca^{2+} pump inhibition on $[Ca^{2+}]_i$ in depolarized NM neurons. Cells were examined in the basal state, then pretreated with drug for 1 min before depolarization with 125 mM KCl for 20 s. Abscissa indicates time postdepolarization. Data shown represent the means ± SE of 3–15 experiments consisting of 4–24 cells per experiment (*$P < 0.05$ or †$P < 0.01$ compared with control).

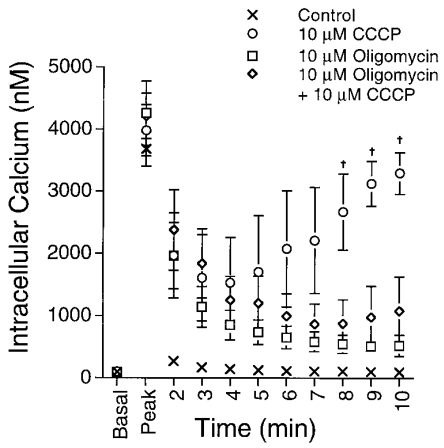

FIG. 7. Effect of mitochondrial or ATP synthase inhibition on $[Ca^{2+}]_i$ in depolarized NM neurons. Cells were again examined in the basal state, then pretreated with either 10 μM CCCP for 1 min before depolarization, 10 μM oligomycin 4 min before depolarization, or both. Abscissa indicates time postdepolarization. Data shown represent the means ± SE of 3–15 experiments consisting of 4–24 cells per experiment (†$P < 0.01$ for 10 μM CCCP alone compared with oligomycin + CCCP).

lar calcium concentrations in 10 μM CCCP-treated cells were 1,966 ± 684 nM 2 min after depolarization, ($P <$ 0.01 vs. control, Fig. 6 and 7). Interestingly, treatment with oligomycin appeared to block the secondary rise in $[Ca^{2+}]_i$ caused by 10 μM CCCP. For example, 10 μM CCCP alone resulted in a rise in $[Ca^{2+}]_i$ to 2,670 ± 616 nM at 8 min postdepolarization. The intracellular calcium concentration for cells treated with 10 μM oligomycin alone at 8 min postdepolarization was 548 ± 149 nM ($P < 0.01$ compared with control and $P < 0.01$ compared with 10 μM CCCP-treated cells, Fig. 7). The intracellular calcium concentration for cells treated with 10 μM oligomycin and 10 μM CCCP at 8 min postdepolarization was 878 ± 382 nM ($P < 0.01$ compared with control and $P < 0.05$ compared with cells treated with 10 μM alone, Fig. 7). Cells treated with oligomycin with or without CCCP are able to maintain glycolytic ATP production. This suggests that the late rise in $[Ca^{2+}]_i$ seen in cells treated with CCCP is a consequence of cellular ATP depletion.

Neurons of the NM possess Ca^{2+}-induced Ca^{2+}-release stores (CICRs) that may contribute to rises in $[Ca^{2+}]_i$ seen after deafferentation (Kato et al. 1996). To determine whether such stores contribute to the secondary rise (6–8 min postdepolarization) in $[Ca^{2+}]_i$ observed after prolonged treatment with 10 μM CCCP, we attempted to block release from CICRs with 100 nM ryanodine. The secondary rise in $[Ca^{2+}]_i$ observed in cells treated with 10 μM CCCP was not blocked by 100 nM ryanodine (data not shown). This indicates that CICRs do not contribute significantly to the rise in $[Ca^{2+}]_i$ caused by prolonged mitochondrial depolarization.

DISCUSSION

Our interest in studying the role of organellar regulation of $[Ca^{2+}]_i$ in NM neurons is based on several observations. First, deafferentation causes morphological and physiological changes in NM neurons comparable with apoptosis, including increases in $[Ca^{2+}]_i$ (Lachica et al. 1996; Zirpel et al. 1995). Second, this apoptotic-like process is potentiated by inhibition of mitochondria, suggesting that they may play a protective role in this process (Garden et al. 1994; Hartlage-Rübsamen and Rubel 1996; Hyde and Durham 1994a). Both mitochondria and the sarco-endoplasmic reticulum have been implicated in the regulation of $[Ca^{2+}]_i$ in other systems (Herrington et al. 1996; Kiedrowski and Costa 1995; Schinder et al. 1996), but little or no correlative data exists between these in vitro studies and in vivo observations. Given the body of in vivo evidence that points to the importance of both mitochondrial function and cellular regulation of $[Ca^{2+}]_i$ in NM neuronal survival, it becomes of particular interest to study the organellar regulation of $[Ca^{2+}]_i$ in NM neurons, both in the basal state and after imposed Ca^{2+} loads.

Depolarization of NM neurons results in a rapid rise in $[Ca^{2+}]_i$ to 0.5–5.0 μM, which normally returns to basal levels within minutes. A number of possible mechanisms for the clearance of Ca^{2+} exist, including sequestration by cellular organelles, such as the mitochondria and the endoplasmic reticulum, and plasma membrane extrusion (e.g., an ATP-driven Ca^{2+} pump) (Gunter et al. 1994; Herrington et al. 1996; Thastrup et al. 1990). Herein we have demonstrated that mitochondria, but not the endoplasmic reticulum, appear to play an important role in buffering $[Ca^{2+}]_i$ after imposed Ca^{2+} loads.

To study the role of mitochondria in this process, we employed drugs that alone or in combination affect $\Delta\Psi_m$, ATP synthesis, or both. In normally functioning cells, mitochondria sequester Ca^{2+} via a uniporter, which relies on the electochemical gradient caused by the largely negative mitochondrial matrix (Gunter and Gunter 1994). The protonophore CCCP dissipates $\Delta\Psi_m$, halting mitochondrial Ca^{2+} accumulation and causing release of any stored Ca^{2+} (Werth and Thayer 1994). Oligomycin, an inhibitor of ATP synthase, inhibits oxidative phosphorylation of ATP but not glycolytic production of ATP. For the purposes of clarity, we will call the treatment of cells with CCCP alone *condition A,* the treatment of cells with oligomycin alone *condition B,* and treatment of cells with both oligomycin and CCCP *condition C.* A number of conclusions can be drawn about cellular energetics under each of these conditions. In *condition A,* both ATP levels and $\Delta\Psi_m$ are reduced. Mitochondrial uptake of calcium is inhibited by reduction of $\Delta\Psi_m$. The reduction in cellular ATP is due to both the inhibition of oxidative phosphorylation (dependent on $\Delta\Psi_m$) and the reversal of ATP synthase in a futile cycle to regenerate $\Delta\Psi_m$. This results in hydrolysis of any cellular ATP stores and ATP produced by glycolysis. Thus we would expect that ATP available for cellular processes such as membrane Ca^{2+} ATPases would be the lowest in *condition A.* In *condition B,* treatment with oligomycin alone results in inhibition of ATP via oxidative phosphorylation, while $\Delta\Psi_m$ remains intact. In this condition, cellular ATP levels are reduced but to a lesser extent than in *condition A,* as $\Delta\Psi_m$ is intact and no reversal of ATP synthase occurs. Mitochondrial uptake of calcium may occur via the calcium uniporter supported by the intact electrochemical gradient. *Condition C,* in which both reduction of $\Delta\Psi_m$ and inhibition of ATP synthase occurs, would be expected to have similar ATP levels as in *condition B* because reversal of ATP synthase is inhibited.

In this condition, mitochondrial uptake of calcium is inhibited by the dissipation of $\Delta\Psi_m$.

Treatment of cells with CCCP alone (*condition A*) results in dissipation of $\Delta\Psi_m$ (thus inhibiting the mitochondrial Ca^{2+} uniporter) but does not cause an immediate elevation of $[Ca^{2+}]_i$ without prior depolarization of the cells, a finding that has been suggested in other neuronal systems (Budd and Nicholls 1996a). Treatment with 2 or 10 μM CCCP before depolarization caused a rapid loss of the ability of the cell to buffer $[Ca^{2+}]_i$. After depolarization, these cells typically exhibited an initial decline in $[Ca^{2+}]_i$, but lost this buffering capacity within 2 min. During this same time course, 2 or 10 μM CCCP had no effect on basal $[Ca^{2+}]_i$ in the absence of depolarization. In addition to early changes in the buffering of $[Ca^{2+}]_i$, CCCP alone also caused a dramatic increase in $[Ca^{2+}]_i$ 6–7 min after depolarization. This was perhaps due to depletion of cellular ATP stores, a consequence of the reversal of mitochondrial ATP synthase in response to mitochondrial depolarization with CCCP (Budd and Nicholls 1996a,b; Herrington et al. 1996).

When treated with oligomycin alone (*condition B*), cellular ATP synthase is inhibited and cellular ATP is reduced but not depleted, as glycolysis provides some ATP production. In this case, $\Delta\Psi_m$ is not affected, and thus the mitochondrial Ca^{2+}-uniporter still operates. Oligomycin alone caused disruption of postdepolarization but not basal $[Ca^{2+}]_i$ regulation in NM neurons. This indicates that some ATP-dependent mechanisms important in regulating $[Ca^{2+}]_i$ in NM neurons are sensitive to decreases in the supply of ATP (i.e., the quantitiy of ATP supplied via oxidative phosphorylation vs. glycolysis). Furthermore, as the cellular buffering of $[Ca^{2+}]_i$ was not completely blocked, it indicates that other mechanisms, such as mitochondrial sequestration or non-ATP dependent exchangers are operating to remove cytoplasmic $[Ca^{2+}]_i$.

When treated with CCCP alone, ATP synthase reverses and rapidly hydrolyzes cellular ATP stores in a futile cycle aimed at maintaining $\Delta\Psi_m$. Oligomycin inhibits ATP synthase, and treatment of cells with CCCP in the presence of oligomycin should result in inhibition of the reversal of ATP synthase, *condition C.* As mentioned above, late (7–8 min postdepolarization) rises in $[Ca^{2+}]_i$ observed in cells depolarized with KCl in the presence of CCCP alone (*condition A*) may be due to depletion of cellular ATP and failure of ATP-dependent clearance mechanisms. Coperfusion of 10 μM oligomycin with 10 μM CCCP inhibited this late increase in $[Ca^{2+}]_i$, *condition C* (Fig. 7). This may be due the ability of oligomycin to prevent the reversal of ATP synthase and subsequent rapid hydrolysis of cellular ATP (Budd and Nicholls 1996a,b; Herrington et al. 1996). These data would suggest that failure of ATP-dependent mechanisms are primarily responsible for the late increases in $[Ca^{2+}]_i$ observed in *condition A.*

It is important to note that inhibition of SERCA pumps, ATP synthase, or mitochondrial Ca^{2+} sequestration, alone or in combination, did not completely block the initial postdepolatization cellular buffering of $[Ca^{2+}]_i$. One explanation for this is that CCCP and/or oligomycin are not completely efficacious. Rhodamine-123 only gives us a relative measure of $\Delta\Psi_m$. Thus we cannot determine quantitatively the change in mitochondrial potential in each cell. Clearly, however, CCCP acts uniformly on NM neurons to cause a rapid drop in mitochondrial potential (Fig. 1). We have not directly measured ATP levels in these neurons under *conditions A–C* but have estimated the *relative* levels of ATP under each of these conditions as described by known bioenergetic pathways. This experimental design has been used in other systems to draw conclusions regarding the role of mitochondria in cellular regulation of $[Ca^{2+}]_i$ (Budd and Nicholls 1996a,b; Kiedrowski and Costa 1995).

Alternatively, the persistence of cellular buffering of $[Ca^{2+}]_i$ despite the presence of organellar inhibitors may indicate the presence of other nonendoplasmic reticular, non-ATP dependent, and nonmitochondrial sequestration/extrusion mechanisms. The plasma membrane possesses both Ca^{2+}-ATPases and Na^+/Ca^{2+} exchange mechanisms, and it is possible that the latter are operating to reduce $[Ca^{2+}]_i$ in these circumstances. However, the inability of such mechanisms to compensate for either reduction of cellular ATP or dissipation of $\Delta\Psi_m$ points toward the central role of mitochondria in the buffering of $[Ca^{2+}]_i$ in response to large Ca^{2+} loads.

One advantage of our system is the in situ nature of the preparation. By the same token, this may introduce complicating variables. For example, the NM neurons in this preparation are relatively intact, including the presence of presynaptic terminals impinging on the neurons. We cannot distinguish between the direct effects our drugs have on the NM neurons themselves and the secondary effects that may occur due to a primary effect on these terminals or surrounding glial cells. As NM neurons depend on afferent input for their long-term survival, we cannot discount the importance of this afferent input in the events surrounding cellular regulation of calcium. However, previous work in this laboratory has examined the role of the primarily glutamatergic input on the regulation of calcium in these neurons (Kato et al. 1996; Lachica et al. 1995, 1996; Zirpel and Rubel 1996). Under no circumstance has the blockade of and/or synaptic activation of the terminals or manipulations of the glutamate receptors themselves resulted in changes in cytosolic calcium as those observed with mitochondrial inhibition (as described herein). Furthermore, as discussed above, a large body of in vivo evidence points to the importance of both mitochondrial function and cellular regulation of $[Ca^{2+}]_i$ in NM neuronal survival.

We have found that normal mitochondrial function plays an important role in regulating $[Ca^{2+}]_i$ in NM neurons exposed to large Ca^{2+} loads. This finding is in agreement with morphological and functional data emphasizing the importance of both mitochondrial function and $[Ca^{2+}]_i$ in deafferentation-induced cell death in the NM. A drop in $\Delta\Psi_m$ is one of the earliest indicators of apoptosis in some cell types (Schinder et al. 1996; Zamzami et al. 1995, 1996). Furthermore, an increase in oxidative metabolism and an increase in mitochondrial number are some of the earliest changes noted in the NM after deafferentation (Durham and Rubel 1985; Hyde and Durham 1990). Taken together, these data would suggest a neuroprotective role for the mitochondria in the NM.

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