

THE MODULATION OF ACTION POTENTIAL GENERATION BY CALCIUM-INDUCED CALCIUM RELEASE IS ENHANCED BY MITOCHONDRIAL INHIBITORS IN MUDPUPPY PARASYMPATHETIC NEURONS

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Abstract—Previously, we demonstrated that outward currents activated by calcium-induced calcium release (CICR) opposed depolarization-induced action potential (AP) generation in dissociated mudpuppy parasympathetic neurons [J Neurophysiol 88 (2002) 1119]. In the present study, we tested whether AP generation by depolarizing current ramps could be altered by dissipating the mitochondrial membrane potential and thus interrupting mitochondrial Ca^{2+} buffering. Exposure to the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 2 μM) alone or in combination with the mitochondrial ATP synthase inhibitor oligomycin (8 $\mu\text{g/ml}$), increased the latency to AP generation. Exposure to the electron transport chain inhibitor rotenone (10 μM) alone or in combination with oligomycin (8 $\mu\text{g/ml}$) similarly increased the latency to AP generation. CCCP and oligomycin or rotenone and oligomycin treatment caused rhodamine 123 loss from mitochondria within a few minutes, confirming that the mitochondrial membrane potential was dissipated during drug exposure. Oligomycin alone had no effect on the latency to AP generation and did not cause loss of rhodamine 123 from mitochondria. The increase in latency induced by CCCP and oligomycin was similar when recordings were made with either the perforated patch or standard whole cell patch recording configuration. Exposure to the endoplasmic reticulum Ca-ATPase inhibitor thapsigargin (1 μM), decreased the latency to AP generation. In cells pretreated with thapsigargin to eliminate CICR, CCCP and oligomycin had no effect on AP latency. Pretreatment with iberiotoxin (IBX; 100 nM), an inhibitor of large conductance, calcium- and voltage-activated potassium channels, reduced the extent of the CCCP- and oligomycin-induced increase in latency to AP generation. These results indicate that treatment with CCCP or rotenone to dissipate the mitochondrial membrane potential, a condition which should minimize sequestration of Ca^{2+} by mitochondria, facilitated the Ca^{2+} -induced Ca^{2+} release activation of IBX-sensitive and IBX-insensitive conductances that regulate AP generation. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: AP, action potential; BAPTA, 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid; BCECF, 2',7'-bis(2-carboxyethyl)-5-(and-6-)carboxyfluorescein; BK, large conductance, calcium- and voltage-activated potassium; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CICR, Ca^{2+} -induced Ca^{2+} release; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis(β -aminoethyl ether)*N,N,N',N'*-tetraacetic acid; ER, endoplasmic reticulum; IBX, iberiotoxin; SMOCs, spontaneous miniature outward currents; VDCC, voltage-dependent Ca^{2+} channels.

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doi:10.1016/j.neuroscience.2003.12.010

Key words: protonophore, rotenone, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), autonomic neurons, membrane excitability.

Calcium-induced Ca^{2+} release (CICR) is a well-documented mechanism by which Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC) can initiate release of Ca^{2+} from internal stores (Marrion and Adams, 1992; Kuba, 1994; Verkhatsky and Shmigol, 1996; Berridge, 1998). In mudpuppy parasympathetic neurons a CICR mechanism stimulates spontaneous miniature hyperpolarizations (in current clamp) and spontaneous miniature outward currents (SMOCs; in voltage clamp) which are initiated by the simultaneous activation of approximately 20 large conductance, calcium- and voltage-activated potassium (BK) channels (Merriam et al., 1999; Scornik et al., 2001). More recently, we showed that CICR-activated conductances regulate the latency to action potential (AP) generation (Parsons et al., 2002). Our earlier studies showed that this modulation of AP latency was reduced significantly or eliminated by conditions that: (a) reduced Ca^{2+} influx through VDCCs, (b) blocked BK channels or (c) depleted the caffeine-sensitive intracellular Ca^{2+} stores in the endoplasmic reticulum (ER; Parsons et al., 2002). Thus, in the proposed model, activation of only a few voltage-gated calcium channels, during subthreshold depolarizations, allows sufficient Ca^{2+} influx to locally activate ryanodine receptors and initiate CICR. The Ca^{2+} released from internal stores raises the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in restricted domains between the ER and the plasma membrane to levels high enough (40 μM ; Scornik et al., 2001) to activate plasma membrane outward currents that decrease the effectiveness of the depolarizing current (Parsons et al., 2002). Thus, CICR acts as an amplification mechanism for Ca^{2+} , allowing activation of Ca^{2+} -dependent outward currents that are not activated directly by Ca^{2+} influx alone.

Cytoplasmic Ca^{2+} levels are tightly regulated by intracellular buffers and active and passive transport mechanisms that extrude Ca^{2+} from cells or accumulate Ca^{2+} in intracellular stores. Mitochondria, in addition to being the major ATP producing organelle in neurons, also play a key role in the homeostasis of intracellular Ca^{2+} (Babcock and Hille, 1998; Rizzuto et al., 1999; Duchon, 2000; Gunter et al., 2000; Nicholls and Budd, 2000; Camello-Almaraz et al., 2002). Mitochondria can be closely associated with the ER and plasma membrane; thus mitochondria are critically

positioned to buffer transient rises in Ca^{2+} within local domains (Landolfi et al., 1998; Rizzuto et al., 1998).

Consequently, we hypothesized that mitochondria might modulate the CICR-induced regulation of AP generation. Mitochondria might be important buffering organelles that regulate the extent of the CICR-induced rise in Ca^{2+} within local domains near the plasma membrane and as a consequence, regulate the ability of depolarizing currents to move the membrane potential to the threshold for AP generation. In the present study, we have tested this hypothesis by measuring the change in AP latency produced by pharmacologically inhibiting the ability of mitochondria to sequester Ca^{2+} . We utilized two drugs that cause dissipation of mitochondrial membrane potential by different mechanisms. Our results demonstrate that treatment with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 2 μM) or the electron transport chain inhibitor rotenone (10 μM), in combination with the mitochondrial ATP synthase inhibitor oligomycin (8 $\mu\text{g/ml}$), reduces the mitochondrial membrane potential. Both treatments cause the mitochondrial membrane potential to dissipate and as a consequence, mitochondrial Ca^{2+} sequestration should be diminished (Friel and Tsien, 1994; Budd and Nicholls, 1996; Nicholls and Budd, 2000; Medler and Gleason, 2002). During both drug treatments, the latency to AP generation was significantly lengthened. Elimination of CICR, by a thapsigargin-induced depletion of intracellular Ca^{2+} stores, blocked the mitochondrial modulation of AP latency; thus, the effect on latency to AP generation required CICR to be intact.

EXPERIMENTAL PROCEDURES

All experiments were performed on parasympathetic neurons that were dissociated from mudpuppy (*Necturus maculosus*) cardiac ganglia and maintained in culture for 12–36 h. Mudpuppies were killed by rapid decapitation, following procedures approved by the University of Vermont Institutional Animal Care and Use Committee and methods described in the National Institutes of Health Guide for the Care and use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. The method of cell dissociation used a combination of collagenase, type I (Sigma Chemical Co., St. Louis, MO, USA) and neutral protease (Roche Molecular Biochemicals, Indianapolis, IN, USA) following methods described previously (Merriam and Parsons, 1995). All experiments were done at room temperature (21–22 °C) using mudpuppy physiological solution that contained: 110 mM NaCl, 3.6 mM CaCl_2 , 2.5 mM KCl, 10 mM NaHEPES, 5 mM glucose, pH 7.3. Tetrodotoxin (0.3 μM) was included in the bath solution in some optical experiments.

Electrophysiological methods

Voltage recordings were made using either the perforated patch or standard configuration of the whole cell patch clamp technique (Hamill et al., 1981; Horn and Marty, 1988) and were controlled using the current clamp bridge mode of an Axoclamp 2A/Digidata 1200/pClamp 6.0.3 acquisition system (Axon Instruments, Union City, CA, USA). Voltage responses were digitized at 1 kHz and acquired on line. For most experiments, the perforated patch recording mode was used with pipette solution composition: 80 mM K aspartate, 40 mM KCl, 5 mM MgCl_2 , 10 mM HEPES–KOH, pH 7.2 and the patch pipettes were backfilled with 0.2 mg/ml amphotericin B (Sigma). For standard whole cell recordings the

internal solution contained: 80 mM K aspartate, 40 mM KCl, 2 mM MgCl_2 , 0.4 mM NaGTP, 3 mM MgATP, 3 mM phosphocreatine, 10 mM HEPES–KOH, pH 7.2 and either 5 mM EGTA or 5 mM 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid (BAPTA).

The average resting membrane potential of the dissociated neurons is approximately -50 mV (Scornik et al., 2001). Although the resting membrane potential varied between cells, control and test whole cell recordings were obtained from the same cell with the membrane potential set between -55 and -60 mV. Following drug treatments, there was no consistent or sustained change in the resting membrane potential. Occasionally, there was a 5–10 mV depolarization, which lasted <1 min, following a switch from control solution to the solution containing CCCP and oligomycin. In these cells, experiments were started after the membrane potential returned to the initial level.

Current ramps (400 ms) were applied to determine the latency to AP generation. The rate of depolarization with the current ramp was adjusted under control conditions to make it possible to measure a decrease or increase in latency to the first AP. The latency, determined as the time interval from onset of the current ramp to the point at which the rising phase of the AP crossed 0 mV, was compared in the same cell prior to and during drug application.

Cell input resistance was monitored from the change in membrane potential produced by 500 ms, 5 pA hyperpolarizing current pulses. The control input resistance was 2.4 ± 0.2 G Ω in 41 cells, when measured with the membrane potential maintained between -55 and -60 mV.

Optical methods

Mitochondrial accumulation of rhodamine 123 was used to assess dissipation of the mitochondrial membrane potential following drug treatment (Johnson et al., 1981). Control cells were treated for 10 min with 5 μM rhodamine 123 in control mudpuppy solution, then washed three times with control solution without rhodamine 123 and imaged after 5 min with the DeltaVision Restoration Microscope (Applied Precision, Issaquah, WA, USA) using a 60 \times (1.4 NA) oil-immersion lens and FITC filters for excitation and emission. Some cells were pretreated with a solution containing CCCP (2 μM), oligomycin (8 $\mu\text{g/ml}$), or a combination of CCCP and oligomycin for 10 min before the addition of rhodamine 123 solution and rhodamine wash-out. Therefore, the total time of drug treatment for these cells was 25 min before imaging. A stack of z-slices 0.2 μm apart was acquired and the entire stack (reported as F/F_0) deconvolved using softWoRx software (Applied Precision). For Fig. 2A–D, a projection of five slices near the cell surface was generated (total thickness of 1 μm) and adjusted for brightness and contrast with Adobe Photoshop 4.0.1, San Jose, USA. In other cells (results shown in Fig. 2E), a time-series was acquired after rhodamine 123 pretreatment. A single optical slice was acquired at a rate of 0.1 Hz during CCCP and oligomycin or rotenone and oligomycin bolus application. Mitochondrial fluorescence was determined from pixel intensity within identified mitochondria and was corrected for any decrease in fluorescence intensity due to bleaching and/or extrusion of rhodamine 123 from mitochondria with a single exponential decay algorithm (Microcal Origin 7.0; Microcal, Northampton, MA, USA), to give F/F_0 .

Changes in $[\text{Ca}^{2+}]_i$ were assessed from variations in fluo-3 fluorescence intensity. Cells were incubated for 15 min in control mudpuppy solution containing 5 μM fluo-3-AM and 0.02% Pluronic F-127. They were rinsed three times with control solution and the AM ester was allowed to cleave for at least 45 min. Confocal images were obtained with a Noran Oz system (Middleton, WI, USA) attached to a Nikon Diaphot 200 inverted microscope. A 60 \times (1.2 NA) water immersion lens was used with an excitation wavelength of 488 nm. The 500 nm long-pass filter was used for data acquisition. Images were acquired at 0.1 Hz with a bath flow of approximately 1 ml/min. Runs were a minimum of 20

min with 5–10 min of control solution followed by 10 min of CCCP (2 μ M) and/or oligomycin (8 μ g/ml) or rotenone (10 μ M) and oligomycin (8 μ g/ml). In some cases (six of nine cells to which CCCP and oligomycin were applied and all cells exposed to rotenone and oligomycin), the drug treatment was followed by a wash-out period of approximately 10 min with control solution. Using InterVision software (Noran), a region corresponding to the cytoplasm was selected and an average brightness over time plot was constructed. The plots were corrected for dye bleaching using a single or double exponential decay algorithm (Microcal Origin 7.0; Microcal) and were normalized to this decay curve to give F/F_0 .

Changes in intracellular pH produced during drug exposure were measured by monitoring changes in 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescence. Cells were incubated in 10 μ M BCECF-AM in control solution for 15–20 min at room temperature, then rinsed three times in dye-free solution and stored at room temperature for at least 20 min before imaging to allow cleavage of the AM ester. During experiments, the BCECF fluorescence was monitored with cells initially in control solution followed by perfusion with drug containing solution as described above for Ca^{2+} imaging. Data were analyzed as described above for Ca^{2+} measurements and change in BCECF fluorescence intensity is reported as F/F_0 .

Drugs

All drugs used in the present study were obtained from commercial sources: rhodamine 123, thapsigargin (Calbiochem, LaJolla, CA, USA); Pluronic F-127, fluo-3-AM and BCECF-AM (Molecular Probes, Eugene, OR, USA); caffeine, CCCP, iberiotoxin (IBX), oligomycin and rotenone (Sigma Chemical Company). CCCP (final concentration 2 μ M), oligomycin (a mixture of oligomycin A, B, and C; final concentration 8 μ g/ml), thapsigargin (final concentration 1 μ M), and rotenone (final concentration 10 μ M) were diluted each day from frozen aliquots of concentrated dimethylsulfoxide (DMSO) stock solutions. Pluronic F-127, fluo3-AM and BCECF-AM were also used from concentrated stock solutions in DMSO. For a vehicle control, DMSO was added at the final concentration to the control solution.

Data analysis

Control and test results were averaged from different cells and the averaged values from a number of cells were expressed as the mean \pm S.E.M. of the control or test group. Data were analyzed with the Student's paired or non-paired *t*-test with $P < 0.05$ considered statistically significant.

RESULTS

Treatment with CCCP or rotenone increases the latency to AP generation

Previously, we showed that CICR-activated conductances oppose AP generation initiated by a depolarizing current ramp (Parsons et al., 2002). We predicted that if CICR is affected by mitochondrial Ca^{2+} buffering, then treatment with a protonophore should change the latency to AP generation. It is well documented that calcium accumulation by mitochondria is reduced by protonophores such as CCCP that cause the mitochondrial membrane potential to dissipate (Friel and Tsien, 1994; Budd and Nicholls, 1996; Nicholls and Budd, 2000). Consequently, we tested, using the perforated patch whole cell recording configuration, the effect of treatment with 2 μ M CCCP and 8 μ g/ml oligomycin on the latency to AP generation during a 400 ms

depolarizing current ramp. Oligomycin was included in the test solution to avoid the compensatory hydrolysis of cytoplasmic ATP by the mitochondrial ATP synthase working in reverse mode (Budd and Nicholls, 1996; Colegrove et al., 2000; Vanden Berghe et al., 2002). During exposure to CCCP and oligomycin, the latency to AP generation was increased by $56 \pm 4\%$ in 13 cells (Fig. 1A). CCCP exposure alone also significantly increased the latency to AP generation ($53 \pm 11\%$; $n=6$ cells; Fig. 1B) whereas oligomycin by itself had no significant effect on latency in 10 cells (Fig. 1C).

In addition to their effect on mitochondria, protonophores can form proton channels in the plasma membrane and membranes of intracellular organelles (Park et al., 2002). Although we did not observe any sustained change in membrane potential during exposure to CCCP and oligomycin or CCCP alone, we considered it possible that protonophore treatment might have effects on the plasma membrane input resistance. Consequently, to test whether the cell input resistance was changed during protonophore exposure, we measured the hyperpolarization induced by a 500 ms, 5 pA constant current pulse prior to and during exposure to CCCP and oligomycin or CCCP alone with the cell membrane potential initially maintained between -55 and -60 mV. After 5 min in CCCP alone or CCCP and oligomycin, there was a decrease in the input resistance. However, we found that the observed changes in input resistance did not correlate consistently with changes in latency to AP generation. For instance, while the increase in latency to AP generation produced by CCCP alone was the same as CCCP and oligomycin together, the decrease in the input resistance was significantly less for cells exposed to CCCP alone ($29 \pm 7\%$, $n=4$ cells) than for cells exposed to CCCP and oligomycin ($50 \pm 6\%$, $n=8$) together. In oligomycin alone, there was no significant change in input resistance ($20 \pm 15\%$ increase, $n=8$ cells).

In four of the 13 cells exposed to CCCP and oligomycin, we tested whether both the increase in latency to AP generation and change in input resistance were reversible. In these four cells, following exposure to CCCP and oligomycin for 5 min, the latency to AP generation increased by $58 \pm 6\%$ and the hyperpolarization produced by a 500 ms 5 pA current step decreased by $51 \pm 9\%$ (Fig. 2). After approximately 10 min in drug-free solution, the latency to AP generation returned to the control value indicating that the drug-induced change in latency had reversed fully (Fig. 2A). In contrast, the amplitude of the current-induced hyperpolarization only partially recovered by the end of a 10 min wash in drug-free solution (Fig. 2B).

To test whether a change in cell input resistance caused the change in AP latency, we determined whether a similar change in latency to AP generation was produced if the mitochondrial membrane potential was dissipated by treatment with a drug more specific to mitochondria that should not affect the plasma membrane input resistance. Rotenone (10 μ M), an electron transport chain inhibitor, was used with or without 8 μ g/ml oligomycin, to specifically dissipate the mitochondrial membrane potential and reduce its ability to accumulate Ca^{2+} (Nicholls and Budd,

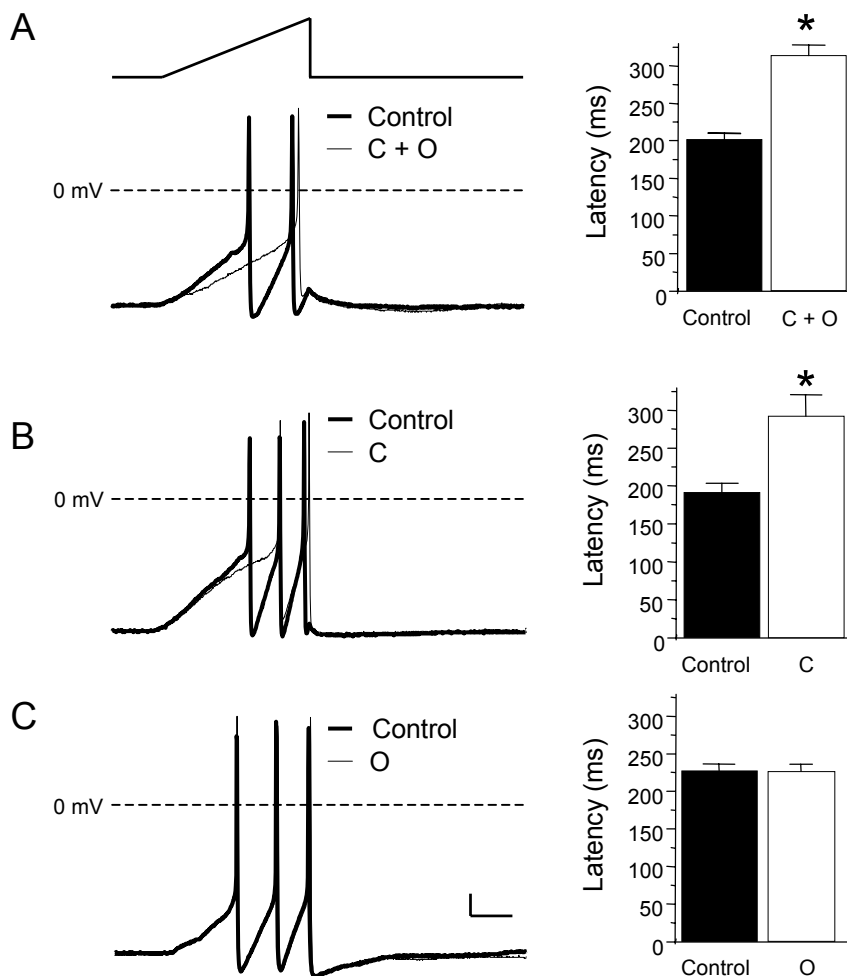


Fig. 1. The latency to AP generation is increased following treatment with CCCP and oligomycin, CCCP alone, but not by oligomycin alone. In each panel (A–C), example recordings from an individual cell prior to and during drug treatment are shown on the left and bar graphs summarizing results from multiple cells are presented on the right. In each example, the latency to AP generation by a 400 ms depolarizing current ramp was determined before (Control) and following a 5 min drug treatment. (A) 2 μ M CCCP and 8 μ g/ml oligomycin (C+O; $n=13$ cells); (B) 2 μ M CCCP alone (C; $n=6$ cells); and (C) 8 μ g/ml oligomycin alone (O; $n=10$ cells). For the AP recordings in this figure and Figs. 3, 5 and 6, the scale bar=10 mV (y axis) and 100 ms (x axis). In this figure and in Figs. 2, 3, 5 and 6, control and test recordings were made from the same cell with averaged results given as the mean \pm S.E.M. Asterisks indicate a significant difference between conditions using a paired t -test.

2000). After a 5 min exposure to drug containing solution, the latency to AP generation was increased by $42 \pm 5\%$ in rotenone and oligomycin ($n=6$ cells) and by $40 \pm 14\%$ in rotenone alone ($n=3$ cells; Fig. 3). In contrast, the input resistance was not significantly changed after 5 min with rotenone alone ($8 \pm 15\%$ increase) or rotenone plus oligomycin ($11 \pm 6\%$ decrease).

We concluded from these results that there was no consistent correlation between the drug-induced increase in latency to AP generation and drug-induced change in the input resistance. Subsequent results also support this conclusion.

Rhodamine 123 accumulation into mitochondria is diminished when cells were exposed to either CCCP or rotenone

Rhodamine 123 will accumulate in mitochondria with negative membrane potentials (Johnson et al., 1981). However, if the mitochondrial membrane potential is

dissipated, mitochondria no longer accumulate rhodamine 123 and dye that was accumulated will diffuse into the cytosol. Consequently, we monitored changes in mitochondrial rhodamine 123 fluorescence to confirm that exposure to CCCP or rotenone dissipated the mitochondrial membrane potential. In control cells, mitochondria accumulated rhodamine 123 and mitochondria could be seen dispersed throughout the cytoplasm (Fig. 4A).

Next, we exposed a different set of cells to either CCCP and oligomycin or CCCP alone. We found that the rhodamine 123 fluorescence was not localized in mitochondria, but rather was diffusely spread throughout the cell (Fig. 4B, D). In other cells exposed to oligomycin alone, mitochondria accumulated rhodamine 123 in a pattern similar to the control cells, indicating that the mitochondrial membrane potential was still intact (Fig. 4C).

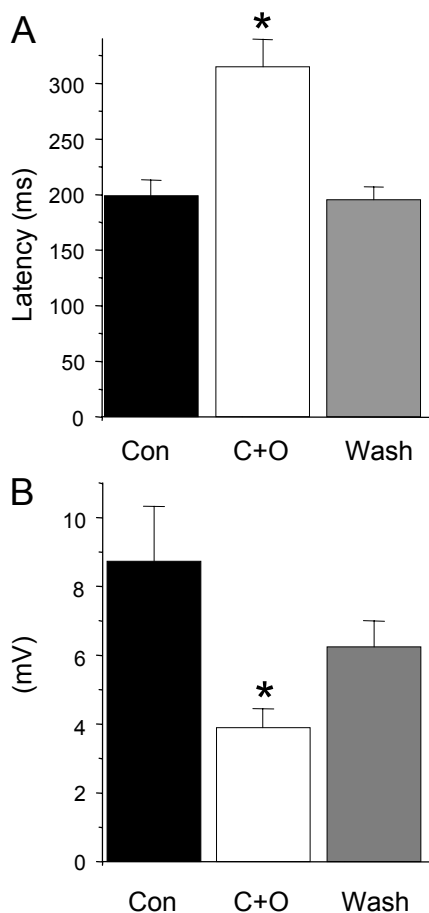


Fig. 2. The CCCP- and oligomycin-induced increase in latency to AP generation was reversible. Results summarized in panel (A) demonstrate in four cells that the increase in latency by CCCP and oligomycin (C+O) completely recovered after 10 min of wash with drug-free solution. In contrast, in these same four cells, the amplitude of the hyperpolarization produced by a 500 ms, 5 pA current pulse did not recover fully after 10 min of wash with drug-free solution.

In a second series of experiments, we determined the time course of loss of rhodamine 123 fluorescence from mitochondria during exposure to CCCP and oligomycin or rotenone and oligomycin. As shown in Fig. 4E, during exposure to either drug treatment, there was a progressive loss of rhodamine 123 fluorescence from mitochondria. However, in CCCP and oligomycin, the mitochondrial fluorescence reached a minimum within 2 min whereas in rotenone and oligomycin the fluorescence took longer to dissipate. These experiments indicated that with bath application of either the CCCP or rotenone, the mitochondrial membrane potential was dissipated, an observation indicating that ability to sequester Ca^{2+} should be greatly diminished. Given that the change occurred more rapidly in CCCP and oligomycin than in rotenone and oligomycin, we used CCCP and oligomycin treatment in the following electrophysiological studies. The results also demonstrated that a 5 min equilibration period in CCCP and oligomycin was adequate to dissipate the mitochondrial membrane potential.

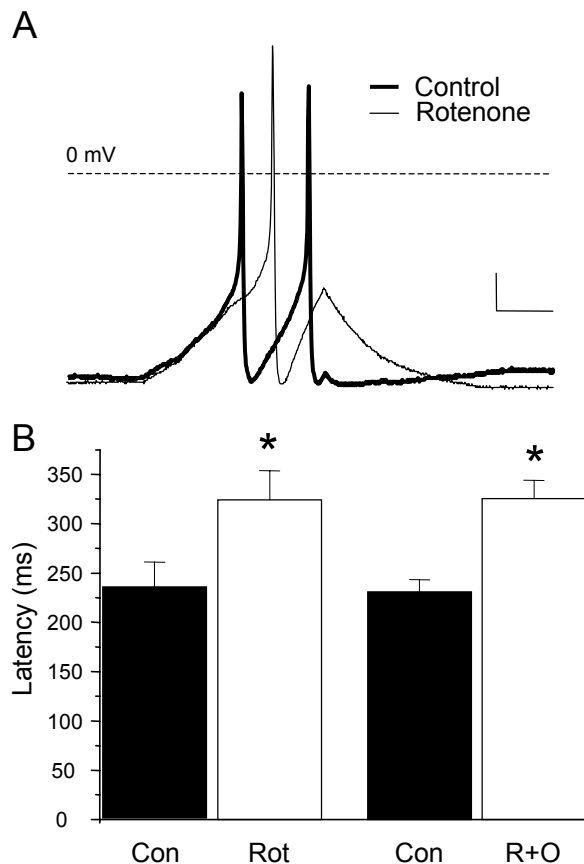


Fig. 3. The latency to AP generation is increased following treatment with rotenone (Rot) alone or Rot and oligomycin. Panel (A) shows a recording from an individual cell prior to and during Rot (10 μ M) treatment. In this example, the latency to AP generation by a 400 ms depolarizing current ramp was determined before (Con) and following a 5 min Rot treatment. Panel (B) presents bar graphs summarizing results from multiple cells prior to and during exposure to 10 μ M Rot ($n=3$ cells) or 10 μ M Rot and 8 μ g/ml oligomycin (R+O; $n=6$ cells).

Treatment with CCCP and oligomycin increases the latency to AP generation in the presence of intracellular EGTA or BAPTA

The initial latency measurements were made with the perforated patch recording configuration in which the intracellular environment remains intact and is not dialyzed by pipette solution. We next determined whether CCCP and oligomycin could affect the latency to AP generation when recordings were made with the standard whole cell recording configuration in which the internal environment is controlled by dialyzing with Ca^{2+} buffers and intracellular mediators including ATP. Two series of current ramp experiments were done with the standard whole cell recording technique. In the first, EGTA (5 mM) was included in the pipette solution whereas in the second, BAPTA (5 mM) was present. The results summarized in Fig. 5 indicate that, during exposure to CCCP and oligomycin, the latency to AP generation was increased as observed with the perforated patch recordings and furthermore, the CCCP- and oligomycin-induced increase in latency was similar whether EGTA (50 \pm 9%, $n=4$ cells; Fig. 5A) or BAPTA

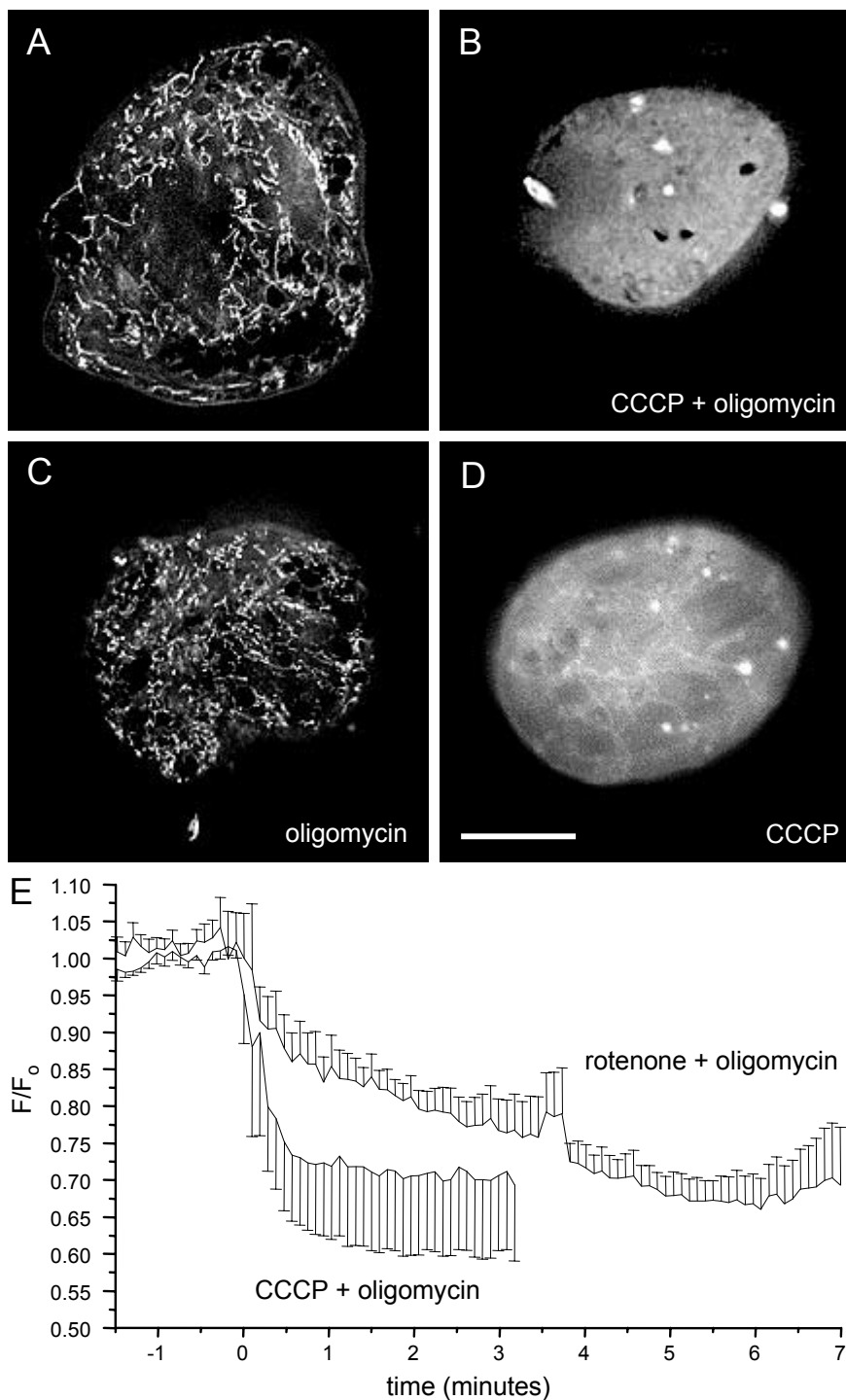


Fig. 4. Changes in rhodamine 123 fluorescence demonstrated drug-induced dissipation of the mitochondrial membrane potential. In (A–D), the pattern of rhodamine 123 fluorescence in four different cells: (A) Control; (B–D), cells pretreated with CCCP and oligomycin, oligomycin, or CCCP, respectively, as described in Experimental Procedures. One micrometer thick projection of 5 slices 0.2 μm thick. Acquired with the Deltavision Restoration Microscope and processed as described in Experimental Procedures. Calibration bar = 10 μm . (E) Change in mitochondrial rhodamine 123 fluorescence during CCCP and oligomycin or rotenone and oligomycin treatment. Fluorescence was normalized to a first order bleaching curve (F_0) as described in the Experimental Procedures. Data presented as mean \pm S.E.M. for five cells in CCCP and oligomycin and seven cells in rotenone and oligomycin. Drugs were added at time = 0.

($48 \pm 12\%$, $n=5$ cells; Fig. 5B) was used to buffer Ca^{2+} in the pipette solution. In these experiments, the input resis-

tance decreased during exposure to CCCP and oligomycin. However, the decrease was significantly less when

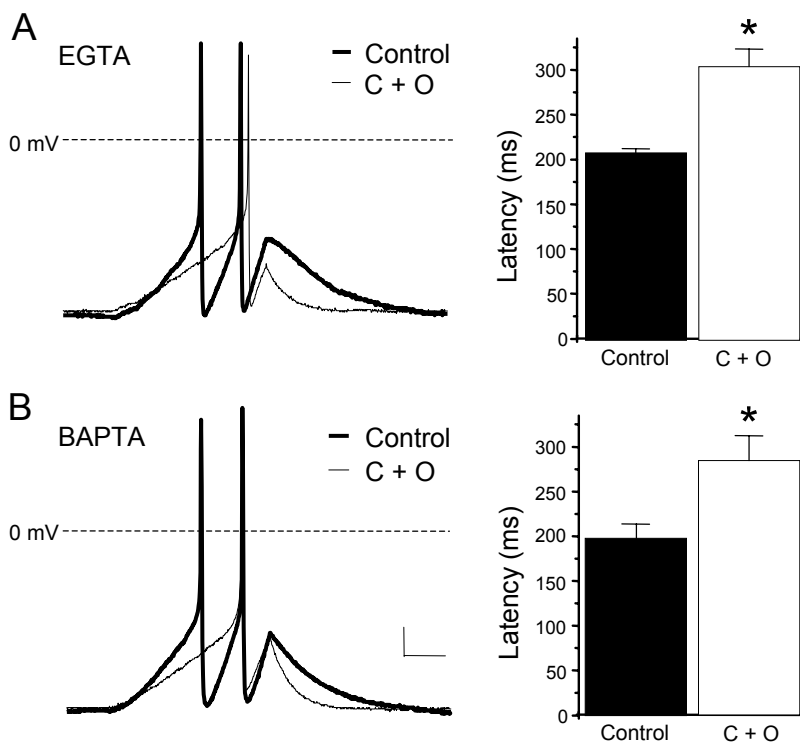


Fig. 5. Treatment with CCCP and oligomycin increases the latency to AP generation in recordings made with the standard whole cell patch configuration. With either EGTA (A) ($n=4$ cells) or BAPTA (B) ($n=5$ cells) in the pipette solution, exposure to CCCP and oligomycin increased the latency to AP generation to the same extent.

BAPTA ($33\pm 5\%$, $n=5$ cells) was present than when EGTA ($61\pm 4\%$, $n=4$ cells) was present.

The increase in latency to AP generation by CCCP and oligomycin treatment requires CICR

We next determined whether the CCCP- and oligomycin-induced increase in latency required that CICR be functional. In these and all subsequent experiments, we used the perforated patch recording configuration to avoid dialyzing the cell interior. Thapsigargin treatment inhibits the ER Ca^{2+} -ATPase resulting in a progressive depletion of internal Ca^{2+} stores (Thomas and Hanley, 1994). Addition of 10 mM caffeine along with thapsigargin empties the stores more rapidly so caffeine was always included in the thapsigargin pretreatment in order to eliminate CICR (Parsons et al., 2002). Similar to the observations in our previous paper (Parsons et al., 2002), treatment with thapsigargin decreased the latency to AP generation ($33\pm 5\%$; $n=4$ cells; Fig. 6A). Thapsigargin treatment did not significantly change the cell input resistance ($2\pm 2\%$, $n=4$ cells).

In subsequent experiments, we pretreated neurons with 1 μM thapsigargin for at least 10 min (with caffeine present for the first 3–4 min) to eliminate CICR and then determined the latency in the same cells prior to and during exposure to the CCCP and oligomycin solution also containing thapsigargin. In six cells pretreated with thapsigargin, CCCP and oligomycin exposure had no effect on latency to AP generation (Fig. 6B). Even

though the latency to AP generation was not increased by CCCP and oligomycin after CICR was eliminated in these six cells, the cell input resistance was significantly decreased by $60\pm 7\%$ during exposure to CCCP and oligomycin.

Pretreatment with IBX reduces, but does not eliminate the effect of CCCP and oligomycin on the latency to AP generation

Previously, we demonstrated that exposure to the potent BK channel blocker IBX, decreases the latency to AP generation suggesting BK channels activated by CICR modulate excitability (Parsons et al., 2002). In the present study we tested whether pretreatment with IBX could eliminate the CCCP- and oligomycin-induced increase in the latency to AP generation. Cells were pretreated for at least 5 min with 100 nM IBX, a concentration previously shown to inhibit the SMOCs generated by synchronous activation of BK channels by CICR (Merriam et al., 1999). We then determined the latency to AP generation in IBX prior to and during exposure to CCCP and oligomycin. In six IBX-pretreated cells, exposure to CCCP and oligomycin increased the latency to AP generation by $37\pm 8\%$. Although CCCP and oligomycin increased the latency to AP generation in IBX-pretreated cells, the change in latency was significantly less than that noted with cells not pretreated with IBX ($56\pm 4\%$, $n=13$). In contrast, the CCCP and oligomycin-induced decrease in the input resistance in the

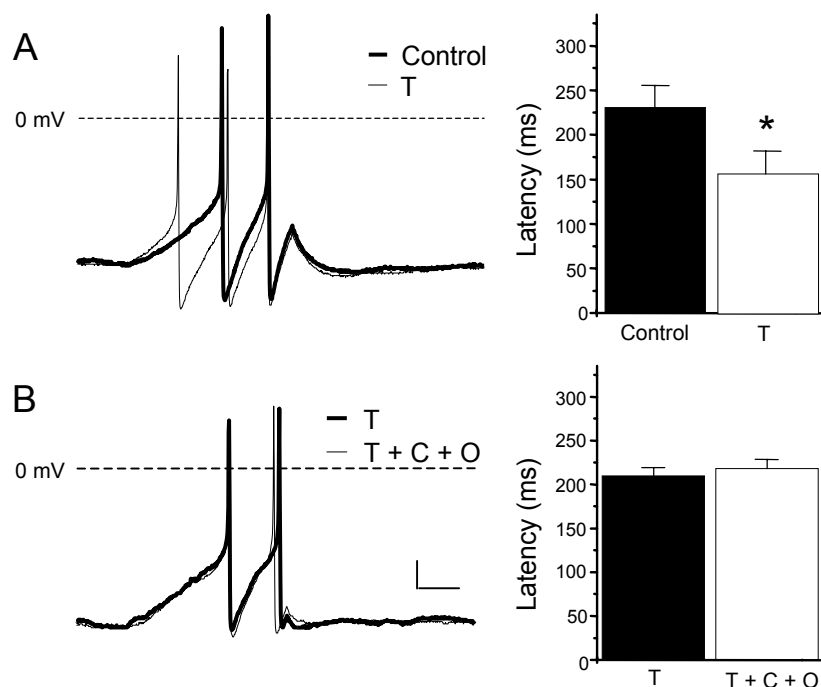


Fig. 6. Treatment with thapsigargin to eliminate CICR decreased the latency to AP generation and eliminated the CCCP- and oligomycin-induced increase in latency to AP generation. (A) Exposure to 1 μ M thapsigargin (T) decreased the latency to AP generation. (B) In another cell pretreated with 1 μ M thapsigargin (T) for at least 10 min, 2 μ M CCCP and 8 μ g/ml oligomycin (T+C+O) exposure did not change the latency to AP generation. In both experiments, caffeine (10 mM) was included during the initial 3–4 min of thapsigargin treatment. Bar graphs in A and B summarize results from four cells and five cells, respectively.

six IBX-pretreated cells ($51 \pm 4\%$) was similar to that determined for 13 cells not pretreated with IBX ($50 \pm 6\%$).

Treatment with CCCP, but not rotenone, increases fluo-3 fluorescence

Ca^{2+} sequestered in mitochondria can be released into the cytoplasm during protonophore exposure and protonophore treatment has been reported to cause a small elevation of $[\text{Ca}^{2+}]_i$ at rest in some neurons (Nohmi et al., 2000; Medler and Gleason, 2002). We used laser scanning confocal measurements of fluo-3 fluorescence to test whether intracellular Ca^{2+} changed during treatment with CCCP or with rotenone. In most experiments oligomycin was also present. During exposure to CCCP and oligomycin, the fluo-3 fluorescence ratio increased from 1.00 to 1.11 ± 0.01 in nine cells (Fig. 7A₁). In six of the nine trials, the CCCP and oligomycin was washed out and in all six cells, the ratio declined toward 1.00 following return to the control solution without drugs. Exposure to CCCP alone also produced a consistent increase in the fluo-3 fluorescence ratio (1.09 ± 0.02 , $n=3$ cells; data not shown). These observations suggested that during CCCP and oligomycin treatment, fluo-3 fluorescence increased by approximately 10% and that this change was reversible.

Similar fluo-3 fluorescence measurements were completed with rotenone and oligomycin or oligomycin alone. No consistent change in fluo-3 fluorescence was seen during exposure to rotenone and oligomycin

(1.00 ± 0.01 , $n=5$; Fig. 7A₂) or oligomycin alone (1.00 ± 0.01 , $n=5$; Fig. 7A₃).

The BCECF fluorescence F/F_o ratio changes during CCCP and oligomycin, but not rotenone and oligomycin treatment

During treatment with protonophores, the cytosol can become acidified, with the decrease in intracellular pH due to loss of protons from intracellular organelles and an equilibration of protons across the plasma membrane (Nicholls and Budd, 2000). Consequently, we tested whether a change in intracellular pH occurred in mudpuppy cardiac neurons during exposure to protonophore alone or in combination with oligomycin, using the pH-sensitive dye BCECF. An increase in the BCECF F/F_o ratio indicates an increase in cytosolic pH. In 15 cells during a 10-min exposure to CCCP and oligomycin, on average there was an initial small increase in the BCECF F/F_o ratio followed by a decrease in the ratio (Fig. 5B₁). However, the pattern of change was quite variable between cells. During exposure to CCCP alone, the BCECF F/F_o ratio also exhibited a similar biphasic change ($n=11$ cells; data not shown).

Additional experiments were done to test whether any change in the BCECF F/F_o ratio occurred when cells were exposed to rotenone and oligomycin or oligomycin alone. During exposure to rotenone and oligomycin ($n=9$ cells; Fig. 7B₂) or oligomycin alone ($n=10$ cells; Fig. 7B₃), there was no consistent change in the BCECF F/F_o ratio.

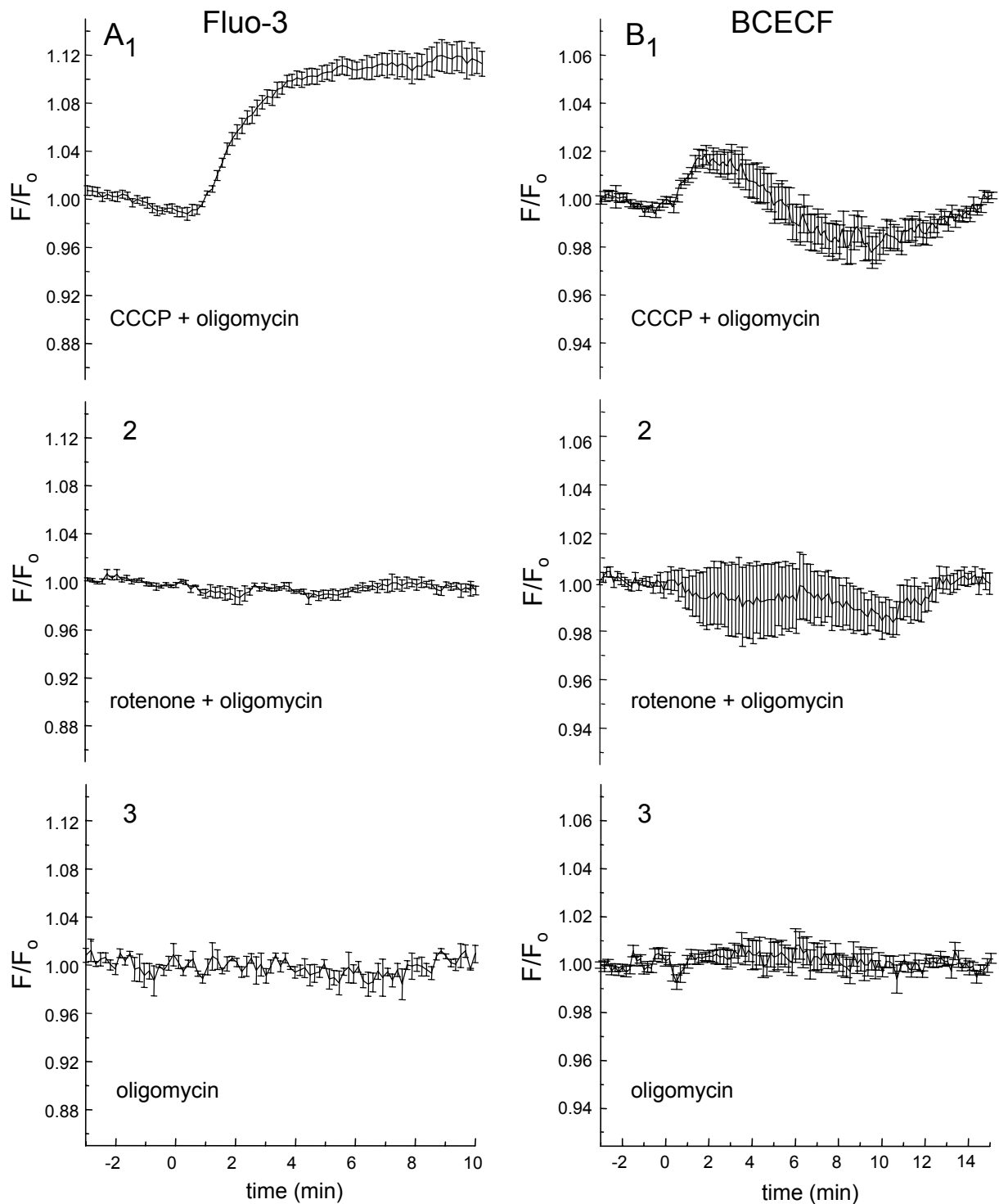


Fig. 7. The change in fluo-3 fluorescence ratio (F/F_0) or BCECF fluorescence ratio (F/F_0) was used to monitor changes in $[Ca^{2+}]_i$ or intracellular pH during drug application. (A_{1–3}) Change in fluo-3 fluorescence ratio in response to CCCP and oligomycin (A₁), rotenone and oligomycin (A₂) or oligomycin alone (A₃). (B_{1–3}) Change in BCECF fluorescence ratio in response to CCCP and oligomycin (B₁), rotenone and oligomycin (B₂), or oligomycin alone (B₃). Drugs were added at time=0. The data are presented as the mean \pm S.E.M. from multiple cells. Data were acquired with the Noran Oz Confocal Microscope and processed as described in the Experimental Procedures.

DISCUSSION

Dissipation of the mitochondrial membrane potential decreases the effectiveness of depolarizing current ramps to generate APs

The present study demonstrated that following treatment with CCCP, CCCP and oligomycin, rotenone and oligomycin or rotenone alone, the latency to AP generation was significantly increased. We attribute the change in latency to AP generation to a CCCP- or rotenone-induced dissipation of the mitochondrial membrane potential and resultant inhibition of Ca^{2+} sequestration, which enhanced CICR. A CCCP- and rotenone-induced dissipation of the mitochondrial membrane potential was confirmed with measurements of rhodamine 123 fluorescence. Rhodamine 123 was concentrated in mitochondria in control cells as well as in cells treated only with oligomycin, but not in cells exposed to CCCP alone, CCCP and oligomycin or rotenone and oligomycin.

The membrane potential change produced by a small hyperpolarizing current step commonly is used to estimate the cell input resistance. During exposure to CCCP and oligomycin, but not rotenone and oligomycin, there was a decrease in the input resistance. This decrease in input resistance could have potentially decreased the effectiveness of the depolarizing current ramp. However, we do not attribute the increase in latency to a decrease in cell input resistance. A consistent decrease in input resistance was only observed in the presence of CCCP, whereas the change in latency to AP generation was still observed with rotenone treatment. Also, in many other experiments, there was no consistent correlation between the change in input resistance determined from hyperpolarizing current steps and the change in latency to AP generation during depolarizing current ramps. For example, in cells pretreated with thapsigargin, exposure to CCCP and oligomycin decreased the input resistance without changing the latency to AP generation.

The results summarized above indicate that the decrease in input resistance was not a critical factor in determining the latency. Two possibilities are suggested to explain this observation. First, the decrease in the hyperpolarization from which input resistance was calculated could represent a drug-induced modulation of a conductance activated by hyperpolarization, such as the inward rectifier, that might have abbreviated the extent of the hyperpolarization. Alternatively, the conductances activated by the depolarizing current ramp near the threshold for AP generation were sufficiently great in magnitude that a passive change in input resistance had minimal effect. An analysis of membrane conductances activated near the threshold for AP generation required to answer this question is beyond the scope of the present study, but will be undertaken in future studies.

The latency to AP generation was not affected by exposure to oligomycin alone. Therefore, we concluded that the CCCP-induced change in latency to AP generation was not due to an inhibition of mitochondrial ATP production. This conclusion is supported by the observation that

CCCP and oligomycin increased the latency to AP generation equally well when studied with either the standard whole cell recording configuration with the cell dialyzed with ATP or the perforated patch configuration which allows the intracellular milieu to remain relatively intact. Also, in all recording modes, glucose was present in the extracellular solution so one might expect that the glycolytic pathway should have maintained the cytosolic ATP/ADP ratio for periods longer than the duration of the experiments (Kauppinen and Nicholls, 1986; Werth and Thayer, 1994; White and Reynolds, 1995; Peng, 1998).

Treatment with CCCP, but not rotenone, increased fluo-3 fluorescence

Mitochondria not only produce metabolic energy, but also are key organelles contributing to the regulation of $[\text{Ca}^{2+}]_i$ (Babcock and Hille, 1998; Duchen, 2000; Nicholls and Budd, 2000). Dissipation of the mitochondrial membrane potential decreases the ability of mitochondria to buffer rises in $[\text{Ca}^{2+}]_i$ (Duchen, 2000; Nicholls and Budd, 2000). In addition, Ca^{2+} within the mitochondria can be released into the cytoplasm during protonophore exposure and protonophore treatment has been reported to cause a small elevation of $[\text{Ca}^{2+}]_i$ at rest in some neurons (Nohmi et al., 2000; Medler and Gleason 2002), but not in others (Vanden Berghe et al., 2002). In the present study, the fluo-3 fluorescence ratio F/F_0 increased by approximately 10% during exposure to CCCP alone or CCCP and oligomycin, but not during treatment with oligomycin alone. The CCCP- and oligomycin-induced increase in fluo-3 fluorescence was maintained throughout a 10-min exposure and reversed following drug removal. As no change in fluo-3 fluorescence occurred with oligomycin alone, the elevation of fluo-3 fluorescence was not thought to be due to an oligomycin-induced inhibition of ATP synthesis or a secondary effect due to inhibition of other ATPases that can occur with high concentrations of oligomycin (Fortes and Lee, 1984). In addition, during exposure to rotenone and oligomycin, there was no consistent change in the fluo-3 ratio. As both CCCP and rotenone should dissipate the membrane potential, both would be expected to cause a change in the fluo-3 ratio if it reflected a rise in $[\text{Ca}^{2+}]_i$ due to the release of Ca^{2+} from mitochondria. Based on the more gradual decrease in rhodamine 123 fluorescence, we assume that rotenone takes longer to fully dissipate the mitochondrial membrane potential than CCCP. This could lead to a slower release of Ca^{2+} from mitochondria, but it should still have been detectable with our Ca^{2+} imaging methods. Therefore, since only CCCP appeared to increase the fluo-3 ratio, we tentatively concluded that the sustained small increase in fluo-3 fluorescence ratio during exposure to CCCP might reflect an undefined effect of the protonophore on dye fluorescence or binding affinity.

Even if global $[\text{Ca}^{2+}]_i$ actually was increased to a small extent (perhaps by 10–15 nM assuming a $[\text{Ca}^{2+}]_i$ of 60–90 nM at rest) during CCCP exposure, it should not have been a factor determining the effect on latency to AP generation. This conclusion is based on the observation that the CCCP-induced increase in latency to AP genera-

tion was similar when recordings were made with either the perforated patch or standard whole cell recording mode. With the standard whole cell recording mode, a slowly developing small elevation in global $[Ca^{2+}]_i$, should be quickly buffered by the Ca^{2+} chelator (EGTA or BAPTA) included in the pipette solution.

During exposure to protonophore, the cytosolic pH can decrease (Nicholls and Budd, 2000). We found that the BCECF fluorescence ratio changed during protonophore exposure, an observation suggesting that the intracellular pH changed. However, the change in the F/F_o ratio was small corresponding to very small shifts in pH units (Wong et al., 2001). In addition, the average change in F/F_o ratio was biphasic, initially an increase indicating an increase in pH followed by a decrease consistent with a fall in pH. In contrast, no consistent change in BCECF fluorescence ratio was noted during exposure to rotenone. Therefore, the increase in latency for AP generation was not considered to be related to small shifts in intracellular pH.

The mitochondrial modulation of the latency to AP generation requires CICR

CICR is an effective mechanism by which Ca^{2+} influx can initiate the release of Ca^{2+} from ER stores into restricted domains near the plasma membrane, causing the local concentration of Ca^{2+} to rise sufficiently to activate Ca-dependent ion channels, including BK channels (Kuba et al., 1983; Kaczorowski et al., 1996; Scornik et al., 2001), which generates the SMOCs recorded in mudpuppy cardiac neurons (Satin and Adams, 1987; Merriam et al., 1999). The combined treatment of thapsigargin and caffeine effectively depletes Ca^{2+} stores in the ER (Thomas and Hanley, 1994) and eliminates CICR. The effect of CCCP and oligomycin on the latency to AP generation was eliminated by thapsigargin, demonstrating that CICR was required for the observed CCCP-induced increase in the latency to AP generation.

CICR-activated IBX-sensitive and IBX-insensitive channels contribute to the regulation of AP latency

We previously showed that the BK channel inhibitor IBX decreases the latency to AP generation, suggesting that currents flowing through BK channels contribute to the CICR-activated outward current opposing the ramp current used to initiate AP generation (Parsons et al., 2002). However, in our prior study, the decrease in latency following IBX treatment ($16 \pm 5\%$) was half that following elimination of CICR by thapsigargin treatment ($32 \pm 6\%$; Parsons et al., 2002). SMOCs, which are generated by the synchronous activation of BK channels, are eliminated equally by treatment with either thapsigargin (Parsons et al., 2002) or by exposure to 100 nM IBX (Merriam et al., 1999). These prior results suggest that activation of IBX-insensitive channels must also contribute to the CICR modulation of latency to AP generation. In the present study, we found that CCCP and oligomycin increased the latency to AP generation by $37 \pm 8\%$ in cells pretreated with 100 nM IBX to block BK channels. This increase in AP latency was less than that obtained for cells not pretreated with IBX ($56 \pm 4\%$). The

present observations provide additional support for the conclusion that both IBX-sensitive and IBX-insensitive ionic conductances, activated by CICR, contribute to the modulation of the latency to AP generation. In our previous study, we also noted that treatment with apamin, an inhibitor of small conductance Ca^{2+} -activated K^+ channels, had no effect on the latency to AP generation produced by a depolarizing current ramp (Parsons et al., 2002). This result suggests that apamin-sensitive small conductance Ca^{2+} -activated K^+ channels were not involved (Parsons et al., 2002). In recent unpublished results, we found that treatment with 20 μ M glibenclamide to inhibit ATP-dependent K^+ channels had no effect on the latency to AP generation (Barstow and Parsons, unpublished observations). Thus, it appears that activation of K_{ATP} channels also does not contribute to the regulation of latency to AP generation. Additional experiments are under way to identify the IBX-insensitive ionic conductance(s) contributing to the CICR-mediated regulation of AP generation in these mudpuppy neurons.

Proposed mechanism by which mitochondrial sequestration of Ca^{2+} might affect the CICR-modulation of the latency to AP generation

Rhodamine 123 staining demonstrated that mitochondria are densely distributed throughout the cytoplasmic compartment; thus they are positioned to potentially affect changes in Ca^{2+} levels by buffering Ca^{2+} entering VDCCs and/or Ca^{2+} domains near ryanodine-sensitive release sites on the ER. Within local domains near VDCCs or ryanodine-sensitive release sites, the Ca^{2+} can reach micromolar concentrations and mitochondria could regulate the amplitude, duration and spread of Ca^{2+} within these domains (Rizzuto et al., 1998), thereby modulating CICR effectiveness (Babcock et al., 1997). Previously, we estimated that the very brief, local rise in Ca^{2+} that occurs during CICR must reach at least 40 μ M (Scornik et al., 2001). This is well above the 200–500 nM thought to be the threshold for Ca^{2+} sequestration into mitochondria (Friel and Tsien, 1994; Werth and Thayer, 1994; Colegrove et al., 2000).

EGTA and BAPTA have comparable Ca^{2+} buffering capacity although BAPTA can buffer fast transient elevations of Ca^{2+} much more efficiently than EGTA (Naraghi, 1997). Therefore, results with EGTA or BAPTA often are compared to distinguish Ca^{2+} transients that are very fast or occur in local domains (Deisseroth et al., 1996; Neher, 1998). In mudpuppy cardiac neurons Ca^{2+} influx through VDCCs directly activates BK channels that contribute to AP repolarization (Parsons et al., 2002). In ongoing experiments we have found that the rate of AP repolarization was consistently slower when 5 mM BAPTA was included in the pipette solution than when 5 mM EGTA was included in the pipette solution (Barstow and Parsons, unpublished observations). This observation suggests that 5 mM BAPTA, but not 5 mM EGTA, can buffer the rise in $[Ca^{2+}]_i$ which directly activates BK channels that participate in AP repolarization. In contrast, the latency to AP generation was increased by CCCP and oligomycin to the same ex-

tent whether the recordings were made with the perforated patch configuration or with the standard whole cell configuration with either 5 mM EGTA or 5 mM BAPTA in the pipette solution. Thus, the elevation of Ca^{2+} due to release from internal stores by CICR must occur in discrete domains where BAPTA at a concentration of 5 mM is ineffective. This observation suggests that mitochondria may be positioned such that they buffer the CICR-activated transient rise in Ca^{2+} that occurs in local domains between the ER Ca^{2+} release sites and opposing plasma membrane. Interruption of mitochondrial sequestration of Ca^{2+} would increase the Ca^{2+} concentration within the restricted domain between the plasma membrane and ER, and as a consequence, could increase the efficiency of CICR, which in turn increases the magnitude of the outward currents that oppose the depolarizing current ramps.

Acknowledgements—This work was supported in part by NSF grant IBN-0076741 and NIH grant HL-65481. The DeltaVision Restoration microscope is provided through the Imaging Core supported by NIH grant P20 RR-16435 from the COBRE program of the National Center for Research Resources. We thank Dr. John Tompkins for reviewing this manuscript.

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(Accepted 11 December 2003)