Mitochondria Support Inositol 1,4,5-Trisphosphate-mediated Ca\textsuperscript{2+} Waves in Cultured Oligodendrocytes*

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We have examined the spatial and temporal nature of Ca\textsuperscript{2+} signals activated via the phosphoinositide pathway in oligodendrocytes and the cellular specializations underlying oligodendrocyte Ca\textsuperscript{2+} response characteristics. Cultured cortical oligodendrocytes were incubated with fluo 3 or fura 2, and digital video fluorescence microscopy was used to study the effect of methacholine on [Ca\textsuperscript{2+}]\textsubscript{i}. Single peaks, oscillations, and steady-state plateau elevations were evoked by increasing agonist concentration. The peaks and oscillations were found to be Ca\textsuperscript{2+} wave fronts, which propagate via distinct amplification regions in the cell where the kinetics of Ca\textsuperscript{2+} release (amplitude and rate of rise of response) are elevated. Staining with 5,5\textquotesingle,6,6\textquotesingle-tetrachloro-1,1\textquotesingle,3,3\textquotesingle-tetraethylbenzimidazolocarbocyanine iodide (JC-1) and 3,3\textquotesingle-dihexyloxacarbocyanine iodide revealed that mitochondria are found in groups of three or more in oligodendrocyte processes and that the groups are distributed with considerable distance separating them. Cross-correlation analysis showed a high degree of correlation between sites where mitochondria are present and peaks in the amplitude and rate of rise of the Ca\textsuperscript{2+} response. Intramitochondrial Ca\textsuperscript{2+} concentration, measured using rhod 2, increased upon treatment with methacholine. Methacholine also evoked a rapid change in mitochondrial membrane potential as measured by the J-aggregate fluorescence of JC-1. Pretreatment with the mitochondrial inhibitors carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (1 \textmu M, 2 min) or antimycin (2 \textmu g/ml, 2 min) altered the methacholine-evoked Ca\textsuperscript{2+} response in most cells studied, responses being either markedly potentiated or inhibited. The results of this study demonstrate that stimulation of phosphoinositide-coupled muscarinic acetylcholinocepotor activates propagating Ca\textsuperscript{2+} wave fronts in oligodendrocytes and that the characteristics of these waves are dependent on mitochondrial location and function.

Cultured oligodendrocytes express a variety of receptors coupled to the mobilization of Ca\textsuperscript{2+} from inositol trisphosphate receptor (InsP\textsubscript{3}R)-expressing intracellular stores. These include M\textsubscript{1} muscarinic cholinceptors (1–3), which are also thought to be expressed on oligodendrocytes in vivo (4). While InsP\textsubscript{3}-evoked Ca\textsuperscript{2+} responses are known to be present in oligodendrocytes (2, 5), the spatial and temporal nature of these has not been characterized. In a wide variety of cell types, including several other types of glial cells, stimulation of InsP\textsubscript{3}-coupled receptors results in activation of Ca\textsuperscript{2+} waves and oscillations (6–10). In astrocytes, Ca\textsuperscript{2+} waves are initiated in several distinct regions, which then propagate throughout the cell via multiple amplification sites, at which the amplitude of the wave and the rate of rise of the response are markedly elevated compared to surrounding regions (9, 10).

The molecular specializations underlying wave initiation and propagation sites in glial and other cell types remain poorly defined. Recent reports have, however, implicated a role for mitochondria as well as endoplasmic reticulum (ER) in the regulation of cytosolic Ca\textsuperscript{2+} signals and the propagation of Ca\textsuperscript{2+} waves (11–15). Endoplasmic reticulum and mitochondria are found in close apposition in several cell types (16, 17). Ca\textsuperscript{2+} released via InsP\textsubscript{3}R is transferred into mitochondria more effectively than [Ca\textsuperscript{2+}]\textsubscript{i}, increases evoked by other means (11, 18), consistent with a close functional relationship between InsP\textsubscript{3}R and mitochondrial Ca\textsuperscript{2+} uptake. Also, Ca\textsuperscript{2+} waves are increased by up-regulation of mitochondrial function in Xenopus oocytes (14). Coordinate activity of mitochondria and ER (14, 15) could enable differences in Ca\textsuperscript{2+} buffering and Ca\textsuperscript{2+} regulation in subcellular microdomains.

The present study was undertaken to determine whether Ca\textsuperscript{2+} signals activated via the phosphoinositide pathway in oligodendrocytes would take the form of Ca\textsuperscript{2+} waves with characteristic initiation sites and propagation sites. Further, we sought to determine the nature of the cellular specializations underlying oligodendrocyte Ca\textsuperscript{2+} signals. For this, we analyzed the pattern of distribution of mitochondria in cells previously stimulated to evoke Ca\textsuperscript{2+} responses and also examined the effect of altering mitochondrial activity on Ca\textsuperscript{2+} signaling. Our findings indicate that oligodendrocytes respond to methacholine (MCh)-evoked InsP\textsubscript{3}R generation with Ca\textsuperscript{2+} waves. These waves include sites of high local Ca\textsuperscript{2+} release kinetics in the same locations as for caffeine-evoked waves in the same cells, consistent with their corresponding to distinct cellular specializations. Mitochondria were found to be present at these wave amplification sites, apparently in intimate association with the ER, but not elsewhere along oligodendrocyte processes. MCh also evokes changes in mitochondrial Ca\textsuperscript{2+} and membrane potential. Inhibition of mitochondrial activity was found to abolish or in some cases potentiate the ability of MCh to evoke a cytosolic Ca\textsuperscript{2+} response. Thus, both mitochondrial location and function appear to be crucial to the generation and characteristics of Ca\textsuperscript{2+} waves in oligodendrocytes.

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‡‡The abbreviations used are: InsP\textsubscript{3}R, inositol 1,4,5-trisphosphate receptor; InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; MCh, methacholine; ER, endoplasmic reticulum; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; JC-1, 5,5\textquotesingle,6,6\textquotesingle-tetrachloro-1,1\textquotesingle,3,3\textquotesingle-tetraethylbenzimidazolocarbocyanine iodide; DiOC\textsubscript{3}(3), 3,3\textquotesingle-dihexyloxacarbocyanine iodide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MCh-evoked InsP\textsubscript{3}R generation with Ca\textsuperscript{2+} waves. These waves include sites of high local Ca\textsuperscript{2+} release kinetics in the same locations as for caffeine-evoked waves in the same cells, consistent with their corresponding to distinct cellular specializations. Mitochondria were found to be present at these wave amplification sites, apparently in intimate association with the ER, but not elsewhere along oligodendrocyte processes. MCh also evokes changes in mitochondrial Ca\textsuperscript{2+} and membrane potential. Inhibition of mitochondrial activity was found to abolish or in some cases potentiate the ability of MCh to evoke a cytosolic Ca\textsuperscript{2+} response. Thus, both mitochondrial location and function appear to be crucial to the generation and characteristics of Ca\textsuperscript{2+} waves in oligodendrocytes.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Caffeine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), antimycin, and n-adamantanemethol chloride (MCh) were obtained from Sigma. Fura-2 AM, fluo-3 AM, and rhod-2 AM were obtained from Research Biochemicals International. 3,3’-Dihexyloxacarbocyanine iodide (DiOC(3)) was a gift from Dr. M. Terasaki. 5,5’,6,6’-Tetrachloro-1,1’,3,3’-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was from Molecular Probes.

**Cell Culture**—Oligodendrocytes were cultured from 2-day-old rat pups as described previously (19). After 8 days in vitro, the flask was vigorously shaken overnight, and the supernatant was repeatedly plated onto plastic dishes. Nonadherent cells were then replated onto glass coverslips coated with polyornithine in DME-N1 containing 0.5% fetal bovine serum and maintained in 10% CO2, 90% air. After 24 h, culture medium was replaced with DME-N1 containing 2% fetal bovine serum, and cells were used 4–8 days after plating on coverslips. More than 90% of these cells were positively labeled with a monoclonal antibody against galactocerebroside, a marker for oligodendrocytes (20).

\([\text{Ca}^{2+}]_i\), and Membrane Potential Measurements—Cells grown on coverslips were incubated with 5 μM fura-2 AM for 20 min at room temperature as described previously (9, 21). The perfusion chamber was positioned on the stage of an inverted microscope, with fluorescence images at 540 nm and excitation wavelengths being acquired at 510 nm emission wavelength through a microchannel plate intensifier with a CCD camera (9). All cells in a field were analyzed individually. For analysis of Ca^{2+} waves in single cells, cells were incubated with 5 μM fluo 3-AM for 20 min, and fluorescence images were acquired with excitation at 495 nm (emission at 525 nm) (9). Images were digitized and averaged in a Trapp 55/2256 image processor. Nonzero pixels, within each slice were averaged and plotted as normalized fluorescence intensities (ΔF/ΔFo) against time or against distance along the cell. Cells were divided for analysis into 0.83-μm-wide regions sequentially along the longitudinal axis. For measurement of Ca^{2+} within mitochondria, cells were incubated with 5 μM rhod-2 AM for 30 min and returned to culture medium for 24 h at 37 °C, which results in removal of most cytosolic rhod 2 while the mitochondria remain stained (15). Images were acquired with excitation at 525 nm and emission at 610 nm. High resolution imaging enabled delimiting of individual mitochondria for analysis. For measurement of mitochondrial membrane potential, cells were incubated with 10 μg/ml JC-1 for 10 min, and red (J-aggregate) fluorescence was measured (33, 34). Rhod 2 and JC-1 fluorescence signals were not calibrated to Ca^{2+} concentration or membrane potential and represent qualitative measures.

**Staining and Analysis of Endoplasmic Reticulum and Mitochondria Distribution**—Cells were incubated in DiOC(3) (0.5 μg/ml) in phosphate-buffered saline for 1 min, washed, and examined under the fluorescence microscope. For JC-1 staining, cells were incubated with 10 μg/ml JC-1 for 10 min in phosphate-buffered saline, washed once in phosphate-buffered saline, and viewed using either fluorescent filters or Cy3 filters sets as appropriate. For high resolution analysis of cells stained with these fluorescent organelle dyes, we used an exhaustively photon reassignment procedure developed jointly by University of Massachusetts and Scannalytics Inc. (22). In this method, digital images from a standard wide field microscope are restored using an algorithm that removes out of focus plane fluorescence (22). The axial resolution of our microscope under our measurement conditions was 0.75 μm. Ca^{2+} waves were evoked in a flou 3-loaded cell using MCh and, after recovery, the cell was incubated with JC-1 or DiOC(3) while still on the microscope stage, and the fluorescence of this dye was measured using appropriate optical filters. Ca^{2+} wave kinetics and mitochondrial distribution patterns were then compared. Fluor 3 fluorescence was negligible (<1% of total fluorescence for both JC-1 and DiOC(3)) in the DiOC(3) or JC-1 channels due to the relatively bright fluorescence of these two dyes. The cross-correlation analysis—the spatial patterns of Ca^{2+} responses and of mitochondrial distribution were compared in the same cells using a cross-correlation function derived from the fast Fourier transform of the data sets as a quantitative test for similarity (23, 24). The measured values of normalized fluorescence intensities were subtracted out, and the resulting zero mean waves were embedded in surrounding zeros. The windowed data were Fourier transformed via a fast Fourier transform algorithm, using standard functions in Mathematica (Wolfram Research, Inc., Champaign, IL). Cross-spectra were then formed as a product of one wave with the complex conjugate transform of a second wave. The cross-correlation function was produced by inverse Fourier transformation of the cross-spectrum. Changing the order of the sequences produced the expected mirror image cross-correlation functions. Performing these operations on a single wave produces the power density spectrum and the autocorrelation function. Data are presented as mean ± S.D.

**RESULTS**

**InsP₃-mediated Ca^{2+} Responses in Oligodendrocytes**—Experiments were performed to examine the spatial and temporal characteristics of Ca^{2+} responses evoked in oligodendrocytes by MCh, an agonist of muscarinic receptors. Single peaks, oscillations, and steady-state plateau [Ca^{2+}]i elevations were evoked by increasing agonist concentration (Fig. 1, A and B; n = 240 cells). 73.3% of cells examined responded to 1 mM MCh. In addition, in individual cells, a graded increase in the magnitude of the peak of the wave was observed (Fig. 1A), unlike the all or none responses to MCh previously reported in several other cell types (25, 26). The EC_{50} of the peak response amplitude was 1.5 ± 0.5 μM, whereas the EC_{50} of the plateau phase was 35.6 ± 26.2 μM; however, these values were not significantly different. Addition of 100 μM MCh in nominally Ca^{2+}-free conditions ([Ca^{2+}]o < 5 μM) evoked an initial peak response of the same magnitude as in normal Ca^{2+}, but whereas in normal Ca^{2+} a plateau phase or oscillations were evoked, in Ca^{2+}-free medium no significant plateau phase (Fig. 1C), or only one or two small oscillations (not shown), was found (n = 10). Oligodendrocytes activatedly stimulated these times with MCh in normal extracellular solution ([Ca^{2+}]o) = 1.5 mM, at >15-min intervals, displayed responses of consistent magnitude. Pre-treatment with the store-depleting agent thapsigargin abolished responses to MCh (n = 34; not shown). These results suggest that the initial peak responses are due to InsP₃-mediated Ca^{2+} mobilization from stores, whereas the sustained oscillations and the plateau phase apparently require Ca^{2+} entry across the plasma membrane.

To investigate the spatial nature of the Ca^{2+} response evoked by InsP₃ generation, we examined the MCh response in fluo 3-loaded oligodendrocytes at higher time resolution (1 image every 66 ms) and sectioning of the cell into successive 0.83-μm-wide segments along the cell axis (see Ref. 9). Stimulation of an oligodendrocyte with 100 μM MCh resulted in the activation of propagating Ca^{2+} wave fronts from several initiation sites (Fig. 2a). Although oligodendrocyte processes are approximately cylindrical (27), the magnitude and rate of rise of the Ca^{2+} response were found to be non-uniform, with some regions of the processes displaying markedly higher amplitude responses or faster rate of rise of response than in surrounding regions (Fig. 2b). The regions of large amplitude and regions with steeply rising responses corresponded closely to each other (see later). These appear highly analogous to the amplification regions previously described for InsP₃-mediated responses in type 1 astrocytes (9), the elevated Ca^{2+} kinetics of which probably contribute to the sustenance and propagation of the wave (10). A plot of the time to 50% peak against cell length clearly shows the wave initiation sites (Fig. 2c, asterisks) which are located near but not colocalized with sites where the higher kinetics in Ca^{2+} release are measured (compare Fig. 2b and Fig. 2c). The specialized regions of wave initiation and amplification varied in diameter from 1 to 5 μM.

**Comparison between InsP₃ and Caffeine-evoked Ca^{2+} Waves**—Oligodendrocytes express a single Ca^{2+} pool sensitive to InsP₃, caffeine, and cyclopiazonic acid (28). We have previously demonstrated that cells of the oligodendrocyte lineage respond to caffeine with propagating Ca^{2+} waves the local kinetics of which vary along the length of the cell (28). Here we

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**Fig. 1.** Concentration-response relationship for MCh-evoked \( [\text{Ca}^{2+}]_{i} \) responses. a, \( \text{Ca}^{2+} \) responses to increasing concentrations of agonist increase in a graded manner. A series of \( \text{Ca}^{2+} \) responses to increasing concentrations of MCh at 30-min intervals. The concentrations (\( \mu \text{M} \)) of the agonist are indicated on the top abscissa, and the durations of agonist treatment are indicated by the rectangles. 1–10 \( \mu \text{M} \) concentrations of MCh activate only a single peak \( [\text{Ca}^{2+}]_{i} \) elevation; 100 \( \mu \text{M} \) concentrations activate a peak followed by a series of oscillations on top of a plateau; 1 \( \text{mM} \) MCh activates a peak followed by a more stable plateau. b, changes in response characteristics with increasing agonist concentration. A series of \( [\text{Ca}^{2+}]_{i} \) responses to increasing concentrations of MCh were measured in oligodendrocytes at 30-min intervals between challenges as in a. Responses from 240 cells from 3 separate experiments in different oligodendrocyte cultures are shown in a column chart displaying the different types of responses. i, percentage of cells that show a single peak or a series of oscillations; II, cells that display a peak and stable plateau; iii, percentage of total cells that respond to MCh. An increasing percentage of cells are recruited as agonist concentration was increased. Low \( \mu \text{M} \) concentrations of MCh typically activated only a single peak or a series of oscillations, with very few plateau responses. c, dependence of plateau response on extracellular \( \text{Ca}^{2+} \). Successive responses to 100 \( \mu \text{M} \) MCh in nominally \( \text{Ca}^{2+} \)-free and 1.5 \( \text{mM} \) \( \text{Ca}^{2+} \)-containing medium in a single fura 2-loaded cell. MCh activated a rapid onset peak elevation of \( [\text{Ca}^{2+}]_{i} \), that develops within 5 s of agonist application, which was similar in magnitude compared with the normal \( \text{Ca}^{2+} \). However, the response in \( \text{Ca}^{2+} \)-free medium decays to basal levels even in the presence of agonist.

compared, in the same cells, InsP3-mediated (MCh-induced) and caffeine-mediated waves. These experiments showed that the local amplitudes and rates of rise of the \( \text{Ca}^{2+} \) signal were similar under each stimulation condition (Fig. 3a). In the cell shown in Fig. 3, at least four specialized regions of high amplitude (marked with an asterisk) are identifiable, which appear in similar locations in the cell for the two responses. An additional high amplitude region for the caffeine response (42–48 \( \mu \text{M} \)) does not appear in the MCh response. Cross-correlation analysis of the caffeine peak amplitude versus MCh peak amplitude showed high correlation values in phase (Fig. 3b). In three separate experiments, the cross-correlation values were 0.70 ± 0.05 (mean ± S.D.). The pattern of cross-correlation is similar to the autocorrelation of the local peak amplitude data for the caffeine response (Fig. 3b). Comparison of the slopes between the two responses also yielded similar high correlation values (Fig. 3b; 0.82 ± 0.17; \( n = 3 \)). This result indicates that a local cellular specialization that supports high \( \text{Ca}^{2+} \) release may be due not only to increased expression of InsP3R, as suggested by us previously (10) but to a generalized specialization of the \( \text{Ca}^{2+} \) release machinery, perhaps including \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \)-release processes and specialization of other organelles (see later).

**Mitochondrial Distribution in Oligodendrocytes—**One possible cause of regions with higher local kinetics of \( \text{Ca}^{2+} \) release in response to InsP3 or caffeine would be mitochondrial regulation of \( \text{Ca}^{2+} \) in cellular microdomains. Mitochondria are known to be able to take up considerable amounts of \( \text{Ca}^{2+} \) in response to InsP3 or caffeine but would be mitochondrial regulation of \( \text{Ca}^{2+} \) in cellular microdomains. Mitochondria are known to be able to take up considerable amounts of \( \text{Ca}^{2+} \) in response to InsP3 or caffeine but would be mitochondrial regulation of \( \text{Ca}^{2+} \). The pattern of distribution of mitochondria and ER in oligodendrocytes was investigated using the lipophilic dyes JC-1 and DiOC6(3).
Role of Mitochondria in Propagation of Ca\(^{2+}\) Waves—To investigate whether the distribution of mitochondria is related to characteristics of the Ca\(^{2+}\) waves evoked by store release, we studied Ca\(^{2+}\) waves induced by MCh (100 \(\mu\)M) and compared the spatial kinetics with the mitochondrial distribution in the same cells. If mitochondrial function is fundamental to Ca\(^{2+}\) signaling in cellular microdomains, the pattern of mitochondrial distribution will be expected to be well correlated with the kinetics of local Ca\(^{2+}\) release. Ca\(^{2+}\) waves were evoked by MCh in fluo 3-loaded cells and, following recovery, they were incubated with JC-1 or DiOC\(_6\)(3) while still on the microscope stage. The fluorescence of this second dye was measured, and the resultant wave and organelle distribution patterns were then compared. Cross-correlation analysis was used to compare the patterns of local amplitudes and the rate of rise of the signals with the patterns of JC-1 or DiOC\(_6\)(3) fluorescence (Fig. 3b). Comparison of the patterns of DiOC\(_6\)(3) staining along the cellular process (distribution of mitochondria) with the patterns of both the amplitude and rate of rise of the Ca\(^{2+}\) response showed high correlation values (Fig. 5b), in this cell \(12-23 \mu\)m from phase. The local amplitude and the rate of rise of the Ca\(^{2+}\) responses when compared with each other showed good correlation values in phase.

Results from six separate experiments are summarized in Table I. JC-1 staining was discrete and appeared at all sites of the cell process where higher Ca\(^{2+}\) release kinetics were measured (Fig. 5c). No peaks in the Ca\(^{2+}\) response were found at sites along processes where

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**Fig. 2.** MCh activates Ca\(^{2+}\) waves in oligodendrocytes. Responses from a single experiment, representative of data from five independent experiments on fluo 3-loaded oligodendrocytes from different cultures. An oligodendrocyte was stimulated with 100 \(\mu\)M MCh. For analysis, the cell image was serially sliced through the cell body and two main processes into 0.8-\(\mu\)m-wide regions. Data illustrate the characteristics and time course of wave fronts traveling along a process, through the cell body, and out along the second process. a, offset plot of Ca\(^{2+}\) waves evoked by MCh from successive regions of an oligodendrocyte process. Four sites of MCh-evoked wave initiation can be identified, and differences in magnitude and rate of rise of peak can also be detected. b, plot of the amplitude and rate of rise of the [Ca\(^{2+}\)] response evoked against the distance along the cell. The signal amplitude and the rate of rise vary greatly through the length of the process showing specialized regions where the kinetics are significantly enhanced. Peak and rate of rise were calculated as described previously (9) and represent \(\Delta F/F\) and \(\Delta F/F\) per s, respectively. c, analysis of the onset of the [Ca\(^{2+}\)] response along the length of the cell. This was measured by a plot of the time taken to reach 50% of the maximum response. The four different sites where Ca\(^{2+}\) waves initiate are identifiable (*), separated by regions along which the waves travel at a non-uniform rate.

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**Fig. 3.** MCh and caffeine (caf) activate Ca\(^{2+}\) waves with similar amplitude (Amp.) characteristics in oligodendrocytes. a, responses from a single experiment, representative of data from three independent experiments on fluo 3-loaded oligodendrocytes from different cultures. Plot of the amplitude (peak \(\Delta F/F\)) of the [Ca\(^{2+}\)] response evoked by MCh or caffeine against distance along the cell. At least four specialized regions of high amplitude (marked with asterisks) are identifiable, which appear to be in very similar locations in the two response types. An additional high amplitude region for the caffeine response (42-48 \(\mu\)m) does not appear in the MCh response. b, cross-correlation analysis comparing the patterns of MCh and caffeine response kinetics show correlation patterns very similar to the autocorrelation for the caffeine amplitude response (---). ●, comparison of peak amplitudes in the MCh response versus peak amplitudes in the caffeine response; ○, slopes of the MCh response versus slopes in the caffeine response.
mitochondria were absent, and mitochondria were not found at sites where response peaks were not observed. With JC-1, the green and red fluorescence intensity patterns of staining were essentially identical with each other (Table I), such that the cross-correlation appears essentially similar to autocorrelation. Comparison of the patterns of Ca$^{2+}$ wave kinetics (amplitude and rate of rise) also resulted in very high correlation values (Fig. 5d). The magnitude and the pattern of cross-correlation were similar between wave characteristics and either JC-1 green fluorescence or red J-aggregates (Fig. 5d; Table I). This result may suggest that the location of functional mitochondria, rather than their local membrane potential at rest, may be most crucial in determining wave characteristics. Mitochondrial location was found to be invariant over $>$15-min periods either at rest or after stimulation with MCh (not shown).

The effects of agonist-induced Ca$^{2+}$ waves on mitochondrial activity were measured by monitoring intramitochondrial Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{m}$) and mitochondrial membrane potential using the fluorescent dyes rhod 2 and JC-1, respectively. Rhod 2 can be selectively loaded into mitochondria, and used as an indicator of mitochondrial free [Ca$^{2+}$]$_{m}$ (see Ref. 15). High resolution imaging allowed measurement of fluorescence within individual mitochondria along oligodendocyte processes. MCh (100 µM) caused a rapid onset increase in mitochondrial rhod 2 fluorescence (43 of 66 mitochondria, 65% (Fig. 6a)). These responses reflect uptake of Ca$^{2+}$ into mitochondria and appear to occur sufficiently rapidly to act as a fast local Ca$^{2+}$ buffering system. Such a mechanism could potentially play a role in modulation of Ca$^{2+}$ sensitivity of nearby InsP$_3$Rs (see “Discussion”). Treatment with the protonophore FCCP (1 µM), which collapses the proton gradient across mitochondrial membranes (12, 38), decreased rhod 2 fluorescence in mitochondria, typically within 2 min of treatment, indicating release of mitochondrial Ca$^{2+}$ (27 of 43 mitochondria, 63% (Fig. 6b)). Similarly, antimycin (2 µg/ml), an inhibitor of electron transport through complex III (14, 39), also decreased rhod 2 fluorescence (i.e. [Ca$^{2+}$]$_{m}$) in 24 of 30 mitochondria (80%, Fig. 6c). The antimycin-induced decrease in rhod 2 fluorescence began within 30 s of treatment and took up to 4 min to reach completion. To examine the effect of MCh on mitochondrial membrane potential, cells were incubated with JC-1, and the level of red J-aggregate formation in response to elevated mitochondrial membrane potential.

To investigate the nature of the role played by mitochondria in Ca$^{2+}$ signaling in oligodendrocytes, the effect of modifying mitochondrial activity on MCh-evoked cytosolic Ca$^{2+}$ signals was studied. After initial stimulation with MCh, cells were treated with FCCP. Relatively brief (2 or 5 min) pretreatment with 1 µM FCCP caused MCh responses in some cells to be abolished (Fig. 7a) but responses in other cells to be markedly potentiated (Fig. 7b), while others were unaffected (Table II). In the cell shown in Fig. 7b, the amplitude of the MCh-evoked peak is increased after FCCP pretreatment, and the peak is now followed by a second peak and then a steady-state plateau. FCCP treatment by itself evoked a slow, persistent elevation of [Ca$^{2+}$]$_{m}$ in oligodendrocytes, probably due to the leak of Ca$^{2+}$ out of mitochondria. More sustained treatment with FCCP (5 µM, 30 min) pretreatment greatly reduced or abolished the
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MCh-evoked $Ca^{2+}$ response (Table II). In most cells examined, a 2-min pretreatment with CCCP (5 μM), a structural analog of FCCP, completely abolished MCh-induced $[Ca^{2+}]_i$ signals (Table II). An alternative approach to modulation of mitochondrial function was treatment of the cells with antimycin. Incubation with antimycin (2 μg/ml) for as little as 2 min inhibited $Ca^{2+}$ responses to MCh stimulation in most cells (Fig. 7c; Table II).

**Fig. 7. Comparison of $Ca^{2+}$ wave characteristics and distribution of mitochondria.**

**a,** plot of the amplitude of the $Ca^{2+}$ signal ($\Delta F/F$ (●) and DiOC$_6$(3) fluorescence intensity (○) both measured in serial slices of the cell against distance (cell length) along an oligodendrocyte process. The cell was sliced into 0.5-μm-wide segments and numbers indicate discrete peaks in both $Ca^{2+}$ wave amplitude and DiOC$_6$(3) fluorescence. Comparison of the wave amplitude and rate of rise of response (○) showed high correlation consistent with these being representations of the same release phenomenon. The rate of rise of response (●) and the peak amplitudes (◇) when compared with DiOC$_6$(3) fluorescence also showed high cross-correlation 2–3 μm from phase. c, measurements of wave amplitude ($\Delta F/F$(●)) and both red (○) and green (◇) JC-1 fluorescence intensities plotted against distance along the process. Peaks of apparent correlation are numbered for both amplitude data and JC-1 fluorescence. Peaks in wave amplitude are found only where peaks of fluorescence occur, and no mitochondria are found in the absence of a peak wave front amplitude. Red JC-1 fluorescence displays particularly high discrimination of mitochondrial signal. d, cross-correlation analysis of patterns of JC-1 fluorescence and $Ca^{2+}$ release kinetics in an oligodendrocyte process. Comparison of wave amplitudes and JC-1 red (○) and green (●) and rate of rise of response and JC-1 red (◇) and green (△) fluorescence. The red and green fluorescence profiles have high cross-correlation with wave characteristics, and also showed high cross-correlation with each other (not shown).

**Table I**

Correlation between distribution of mitochondria and $Ca^{2+}$ wave propagation characteristics

Results of cross-correlation analysis of $Ca^{2+}$ wave patterns and mitochondrial staining with carbocyanine dyes. Data are presented as mean ± S.D. High correlation is found between mitochondrial location and two parameters of high $Ca^{2+}$ release kinetics, peak (amplitude of $Ca^{2+}$ response) and slope (rate of rise of $Ca^{2+}$ response). Correlation occurs very close to phase, indicating that the locations of maxima in these kinetic parameters are very close to maxima in dye fluorescence, i.e. mitochondrial loci.

| Comparison | Peak correlation | Location of peak correlation (μm) | Correlation in phase | n |
|------------|------------------|----------------------------------|---------------------|---|
| Peak vs. slope | 0.76 ± 0.13 | 0.00 ± 0.00 | 0.76 ± 0.13 | 11 |
| JC-1 red vs. green | 0.65 ± 0.24 | 0.11 ± 0.29 | 0.65 ± 0.24 | 7 |
| Peak vs. JC-1 red | 0.59 ± 0.16 | 1.90 ± 2.41 | 0.34 ± 0.29 | 7 |
| Peak vs. JC-1 green | 0.46 ± 0.29 | 0.60 ± 0.79 | 0.40 ± 0.32 | 7 |
| Peak vs. DiOC$_6$(3) | 0.67 ± 0.21 | 5.23 ± 3.53 | 0.35 ± 0.47 | 6 |
| Slope vs. JC-1 red | 0.47 ± 0.16 | 2.01 ± 2.54 | 0.29 ± 0.13 | 7 |
| Slope vs. JC-1 green | 0.34 ± 0.29 | 1.10 ± 1.24 | 0.25 ± 0.32 | 7 |
| Slope vs. DiOC$_6$(3) | 0.47 ± 0.24 | 5.00 ± 3.70 | 0.18 ± 0.24 | 6 |

Fig. 7c shows an example trace of a single oligodendrocyte which initially displayed a large oscillatory response to MCh (100 μM). Antimycin (2 μg/ml, 2 min) pretreatment completely abolished this response. Prolonged treatment with antimycin had no greater effect on $Ca^{2+}$ signals (Table II), while a lower concentration (0.2 μg/ml, 15 min) also inhibited MCh-evoked responses to a marked extent (Table II).
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**Fig. 6.** Measurement of mitochondrial \([\text{Ca}^{2+}]_{\text{mito}}\) and membrane potential in oligodendrocytes. a, oligodendrocytes were loaded with rhod 2 (see "Experimental Procedures") and kept overnight at 37°C to ensure loss of nonmitochondrial dye (15). Cells were positioned on the microscope stage and then stimulated with MCh. Rhod 2 fluorescence was measured in single mitochondria within oligodendrocyte processes. Example trace from an oligodendrocyte mitochondrion demonstrates that MCh causes a rapid onset increase in rhod 2 fluorescence, i.e., an elevation in \([\text{Ca}^{2+}]_{\text{mito}}\). b, c, FCCP (1 mM) or antimycin (2 mM) decrease rhod 2 fluorescence in oligodendrocyte mitochondria. Rhod 2-loaded oligodendrocytes were perfused with FCCP or antimycin. Example traces from individual mitochondria illustrate a gradual decline in fluorescence levels beginning 5-30 s after drug exposure and reaching a plateau after 1-4 min. Although a number of reports have shown that cells of the oligodendrocyte lineage in culture possess functional receptor systems for neurotransmitters and that their stimulation results in cellular \([\text{Ca}^{2+}]\) signals (2, 5, 40-42), the spatiotemporal characteristics of these responses have not previously been examined. We show here that MCh-evoked \([\text{Ca}^{2+}]\) signals in oligodendrocytes are propagating \([\text{Ca}^{2+}]\) wave fronts. Such waves might enable oligodendrocytes to convey signaling information over relatively long distances in brain (43). A possible role for long distance signaling by astrocytes in brain function has already been hypothesized (6, 44).

As in astrocytes (9, 10), propagation of \([\text{Ca}^{2+}]\) waves in oligodendrocytes occurs with significant differences in \([\text{Ca}^{2+}]\) release kinetics in certain regions of the cell. These regions of cellular specializations, where the local signal amplitude and rate of rise are higher, were highly correlated in both InsP_3_ and caffeine-evoked \([\text{Ca}^{2+}]\) waves. Thus, microdomains that may support wave propagation (10) can apparently do so via both InsP_3_ and ryanodine receptors, giving the oligodendrocyte a generalized "\([\text{Ca}^{2+}]\) fingerprint" for signal propagation. These sites appear to be distinct from sites of wave initiation, of which each cell can possess five or more throughout its arborization. The changes in local wave kinetics are not due to large changes in cell shape, since oligodendrocyte processes have been shown to be approximately uniformly cylindrical (27). Our results are consistent with the idea that oligodendrocytes possess a single

**DISCUSSION**

The results of this study demonstrate that stimulation of a phosphoinositide-coupled muscarinic acetylcholine receptor activates propagating \([\text{Ca}^{2+}]\) wave fronts in oligodendrocytes and that the characteristics of these waves are dependent on mitochondrial location and function. MCh activates a rapid onset \([\text{Ca}^{2+}]\) peak in a concentration-dependent manner in \(~73\%\) of oligodendrocytes tested. Plateau and sustained oscillations, but not initial peak, elevations were dependent on extracellular \([\text{Ca}^{2+}]\), consistent with store-operated channel activation and the need for store refilling (8). Although a number of reports

**Fig. 7.** Effects of inhibition of mitochondrial activity on MCh-evoked \([\text{Ca}^{2+}]\) signals. a, b, example traces of MCh induced \([\text{Ca}^{2+}]\) signals in two separate fura 2-loaded cells from the same experimental field before (left) and after (right) exposure to FCCP (1 mM, 2 min). The cell in a is inhibited by this treatment, whereas the response of the cell in b is enhanced by FCCP. c, pretreatment with antimycin (2 mM, 2 min) abolished the \([\text{Ca}^{2+}]\), elevation evoked by MCh (100 \(\mu\text{M}\)) in this fura 2-loaded oligodendrocyte. In all panels MCh treatment is indicated by open rectangles and perfusion of mitochondrial inhibitors is shown as black bars on the abscissas.
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**Table II**

Effects of mitochondrial inhibitors on MCh-evoked Ca\(^{2+}\) responses in oligodendrocytes

Fura-2-loaded oligodendrocytes were challenged with MCh (100 \(\mu\)M) first; after recovery, they were exposed to FCCP, CCCP, or antimycin for different times, and the cells challenged with MCh a second time. Data represent response to the second stimulation with MCh. \(n\) is total number of cells responding to MCh that were examined under each condition.

| Treatment | Time (min) | Potentiated cells | Unaffected cells | Inhibited cells | \(n\) |
|-----------|------------|-------------------|------------------|----------------|------|
| FCCP (1 \(\mu\)M) | 2 | 41 | 44 | 14 | 133 |
| | 5 | 36 | 40 | 24 | 116 |
| | 15 | 19 | 35 | 47 | 75 |
| FCCP (5 \(\mu\)M) | 15 | 0 | 0 | 100 | 41 |
| Antimycin (2 \(\mu\)g/ml) | 2 | 2 | 13 | 85 | 130 |
| | 30 | 9 | 9 | 88 | 121 |
| Antimycin (0.2 \(\mu\)g/ml) | 30 | 10 | 26 | 64 | 39 |
| CCCP (5 \(\mu\)M) | 2 | 4 | 16 | 80 | 56 |

Functional Ca\(^{2+}\) pool that is specialized in certain regions of the cell as wave amplification sites. While it is possible that InsP\(_3\)Rs and ryanodine receptors are colocalized at these specialized sites, an alternative or complementary explanation would be that the major factor or factors governing this Ca\(^{2+}\) fingerprint is related to cellular characteristics other than the density of ER receptor channels alone.

Fluorescent dyes targeted to either ER and mitochondria (DiOC\(_{6}\)) or mitochondria alone (JC-1) showed that in oligodendrocyte processes mitochondria are typically found in closely associated groups at cellular sites that closely correspond to the sites of high Ca\(^{2+}\) wave kinetics. Mitochondria were always located at such sites and were not found elsewhere along the processes. This indicates that mitochondria are likely to be important for regulation of Ca\(^{2+}\) response characteristics and that regulation of the spatial distribution of mitochondria could be one way in which spatially discrete signaling is achieved in glial cells. Mitochondrial location in oligodendrocyte processes did not vary over >15-min periods under resting or agonist-stimulated conditions (not shown). Evidence from this and previous (11, 16–18, 36) studies show that a close spatial relationship exists between mitochondria and ER. One possible explanation of mitochondrial effects on InsP\(_3\)-mediated waves is that the proximity of mitochondria to the ER InsP\(_3\)Rs alters gating kinetics of InsP\(_3\)R channels. Mitochondria located close to InsP\(_3\)Rs could buffer considerable quantities of Ca\(^{2+}\), thus maintaining a microdomain of Ca\(^{2+}\) near the receptor that is at a favorable concentration for channel activation. When mitochondria are inhibited (or physiologically when mitochondrial Ca\(^{2+}\) load is already high), the local cytosolic Ca\(^{2+}\) concentration rises to a level at which Ca\(^{2+}\) is inhibitory to InsP\(_3\)R activation, such that even during InsP\(_3\) generation the InsP\(_3\)R is not activated. Thus, by altering the excitability of the medium, mitochondria could shape the characteristics of the signal that InsP\(_3\) can evoke (14, 29, 45, 46). This hypothesis is supported by the finding that MCh evokes a rapid elevation of \([\text{Ca}^{2+}]_i\) and a depolarization of mitochondrial membranes in oligodendrocytes. Rapid mitochondrial Ca\(^{2+}\) uptake enables mitochondria to sequester Ca\(^{2+}\) during the early stages of Ca\(^{2+}\) oscillations (29). The ability of mitochondria to buffer Ca\(^{2+}\) loads could enable them to decrease the magnitude of an agonist-evoked Ca\(^{2+}\) peak by uptake of Ca\(^{2+}\) from the cytoplasm or to modify the Ca\(^{2+}\)-dependent sensitivity of the release channel.

In addition to spatial distribution, evidence for a functional role for mitochondria in oligodendrocyte signaling comes from the fact that inhibitors of mitochondrial activity typically inhibited or abolished Ca\(^{2+}\) responses. Brief treatment with FCCP revealed a bimodal effect of mitochondrial modulation, causing either an increase or a decrease in the amplitude of the response. The bimodal effect of FCCP pretreatment on Ca\(^{2+}\) responses in oligodendrocytes appears consistent with a role for mitochondria in altering the Ca\(^{2+}\) “setpoint” for InsP\(_3\)Rs, thus either increasing or decreasing the sensitivity of the receptor to InsP\(_3\) (8, 30). FCCP was found to evoke decreases in \([\text{Ca}^{2+}]_i\) as measured by rhod 2 and increases in \([\text{Ca}^{2+}]_i\), as measured by fluo 3 over 2–3 min of incubation (Fig. 6b). No consistent relationship was evident between the magnitude of \([\text{Ca}^{2+}]_i\), elevation evoked by FCCP and its effect on Ca\(^{2+}\) signals. The important modulatory effect of mitochondrial Ca\(^{2+}\) release, however, would be expected to be very localized, i.e. effects on \([\text{Ca}^{2+}]_i\) around nearby InsP\(_3\)Rs, rather than via bulk cytosolic \([\text{Ca}^{2+}]_i\), elevation, and as such may not be readily measured. Potentiation of responses was also found in a small percentage of cells treated with antimycin. However, some cells appeared relatively resistant to the effects of mitochondrial inhibitors, with ~25% of cells unaffected by antimycin and ~40% unaffected by 1 \(\mu\)M FCCP, and after MCh stimulation only a proportion of mitochondria showed changes in membrane potential and \([\text{Ca}^{2+}]_i\). Such differences may indicate either that mitochondria are more important for Ca\(^{2+}\) signaling in some cells than in others or that the interaction between cytosolic and mitochondrial responses depends on several independent factors. Possible factors modulating the consequences of mitochondrial inhibition on cytosolic responses include: (i) the amount of Ca\(^{2+}\) loaded into mitochondria in a given cell; (ii) the concentration of cytosolic Ca\(^{2+}\) around InsP\(_3\)Rs near the mitochondria; and (iii) the spatial relationship between the mitochondria and InsP\(_3\)Rs. It remains possible that a primary role of mitochondria in modulating Ca\(^{2+}\) signaling is simply to produce sufficient ATP to maintain Ca\(^{2+}\) uptake into ER via the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\) ATPase. Indeed, the effects of long term treatment with mitochondrial inhibitors on Ca\(^{2+}\) responses may be due to a combination of modulation of release and ATP depletion. However, only brief periods (2 min) of FCCP, CCCP, or antimycin pretreatment were required to modify or abolish Ca\(^{2+}\) responses in oligodendrocytes, a time period no greater than that required to inhibit mitochondrial function as measured by \([\text{Ca}^{2+}]_i\) or mitochondrial membrane potential.

Our results detail several novel functional consequences of colocalization of mitochondria and ER: (i) the present study demonstrates that active mitochondria/ER interactions appear to be crucial to Ca\(^{2+}\) wave propagation, such that in the absence of functional mitochondrial Ca\(^{2+}\) uptake, oligodendrocyte Ca\(^{2+}\) responses are modified, resulting in enhanced release kinetics, or inhibition or even abolition of wave generation, depending on the particular cell examined; (ii) the location of mitochondria appears to underlie the spatial distribution of sites of enhanced Ca\(^{2+}\) release, which are thought to be crucial to the maintenance of wave propagation (10); (iii) changes in both intramitochondrial Ca\(^{2+}\) and mitochondrial membrane...
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potential occur upon agonist stimulation of oligodendrocytes. A variety of factors may govern $\text{Ca}^{2+}$ waves in glia, including regional heterogeneities in ER protein expression, but clearly sites of mitochondrial expression play a major role in shaping $\text{Ca}^{2+}$ responses.

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