Stimulation-Evoked Increases in Cytosolic [Ca\textsuperscript{2+}] in Mouse Motor Nerve Terminals Are Limited by Mitochondrial Uptake and Are Temperature-Dependent

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Increases in cytosolic [Ca\textsuperscript{2+}] evoked by trains of action potentials (20–100 Hz) were recorded from mouse and lizard motor nerve terminals filled with a low-affinity fluorescent indicator, Oregon Green BAPTA 5N. In mouse terminals at near-physiological temperatures (30–38°C), trains of action potentials at 25–100 Hz elicited increases in cytosolic [Ca\textsuperscript{2+}] that stabilized at plateau levels that increased with stimulation frequency. Depolarization of mitochondria with carbonyl cyanide m-chloro-phenylhydrazone (CCCP) or antimycin A1 caused cytosolic [Ca\textsuperscript{2+}] to rise to much higher levels during stimulation. Thus, mitochondrial Ca\textsuperscript{2+} uptake contributes importantly to limiting the rise of cytosolic [Ca\textsuperscript{2+}] during repetitive stimulation.

In mouse terminals, the stimulation-induced increase in cytosolic [Ca\textsuperscript{2+}] was highly temperature-dependent over the range 18–38°C, with greater increases at lower temperatures. At the lower temperatures, application of CCCP continued to depolarize mitochondria but produced a much smaller rise in the cytosolic [Ca\textsuperscript{2+}] transient evoked by repetitive stimulation. This result suggests that the larger amplitude of the stimulation-induced cytosolic [Ca\textsuperscript{2+}] transient at lower temperatures was attributable in part to reduced mitochondrial Ca\textsuperscript{2+} uptake.

In contrast, the stimulation-induced increases in cytosolic [Ca\textsuperscript{2+}] measured in lizard motor terminals showed little or no temperature-dependence over the range 18–33°C.

Key words: mitochondria; presynaptic terminal; motor nerve terminal; calcium indicator dyes; calcium sequestration; neuromuscular junction; lizard; temperature

When certain nerve terminals are stimulated with prolonged depolarizing pulses or trains of action potentials, the average cytosolic [Ca\textsuperscript{2+}] measured with fluorescent indicators increases rapidly at first but then stabilizes at a plateau level until stimulation ceases (Steunkel, 1994; Ravin et al., 1997; David et al., 1998). This stabilization of average cytosolic [Ca\textsuperscript{2+}] during continued Ca\textsuperscript{2+} influx is disrupted by agents that inhibit mitochondrial Ca\textsuperscript{2+} uptake (Steunkel, 1994; David et al., 1998), suggesting that mitochondrial Ca\textsuperscript{2+} uptake contributes importantly to sequestration of the Ca\textsuperscript{2+} loads entering stimulated nerve terminals. Ca\textsuperscript{2+} uptake via the mitochondrial uniporter is driven by the large negative potential (approximately −150 to −200 mV) created by proton transport across the inner mitochondrial membrane (for review, see Gunter and Pfeiffer, 1990).

For some secretory cells, additional evidence for mitochondrial Ca\textsuperscript{2+} uptake has been obtained using fluorescent or luminescent indicators localized within the mitochondrial matrix. Increases in matrix [Ca\textsuperscript{2+}] evoked by depolarization and/or hormones have been demonstrated in adrenal chromaffin cells (Babcock et al., 1997; Montero et al., 2000) and lizard motor nerve terminals (David et al., 1998). Mitochondrial Ca uptake has also been demonstrated by electron probe microanalysis of total Ca in frog sympathetic ganglion neurons fast-frozen after a 45 sec bath application of 50 mM K\textsuperscript{+} (Pivovarova et al., 1999). Simultaneous imaging of cytosolic and mitochondrial [Ca\textsuperscript{2+}] showed that, in lizard motor nerve terminals, mitochondrial Ca\textsuperscript{2+} uptake begins after as few as 25–50 action potentials delivered at 50–100 Hz, at approximately the same time that cytosolic [Ca\textsuperscript{2+}] reaches a plateau (David et al., 1998). In this preparation, as in crayfish motor nerve terminals, adrenal chromaffin cells, and several types of neuronal somata, mitochondria have been shown to be the dominant means of sequestering moderate to large Ca\textsuperscript{2+} loads (Friel and Tsien, 1994; Werth and Thayer, 1994; White and Reynolds, 1995; Her- rington et al., 1996; Park et al., 1996; Tang and Zucker, 1997; David, 1999; Colegrove et al., 2000).

The present study was undertaken to measure cytosolic [Ca\textsuperscript{2+}] transients evoked by physiological stimulation in mammalian (mouse) motor nerve terminals and to determine whether mitochondrial Ca\textsuperscript{2+} sequestration contributes to limiting the magnitude of these transients. We demonstrate that, at temperatures near physiological (33–38°C), the elevation of average cytosolic [Ca\textsuperscript{2+}] stabilizes during trains of action potentials (25–100 Hz) and that this stabilization is blocked by agents that depolarize mitochondria. This ability to limit stimulation-induced increases in cytosolic [Ca\textsuperscript{2+}] during high-frequency stimulation is impaired at cooler temperatures, attributable in part to decreased mitochondrial Ca\textsuperscript{2+} sequestration. In contrast, cytosolic [Ca\textsuperscript{2+}] transients in lizard motor nerve terminals show no detectable temperature-dependence over the range 18–33°C.

MATERIALS AND METHODS

Most experiments were performed on motor terminals innervating the internal oblique muscle, a fast-twitch neuromuscular preparation that is only one to two muscle fibers thick. Male mice (C57L, 2–4 months) were killed by an overdose of ether, followed by rapid cervical dislocation, and a piece of the abdominal wall containing the internal and external oblique muscles and the lumboinguinal nerve was removed and pinned to a layer of Sylgard. The overlying external oblique muscle was removed to permit access to axons and visualization of terminals on the underlying internal oblique muscle. In most experiments, the preparation was bathed for 5 min in a HEPES-buffered physiological saline (see below) containing 5 mg/ml collagenase (type I, Sigma, St. Louis, MO) to facilitate removal of connective tissue. (Recorded fluorescence transients were similar in collagenase-treated and nontreated preparations.) The experiments of Figure 3, C and D, used motor nerve terminals of the external intercostal muscle of lizards (Anolis sagrei), killed by decapitation and pitting after ether anesthesia as in David et al. (1998).

Identical physiological salines were used for mice and lizards. During dissection and ionophoretic injection of Oregon Green BAPTA 5N (OG-5N), the physiological saline contained (in mM): 157 NaCl, 4 KCl, 2 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 1 HEPE\textsubscript{S}, and 5 glucose. Preparations were mounted in a small...
chamber (0.2–0.5 ml) constructed on a thin (No. 1) glass coverslip and imaged with an inverted microscope using a 20 or 40× water immersion lens. During recordings, the physiological saline contained (in mM): 137 NaCl, 15 NaHCO3, 4 KCl, 1.8 CaCl2, 1.1 MgCl2, 0.33 NaH2PO4, and 11.2 glucose, equilibrated to pH 7.2–7.4 by maintaining a constant flux of 95% O2–5% CO2 above the bathing solution.

Motor nerves were stimulated by applying suprathreshold depolarizing current pulses (0.3 msec) via a suction electrode. Muscle contractions were subsequently blocked using 1–3 mg/l 1-tubocurarine. Stimulation trains were separated by 5–10 min intervals, the time required for mitochondrial [Ca2+]i to return to resting levels after comparable stimulation in lizard motor nerve terminals at room temperature (David, 1999). Drugs were applied by exchanging the bathing solution ~10 times with the desired solution. The temperature of the preparation was monitored by measuring the voltage across a small (1 mm) thermistor probe in the bath, calibrated over the range 0–40°C. The preparation was heated by passing current through a nichrome heating element embedded in a ring-shaped copper lens. During recordings, the physiological saline contained (in mM): 137 NaCl, 15 NaHCO3, 4 KCl, 1.8 CaCl2, 1.1 MgCl2, 0.33 NaH2PO4, and 11.2 glucose, equilibrated to pH 7.2–7.4 by maintaining a constant flux of 95% O2–5% CO2 above the bathing solution.

Changes in the membrane potential across the inner mitochondrial membrane were assessed using tetramethyl rhodamine ester (TMRE) (Ehrenberg et al., 1998), present at 1 μM throughout the experiment. Other experiments used 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), added to the preparation at 5 μg/ml for 20 min and then washed out before imaging (as by David, 1999). TMRE was excited at 530 nm and emissions >550 nm; JC-1 was excited at 488 nm, and emissions at <570 and >570 nm were ratioed. Statistical analyses were performed using Instat (Graph Pad, San Diego, CA). Pairwise comparisons used a two-tailed t test and n values indicate the number of ΔF/F transients analyzed. Results presented here came from 12 mice and 9 lizards. Other statistical tests are described in the legend of Figure 3. Averages are reported as mean ± SEM.

RESULTS

Stimulation-induced increases in cytosolic [Ca2+]i are more temperature-dependent in mouse than in lizard motor terminals

Figure 1 shows pseudocolor fluorescent images (A, B) and ΔF/F transients (C, D) from a representative mouse motor terminal filled with OG-5N and stimulated with a train of 500 action potentials (100 Hz for 5 sec). During stimulation at a near-physiological temperature (32°C; A, C) the rate of increase in fluorescence fell sharply within a few seconds, so that fluorescence stabilized at a plateau or near-plateau level that persisted until stimulation stopped (open symbols in C). In contrast, trains administered at a cooler temperature (18°C; B, D) produced a larger increase in fluorescence that continued to increase throughout the train (open symbols in D). Three different regions within the terminal showed similar ΔF/F transients (compare open triangles, squares, and diamonds in C and D), with amplitudes much greater than that in the
preterminal axon (filled triangles). This evidence for a localized, uniform \( \Delta F/F \) increase is similar to that demonstrated in lizard motor nerve terminals, which have a similar morphology (David et al., 1997, their Fig. 1). When stimulation ceased, \( \Delta F/F \) showed an early rapid decay, followed by a slower decay, similar to patterns demonstrated previously in a variety of tissues (e.g., adrenal chromaffin cells [Babcock et al., 1997]; crayfish and lizard motor nerve terminals [Tang and Zucker, 1997; David, 1999]).

Figure 2 shows that the effect of temperature on \( \Delta F/F \) transients was greatest at higher stimulation frequencies. At both near-physiological (A) and cool (B) temperatures, the amplitude of the \( \Delta F/F \) transient evoked by 500 stimuli increased as the frequency increased from 25 to 100 Hz. This frequency-dependence is similar to that measured in other nerve terminal preparations (Ravin et al., 1997; David, 1999).

The peak amplitude of the \( \Delta F/F \) transient was always greater at the cooler temperature, but the difference was much greater at 100 Hz than at 25 Hz. This temperature-dependence was observed in every studied mouse terminal, as is evident from the averaged \( \Delta F/F \) peak amplitudes plotted in Figure 2C. The greater the stimulation frequency (i.e., the more rapidly the terminal \( \text{Ca}^{2+} \) load was delivered), the greater the difference in peak \( \Delta F/F \) amplitude between warm (28–35°C; open symbols) and cool (17–24°C; filled symbols) temperatures. The effects of temperature changes over this range were usually reversible (data not shown).

Comparison of \( \Delta F/F \) transients recorded in mouse and lizard terminals (Fig. 3) indicates that the temperature-dependence recorded in mouse terminals is not attributable to temperature-dependent properties of OG-5N (also see Materials and Methods). Figure 3A shows the marked temperature dependence of \( \Delta F/F \) transients recorded in a mouse terminal after 1000 stimuli delivered at 50 and 100 Hz at 19 or 31°C (open and filled symbols, respectively). Figure 3C shows that, in contrast, the \( \Delta F/F \) transients recorded from a similarly stimulated lizard motor terminal were not detectably temperature-dependent over the same temperature range. Temperature also had no consistent effect on the fluorescence of resting mouse or lizard terminals, but the low-affinity dye used here might fail to detect small differences.

The histograms in Figure 3 summarize the results of similar experiments: open and filled bars plot, respectively, \( \Delta F/F \) amplitudes measured after 1 or 10 sec of stimulation at 50 Hz, for four different temperature ranges in mouse (B) and two temperature ranges in lizard (D). At near-physiological temperatures, mouse \( \Delta F/F \) amplitudes after 1 and 10 sec of stimulation were similar, indicating good stabilization of cytosolic \([\text{Ca}^{2+}]_o\). As temperatures were lowered, mouse \( \Delta F/F \) amplitudes measured at 1 sec changed relatively little, but the amplitudes measured at 10 sec increased markedly. Thus, the effect of temperature on mouse \( \Delta F/F \) transients was greater for both higher rates of \( \text{Ca}^{2+} \) influx (Fig. 2C) and larger total \( \text{Ca}^{2+} \) loads (Fig. 3B). The average magnitude of the \( \Delta F/F \) increase in lizard terminals over the range 18–33°C was similar after 1 or 10 sec of stimulation. These lizard values were comparable with the \( \Delta F/F \) increases recorded in mouse terminals at mouse physiological temperature and correspond to a stimulation-induced increase in cytosolic \([\text{Ca}^{2+}]_o\) of \( \sim 0.4 \mu M \) (see legend of Fig. 3).

Note that the first \( \Delta F/F \) value measured after the onset of stimulation was similar at near-physiological and cool temperatures (Figs. 3A, 3B, compare 19 and 31–32°C mouse records). This finding suggests that the temperature-dependence of mouse \( \Delta F/F \) transients was not simply attributable to greater \( \text{Ca}^{2+} \) influx per action potential at cooler temperatures (also see Discussion). In addition, Figure 4A shows that application of an agent that increases \( \text{Ca}^{2+} \) influx per action potential produced a \( \Delta F/F \) transient whose shape was much different from that produced by cooling, 3,4-Diaminopyridine (3,4-DAP) (10 \( \mu M \)) is a K⁺ channel blocker that increases by more than fivefold the \( \text{Ca}^{2+} \) influx associated with each action potential in lizard motor terminals (David et al., 1997). At 32°C, the \( \Delta F/F \) transient in 3,4-DAP exceeded the control transient, even at the first sampled point, and did not display the continuous rise during stimulation that was seen at cool temperatures. Thus, it appears that, at near-physiological temperatures, mouse motor terminals are able to limit the increase in cytosolic \([\text{Ca}^{2+}]_o\), even when \( \text{Ca}^{2+} \) influx per action potential is increased, but that this ability is compromised at cool temperatures.

To test whether the plateauing of cytosolic \([\text{Ca}^{2+}]_o\) measured at physiological temperatures might have been attributable to partial or intermittent failure of the action potential depolarization to invade terminals, end-plate potentials (EPPs) were recorded during repetitive stimulation in a preparation at 33°C. Figure 4B shows that each nerve stimulus produced an EPP at the beginning, middle, and end of a 20 sec 50 Hz train. Thus, the plateauing of cytosolic \([\text{Ca}^{2+}]_o\) during repetitive stimulation was not attributable to failure of axonal action potential propagation.

In mouse motor terminals, mitochondrial uptake contributes more to stabilization of cytosolic \([\text{Ca}^{2+}]_o\) at physiological than at cooler temperatures

Figure 5 presents evidence that mitochondrial \( \text{Ca}^{2+} \) uptake contributes importantly to the stabilization of cytosolic \([\text{Ca}^{2+}]_o\) during maintained stimulation in mouse terminals at near-physiological temperatures (33–34.5°C). Figure 5A shows that, when the mitochondrial proton gradient was dissipated by application of a protonophore (CCCP, 1 \( \mu M \); filled circles), cytosolic \([\text{Ca}^{2+}]_o\) no longer stabilized at a plateau value but rather continued to increase throughout the period of stimulation. Figure 5B shows that another
method for dissipating this proton gradient, application of an inhibitor of complex III of the electron transport chain (antimycin A1, 2 μM; filled circles), had a similar effect. This latter finding demonstrates that the crucial proton gradient is that across mitochondrial membranes rather than across the membranes of other intracellular organelles. In Figure 5, the CCCP or antimycin was added in the presence of oligomycin (5 μg/ml), an inhibitor of the mitochondrial \( F_{m} \) \( F_{o} \) ATP synthase. A 20 min exposure to oligomycin alone (triangles) had no significant effect on the recorded transients, indicating that the effects of subsequent addition of CCCP or antimycin were more attributable to loss of the mitochondrial membrane potential than to interruption of oxidative ATP synthesis. Oligomycin also prevents the extra ATP loss that would otherwise occur in the presence of CCCP and antimycin because of reverse operation of \( F_{m} \) \( F_{o} \) ATP synthase (Budd and Nicholls, 1996).

In lizard motor terminals, it was also possible to demonstrate mitochondrial Ca\(^{2+}\) uptake by imaging fluorescent indicators localized within the mitochondrial matrix (David et al., 1998). However, we have not yet succeeded in loading indicator dyes selectively and consistently into mouse motor terminal mitochondria.

Comparison of records in Figure 5, A and C, demonstrates that the mitochondrial contribution to limiting stimulation-induced increases in cytosolic Ca\(^{2+}\) is more prominent at near-physiological than at cooler temperatures. These terminals were selected for comparison because they reached similar plateau levels (and thus similar elevations in cytosolic Ca\(^{2+}\)) during stimulation. The warm (34.5°C) terminal in A reached this plateau level during 50 Hz stimulation, whereas the cool (25°C) terminal in C attained this level during stimulation at a lower frequency (20 Hz), consistent with the temperature-dependent effects shown in Figure 2C. Brief (10–15 min) exposure to CCCP (with oligomycin) at the cool temperature had little or no effect on the \( \Delta F/F \) transient, in contrast to the marked effect of CCCP at the warm temperature. These results suggest that inhibition of Ca\(^{2+}\) influx into mitochondria at cool temperatures contributes to the marked temperature-dependence of mouse \( \Delta F/F \) transients shown in Figures 1–4.

Figure 6B shows that brief application of 1 μM CCCP to mouse mitochondria at 20°C reversibly depolarized mitochondrial membrane potentials, assayed using TMRE (1 μM), a fluorescent dye that localizes into polarized mitochondria (Ehrenberg et al., 1988). CCCP also changed the pattern of terminal TMRE staining from punctate to diffuse (data not shown), another indication of mitochondrial depolarization. This result argues that the temperature-dependence of the effects of CCCP on stimulation-induced mouse \( \Delta F/F \) transients shown in Figure 5 was not simply attributable to collapse of the mitochondrial membrane potential by cool temperatures or to inhibition of CCCP to depolarize mitochondria at cool temperatures (also see Discussion). Figure 6A shows that stimulation (500 stimuli at 50 Hz) applied to this mouse terminal before the CCCP exposure did not detectably depolarize mitochondria at either warm (33°C) or cool (20°C) temperatures.

**DISCUSSION**

**Mitochondria contribute importantly to limiting stimulation-induced increases in cytosolic [Ca\(^{2+}\)] in mouse motor terminals**

Our findings demonstrate that, at near-physiological temperatures (>30°C), mouse motor nerve terminals exert tight control over the elevation in average cytosolic [Ca\(^{2+}\)] produced by trains of action potentials; the elevation of cytosolic [Ca\(^{2+}\)] after 10 sec of 50 Hz stimulation was only slightly greater than that after 1 sec of stimulation. Experiments using mitochondrial inhibitors demonstrated that mitochondrial Ca\(^{2+}\) uptake contributes importantly to this control. Further work will be required to determine the relative contributions of other Ca\(^{2+}\) extrusion–uptake mechanisms in these terminals.

The amplitudes of stimulation-induced \( \Delta F/F \) increases (and therefore of increases in cytosolic [Ca\(^{2+}\)]) in mouse motor termi-
nals at near-physiological temperatures (Figs. 2C, 3B) were similar to those recorded at comparable stimulation frequencies in lizard motor terminals at room temperature (David et al., 1998, their Fig. 1C). For example, during 50 Hz stimulation the cytosolic $[\text{Ca}^{2+}]$ at plateau exceeded resting levels by $0.4 - 0.5$ $\mu$M in mouse (33–38°C) compared with $0.4$ $\mu$M in lizard (18–38°C). For compari-

Figure 4. Cooling and 3,4-DAP have differential effects on the time course of $\Delta F/F$ transients (A), and EPP generation is reliable throughout stimulus trains (B). A, Three superimposed $\Delta F/F$ transients produced by 5 sec of 100 Hz stimulation in a mouse terminal, first at 19°C (open circles), then after heating to 32°C (filled circles), and then after addition of $10$ $\mu$M 3,4-DAP to prolong the action potential (open triangles). Cooling and 3,4-DAP both increase the amplitude of the $\Delta F/F$ transient, but $\Delta F/F$ reaches a limiting plateau value in 3,4-DAP, whereas it continues to increase throughout the train at 19°C. Note also that the first $\Delta F/F$ value sampled after the onset of stimulation is similar for warm and cool temperatures but is larger in 3,4-DAP. B, Each trace shows a sample of five successive EPPs recorded in a muscle fiber at the beginning (a), middle (b), and end (c) of a 1000 stimulus train delivered at 50 Hz at 33°C. Reliable transmission throughout the stimulus train was also verified at lower temperatures (22–25°C) at other terminals in this muscle (data not shown). In this experiment, muscle contractions were blocked using $\alpha$-conotoxin GIIIB (2 $\mu$g/ml), which blocks muscle (but not axonal) Na$^+$ channels. Use of this drug (instead of tubocurare) minimized the rundown of EPP amplitudes usually measured during repetitive stimulation in the presence of nicotinic antagonists. EPPs were recorded using standard intracellular recording techniques, as detailed by David (1999). The downward and upward deflections preceding each EPP are calibrating pulses and stimulus artifacts, respectively. The resting potential was $-75$ mV.

Figure 5. Agents that depolarize mitochondria increase the amplitude of stimulation-induced $\Delta F/F$ transients in mouse terminals more at warm than at cool temperatures. Superimposed $\Delta F/F$ transients produced by 10 sec of 50 (A, B, 33–34.5°C) or 20 (C, 25°C) Hz stimulation were recorded in three terminals in control saline (open circles), 15–20 min after addition of $5$ $\mu$g/ml oligomycin (open triangles), and after the further addition of $1$ $\mu$M CCCP (10–15 min exposure; A, C, filled circles) or $2$ $\mu$M antimycin A1 (6 min exposure; B, filled circles). The warm 50 Hz records in A and the cool 20 Hz records in C were chosen for comparison because the stimulation-induced increases in cytosolic $[\text{Ca}^{2+}]$ in control saline were similar. The effects of brief CCCP exposures were partially reversible, but more prolonged exposures ($>30$ min) resulted in a marked increase in resting fluorescence accompanied by failure of action potential conduction at both warm and cold temperatures (data not shown). The effects of antimycin exposure were not reversible. Two control $\Delta F/F$ transients are shown in B.
cells dialyzed by whole-cell recording (Engisch et al., 1997), and (2) mitochondrial contributions to Ca^{2+} regulation are more evident in intact than in dialyzed retinal bipolar terminals (Zenisek and Matthews, 2000). The polyamine spermine increases mitochondrial Ca^{2+} uptake (Lenzen et al., 1986), and Murphy et al. (1996) found that overexpression of Bel-2 in a neural cell line enhanced both the maximal Ca^{2+} uptake capacity of mitochondria and the ability of mitochondria to sequester large quantities of Ca^{2+} without undergoing profound respiratory impairment. If cytosolic factors do indeed have a major influence on mitochondrial Ca^{2+} uptake, then the affinity and/or capacity of mitochondrial Ca^{2+} uptake mechanisms may well vary from cell to cell and/or from one region of the cell to another.

**Possible mechanisms underlying temperature-dependence of ΔF/ΔF transients in mouse terminals**

At temperatures below 30°C, the amplitude of the ΔF/ΔF transient in mouse terminals increased progressively during 100 Hz stimulation instead of stabilizing at plateau (or near-plateau) values. Evidence summarized in Materials and Methods and Figure 3 indicates that this effect of cool temperatures on mouse ΔF/ΔF transients was not attributable to temperature-dependent properties of the indicator dye. Results with 3,4-DAP (Fig. 4A) suggested that the effect of cool temperatures on mouse ΔF/ΔF transients was also unlikely to be simply attributable to increased Ca^{2+} entry per action potential. Further evidence against a major role for increased Ca^{2+} entry comes from measurements made in other neurons. Cooling produces some changes expected to increase Ca^{2+} entry and/or accumulation, such as increased action potential amplitude and duration and reduced rates of Ca^{2+} extrusion (Helmcen et al., 1997), but produces other changes expected to decrease Ca^{2+} entry, such as slowed channel activation kinetics and reduced open probability (Nobile et al., 1990). Kenyon and Goff (1998) found that lowered temperatures actually reduced depolarization-induced Ca^{2+} influx in cultured chick DRG neurons. Borst and Sakmann (1998) found that in the rat calyx of Held terminal the integrated Ca^{2+} current in response to an action potential-like voltage-clamp signal increased only 13% after cooling from 36 to 23°C. This measured difference is much smaller than the ~100% increase in Ca^{2+} entry per action potential that would be needed to explain our finding that the amplitude of the ΔF/ΔF transient during 50 Hz stimulation at cooler temperatures was similar to that recorded during 100 Hz stimulation at near-physiological temperatures (Fig. 2C). Also, the difference between ΔF/ΔF amplitudes recorded at near-physiological and cool temperatures was minimal at shorter train durations and low frequencies (25 Hz) but became greater with longer durations and higher frequencies (100 Hz) (Fig. 2C). This pattern would not be predicted if cooling acted simply to increase action potential-associated Ca^{2+} entry by a fixed amount or percentage. Present data cannot, however, exclude the possibility that cooling activates a mechanism that progressively increases Ca^{2+} entry per action potential during high- but not low- frequency stimulation.

We hypothesize that the effects of cooling on mouse ΔF/ΔF transients are primarily attributable to a reduced ability of mouse terminals to buffer-extrude large Ca^{2+} loads at cool temperatures. Mechanisms to limit stimulation-induced increases in cytosolic Ca^{2+} did not fail altogether at cool temperatures, because mouse ΔF/ΔF transients continued to stabilize during maintained stimulation at frequencies up to at least 50 Hz (albeit at levels higher than those measured at near-physiological temperatures). Rather, it appears that, at cool temperatures, the mechanisms for limiting the increase in mouse cytosolic Ca^{2+} are less powerful, becoming overwhelmed during intense or prolonged stimulation.

**Mitochondria in mouse terminals may take up less Ca^{2+} at cool temperatures**

Our results with potential-sensitive dyes indicate that, at cool temperatures, mouse motor terminal mitochondria retain a membrane potential and that this membrane potential is not reduced during nerve stimulation but is reduced by CCCP. Although our
measurements cannot rule out the possibility that cooling partially depolarized motor terminal mitochondria, our findings are consistent with the lack of effect of temperature on the membrane potential of isolated rat liver mitochondria measured using a different technique (Dufour et al., 1996). The lack of effect of nerve stimulation on mitochondrial membrane potential agrees with measurements in lizard motor terminal mitochondria loaded with JC-1 (David, 1999). Thus, it appears that mitochondria can take up physiological Ca\(^{2+}\) loads with little or no loss of their membrane potential (Magnus and Keizer, 1997; Hoyt et al., 1998; Kavanagh et al., 2000).

Our finding that, at cool temperatures, CCCP had a reduced effect on mouse \(\Delta F/\Phi\) transients is thus consistent with the hypothesis that cold temperatures reduce the amount of mitochondrial Ca\(^{2+}\) sequestration for a given cytosolic [Ca\(^{2+}\)]. The results of Biscoe and Duchen (1990) on rabbit carotid body chemoreceptors are also consistent with the hypothesis of reduced mitochondrial Ca\(^{2+}\) sequestration at cool temperatures. Perhaps cool temperatures reduce Ca\(^{2+}\) uptake via the mitochondrial uniporter or increase Ca\(^{2+}\) extrusion via exchangers (e.g., the Na\(^+\)/Ca\(^{2+}\) exchanger) in the mitochondrial membrane. The former explanation seems more likely on energetic grounds. Further work will be required to determine the temperature-dependence of mitochondrial and other Ca\(^{2+}\) sequestration-extrusion mechanisms in mouse motor terminals.

Many \textit{in vitro} studies of Ca\(^{2+}\) handling in mammalian neurons are performed at room temperature. If our findings in mouse motor nerve terminals apply also to other mammalian neurons and terminals, this use of temperatures ~15°C cooler than physiological may tend to exaggerate the increase in cytosolic [Ca\(^{2+}\)] and to underestimate the mitochondrial contributions to Ca\(^{2+}\) sequestration that would accompany a given depolarizing stimulus \textit{in vivo}.

Whereas \(\Delta F/\Phi\) transients in mouse motor terminals were markedly temperature-dependent, stimulation-induced \(\Delta F/\Phi\) transients in lizard motor nerve terminals studied under identical experimental conditions were amazingly independent of temperature over the range 18–33°C. Because mitochondria are the dominant mechanism limiting the increase in cytosolic [Ca\(^{2+}\)] during prolonged stimulation in lizard (David, 1999), this temperature-independence suggests that lizard motor terminal mitochondria take up Ca\(^{2+}\) equally rapidly over this range of temperatures. This ability to limit the elevation of average cytosolic [Ca\(^{2+}\)] may contribute importantly to the ability of this ectotherm to survive and function over a range of tissue temperatures.

**REFERENCES**


