High Density Distribution of Endoplasmic Reticulum Proteins and Mitochondria at Specialized Ca\textsuperscript{2+} Release Sites in Oligodendrocyte Processes*

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**Peter B. Simpson‡, Surabhi Mehrotra, G. David Lange§, and James T. Russell¶**

From the Laboratory of Cellular and Molecular Neurophysiology, NICHD, and §Instrumentation and Computers Section, NINDS, National Institutes of Health, Bethesda, Maryland 20892-4995

In oligodendrocyte processes, methacholine-evoked Ca\textsuperscript{2+} waves propagate via regions of specialized Ca\textsuperscript{2+} release kinetics (wave amplification sites) at which the amplitude and rate of rise of local Ca\textsuperscript{2+} signals are markedly higher than in surrounding areas (Simpson, P. B., and Russell, J. T. (1996) J. Biol. Chem. 271, 33493–33501). In the present study we have examined the effects of other phosphoinositide-coupled agonists on Ca\textsuperscript{2+} in these cells, and the structural specializations underlying regenerative wave amplification sites. Both bradykinin and norepinephrine evoke Ca\textsuperscript{2+} waves, which initiate at the same loci and propagate through the cell body and multiple processes via identical wave amplification sites. Antibodies against type 2 inositol 1,4,5-trisphosphate receptors (InsP\textsubscript{3}R\textsubscript{2}) and calreticulin identify expression of these proteins in oligodendrocyte membranes in Western blots. Immunocytochemistry followed by high resolution fluorescence microscopy revealed that both InsP\textsubscript{3}R\textsubscript{2} and calreticulin are expressed in high intensity patches along processes. Cross-correlation analysis of the profiles of local Ca\textsuperscript{2+} release kinetics during a Ca\textsuperscript{2+} wave and immunofluorescence for these proteins along cellular processes showed that the domains of high endoplasmic reticulum protein expression correspond closely to wave amplification sites. Staining cells with the mitochondrial dye, Mito-Tracker\textsuperscript{®}, showed that mitochondria are only found in intimate association with these sites possessing high density endoplasmic reticulum proteins, and they remain in the same locations over relatively long periods of time. It appears, therefore, that multiple specializations are found at domains of elevated Ca\textsuperscript{2+} release in oligodendrocyte processes, including high levels of calreticulin, InsP\textsubscript{3}R\textsubscript{2} Ca\textsuperscript{2+} release channels, and mitochondria.

Endoplasmic reticulum (ER) Ca\textsuperscript{2+}-binding proteins provide a high capacity buffering mechanism which results in the lowering of [Ca\textsuperscript{2+}]\textsubscript{free} in the ER, and thus a reduction in the gradient against which pumps must transport cytoplasmic Ca\textsuperscript{2+} into the store. They are also thought to be important in localizing Ca\textsuperscript{2+} to sites of release, and in modulating release activity, via protein-protein interactions with release channels (1–4). The best described of these Ca\textsuperscript{2+}-binding proteins are calsequestrin and calreticulin. In many cells, calreticulin is the major calcium-binding protein of the ER lumen (2, 5). Three subtypes of inositol 1,4,5-trisphosphate receptor (InsP\textsubscript{3}R) are now known (see Ref. 6 for review), which can have different modulatory properties and discrete functions even when expressed together in the same cell (7–9). Localization of calreticulin to InsP\textsubscript{3}R-containing membrane vesicles has been reported in some cell types using density gradient techniques (2, 10). The function of this coexpression, however, has remained controversial. Recent reports have indicated that calreticulin may play a role in regulating Ca\textsuperscript{2+} signals, including perhaps serving as a luminal sensor for Ca\textsuperscript{2+} store depletion (4, 11).

InsP\textsubscript{3}-mediated Ca\textsuperscript{2+} waves in several cell types propagate over long distances by regenerative Ca\textsuperscript{2+} release at specialized cellular domains (12–15). In glial cells, these specialized wave amplification sites are characterized by significantly higher amplitude in local Ca\textsuperscript{2+} signals and steeper rate of rise of the signals (12, 14, 16). Ca\textsuperscript{2+} waves typically travel in complex nonlinear paths through three-dimensional space (12, 15, 17–19), making analysis of their mechanisms of propagation problematical. In recent studies, however, we have investigated Ca\textsuperscript{2+} waves in cultured cortical oligodendrocytes which because of their long, thin, relatively linear processes, allow for the analysis of Ca\textsuperscript{2+} waves as one dimensional propagatory entities. Oligodendrocytes express a variety of receptors coupled to the hydrolysis of phosphoinositides and consequent mobilization of Ca\textsuperscript{2+} from InsP\textsubscript{3}R-containing intracellular stores. These include α\textsubscript{1A} adrenoreceptors, M1 muscarinic cholinoreceptors, and bradykinin receptors (20–23). We have previously demonstrated that oligodendrocytes respond to the muscarinic receptor agonist methacholine (MCh) by the induction of Ca\textsuperscript{2+} waves initiating in several distinct regions of oligodendrocyte processes. These waves travel along each process and into the cell body via multiple amplification sites (14). One or more mitochondria are closely associated with each of these specialized Ca\textsuperscript{2+} wave amplification sites in oligodendrocytes, and inhibition of mitochondrial activity markedly affects methacholine-evoked Ca\textsuperscript{2+} responses (14).

The present study was undertaken to investigate whether specializations in ER protein distribution as well as mitochondrial location would underlie the specialized Ca\textsuperscript{2+} release sites found during InsP\textsubscript{3}R-evoked Ca\textsuperscript{2+} waves. Our results indicate that type 2 InsP\textsubscript{3}R (InsP\textsubscript{3}R\textsubscript{2}), and calreticulin, are expressed in much higher intensity at Ca\textsuperscript{2+} wave amplification sites along oligodendrocyte processes compared with other regions. Furthermore, in the processes stationary mitochondria were found only at these specialized Ca\textsuperscript{2+} release sites in close proximity to the wave amplification sites.
association with high density of ER proteins. These findings suggest that wave propagation in glia may be modulated by special microdomains of Ca\(^{2+}\) release involving both mitochondria and ER proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—**(-)-Norepinephrine hydrochloride and acetyl-b-methylcholine chloride were obtained from Sigma. Bradykinin, fura 2-AM and fluo 3-AM were obtained from Research Biochemicals International. MitoTracker CMXRs was from Molecular Probes. PA3-900 was from Affinity Bioreagents Inc. AP42 was a gift from Dr. A. Sharp (Johns Hopkins University, Baltimore, MD).

**Cell Culture—**Oligodendrocytes were prepared from 2-day-old rat pups as described previously (14, 24). Briefly, cortices were removed and manually dissociated, and cells cultured in plastic flasks (25). After 8 days in vitro, the flakes were vigorously shaken overnight. The supernatant was repeatedly plated onto plastic dishes, to which endothelial cells, microglia, and fibroblasts quickly attach. Non-adherent cells were then replated onto glass coverslips coated with 0.1 mg/ml polyornithine. Cells were cultured in DME-N1 containing 0.5% fetal bovine serum for 24 h, and thereafter in DME-N1 containing 2% fetal bovine serum, and maintained in 10% CO\(_2\), 90% air, under which conditions the bipotential cells developed into oligodendrocytes. Cells were >85% positive for the oligodendrocyte marker galactocerebroside. Culture medium was replaced every 3 days, and all cells were used 4–8 days after replating.

**(Ca\(^{2+}\))\(_i\), Measurement—**For the study of Ca\(^{2+}\) wave propagation in glial cell processes, cells were incubated with 5 \(\mu\)M fluo 3-AM for 20 min at room temperature as described previously (12, 26). Experiments were performed in a Leiden coverslip chamber continuously perfused with balanced salt solution. The perfusion chamber was positioned on the stage of an inverted microscope, and fluorescence images acquired at 495 nm excitation (510 nm emission) wavelength through a microchannel plate intensifier with a CCD camera (12). Images were digitized and averaged (2 frames at each wavelength) in a Trappix 55/4256 image processor. Cells were divided for analysis into 0.8–2.0-µm-wide regions sequentially along the longitudinal axis of the cell (14). Fluorescence intensity values in the nonzero pixels within each slice were averaged (\(F\)) and plotted as normalized fluorescence intensities (\(\Delta F/F\)) against time, where \(\Delta F\) was calculated as the difference between the average value of the first 20 data points prior to stimulation of the cell and \(F\).

**Membrane Preparation, SDS-Polyacrylamide Gel Electrophoresis, and Immunoblot Analyses—**Oligodendrocyte membranes were prepared and Western blots performed according to previously published methods (7). Membrane proteins were separated in 7.5% SDS-polyacrylamide gels using the Phastgel system (Pharmacia Biotech Inc.). Proteins were electrophoretically transferred to nitrocellulose and blots were incubated overnight at 4°C in Tris-buffered saline-Tween 20 containing 5% nonfat dried milk, followed by incubation in primary antibody (1:1000 dilution). After incubation in peroxidase-conjugated secondary antibodies (Amersham Corp.), blots were developed using enhanced chemiluminesence reagents. Rabbit polyclonal antibody AP42, raised against a peptide sequence corresponding to the C-terminal region of mouse InsP\(_3\)R subtype 2 (GSNTPHENHHMPPH) (27), was a gift from Dr. A. Sharp. This sequence is unconserved, i.e. it is not present in the other known subtypes of InsP\(_3\)R or in any other known oligodendrocyte protein. PA3-900, a rabbit polyclonal antibody raised against recombinant human calreticulin produced in the baculovirus insect cell system (28), was from Affinity Bioreagents.

**Immunocytochemistry and Mitochondria-specific Staining—**Immunocytochemistry on either naive cells or cells in which Ca\(^{2+}\) waves were first measured was performed as described previously (14). Cy3- or fluorescein isothiocyanate-labeled anti-rabbit secondary antibodies (Jackson Immunocytochemical Laboratories) were used as appropriate. Briefly, cells were fixed in 100% methanol at −20 °C for 3 min, washed three times in phosphate-buffered saline (PBS) (pH 7.1), then incubated in primary antibody (1:200 for anti-InsP\(_3\)R antibodies, 1:300 for anti-calreticulin) overnight at 4°C. After further PBS washes, cells were incubated for 1–2 h in secondary antibody (1:400 dilution in 10% goat serum). Cells were then washed three times, and the coverslips were mounted on a glass microscope slide using Mowiol. Controls consisted of substitution of the primary antibody with normal serum from the same host species, used at a dilution equal to that of the primary antibody, and consistently showed negligible fluorescence levels. For the study of mitochondrial distribution, living cells were incubated with MitoTracker Red CMXRos (500 nm, 30 min, Molecular Probes) at 37 °C, then washed in prewarmed PBS. Cells were then imaged using a Cy3 filter set in a fluorescence microscope. To examine the spatial relationship between mitochondria and ER markers, MitoTracker-loaded cells were fixed in 2% paraformaldehyde (4 min, 4°C) and 100% methanol (−20 °C, 3 min), washed, and developed for immunocytochemistry as above. Use of fluorescein isothiocyanate-labeled secondary antibodies enabled good resolution and separation of antibody fluorescence from that of MitoTracker. Cells previously subjected to [Ca\(^{2+}\)]\(_i\), measurements were processed for immunocytochemistry or organelle labeling on the microscope stage following the same procedures described for naive cells.

**Fluorescence Microscopy for Molecular Analysis—**For comparison of immunofluorescence or mitochondrial staining with Ca\(^{2+}\) release kinetics, cells were imaged in the Ca\(^{2+}\) imaging system using a Cy3 filter set (Chroma Technologies, Inc., Brattleboro, VT). For analysis of subcellular immunofluorescence with high resolution, a digital confocal microscopy technique was employed (14). For this, cells were imaged with a cooled CCD camera (Photometrics, Inc., Tucson, AZ) using the Cellscan software environment (Scanalytics, Inc., Billerica, MA). The Cellscan environment allows for acquisition of wide angle fluorescence microscopy images at all the focal planes through cells (z-series). Images are then restored using a deconvolution procedure (extensive photon reassignment protocol, EPR) into confocal images by removing out-of-focus light. The software system is based on the algorithm developed by Dr. Fay and co-workers (14, 29). The algorithm used the point-spread function of the microscope obtained by acquiring a z-series images of a subresolution polystyrene bead 200 nm in diameter filled with fluorescein. This data set was used by the algorithm to reassign out-of-focus plane light, which causes blurring of confocal images (29). Under our measurement conditions, the z-resolution by our optics was 0.48 µm (measured as full width at half-maximum intensity).

**Cross-correlation Analysis—**The spatial patterns of local Ca\(^{2+}\) release kinetics measured in 0.83-µm-wide subregions of oligodendrocyte processes and of mitochondrial distribution measured by fluorescently tagging mitochondria in the same cells were compared using a cross-correlational statistics (mean, variance, and all other parameters of the correlation function) as a quantitative test for similarity (14, 30). Cross-correlation function as a quantitative test for similarity (14, 30). Cross-correlation function is derived from the fast Fourier transform of the two data sets being compared. For this analysis the mean values of the amplitudes in the patterns were subtracted out and the resulting zero mean waves were embedded in surrounding zeros, to eliminate circular correlations, i.e. between the beginning and end of the data sets, due to the periodic nature of Fourier series (30). The data were then analyzed via a Fast Fourier Transform algorithm, using standard functions in Mathematica (Wolfram Research Inc.). Cross-spectra were formed as a product of one data set with the complex conjugate transform of a second data set. The cross-correlation function was produced by inverse Fourier transformation of the cross-spectrum. Performing these operations on a single data set produced the power density spectrum and the auto-correlation function. Results are presented as mean ± S.D. The validity of this quantitative analysis technique was evaluated in two different control experiments. In one, we compared synthesized noisy sine waves of identical frequency but slightly out of phase with each other (Fig. 4A, bottom traces). The cross-correlation of these waveforms (Fig. 4B, solid circles) is a wave pattern of the same frequency as the original waves, with peak correlation shifted from phase to a position equivalent to the delay between the two signals. The noise experimentally inserted into the two sine waves (see figure legend) has minimal effect on the outcome of the cross-correlation. In the second control experiment, a real data set was compared with a scrambled data set (Fig. 4A, top traces) to determine if chance alone would cause high cross-correlation. The method used was a random permutation in the order of the data points, generated by the program Random Permuta-

in the Discrete package of Mathematica. By this method non-

correlated statistics (mean, variance, and all other parameters of the distribution) were kept constant, but spatial correlations were deleted via a random permutation in the order of the data points. The bottom patterns in Fig. 4A are equivalent to the data presented in Fig. 4C (see below), except that one of the patterns has been scrambled with respect to position. The resultant cross-correlation (Fig. 4B, open circles) is a somewhat noisy, relatively flat function that does not greatly deviate from zero correlation at any delay, unlike the cross-correlation function of the original data sets shown in Fig. 4D. These results are consistent with cross-correlation analysis conservatively detecting genuine but not coincidental correlations within related data sets.
In contrast wave amplification occurs at other more numerous loci. At these wave amplification sites (marked with arrows), the magnitude and rate of Ca^{2+} rise were substantially higher than in surrounding regions (Fig. 1, D and E).

We have previously described similar distinct initiation and propagation domains in oligodendrocytes responding to methacholine (14) and in astrocytes responding to norepinephrine (12, 13). Applying the cross-correlation analysis also used in the previous work, we have now examined the distribution of sites associated with responses to norepinephrine and bradykinin. Local Ca^{2+} peak amplitudes had a maximal cross-correlation coefficient of 0.81 at 0.0 μm from phase. Similarly, the comparison between the half-rise time and the rates of Ca^{2+} rise for norepinephrine and bradykinin responses gave peak coefficients of 0.79 and 0.75 at 2.0 μm from phase. Such analysis also confirms that wave initiation and amplification sites

**Fig. 1.** Two different phosphoinositide-coupled receptors activate Ca^{2+} waves with identical propagation characteristics in oligodendrocytes. A, offset plot of [Ca^{2+}]i responses to norepinephrine from successive regions of a cell along the cell axis. A fluo 3-AM-loaded oligodendrocyte was stimulated with norepinephrine (200 nM), and 2.0-μm-wide successive sections were made along the cell image for analysis of fluorescence change (ΔF/Fo). Traces from each region are plotted as a stack, offset by an equal distance along the ordinate axis. The bottom traces on the plot correspond to responses from one end of a process; traces above these are from the proximal process, cell body, then out along another process on the opposite side of the cell. The abscissa represents increasing time from left to right. Distance scale (μm) corresponds to x axis scale in C–E. Four sites of norepinephrine-evoked Ca^{2+} wave initiation can be identified, and differences in the amplitude and rate of rise of peaks can be detected. After an interval of 30 min, the cell was stimulated with bradykinin and the response was analyzed in the same sections as follows. B, offset plot of [Ca^{2+}]i responses to bradykinin 30 min following the norepinephrine treatment. The fluo 3 fluorescence changes (ΔF/Fo) from the same successive regions of the cell shown in A are plotted. Note that Ca^{2+} waves initiate with different latencies but in the same cellular sites of wave initiation seen in response to norepinephrine, and the overall spatial pattern of the response is similar. Four separate sites of Ca^{2+} wave initiation are identifiable as the local “minima” in Fig. 1C. Note that the responses appear earlier than in surrounding areas, appear at local minima (asterisks). C, analysis of the onset of the [Ca^{2+}]i response along the length of the cell. The time taken to reach 50% of maximal response to norepinephrine (open circles) or bradykinin (filled circles) was determined at 2-μm intervals. Wave initiation sites, where responses appear earlier than in surrounding areas, appear at local minima (asterisks). Note that the responses to successive stimulations by the two different agonists begin in virtually the same cellular regions. D) Plot of the peak amplitude (ΔF/Fo) of the evoked [Ca^{2+}]i response in the successive slices of the cell against cell length (μm). The local response amplitudes in the traces shown in A and B were measured and plotted against cell length. Local amplitudes vary greatly along the length of the processes, and the lowest amplitude was measured in the cell body (CB) region (58–78 μm). Specialized regions in which the response amplitude is higher than in surrounding areas are evident (arrows) along the processes. Note the similarity in the local amplitudes in response to consecutive stimulations with norepinephrine (filled circles) and bradykinin (open circles). E, plot of the rate of rise of the evoked [Ca^{2+}]i response against the length of the cell. The rate of rise of response is severalfold higher in discrete regions of the cell and these regions correspond to regions where high amplitude signals were measured (D). The profiles of rates of rise were also similar for norepinephrine (open circles) and bradykinin (filled circles). Rate of rise was calculated as described previously (12) and represents ΔF/F per second.
Neither rapid movement nor slow migration of mitochondria with high spatial resolution in cellular processes overmitochondria-specific fluorescent dye, and imaged mitochondria in oligodendrocytes with MitoTracker CMXRos (MitoTracker), a

experiments were performed to investigate if mitochondria are

tudes had a maximal coefficient of 0.08 at 0.0 μm. Other control

analyses (see “Experimental Procedures”) established that this

analytical protocol does not readily identify false positive

relationships.

Distribution of ER Proteins and Mitochondria in Oligodendrocytes—The expression of InsP₃R2 and calreticulin in oligodendrocytes was investigated using Western blotting and immunocytochemical analyses. The antibody AP42, raised against an unique sequence in the C-terminal region of

R2, detected a single band of approximately 250 kDa in

oligodendrocyte membranes (Fig. 2A), consistent with the ex-

pected size of this protein from previous reports (8, 27). Previ-

ous experiments clearly showed that this antibody does not

cross-react with either InsP₃R1 or InsP₃R3. Antibody PA3-900,

raised against recombinant human calreticulin, reacted against a single band at approximately 60 kDa (Fig. 2B) (28), indicating the expression of calreticulin in or associated with oligodendrocyte membranes. These antibodies were then used to investigate the distribution of InsP₃R2 and calreticulin in oligodendrocytes using standard immunocytochemical tech-

iques and high resolution fluorescence microscopy (see “Ex-

perimental Procedures”) (Fig. 3). InsP₃R2 immunofluorescence

(Fig. 3A) was found distributed in a variegated manner throughout the cell body, except the nucleus, and along the length of the cell processes. Similarly, calreticulin immunofluo-

rescence was also found in the cell body and in a punctate pattern along oligodendrocyte processes (Fig. 3B). The size of these clusters of immunofluorescence varied between different cells, being typically longer and more graded in thick processes, but small and highly punctate in thin processes (see also Fig. 5).

Since, in oligodendrocyte processes, mitochondria appear to be always associated with Ca²⁺ wave amplification sites (14), experiments were performed to investigate if mitochondria are mobile or stationary in these processes. For this, we incubated oligodendrocytes with MitoTracker CMXRos (MitoTracker), a mitochondria-specific fluorescent dye, and imaged mitochondria with high spatial resolution in cellular processes over time. Cells were imaged every 2 s over a period of up to 15 min. Neither rapid movement nor slow migration of mitochondria were observed during this period of time under either resting (Fig. 3C) or agonist-stimulated (data not shown) conditions.

Comparison of Ca²⁺ Wave Kinetics and Local Specializa-

tions—We have previously demonstrated that the distribution of ER in oligodendrocyte processes appears approximately uniform (14). This finding, however, does not preclude the possi-

bility of specializations in the distribution of ER proteins. To examine whether the sites of high density ER protein distribu-
tion were related to the sites of high Ca²⁺ release kinetics, we performed immunocytochemistry in cells after measurement of wave kinetics in the same cells. We stimulated cells with InsP₃-generating agonists, and measured the kinetics of the resulting wave in serial x, y sections of the cell along its axis, as in data

shown in Fig. 1. We then fixed the cell on the microscope stage and incubated with appropriate primary antibody and fluores-
cent secondary antibodies. The pattern of immunofluorescence

was then imaged, and the intensities were measured within the same serial sections of the cell in which the Ca²⁺ wave kinetics were measured. The resultant profile was then compared with the profile of Ca²⁺ wave kinetics using cross-correlation analysis.
Specialized Ca\(^{2+}\) Release Sites in Oligodendrocytes

Fig. 4. Comparison of Ca\(^{2+}\) waves with distribution of calreticulin and InsP\(_3\)R2 in oligodendrocytes. A fluo 3-loaded cell was stimulated with MCh (0.1 mM), and the resultant Ca\(^{2+}\) wave was measured as described in legend to Fig. 1. The cell was fixed, and the ER protein distribution was analyzed using immunofluorescence while the cell remained on the microscope stage. Local Ca\(^{2+}\) wave and intensity of immunofluorescence were measured in the same series of cellular slices in the longitudinal axis of the cell. The profiles of wave kinetics and immunofluorescence were then compared using cross-correlation analysis. Panels A and B show control experiments to validate the cross-correlation analysis procedure. A, two sets of control data are presented. Top, local Ca\(^{2+}\) release amplitudes during the Ca\(^{2+}\) wave (closed circles) presented as experimental data in C, along with calreticulin fluorescence data (open circles), which has the same intensity values in the ordinate scale as in C but which has been scrambled with respect to distance using Mathematica such that an irregular pattern emerges (see “Experimental Procedures”). Bottom, two sine waves were generated, with random noise added independently to each using a randomization function. The sine waves are of the same period but are out of phase with each other. B, the two pairs of control data in A were cross-correlated. The noisy sine waves (bottom trace in A) produced a cross-correlation (closed circles), which has the same period as the original data. The cross-correlation has a central maximum value located at a distance from phase equal to the displacement between the two original non-noisy signals, i.e. 4.2 \(\mu\)m from phase. The scrambled data (open circles, top trace in A) had little or no correlation with the other unscrambled data set (open circles), as evidenced by a cross-correlation function that is flat and close to zero, unlike the cross-correlation between the two original data sets (see C). It is from such a non-correlative function that the reader should derive a visual base line for evaluating the other correlation functions presented here. C, local peak Ca\(^{2+}\) amplitudes during the MCh-evoked Ca\(^{2+}\) wave along an oligodendrocyte process are shown (closed circles), compared with subsequent calreticulin immunofluorescence measurement (open circles) in the same cellular sections. Peaks in local amplitude of Ca\(^{2+}\) release were found at several sites along the process. Comparable high density patches of calreticulin immunofluorescence are found at nearby sites. D, cross-correlation analysis of the patterns of local peak Ca\(^{2+}\) amplitudes and calreticulin immunofluorescence shown in C. This analysis shows high cross-correlation (closed circles), centered close to 0 \(\mu\)m and thus approximately in phase. E, in a different cell from that shown in C and D, local peak amplitudes of Ca\(^{2+}\) release evoked by MCh along an oligodendrocyte process (closed circles) are plotted and compared with the distribution of AP42 (anti-InsP\(_3\)R2) immunofluorescence (open circles). Peaks in local Ca\(^{2+}\) amplitudes were found at several cellular domains. Comparable elevated levels of InsP\(_3\)R2 are found at nearby sites. F, cross-correlation analysis of the local peak Ca\(^{2+}\) amplitude profile and InsP\(_3\)R2 immunofluorescence in this cell shows high correlation values (closed circles), centered around 0 \(\mu\)m (in phase). The cross-correlation is similar to the autocorrelation of AP42 fluorescence (dotted line) shown for comparison.

Fig. 4 (C–F) shows the results of two experiments where the local kinetics of Ca\(^{2+}\) release (half-rise time, local peak Ca\(^{2+}\) amplitudes, and rates of Ca\(^{2+}\) rise) were measured as described in the legend to Fig. 1. A plot of the local Ca\(^{2+}\) amplitudes against the length of the process, together with the intensity of calreticulin staining measured in the same cellular sites, showed that the regions with high intensity calreticulin immunofluorescence corresponded closely with the regions of the process where the local peak Ca\(^{2+}\) amplitudes were highest (Fig. 4C). Local peak Ca\(^{2+}\) amplitudes are found either coincident with or within 1–4 \(\mu\)m of peaks in calreticulin immunofluorescence. Cross-correlation coefficient was high and was similar to the auto-correlation function of the calreticulin fluorescence pattern (Fig. 4D). In parallel experiments, the intensity of InsP\(_3\)R2 immunofluorescence in oligodendrocyte processes was similarly compared with the pattern of local Ca\(^{2+}\) peak amplitudes. In the cell shown in Fig. 4E, high intensity InsP\(_3\)R2 fluorescence was found close to sites where high local peak Ca\(^{2+}\) amplitudes were measured and comparison yielded high degree of correlation coefficients in phase (Fig. 4F).
In several experiments, we consistently found similar high cross-correlation values between the patterns of local Ca\textsuperscript{2+} release amplitudes and the patterns of ER protein (calreticulin and InsP\textsubscript{3}R2) distribution (Table I). In particular, cell regions with elevated levels of InsP\textsubscript{3}R2 and calreticulin consistently displayed significantly higher amplitude local Ca\textsuperscript{2+} release signals than were found in surrounding regions (Table I). The pattern of rates of rise of the responses (i.e. slopes), however, showed more modest cross-correlation values (Table I), probably reflecting the noise level inherent in that measurement.

In another series of experiments, we compared the distribution pattern of ER proteins with the location of mitochondria in oligodendrocyte processes. We have shown previously that, in oligodendrocyte processes, mitochondria are distributed singly or in groups along processes only at sites of wave amplification (14). Since the specialized wave amplification sites in these processes also contain accumulation of calreticulin and InsP\textsubscript{3}R2, we wanted to investigate the spatial relationship between the ER proteins and mitochondria. In these experiments, we again used MitoTracker to selectively stain mitochondria. MitoTracker reacts with accessible thiol groups to form an aldehyde-fixable conjugate and so, unlike other mitochondrial dyes, is retained within mitochondria after fixation and permeabilization (31, 32). Cells were incubated with MitoTracker, washed, fixed, and then incubated with antibodies against InsP\textsubscript{3}R2 or calreticulin. High resolution analysis of the results of these experiments demonstrated that, in oligodendrocyte processes, high concentrations of the ER proteins were always found in close apposition to mitochondria (Figs. 5 and 6). Calreticulin immunofluorescence (green) was found predominantly in several intense patches with little fluorescence in between (Fig. 5A) (cell shown was typical of 4 cells analyzed by digital confocal microscopy). In this cell, rendered from multiple z-plane images in three-dimensional voxel, MitoTracker staining (red) closely corresponded with the regions of high calreticulin immunofluorescence (Fig. 5A). A side-on view of this process (Fig. 5B) illustrates that calreticulin and mitochondria were found in closely apposed z-planes, consistent with a close spatial correspondence between ER and mitochondria. A zoomed-in, single optical plane view of the area within the box in Fig. 5A reveals that even within the 0.09-μm z-dimensional plane, calreticulin fluorescence entwines closely around a group of mitochondria (Fig. 5C). Only occasionally was a strongly staining bead of calreticulin found without a nearby mitochondrion.

Fig. 6 shows a three-dimensional voxel rendering of multiple z-plane images of a cell in which mitochondria were stained with MitoTracker, and InsP\textsubscript{3}R2 was identified by AP42 immuno-fluorescence. InsP\textsubscript{3}R2 fluorescence extended throughout the ER in these cells with significant perinuclear staining (Fig. 6A) (cell shown was typical of 3 cells using digital confocal microscopy). In processes, however, InsP\textsubscript{3}R2 immunofluorescence consistently appeared in high density patches (Fig. 6A). These regions of high density InsP\textsubscript{3}R2 staining in processes were closely associated with mitochondria, as shown by MitoTracker fluorescence in the same regions of the cell (Fig. 6B). A zoomed-in view of one such site (Fig. 6C) illustrates a convolution of mitochondria (red) such as are typically found in thicker oligodendrocyte processes (see also Fig. 5C and Ref. 14), surrounded by multiple InsP\textsubscript{3}R2 "hot-spots" (green) located within ~1 μm of the mitochondria. A side-on view of the region in C is shown in Fig. 6D. Mitochondria and InsP\textsubscript{3}R2 are found to be in similar z-planes to each other (Fig. 6D), consistent with a close spatial apposition in three-dimensional space. Numerous mitochondria, relatively short and rounded in shape, were observed in the cell body (Fig. 6B). Unlike the close similarity in the patterns of distribution between the ER proteins and mitochondria along processes in the cell body, both calreticulin (data not shown) and InsP\textsubscript{3}R2 distribution did not closely mirror the distribution of mitochondria (Fig. 6, A and B).

These results demonstrate that both calreticulin and

| Comparison       | Peak correlation | Location of peak correlation | n  |
|------------------|------------------|------------------------------|----|
| Peak vs. InsP\textsubscript{3}R2 | 0.57 ± 0.25 | 1.88 ± 1.04 | 4  |
| Peak vs. calreticulin | 0.64 ± 0.25 | 1.66 ± 1.04 | 6  |
| Slope vs. InsP\textsubscript{3}R2 | 0.47 ± 0.27 | 0.85 ± 0.98 | 4  |
| Slope vs. calreticulin | 0.23 ± 0.54 | 1.26 ± 1.57 | 6  |

**FIG. 5. Comparison of MitoTracker staining with calreticulin immunofluorescence in an oligodendrocyte process.** Live cells were incubated with 500 nM MitoTracker in PBS for 30 min at 37 °C, fixed, and processed for immunocytochemistry as described under "Experimental Procedures." 42 serial optical sections (every 0.09 μm in the z-dimension) obtained by digital EPR restoration were rendered into a three-dimensional voxel as shown in A and B. Scale bar = 20 μm. A, an oligodendrocyte process reconstructed from a z-series through the cell is depicted, labeled with PA3-900 to detect localization of calreticulin (green). Calreticulin immunofluorescence in this process is predominantly concentrated in multiple high intensity patches. Mitochondria labeled with MitoTracker are found closely associated with these sites of high concentrations of calreticulin. Only very low calreticulin immunofluorescence is found in process regions without mitochondria. Image shown was rendered into a three-dimensional voxel from 31 serial optical sections (every 0.09 μm in the z-dimension) obtained by digital confocal restoration. B, a side-on (X-Z) view of the process depicted in A. The close interrelationship in three-dimensions between calreticulin and mitochondria can be seen, with mitochondria being closely surrounded by high levels of calreticulin immunofluorescence. C, a single optical plane is shown, in a zoomed-in view of the region within the box in A. This illustrates that even within a single 0.09-μm z-dimensional plane, calreticulin fluorescence entwines around a group of mitochondria.

**TABLE I**

| Comparison       | Peak correlation | Location of peak correlation | n  |
|------------------|------------------|------------------------------|----|
| Peak vs. InsP\textsubscript{3}R2 | 0.57 ± 0.25 | 1.88 ± 1.04 | 4  |
| Peak vs. calreticulin | 0.64 ± 0.25 | 1.66 ± 1.04 | 6  |
| Slope vs. InsP\textsubscript{3}R2 | 0.47 ± 0.27 | 0.85 ± 0.98 | 4  |
| Slope vs. calreticulin | 0.23 ± 0.54 | 1.26 ± 1.57 | 6  |
Specialized Ca\textsuperscript{2+} Release Sites in Oligodendrocytes

**DISCUSSION**

We have investigated the structural specializations that underlie enhanced Ca\textsuperscript{2+} release sites in oligodendrocyte processes that support long distance propagation of Ca\textsuperscript{2+} waves. Our results demonstrate that in oligodendrocytes wave initiation and amplification sites remain invariant during Ca\textsuperscript{2+} waves activated by different InsP\textsubscript{3}-generating agonists in the same cell. This is in contrast to a report that different InsP\textsubscript{3}-coupled agonists evoke Ca\textsuperscript{2+} waves that initiate in different subcellular domains within pancreatic acinar cells (33). The molecular basis of wave initiation sites in oligodendrocytes remains undetermined, although locally elevated resting Ca\textsuperscript{2+} levels have been suggested to underlie the sites of wave initiation in astrocytes (12, 13, 16), and spatial compartmentalization of Ca\textsuperscript{2+} signaling complexes to determine wave initiation sites in pancreatic acinar cells (33) (for review, see Ref. 34).

Our results are consistent with the existence of cellular specializations supporting enhanced Ca\textsuperscript{2+} release function at discrete wave amplification sites, whereas no such specializations were identified at wave initiation sites. When we compared local Ca\textsuperscript{2+} release kinetics during agonist-induced Ca\textsuperscript{2+} waves with distribution of ER proteins and mitochondria, we found that a number of cellular factors appear to colocalize with wave amplification sites. These include high density patches of InsP\textsubscript{3}R2, accumulation of calreticulin, and the presence of mitochondria singly or in convoluted groups. It appears, therefore, that in oligodendrocyte processes, multiple cellular specializations may underlie enhanced Ca\textsuperscript{2+} release sites, and mitochondria may function together with the ER to generate the local cytosolic Ca\textsuperscript{2+} signals that support wave propagation.

A number of previous studies have shown that interactions occur between mitochondria and ER-dependent Ca\textsuperscript{2+} signals in various types of cells (18, 19, 35–37). Our present study demonstrates an intimate and regionally specialized relationship between ER and mitochondria, which has apparent functional consequences for wave propagation. Previous findings suggest that the ER membrane system extends approximately uniformly throughout oligodendrocytes (14). However, regions of ER near mitochondria display accumulations of the Ca\textsuperscript{2+}-binding protein, calreticulin, and a high density of InsP\textsubscript{3}R2 Ca\textsuperscript{2+} release channels in the membrane. These regions display larger amplitude and more rapidly rising Ca\textsuperscript{2+} responses and regeneratively support wave propagation. Mitochondria take up Ca\textsuperscript{2+} during cytosolic Ca\textsuperscript{2+} waves in oligodendrocytes (14).

The localization of mitochondria in intimate association with Ca\textsuperscript{2+} release sites (InsP\textsubscript{3}Rs) could thus regulate the Ca\textsuperscript{2+}-dependent gating kinetics of the InsP\textsubscript{3}R Ca\textsuperscript{2+} release channels. Indeed, inhibition of mitochondrial activity using p-trifluoromethoxyphenyl hydrazone or antimycin often inhibits or abolishes cytosolic Ca\textsuperscript{2+} responses (14). While the precise mechanisms involved remain to be elucidated, the functional consequence of the ER/mitochondria specialization in regions of oligodendrocyte processes may be amplification of local Ca\textsuperscript{2+} release kinetics, an important feature of long distance Ca\textsuperscript{2+} wave propagation.

The finding of calreticulin and InsP\textsubscript{3}R2 together in the ER in the present study is consistent with findings in several other cell types (2, 10). Previous studies have also shown that overexpression of calreticulin in *Xenopus* oocytes reduced the frequency of InsP\textsubscript{3}-mediated Ca\textsuperscript{2+} waves (4), whereas overexpression in HeLa cells decreased the rate of return to basal [Ca\textsuperscript{2+}] after InsP\textsubscript{3}-evoked Ca\textsuperscript{2+} release (11). In addition, decreasing calreticulin levels has been reported to lower the amplitude of the Ca\textsuperscript{2+} response to bradykinin in NG-108-15 neuroblastoma cells (38). Although calreticulin is believed to have a variety of functions, including an apparently complex role in regulating

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In *InsP*\textsubscript{3}R2 are expressed in accumulated patches in a close spatial relationship with one or more mitochondria along oligodendrocyte processes. This finding is consistent with our previous observation that mitochondria are always found at regions of high Ca\textsuperscript{2+} release kinetics (14), and indicates that ER specializations occur at sites close enough to single or a cluster of mitochondria to potentially permit complex functional interactions between mitochondria and Ca\textsuperscript{2+} stores during intracellular Ca\textsuperscript{2+} signaling.

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**FIG. 6. Comparison of MitoTracker staining with InsP\textsubscript{3}R2 immunofluorescence.** Live oligodendrocytes were incubated with MitoTracker (500 nM), fixed, and then processed for immunocytochemistry. 62 serial optical sections (every 0.09 μm in the z-dimension) obtained by digital confocal restoration were rendered into a three-dimensional voxel as shown. *Scale bar = 10 μm*. A, an oligodendrocyte cell body and a single process reconstructed from the z-series through the cell are depicted, labeled with AP42. InsP\textsubscript{3}R2 immunofluorescence is punctate in nature and is found in high intensity in the perinuclear region. Significant staining is also observed in the rest of the cell body and in high density patches at several sites along the process. B, the same cell from A is shown stained with MitoTracker. Staining in the cell body appears very different from InsP\textsubscript{3}R2 immunofluorescence (shown in A), being absent from the immediate perinuclear region. Along the process, however, several single and groups of mitochondria are found at regions close to where InsP\textsubscript{3}R2 immunofluorescence is found in A. C: the region shown in the box in B was rendered in pseudocolor (red, MitoTracker) and fused with the same region in A (green, AP42 fluorescence). A convoluted group of mitochondria are found to be surrounded by several hot-spots of InsP\textsubscript{3}R2 immunofluorescence, creating an entangled ER/mitochondrial cellular domain. Mitochondria appear as red convoluted rods enclosed within patchy green staining of InsP\textsubscript{3}R2. D, a side-on (X-Z) view of the region depicted in C. The close interrelationship in three dimensions between InsP3R2 expression and mitochondria can be seen, with high AP42 immunofluorescence being concentrated at sites located around and between individual mitochondria.
Ca\(^{2+}\) responses (2, 34, 39), the precise molecular role it plays in signaling is not understood. Our observation that calreticulin is found in high concentrations near InsP\(_3\)Rs in oligodendrocyte processes is suggestive of a role in localizing Ca\(^{2+}\) to release sites domains, thereby contributing to enhanced release kinetics at specialized Ca\(^{2+}\) release sites. A physical interaction between activated InsP\(_3\)Rs and calreticulin, which results in a conformation change in calreticulin, thus releasing bound Ca\(^{2+}\), has been previously suggested (4).

Recent experiments show that oligodendrocytes in both brain and spinal cord express at least type 1 InsP\(_3\)Rs, with the expression in brain oligodendrocytes being transient during development while in the spinal cord it is more persistent (40). The expression of type 2 and type 3 InsP\(_3\)Rs in oligodendrocytes, however, has not previously been studied. We show here that, in culture, oligodendrocytes express InsP\(_3\)R2 in the cell body and throughout the process arborizations, and that they are found in high density at specialized Ca\(^{2+}\) release sites. Type 1 and type 3 InsP\(_3\)Rs are also expressed in oligodendrocytes but are only found in the perinuclear region of the cell, suggesting that they are unlikely to be important for Ca\(^{2+}\) wave propagation along processes in these cells.\(^2\) Such spatially discrete functional roles for InsP\(_3\)R subtypes has been suggested recently in Xenopus oocytes (9).

The results presented here show that in oligodendrocyte processes, Ca\(^{2+}\) wave propagation is supported by specialized Ca\(^{2+}\) release sites with enhanced Ca\(^{2+}\) release kinetics. These sites are identified by accumulation of the ER proteins, calreticulin and InsP\(_3\)R2, and the presence of mitochondria, which may modulate the Ca\(^{2+}\) release process. A combination of ER proteins and intimate mitochondrial involvement may facilitate large localized changes in Ca\(^{2+}\) response characteristics and support long distance signaling. While several aspects of these structural specializations have now been identified, the precise molecular mechanisms and the interactions between the individual components that underlie the enhanced kinetic behavior of the local machinery remain to be investigated.

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