Role of Mitochondrial Dysfunction in the Ca$^{2+}$-Induced Decline of Transmitter Release at K$^+$-Depolarized Motor Neuron Terminals

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Calupca, Michelle A., Gregory M. Hendricks, Jean C. Hardwick, and Rodney L. Parsons. Role of mitochondrial dysfunction in the Ca$^{2+}$-induced decline of transmitter release at K$^+$-depolarized motor neuron terminals. J. Neurophysiol. 81: 498–506, 1999. The present study tested whether a Ca$^{2+}$-induced disruption of mitochondrial function was responsible for the decline in miniature endplate current (MEPC) frequency that occurs with nerve-muscle preparations maintained in a 35 mM potassium propionate (35 mM KP) solution containing elevated calcium. When the 35 mM KP contained control Ca$^{2+}$ (1 mM), the MEPC frequency increased and remained elevated for many hours, and the mitochondria within twitch motor neuron terminals were similar in appearance to those in unstimulated terminals. In contrast, when Ca$^{2+}$ was increased to 3.6 mM in the 35 mM KP solution, the MEPC frequency initially reached frequencies >350 s$^{-1}$ but then gradually fell approaching frequencies <50 s$^{-1}$. A progressive swelling and eventual distortion of mitochondria within the twitch motor neuron terminals occurred during prolonged exposure to 35 mM KP with elevated Ca$^{2+}$. After ~300 min in 35 mM KP with elevated Ca$^{2+}$, only 58% of the twitch terminals accumulated FM1–43, and the frequency of MEPC occurrence decreased to 35 mM KP with elevated Ca$^{2+}$ was less when 15 mM glucose was present or when preparations were pretreated with 10 mM oligomycin and then bathed in the 35 mM KP with glucose. When glucose was present, with or without oligomycin pretreatment, a greater percentage of twitch terminals accumulated FM1–43. However, the mitochondria in these preparations were still greatly swollen and distorted. We propose that prolonged depolarization of twitch motor neuron terminals by 35 mM KP with elevated Ca$^{2+}$ produced a Ca$^{2+}$-induced decrease in mitochondrial ATP production. Under these conditions, the cytosolic ATP/ADP ratio decreased, thereby compromising both transmitter release and refilling of recycled synaptic vesicles. The addition of glucose stimulated glycolysis which contributed to the maintenance of ATP levels.

INTRODUCTION

Previous studies from our laboratory have demonstrated a calcium (Ca$^{2+}$)-dependent decline in transmitter release at potassium (K$^+$)-stimulated snake twitch fiber endplates (Connor et al. 1997). With control Ca$^{2+}$ (1 mM) in the K$^+$-depolarizing solution (35 mM potassium propionate, KP), miniature endplate current (MEPC) frequency increased and remained elevated for many hours (Connor et al. 1997). In contrast, with the Ca$^{2+}$ concentration elevated in the 35 mM KP solution to 3.6 mM, the MEPC frequency increased initially to many hundreds per second but then declined progressively to much lower frequencies, although MEPC amplitudes remained unchanged. On the basis of these observations, it was hypothesized that the progressive decline in transmitter release was related to an intraterminal accumulation of Ca$^{2+}$, which, in turn, either depressed some step in the release process or inhibited endocytosis leading to depletion of synaptic vesicles (Connor et al. 1997).

In an earlier study, Coniglio et al. (1993) had demonstrated that during exposure to isotonic KP with both control and elevated Ca$^{2+}$, the number of synaptic vesicles present in the chronically depolarized nerve terminals was consistently less than that in resting terminals. More recently, Lindgren et al. (1997) demonstrated that exposure to solutions that contained organic anions such as propionate in place of chloride resulted in acidification of the nerve terminal cytosol. Acidification of the terminal produced by concentrations of K propionate >100 mM reversibly inhibited endocytotic retrieval of synaptic vesicle membrane (Lindgren et al. 1997). Thus the progressive decline in transmitter release observed in the study of Coniglio et al. (1993) very likely resulted, at least in part, from a low pH-induced inhibition of endocytosis and depletion of synaptic vesicles. In contrast, with 35 mM KP, the extent of acidification of the nerve terminal cytosol did not affect endocytosis (Lindgren et al. 1997). Thus the Ca$^{2+}$-induced decrease in transmitter release produced during continued exposure to 35 mM KP very likely was due to other mechanisms (Connor et al. 1997).

von Gersdorff and Matthews (1994) reported that an elevation of intracellular Ca$^{2+}$ directly depressed endocytosis in goldfish bipolar cells. Thus with prolonged depolarization, one mechanism considered previously by Connor et al. (1997) was that endocytosis was gradually inhibited as Ca$^{2+}$ accumulated in the nerve terminal. However, recent results of Reuter and Prozig (1995) and Wu and Betz (1996) suggested that the rate of endocytosis was not correlated with the intracellular Ca$^{2+}$ concentration for cultured hippocampal neurons and frog motor neuron terminals. Thus direct inhibition of endocytosis by sustained elevation of intraterminal Ca$^{2+}$ might not be the underlying mechanism.

Coniglio et al. (1993) also noted that with continued exposure to isotonic KP (with either control or elevated Ca$^{2+}$), the mitochondria within the chronically depolarized nerve terminals progressively became swollen and distorted. Mitochondria take up Ca$^{2+}$ when intracellular Ca$^{2+}$ concentrations rise to levels >500 nM, a mechanism that is now recognized as an important aspect of cytosolic Ca$^{2+}$ homeostasis (Gunter and...
Gunter 1994; Gunter et al. 1994; Herrington et al. 1996; Park et al. 1996; Werth and Thayer 1994). However, when the intracellular Ca\(^{2+}\) concentration remains elevated for prolonged periods causing mitochondria to accumulate excessive Ca\(^{2+}\), the mitochondrial membrane potential begins to diminish (Isaev et al. 1996; Schinder et al. 1996; White and Reynolds 1996). Excessive accumulation of Ca\(^{2+}\) coupled with the loss of the potential gradient can activate the mitochondrial transition pore, which leads to mitochondrial swelling and interruption of ATP production (Isaev et al. 1996; Kristal and Dubinski 1997; Zamzami et al. 1997). Given that mitochondria produce the ATP required to support energy-dependent processes in motor neuron terminals, a progressive loss of ATP production potentially could compromise nerve terminal function.

We considered that an alteration in mitochondrial function might be involved in the Ca\(^{2+}\)-induced depression of transmitter release at nerve terminals exposed to 35 mM KP. Consequently, we hypothesized that in 35 mM KP with elevated Ca\(^{2+}\), but not control Ca\(^{2+}\), the depolarization-induced influx of Ca\(^{2+}\) into twitch motor neuron terminals might elevate intraterminal Ca\(^{2+}\) to levels high enough to promote excessive accumulation of Ca\(^{2+}\) within mitochondria. Furthermore we proposed that as the mitochondria continued to accumulate Ca\(^{2+}\), mitochondrial ATP production gradually became depressed, which led to a progressive decline in nerve terminal ATP levels and depression of ATP-dependent mechanisms required to sustain transmitter release.

In the present study, we have tested whether a Ca\(^{2+}\)-induced alteration of mitochondrial morphology and depression of ATP production contributed to the Ca\(^{2+}\)-induced decline in transmitter release at snake twitch motor neuron terminals maintained in 35 mM KP. The study combines voltage-clamp recordings of MEPCs, optical assay of vesicle membrane recycling using the activity-dependent dye FM1–43, and electron microscopic examination of mitochondrial morphology. Ultrastructural studies were needed because change in intraterminal organelle morphology was not examined by Connor et al. (1997) in their study of the decline in MEPC frequency during exposure to 35 mM KP with elevated Ca\(^{2+}\).

The results of this study demonstrated that in twitch nerve terminals exposed to 35 mM KP with elevated Ca\(^{2+}\), but not with control Ca\(^{2+}\), the mitochondria progressively became swollen and distorted. Furthermore the alteration in mitochondrial morphology occurred when MEPC frequency began to decline. In addition, exposure to glucose to stimulate nerve terminal glycolysis, and thus increase ATP production, reduced the decline in transmitter release.

**Methods**

Experiments were performed on visually identified twitch muscle fiber endplates in the costocutaneous muscle of garter snakes (Thamnophis) at room temperature (21–23°C). Snakes were killed by rapid decapitation, and muscle preparations were dissected and pinned to the bottom of silicone elastomer (Sylgard)-coated plastic dishes containing a N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-buffered control physiological solution, which contained (in mM) 159 NaCl, 2.5 KCl, 1.0 CaCl\(_2\), 4.2 MgCl\(_2\), and 5.0 HEPES, pH 7.3 (Connor et al. 1993; Connor et al. 1984, 1997). Transmitter release was induced by exposure to a potassium depolarizing solution in which 35 mM NaCl was replaced by 35 mM potassium propionate (KP). The 35 mM KP solution with “control” Ca\(^{2+}\) contained (in mM) 126 NaCl, 35 K propionate, 1.0 CaCl\(_2\), 4.2 MgCl\(_2\), 5.0 CsCl, and 5.0 HEPES, pH 7.3 (Connor et al. 1997). The cesium chloride was included to facilitate voltage clamping depolarized muscle fibers to hyperpolarized potentials (Coniglio et al. 1993; Connor et al. 1984, 1997). For most experiments in the present study, the 35 mM KP solution contained an “elevated” Ca\(^{2+}\) concentration (3.6 mM) and no added magnesium. In a few experiments, 1.6 mM magnesium was present with 3.6 mM Ca\(^{2+}\) in the 35 mM KP solution. Because the results were not affected by the presence of magnesium, data obtained using solutions with or without magnesium were presented together. In one series of experiments, sodium (Na\(^{+}\)) was replaced by lithium (Li\(^{+}\)) in the 35 mM KP solution containing 3.6 mM Ca\(^{2+}\) (Li-KP) to minimize Ca\(^{2+}\) extrusion by Na\(^{+}\)-Ca\(^{2+}\) exchange from the motor neuron terminals (Blaustein 1988; Missiaen et al. 1993).

For other experiments, muscle preparations were treated with oligomycin (10 μM, a mixture of oligomycin A, B, and C, Sigma, St. Louis, MO) to inhibit the mitochondrial ATP synthase. Oligomycin was dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock aliquots, frozen, and diluted each day to 10 μM in either the control physiological solution or the 35 mM KP solution. Generally, the muscle preparations were pretreated with oligomycin in the control physiological solution for ~20 min before exposure to the 35 mM KP solutions, which also contained oligomycin. In other experiments, 15 mM glucose was added to the 35 mM KP solution.

**Electrophysiology**

Twitch muscle fibers were identified using criteria described in previous reports (Coniglio et al. 1993; Connor et al. 1984, 1997; Dionne and Parsons 1981). There are two types of twitch fibers in snake muscle: slower twitch and faster twitch fibers (Lichtman and Wilkinson 1987; Wilkinson and Lichtman 1985). In these experiments, we did not distinguish between twitch fiber types. MEPCs were recorded from individual twitch fiber endplates bathed in the control physiological solution or kept for various lengths of time in 35 mM KP (Coniglio et al. 1993; Connor et al. 1984, 1997). The fibers were voltage clamped to −150 mV to increase the driving force for MEPCs and thus increase the signal-to-noise ratio. Current records were stored on a PCM recorder (A. R. Vetter, Rebersburg, PA) for subsequent digitization and analysis. Current records were digitized using the SCAN program (generously provided by Dr. John Dempster, University of Strathclyde, Glasgow, Scotland), and the frequency of MEPCs for a given recording was determined by counting events displayed on computer traces (Connor et al. 1997). Averaged data are expressed as means ± SE.

**Optical identification of nerve terminals and assay of synaptic vesicle recycling**

Twist neuromuscular junctions were visualized by staining with rhodamine-conjugated peanut agglutinin (PNA, Sigma), which marks synaptic and terminal Schwann cell basal laminae (Connor et al. 1997; Ko 1987). Muscle preparations were exposed to PNA (33 μg/ml; dissolved in the control physiological solution) for ~15 min and then rinsed. Optical estimates of vesicle release and recycling were made with the use of K\(^{+}\)-stimulated transmitter release coupled with accumulation of the fluorophore FM1–43 (2 μM; Molecular Probes, Eugene, OR) into recycling synaptic vesicles (Betz and Bewick 1992, 1993; Betz et al. 1992). Muscle preparations were exposed to FM1–43 during the last 5–6 min of an ~300-min exposure to the 35 mM KP solutions, then washed for ≥15 min in the control physiological solution before viewing.

Nerve terminals were examined with a Zeiss fluorescence photomicroscope equipped with filter sets appropriate for FM1–43 (green emission filter, 520–560 nm) or rhodamine (red emission filter, >590 nm). A ×40 water immersion lens was used to locate individual nerve terminals. Background levels of fluorescence were established by
examining endplates that had been exposed to FM1–43 for 5–6 min in physiological solution, a condition in which snake nerve terminal boutons are not stained by FM1–43 (Connor et al. 1997). FM1–43 staining was used at twitch nerve terminals maintained for >300 min in a 35 mM KP solution, which contained control Ca$^{2+}$, to establish positive FM1–43 staining. The FM1–43 was present for the final 5–6 min of exposure to the KP solution. Previously Connor et al. (1997) demonstrated that transmitter release continued at high rates for many hours in a 35 mM KP solution containing control Ca$^{2+}$ (1.0 mM Ca$^{2+}$) and 35 mM twitch nerve terminals were uniformly stained by FM1–43. In each experiment, results from endplates stained with FM1–43 after exposure to the experimental 35 mM KP solutions were compared with endplates maintained only in the control physiological solution or endplates exposed to the 35 mM KP solution with control Ca$^{2+}$. Endplates were examined visually and scored, as in our previous study, as uniformly stained, partially stained, or unstained (Connor et al. 1997). A nerve terminal was considered uniformly stained if all boutons within a nerve terminal were stained and considered partially stained if at least one, but not all boutons, were stained.

Electron microscopy

Nerve-muscle preparations were fixed and prepared for ultrastructural examination. The muscles used for electron microscopy were taken from the same snakes that had been used to provide muscles for MEPC recordings. The methods used to examine the ultrastructure of the motor neuron terminals at twitch fiber endplates followed those described previously (Congilio et al. 1993). Snake muscles were pinned to the bottom of Sylgard-coated plastic dishes and exposed for different durations of time either to the control physiological solution or to 35 mM KP solutions. Endplates from at least two different muscle preparations from two different snakes were examined for each condition. Preparations were fixed for 15 min in 2% glutaraldehyde, washed in fresh Millonig’s phosphate buffer, postfixed for 30 min in 1% osmium tetroxide, and washed again in buffer. The preparations were dehydrated in a graded series of ethanols to 100% and embedded in a resin mixture of Embed 812–Araldite. Araldite was removed from between the slides and examined on a compound light microscope to identify thick sections that contained endplates. Appropriate thick sections were remounted onto the ends of precast Embed–Araldite blocks. The blocks were polymerized, trimmed, and ultrathin sectioned (78–80 nm). Thin sections were examined and photographed on either a JEOL 100CX or 100S electron microscope.

RESULTS

MEPC frequency and nerve terminal morphology during prolonged exposure to 35 mM KP with elevated Ca$^{2+}$

MEPC frequency remained elevated for many hours in muscles bathed in 35 mM KP with control Ca$^{2+}$ (Fig. 1A). In contrast, for muscles kept in 35 mM KP with elevated Ca$^{2+}$ (3.6 mM), the MEPC frequency initially rose to high values but then declined. The time course of this decline in MEPC frequency is presented in Fig. 1A. These results were obtained by recording MEPCs from different twitch fiber endplates at different times in the KP solution.

The morphology of the nerve terminals from muscle preparations maintained for ~480 min in 35 mM KP with normal Ca$^{2+}$ (Fig. 1F) was similar to that of twitch nerve terminals in muscles maintained in the physiological solution (Fig. 1B). Generally, synaptic vesicles still were present in abundant numbers, and the mitochondria, although often larger, were not extensively swollen or distorted. In addition, the majority of the mitochondria were located some distance from the side of the boutons facing the synaptic cleft. In contrast, there was a marked time-dependent change in the morphology of the mitochondria in twitch motor neuron terminals exposed to 35 mM KP with elevated Ca$^{2+}$. The mitochondria were consistently swollen but were readily discernible after ~150 min in 35 mM KP with high Ca$^{2+}$. The mitochondria became more swollen (Fig. 1D) with longer exposures and often were distorted to such an extent that their internal organization was no longer apparent (Fig. 1E). In these same preparations, there was no obvious change in the morphological appearance of mitochondria in the muscle fibers (Fig. 1C). Thus the progressive swelling of the mitochondria was limited to mitochondria located in the nerve terminal. In addition, in many boutons, numerous synaptic vesicles were present both after an ~150- and ~480-min exposure to 35 mM KP with elevated Ca$^{2+}$. However, the number of synaptic vesicles varied between terminals and in some boutons the density of synaptic vesicles was reduced.

The consistent change in mitochondrial morphology provided support for the hypothesis that a gradual loss of mitochondrial function might be a factor contributing to the progressive decline in MEPC frequency that occurred when preparations were kept in 35 mM KP with elevated Ca$^{2+}$. Consequently, additional electrophysiological and morphological experiments were done to test this hypothesis further. In all subsequent experiments, the exposure to the 35 mM K$^+$ solution with 3.6 mM Ca$^{2+}$ ranged between 300 and 360 min because both the decline in MEPC frequency and alteration in mitochondrial morphology were evident by this time.

Exocytosis and mitochondrial morphology with lithium (Li$^+$) substituted for sodium (Na$^+$) in 35 mM KP with elevated Ca$^{2+}$

A number of organelles and cellular processes contribute to the regulation of the intracellular Ca$^{2+}$ concentration (Gunter et al. 1994; Missiaen et al. 1993). With sustained intraterminal Ca$^{2+}$ loading, an important pathway for Ca$^{2+}$ extrusion should be the low-affinity, high-capacity surface membrane Na$^+$–Ca$^{2+}$ exchange mechanism (Blaustein 1988). Substitution of Li$^+$ for extracellular Na$^+$ is known to effectively inhibit Na$^+$–Ca$^{2+}$ exchange (Park et al. 1996). We proposed that elimination of this high-capacity extrusion pathway in the motor neuron terminal might facilitate intraterminal Ca$^{2+}$ loading and lead to a more rapid accumulation of Ca$^{2+}$ into mitochondria and dissipation of the mitochondrial membrane potential. Thus mitochondria might become compromised more quickly, leading to a more rapid decline in MEPC frequency. Consequently we initiated experiments to determine the time course of decline in MEPC frequency when Li$^+$ was replaced for Na$^+$ in the 35 mM KP solution containing elevated Ca$^{2+}$ (Li-KP).

We recorded MEPCs from different twitch fibers during continuous exposure to Li-KP. After ~20 min in Li-KP, the MEPC frequency appeared to be <100 s$^{-1}$ (Fig. 2A). How-
ever, even with exposure times <20 min, many MEPCs were very small. With longer durations in Li-KP, the amplitude of most MEPCs continued to decline (Fig. 2, B–D). Eventually, it became virtually impossible to accurately estimate the MEPC frequency, although inward current fluctuations in the noise, like those shown in Fig. 2D, were evident in recordings from twitch endplates exposed to Li-KP for periods ≥300 min. Comparable current fluctuations were not observed when recordings were made from nonjunctional areas of the muscle fiber (Fig. 2E).

We compared FM1–43 incorporation into recycling synaptic vesicles at twitch nerve terminals after an ∼300-min exposure to either 35 mM KP with elevated Ca²⁺ or Li-KP, also with elevated Ca²⁺. The FM1–43 was included in the respective KP solutions for the final 5–6 min of exposure, and the muscle preparations then were bathed in physiological solution containing PNA for ∼15 min to locate the endplates on individual muscle fibers (Connor et al. 1997). All PNA-identified nerve terminals innervating twitch endplates in preparations maintained for ≥300 min in Li-KP were stained by FM1–43. Intensity of the staining varied between nerve terminals with 94% of the terminals uniformly stained and 6% partially stained (Table 1). In contrast, as previously shown by Connor et al. (1997), only 58% of the nerve terminals innervating twitch endplates in preparations exposed to 35 mM KP with

![Figure 1](http://jn.physiology.org/)

**FIG. 1.** Nerve terminal morphology and miniature endplate current (MEPC) frequency at twitch fiber endplates during prolonged exposure to 35 mM potassium propionate (KP). A: change in MEPC frequency at twitch endplates when snake muscle preparations are depolarized by exposure to an elevated potassium (35 mM K⁺) solution containing either control Ca²⁺ (1 mM Ca²⁺ and 4.2 mM Mg²⁺; ○) or elevated Ca²⁺ (3.6 mM) (○). B–F: electron micrographs of motor neuron terminals; B: terminal kept in the sodium solution. C: terminal after 2 h in the 35 mM K⁺ solution with elevated Ca²⁺. D and E: 2 terminals after 8 h in the 35 mM K⁺ solution with elevated Ca²⁺. F: terminal kept in the 35 mM K⁺ solution with control Ca²⁺ for 8 h. M, mitochondria; SV, synaptic vesicles.

![Figure 2](http://jn.physiology.org/)

**FIG. 2.** MEPC frequency and amplitude decrease during exposure to Li-KP solution, which also contained elevated Ca²⁺. A–E: current records obtained at different times in Li-KP. A–D were recorded after 17, 52, 65, and 179 min, respectively. E was recorded from a nonjunctional region of the muscle fiber after 72 min in Li-KP. Calibration equals: y axis, 5 nA; x axis, 10 ms.
Ultrastructural studies were completed to compare the morphology of motor neuron terminals innervating twitch endplates in preparations that had been kept for ~300 min in 35 mM KP with control Ca\(^{2+}\) (Fig. 3A), 35 mM KP with elevated Ca\(^{2+}\) (Fig. 3B), or Li-KP (Fig. 3C). Synaptic vesicles were present in all three examples. Omega figures commonly were noted in nerve terminals maintained in 35 mM KP with control Ca\(^{2+}\) and Li-KP (Fig. 3, A and C). This latter observation indicated exocytosis still was occurring, consistent with the high percentage of terminals stained by FM1–43 under these two conditions (Table 1). The morphology of mitochondria varied among the different nerve terminals. The mitochondria were not markedly enlarged in terminals maintained in 35 mM KP with control Ca\(^{2+}\) for ~300 min (Fig. 3A). In contrast, the majority of mitochondria in terminals maintained in 35 mM KP with elevated Ca\(^{2+}\) or Li-KP for ~300 min were swollen. Furthermore in some boutons, some mitochondria were distorted to the extent that the internal organization could no longer be discerned. The distortion appeared to be greater for terminals maintained in 35 mM KP with elevated Ca\(^{2+}\) than in terminals kept in Li-KP. In addition, for preparations kept in Li-KP, the morphology of individual mitochondria within

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<th>Condition</th>
<th>Uniform Stain, %</th>
<th>Partial Stain, %</th>
<th>Unstained, %</th>
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<tr>
<td>1.0 mM Ca(^{2+})</td>
<td>100*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.6 mM Ca(^{2+})/Na(^{+})</td>
<td>44 ± 13</td>
<td>14 ± 5</td>
<td>42 ± 11</td>
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<tr>
<td>3.6 mM Ca(^{2+})/Li(^{+})</td>
<td>94 ± 6</td>
<td>6 ± 6</td>
<td>0</td>
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<tr>
<td>3.6 mM Ca(^{2+}) + oligo</td>
<td>59 ± 26</td>
<td>3 ± 3</td>
<td>37 ± 24</td>
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<tr>
<td>3.6 mM Ca(^{2+}) + oligo + glucose</td>
<td>95 ± 3</td>
<td>5 ± 3</td>
<td>0</td>
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<td>3.6 mM Ca(^{2+}) + glucose</td>
<td>73 ± 10</td>
<td>4 ± 3</td>
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*Values are percentages of neuromuscular junctions stained or not stained by exposure to FM1–43 during the final 5–6 min of the ~5-h exposure to 35 mM potassium propionate (KP). The muscles then were washed with a physiological sodium solution and stained with peanut agglutinin (PNA) to identify junctions. Data obtained from 7 to 25 endplates per muscle preparation: 1 mM Ca\(^{2+}\), 3 muscle preparations; 3.6 mM Ca\(^{2+}\)/Na\(^{+}\), 6 muscle preparations; 3.6 mM Ca\(^{2+}\)/Li\(^{+}\), 3 muscle preparations; 3.6 mM Ca\(^{2+}\) + oligo, 3 muscle preparations; 3.6 mM Ca\(^{2+}\) + oligo + glucose, 3 muscle preparations; 3.6 mM Ca\(^{2+}\) + glucose, 4 muscle preparations.
the same bouton varied noticeably with some only slightly swollen, whereas others appeared to be completely distorted.

Stimulation of glycolysis can sustain transmitter release during prolonged exposure to 35 mM KP with elevated Ca$^{2+}$

The dependence of transmitter release on ATP production within the nerve terminals was investigated by testing whether inhibition of the mitochondrial ATP synthase or stimulation of glycolysis affected the decline in MEPC frequency. Preparations were treated with 10 μM oligomycin alone to inhibit the mitochondrial ATP synthase, oligomycin and 15 mM glucose to inhibit the ATP synthase and to stimulate glycolysis, or glucose alone. The muscle preparations were pretreated with 10 μM oligomycin in the control physiological solution for ~15 min before exposure to the KP solution, which also contained oligomycin. Glucose (15 mM) was present only during exposure to the 35 mM KP solution.

During the initial exposure to 35 mM KP with elevated Ca$^{2+}$, MEPC frequency at twitch endplates in untreated muscle preparations reached values >350 s$^{-1}$. The frequency then declined progressively during a 360-min recording period (Fig. 1A), remaining elevated above ~200 s$^{-1}$ at many endplates until ~120 min and then falling progressively to much lower values. For example, the MEPC frequency recorded from 10 twitch endplates in untreated muscles during the first 120 min of exposure to 35 mM KP with high Ca$^{2+}$ averaged 209 ± 31.7 s$^{-1}$. However, after ~200 min in the 35 mM KP solution with elevated Ca$^{2+}$, MEPC frequency was consistently <50 s$^{-1}$ (Table 2).

The MEPC frequency recorded at twitch endplates from muscles treated with oligomycin alone or oligomycin plus glucose also was >200 s$^{-1}$ during the first 120 min in 35 mM KP containing Ca$^{2+}$. In those preparations treated with only oligomycin, the MEPC frequency after 200 min declined to values even lower than those recorded just in 35 mM KP with elevated Ca$^{2+}$ (Table 2). In contrast, the combination of oligomycin treatment and addition of glucose significantly reduced the decline in MEPC frequency at many endplates (Table 2). At 15 of 24 oligomycin-treated endplates, the MEPC frequency during a 240- to 360-min exposure to 35 mM KP with oligomycin and glucose remained >150 s$^{-1}$; whereas MEPC frequency was <50 s$^{-1}$ at only eight endplates. After a 240- to 360-min exposure to 35 mM KP with glucose alone, the MEPC frequency remained >150 s$^{-1}$ at 10 of 28 endplates, whereas the frequency was <50 s$^{-1}$ at 15 endplates (Table 2).

FM1-43 incorporation was determined at twitch nerve terminals after an ~300-min exposure to the 35 mM KP with elevated Ca$^{2+}$ in muscle preparations that had been treated with 10 μM oligomycin alone, oligomycin in combination with 15 mM glucose, or just 15 mM glucose. Approximately 60% of the nerve terminals innervating twitch endplates in the three preparations treated with oligomycin alone and maintained for >300 min in KP with high Ca$^{2+}$ were stained uniformly by FM1-43 (Table 1). Another 3% were partially stained, and the remaining 37% did not accumulate FM1-43. In contrast, all twitch nerve terminals in the three preparations treated with oligomycin and glucose accumulated FM1-43. The staining was generally very strong with 95% of the nerve terminals uniformly stained and 5% partially stained (Table 1). For those preparations bathed in 35 mM KP containing just glucose, ~77% of the twitch nerve terminals were stained by FM1-43: 74% uniformly and 3% partially. The remaining PNA-identified twitch nerve terminals did not appear to be stained by FM1-43.

Ultrastructural studies also were done to compare the morphology of motor neuron terminals innervating twitch endplates for the aforementioned three treatments. All muscle preparations were fixed after an ~300 min exposure to 35 mM KP with elevated Ca$^{2+}$. There was no qualitative difference in the morphology of the boutons within twitch motor neuron terminals in these three groups of muscles (Fig. 3, D–F). Synaptic vesicles were present within most nerve terminals. In all cases, the mitochondria were swollen (Fig. 4), suggesting that none of the conditions reversed the progressive alteration of mitochondrial morphology that occurred with prolonged exposure to 35 mM KP with elevated Ca$^{2+}$.

![Figure 4](image-url)

**Figure 4.** High-power electron micrograph demonstrating the alternation in mitochondrial morphology evident after ~300 min in 35 KP. A: bouton from a preparation bathed in the KP solution with elevated Ca$^{2+}$. B: bouton from a preparation pretreated with 10 μM oligomycin and then bathed in KP with elevated Ca$^{2+}$, 10 μM oligomycin, and 15 mM glucose. M, mitochondria; SV, synaptic vesicles; →, omega figures.
DISCUSSION

The objective of the present study was to elucidate mechanisms underlying the Ca\textsuperscript{2+}-induced inhibition of transmitter release at 35 mM KP-depolarized motor neuron terminals. Two key observations were that coincident with the decline in MEPC frequency was a progressive swelling and distortion of nerve terminal mitochondria and stimulation of glycolysis allowed transmitter release to continue at high rates in many K\textsuperscript+-depolarized terminals even though mitochondria were distorted. On the basis of these observations, we propose that a major factor in the Ca\textsuperscript{2+}-induced progressive decline in MEPC frequency was a gradual decrease in mitochondrial ATP production and consequent decrease in the cytosolic ATP/ADP ratio, which progressively impaired ATP-dependent processes in the nerve terminal.

Mitochondria can accumulate significant amounts of Ca\textsuperscript{2+} as cytosolic levels exceed 500 nM (Friel and Tsien 1994; Gunter and Gunter 1994; Gunter et al. 1994; Herrington et al. 1996; Kriedowski and Costa 1995; Park et al. 1996; Wang and Thayer 1994; White and Reynolds 1995). However, if mitochondrial Ca\textsuperscript{2+} exceeds mM levels, morphological alterations, similar to those observed in this study, were observed in cerebellar granule cells (Isaev et al. 1996). Thus when bath Ca\textsuperscript{2+} was elevated in the present study, Ca\textsuperscript{2+} accumulation within the nerve terminal must have been high enough to exceed the various Ca\textsuperscript{2+}-buffering mechanisms within the nerve terminal. The result of the excess Ca\textsuperscript{2+} in the terminal cytosol was a progressive Ca\textsuperscript{2+} overload of the mitochondria. In contrast, even though Ca\textsuperscript{2+} influx continued with prolonged depolarization in 35 mM KP with control Ca\textsuperscript{2+}, the Ca\textsuperscript{2+} buffering mechanisms that regulate intraterminal Ca\textsuperscript{2+} must have been sufficient to avoid extensive Ca\textsuperscript{2+} accumulation.

Accumulation of FM1–43 into the nerve provided evidence that endocytotic retrieval of synaptic vesicle membrane was occurring (Betz and Bewick 1992, 1993). Previously, we demonstrated that if transmitter release is not occurring at rates well above the resting level, then constitutive endocytosis of vesicle membrane is not sufficient to stain snake motor neuron terminals (Connor et al. 1997). Thus we have interpreted positive FM1–43 staining to indicate that both exocytosis and endocytosis were occurring at a highly accelerated rate. Under most experimental conditions used in the present study, the percentage of twitch motor neuron terminals positively stained by FM1–43 was consistent with the MEPC frequency values obtained after >300 min in the 35 mM KP with 3.6 mM Ca\textsuperscript{2+}; i.e., the higher the mean MEPC frequency, the greater the percentage of terminals positively stained (compare results in Tables 1 and 2). The exceptions to this general correlation were that in the oligomycin-treated preparations, ~60% of the terminals were FM1–43 positive and in the Li-KP preparations 100% of the terminals were stained. In both cases, FM1–43 staining occurred when MEPC frequency was below the rate normally required to stain terminals. It could be, although we consider it unlikely, that in the oligomycin-treated nerve terminals, endocytosis was stimulated independently of exocytosis. However, the number of synaptic vesicles present in the nerve terminals in the oligomycin-treated preparations was not greater than that seen in nerve terminals in other preparations, which also were exposed to 35 mM KP with elevated Ca\textsuperscript{2+} but not treated with oligomycin (Fig. 3). A greater number of vesicles would be expected if endocytosis was occurring at a high rate but exocytosis was not equally enhanced. We favor an alternative explanation. We suggest that exocytosis was occurring at numerous terminals, but the synaptic vesicles had not been refilled, and the only MEPCs recorded were those from vesicles still remaining in preformed quantal stores. ATP is required for refilling of recycling synaptic vesicles (Parsons et al. 1993). Given that the preparations were pretreated with oligomycin for ~15 min in control solution, mitochondrial ATP production should have been inhibited before exposure to 35 mM KP. We propose that with ATP production inhibited, intraterminal ATP levels very likely would have fallen substantially after ~240 min in the elevated K\textsuperscript{+} solution, and as a consequence, recycling vesicles would not have been refilled with acetylcholine (ACh) in these terminals.

MEPC amplitudes decreased progressively during exposure to the Li-KP solution, which also contained elevated Ca\textsuperscript{2+}. We attributed this to a gradual depletion of choline stores in the nerve terminal and a reduced filling of synaptic vesicles. Choline uptake into nerve terminals is a Na\textsuperscript{+}-dependent process and is inhibited when Li\textsuperscript{+} is substituted for Na\textsuperscript{+} (van der Kloot and Molgo 1994). Therefore we suggest that the apparent decrease in MEPC frequency resulted mostly from the progressive release of increasingly smaller quanta of ACh until most events were lost in the recording noise and not from an inhibition of exocytosis. This very progressive decrease in MEPC amplitude is similar to that produced when choline uptake is inhibited pharmacologically, such as with hemicholinium (Elmqvist and Quastel 1965). Evidence for continued exocytosis of partially filled or empty vesicles was obtained both from the positive FM1–43 staining (Table 1) and presence of synaptic vesicles and omega figures (Fig. 3). Also we suggest that the current fluctuations recorded after prolonged exposure to Li-KP most likely represented current responses produced by ACh released from partially filled synaptic vesicles.

At present, we can only speculate why exocytosis continued in motor neuron terminals exposed to Li-KP. From inspection of mitochondria in nerve terminals exposed to the different conditions, it appeared that the numbers and extent of severely distorted mitochondria might be less in the terminals exposed to the Li\textsuperscript{+}-substituted solution. Consequently, it is possible that exocytosis continued simply because there was slightly less extensive physical disruption of the mitochondria and ATP production continued for longer periods. Even though this possibility could not be adequately quantitated with the present data, a potential mechanism by which mitochondria might be spared somewhat in Li-KP can be considered. External Na\textsuperscript{+} is required to sustain cell membrane Na\textsuperscript{+}-H\textsuperscript{+} exchange, which would transport H\textsuperscript{+} out of the nerve terminal to maintain cytoplasmic pH within a physiological range. With prolonged exposure to the Li\textsuperscript{+}-substituted solution, the intraterminal H\textsuperscript{+} concentration should increase progressively, causing the intraterminal pH to decrease. A decrease in pH protects against activation of the mitochondrial transition pore, thus decreasing the likelihood of mitochondrial swelling and loss of membrane potential (Bernardi et al. 1994; Nicoll et al. 1993). Therefore in Li-KP, the integrity of some mitochondria might be prolonged and ATP production might have continued longer even though the mitochondria had accumulated amounts of Ca\textsuperscript{2+} that normally would cause activation of the transition pore. If this explanation is valid, then the cytoplasmic ATP/ADP ratio...
might have been maintained longer, which could support ATP-dependent mechanisms.

The most extensive decline in MEPC frequency was observed when the preparations were pretreated with oligomycin to inhibit the ATP synthase (Table 2). The mitochondria in motor neuron terminals in these preparations were very distorted. We postulate that when the ATP synthase was inhibited, the mitochondria had no mechanism available to maintain the membrane potential. Thus the combination of the extreme Ca\(^{2+}\) accumulation and decline in membrane potential facilitated activation of the transition pore and led to the marked alteration in mitochondrial morphology and loss of function.

MEPC frequency remained high at many terminals when glucose was added to the 35 mM KP solution with elevated Ca\(^{2+}\), even though marked morphological changes in the mitochondria were still evident. We suggest that the presence of glucose did not protect mitochondrial function but rather stimulated glycolysis, which helped to maintain cytoplasmic ATP levels. The combination of glucose and pretreatment with oligomycin further enhanced the ability of terminals to maintain transmitter release at high rates. Therefore when the ATP synthase was inhibited and thus could not operate in a reverse mode, cytosolic ATP was not consumed by the mitochondria in an attempt to maintain the membrane potential (Budd and Nichols 1996) and the glucose-stimulated glycolysis must have sustained cytosolic ATP levels more effectively.

In recent years, evidence has accumulated that strongly suggests that an alteration in mitochondrial function may be a critical factor in programmed cell death and in the etiology of many neurodegenerative diseases (Beal 1992; Kristal and Duibinski 1997; Orrenius and Nicotera 1994; Petit et al. 1996; Zamzami et al. 1997). Impairment of energy metabolism and loss of Ca\(^{2+}\) buffering by mitochondria have been suggested to be key factors in excitotoxic neuronal death (Kiedrowski and Costa 1995; Schinder et al. 1996; White and Reynolds 1996). Mitochondria are a critical Ca\(^{2+}\) buffering mechanism during glutamate-induced Ca\(^{2+}\) loading (Kiedrowski and Costa 1995; Wang and Thayer 1996; White and Reynolds 1995). However, if the buffering capacity of mitochondria is overwhelmed, then the mitochondrial membrane potential can collapse and marked ultrastructural changes in the mitochondria occur (Isaev et al. 1996; Schinder et al. 1996). Thus accumulation of excess Ca\(^{2+}\) by mitochondria and subsequent activation of the permeability transition pore may be a critical early event in the development of glutamate-induced excitotoxicity of neurons within or derived from the CNS (Kristal and Dubinski 1997; Zamzami et al. 1997). The alteration in mitochondrial morphology and disruption of ATP metabolism produced in cultured neurons during glutamate-induced excitotoxicity are similar to those observed in the present study for motor neuron terminals exposed to the 35 mM KP solution with elevated Ca\(^{2+}\) (Isaev et al. 1996; Schinder et al. 1996).

It is also well documented that after disruption of blood flow to areas within the brain, ischemia develops and extracellular K\(^{+}\) levels rise dramatically in the affected area (Kristian and Siesjo 1996; Sweeney et al. 1995). Therefore in addition to becoming deprived of oxygen and glucose, the elevation in extracellular K\(^{+}\) very likely causes a prolonged depolarization of neurons and axons passing through this region. The effects of prolonged depolarization on the nerve terminals in an ischemic region within the brain would be extremely difficult to study because of their small size. Thus we suggest that the isolated motor neuron nerve terminal is a convenient model system to analyze changes in presynaptic function by conditions that produce mitochondrial dysfunction.

In conclusion, the results of the present study show that a Ca\(^{2+}\)-dependent disruption of mitochondrial morphology and function could be produced in isolated, chronically depolarized motor neuron terminals. The alteration in presynaptic function was probably not due to a direct effect of the elevated intraterminal Ca\(^{2+}\) concentration per se but more likely was a secondary consequence of the Ca\(^{2+}\)-induced disruption of mitochondrial metabolic activity.

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