Role of sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPases in mediating Ca^{2+} waves and local Ca^{2+} -release microdomains in cultured glia

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We have characterized the sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pumps in cultured rat cortical type-1 astrocytes, type-2 astrocytes and oligodendrocytes. Perfusion with 10 μM cyclopiazonic acid (CPA) or 1 μM thapsigargin evoked a large and persistent elevation in cytosolic $[\text{Ca}^{2+}]$ in normal Ca^{2+} -containing medium and a small and transient increase in nominally Ca^{2+} -free medium. Subtraction of the response in Ca^{2+} -free medium from that in the control revealed a slow-onset Ca^{2+} -entry response to SERCA inhibition, which began after most of the store depletion had occurred. Thapsigargin- and CPA-induced responses propagated as Ca^{2+} waves, which began in several distinct cellular sites and travelled throughout the cell and through nearby cells, in confluent cultures. Propagation was supported by specialized Ca^{2+} -release sites where the amplitude of the response was significantly higher

and the rate of rise steeper. Such higher Ca^{2+} -release kinetics were observed at these sites during $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} waves in the same cells. Fluorescently tagged thapsigargin labelled SERCA pumps throughout glial cell bodies and processes. In oligodendrocyte processes, multiple domains with elevated SERCA staining were always associated with mitochondria. Our results are consistent with a model in which only a single Ca^{2+} store, expressing $\text{Ins}(1,4,5)\text{P}_3$ receptors and SERCAs sensitive to both thapsigargin and CPA, is present in rat cortical glia, and indicate that inhibition of SERCA activates both Ca^{2+} release as a wavefront and Ca^{2+} entry via store-operated channels. The spatial relationship between SERCAs and mitochondria is likely to be important for regulating microdomains of elevated Ca^{2+} -release kinetics.

INTRODUCTION

In eukaryotic cells, release of Ca^{2+} from intracellular stores associated with the endoplasmic reticulum (ER) or with 'calciosomes', appears to be a ubiquitous mechanism by which Ca^{2+} signals are evoked [1–3]. Release typically occurs via $\text{Ins}(1,4,5)\text{P}_3$ receptors (InsP_3Rs) or ryanodine receptors, and Ca^{2+} is subsequently resequestered into stores via sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPases (SERCAs) [1,3]. Complex spatiotemporal regulation of Ca^{2+} mobilization by a variety of mechanisms, including Ca^{2+} -feedback inhibition of Ca^{2+} release and changes in reuptake or extrusion rates, causes Ca^{2+} waves and/or oscillations in a wide variety of cell types [1].

Ca^{2+} waves can be activated in glia via several mechanisms, including release from intracellular stores [4,5] and modulation of activity of the plasmalemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger [6], depending on the nature of the stimuli used. It is now established that glial cells, including astrocytes [4,7,8] and oligodendrocytes [9], respond to activation of phosphoinositidase C-coupled receptors with Ca^{2+} waves, which travel along a cell and through intercellular networks at non-uniform rates. These waves appear to be propagated by a regenerative saltatory process via specialized intracellular wave-amplification sites at which the kinetics of Ca^{2+} release from stores are elevated [8–10]. Such wave-propagation mechanisms in astrocytes appear likely to be of importance in the brain for long-distance signal propagation and glio-neuronal interactions [11,12]. In oligodendrocyte processes, mitochondria are only found in wave-amplification domains, and appear to be important in local Ca^{2+} regulation at these sites [9]. Spatially and temporally complex Ca^{2+} -response characteristics in oligodendrocytes [9,13] may also enable these

cells to interact functionally with neuronal axons and other glia [14].

The discovery of potent and relatively selective SERCA inhibitors such as thapsigargin and cyclopiazonic acid (CPA) has enabled the widespread study of the roles of SERCA pumps in Ca^{2+} signalling [1,15]. In many cell types, SERCA inhibition leads to elevation of cytosolic $[\text{Ca}^{2+}]$ secondary to leakage of Ca^{2+} from stores. Several different SERCA subtypes are known to be expressed together in some cells [2,16,17], which may identify functionally distinct Ca^{2+} stores [17]. Both thapsigargin and CPA have previously been reported to be effective in inhibiting SERCA pumps in cultured glia [18,19]. In the present study, we have characterized the expression and function of SERCA pumps expressed in cultured rat cortical astrocytes and oligodendrocytes. We demonstrate that SERCA-mediated store depletion activates store-operated Ca^{2+} entry in these glia, and results in activation of Ca^{2+} -wave propagation in the absence of InsP_3 generation. Wave propagation is supported by wave-amplification sites where the kinetics of Ca^{2+} release are higher, and these sites were found to be well correlated for both thapsigargin- and InsP_3 -mediated waves. Furthermore, in oligodendrocyte processes, mitochondria were always found in association with sites where SERCA expression was elevated. This suggests that high concentrations of SERCA pumps together with other cellular specializations may be important in supporting elevated local Ca^{2+} -release kinetics in oligodendrocytes.

EXPERIMENTAL

Materials

Thapsigargin, CPA, noradrenaline and acetyl- β -methylcholine

Abbreviations used: SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; InsP_3R , InsP_3 receptor; MCh, acetyl- β -methylcholine chloride; AM, acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; $[\text{Ca}^{2+}]_o$, nominally Ca^{2+} -free.

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chloride (MCh) were obtained from Sigma. BODIPY FL thapsigargin and MitoTracker CMXRos were from Molecular Probes. Fura 2-acetoxymethyl ester (AM) and fluo 3-AM were from Research Biochemicals International.

Cell culture

Primary cultures of cortical astrocytes and oligodendrocytes were prepared from 2-day-old rats by previously described methods [8,9]. Briefly, cells were grown in plastic flasks to confluence. The flasks were shaken to remove microglia, the medium was discarded and replaced with fresh medium, and the flasks were then shaken vigorously overnight. Type-1 astrocytes, which remain firmly attached to the culture flasks, were removed by treatment with trypsin/EDTA, centrifuged, resuspended in culture medium, plated on to glass coverslips and maintained at 37 °C in 5% CO₂/95% air. The cells dislodged by the overnight shake were replated twice on to plastic Petri dishes for 1 h. Cells not attaching after the second plating were centrifuged, resuspended and plated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum on to glass coverslips coated with polyornithine, and maintained at 37 °C in 5% CO₂/95% air. These cells developed into type-2 astrocytes, as demonstrated by immunocytochemical staining with GFAP and A2B5 antibodies. Alternatively, the unattached cells were replated into DME-N1 containing 0.5% fetal bovine serum, and maintained in 10% CO₂/90% air. After 24 h, the medium was replaced with DME-N1 containing 2% fetal bovine serum. These cells developed into oligodendrocytes [positively stained with anti-(galactocerebrosidase C) antibody]. All cells were used 4–8 days after replating. Cultures consisted of approx. 90% of the identified cell type.

Measurement of cytosolic Ca²⁺ concentration ([Ca²⁺]_i)

Cells grown on coverslips were incubated with 5 μM fura 2-AM for 20 min at room temperature as described previously [8], placed in a Leiden coverslip chamber mounted on the microscope stage, and maintained under perfusion with the physiological salt solution at a rate of 1.5 ml/min. [Ca²⁺]_i measurements were carried out as previously described [8]. Briefly, fura 2 fluorescence was imaged with an inverted microscope on a vertical optical bench using a Nikon 40 × /1.3 NA CF Fluor DL objective lens. Images were digitized and averaged (two frames at each wavelength) in a Trapix 55/4256 image processor (Recognition Concepts, Incline Village, NV, U.S.A.). For analysis of fluorescence in whole cells, non-zero pixels within each cell image were averaged and plotted as calibrated [Ca²⁺]_i against time [8]. For analysis of wave-propagation and local Ca²⁺-release kinetics, cells were divided (from margin to margin) into 0.83 μm- or 1.6 μm-wide slices sequentially along the longitudinal axis [8,9]. Pixel intensities within each slice were then averaged and normalized. From these data, local Ca²⁺-release kinetics were calculated as described previously [8,9]. Specifically, the peak amplitude ($\Delta F/F$) of the response in each slice was calculated by subtraction of average resting fluorescence values from the peak fluorescence. Rate of rise of response [$(\Delta F/F)/s$] was the maximum slope of the rising phase of the Ca²⁺ response between 10% and 90% of the peak. Time to 50% of peak (s) was measured as the time point at which the local Ca²⁺ increase was half-maximal. All calculations were performed using mathematical macros within Synapse (Synergistic Research Systems, Silver Spring, MD, U.S.A.), and values were represented graphically using Kaleidagraph (Abelbeck Software).

Fluorescence microscopy

For high-resolution analysis of SERCA and mitochondrial distributions, we used an exhaustive photon reassignment procedure developed jointly by University of Massachusetts and Scanalytics Inc. [20]. Cells positioned on the stage of a standard wide-field microscope were incubated with BODIPY FL thapsigargin (1 μM) for 1 min, washed, and images acquired through a ×63 lens and FITC filter. In some experiments, cells were first incubated with 25 nM MitoTracker CMXRos for 30 min, then washed and incubated in BODIPY FL thapsigargin. Digital images were restored using an algorithm that removes out-of-focus plane fluorescence light to achieve high spatial resolution [9,20]. The maximum axial resolution of the microscope under our measurement conditions was 0.45 μm. Images were exported into Adobe PhotoShop for printing.

Cross-correlation analysis of wave characteristics

The spatial patterns of Ca²⁺-response characteristics 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 [9,21,22]. Wave characteristics were determined as previously described [8]. The mean values of normalized fluorescence intensities were subtracted out, and the resulting zero mean waves were embedded in surrounding zeros. The windowed data were transformed via a Fast Fourier Transform algorithm, using standard functions in Mathematica (Wolfram Research Inc.). 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. Data are presented as means ± S.D.

RESULTS

Elevation of [Ca²⁺]_i by SERCA inhibition

Thapsigargin and CPA are structurally unrelated inhibitors of SERCA pumps [23,24]. The Ca²⁺ response to SERCA inhibition by these agents was examined in type-1 astrocytes, type-2 astrocytes and oligodendrocytes. Both CPA (10 μM) and thapsigargin (1 μM) in normal Ca²⁺-containing (1.5 mM) medium evoked a sustained [Ca²⁺]_i increase in all three types of glial cell (Figures 1 and 2). This sustained increase in [Ca²⁺]_i is similar to previous observations in neurons [25]. One explanation for the sustained response is activation of Ca²⁺ entry across the plasma membrane, subsequent to store depletion [15]. The presence of store-operated entry mechanisms in cultured astrocytes has, however, recently been questioned [26].

The effect of extracellular Ca²⁺ on CPA and thapsigargin responses was therefore examined. Parallel glial cultures were treated with SERCA inhibitors in normal (1.5 mM) or nominally Ca²⁺-free ([Ca²⁺]_o < 5 μM) medium, and the Ca²⁺ response was measured. In Figure 1, population responses from type-1 astrocytes (a), oligodendrocytes (b) and type-2 astrocytes (c), and the net differences due to [Ca²⁺]_o, are shown. Perfusion with 10 μM CPA or 1 μM thapsigargin in nominally Ca²⁺-free medium evokes a slow-onset transient elevation of [Ca²⁺]_i in all the glial cell types. This response was typically small in amplitude, particularly in oligodendrocytes (Figure 1b). In parallel cultures, thapsigargin and CPA both elicited a large and persistent [Ca²⁺]_i increase in the presence of normal [Ca²⁺]_o (1.5 mM). Subtraction of responses in nominally Ca²⁺-free medium from those in normal [Ca²⁺]_o reveals a slow-onset [Ca²⁺]_o-dependent response to SERCA inhibition (Figures 1a–1c), which begins after most of the store depletion (Ca²⁺ response in Ca²⁺-free medium) has occurred. The majority of

Figure 1 SERCA inhibition evokes Ca^{2+} -store mobilization and Ca^{2+} entry in cultured glia

Perfusion with $10 \mu\text{M}$ CPA (**a, b**) or $1 \mu\text{M}$ thapsigargin (**c**) in nominally Ca^{2+} -free medium (○) evokes a slow-onset transient elevation of $[\text{Ca}^{2+}]_i$ in fura 2-loaded type-1 astrocytes (**a**), oligodendrocytes (**b**) and type-2 astrocytes (**c**). This response is typically of small amplitude, particularly in oligodendrocytes. In each cell type, in parallel cultures, the same experiment was repeated in the presence of normal $[\text{Ca}^{2+}]_o$ (—), which resulted in a larger persistent elevation of $[\text{Ca}^{2+}]_i$. Subtraction of responses in nominally Ca^{2+} -free medium from those in normal $[\text{Ca}^{2+}]_o$ reveals a slow-onset $[\text{Ca}^{2+}]_i$ -dependent response to SERCA inhibition (●). Most of this response occurs after most of the store depletion has occurred, and corresponds to Ca^{2+} entry across the plasma membrane via store-operated channel activation. Results are mean $[\text{Ca}^{2+}]_i$ responses from 13–50 cells in a single field for each condition.

the sustained component of the Ca^{2+} response evoked by thapsigargin or CPA in cortical glia was therefore due to Ca^{2+} entry, occurring with a slow onset after store depletion. This is consistent with activation of a store-operated Ca^{2+} -permeant channel. The time courses of the two phases of the response, i.e. release from intracellular stores and Ca^{2+} entry across the cell membrane, were different in the three cell types (Figure 1).

Previously published work has argued that several distinct Ca^{2+} stores may exist in some cell types, which are endowed with different subtypes of SERCA pumps differing in their sensitivity to thapsigargin and CPA [16,17]. Two distinct Ca^{2+} stores have been inferred to exist in mouse astrocytes [19]. To test for such a possibility in cultured rat glial cell types, we exposed cells to a maximally effective concentration of CPA ($10 \mu\text{M}$) for 4–6 min, followed by the addition of $1 \mu\text{M}$ thapsigargin in the continued presence of CPA. An example of this experimental paradigm in type-2 astrocytes is shown in Figure 2(a). CPA ($10 \mu\text{M}$) evoked a slow-onset Ca^{2+} release in Ca^{2+} -free medium, and addition of thapsigargin ($1 \mu\text{M}$) did not lead to an additional increase in $[\text{Ca}^{2+}]_i$ (Figure 2a). In normal Ca^{2+} -containing medium, CPA evoked a large and sustained increase in $[\text{Ca}^{2+}]_i$, and again the effects of application of thapsigargin together with CPA were not

Figure 2 CPA and thapsigargin mobilize the same Ca^{2+} store in glia

(**a**) Example trace from single fura 2-loaded type-2 astrocytes in normal Ca^{2+} and Ca^{2+} -free conditions. Cells incubated with $10 \mu\text{M}$ CPA (open bar) respond with a slow-onset increase in $[\text{Ca}^{2+}]_i$. Subsequent addition of $1 \mu\text{M}$ thapsigargin (solid bar) results in a negligible increase in either Ca^{2+} release or entry. (**b**) Example trace from single fura 2-loaded oligodendrocytes in normal Ca^{2+} and Ca^{2+} -free conditions. Cells incubated with $1 \mu\text{M}$ thapsigargin (open bar) respond with a slowly rising increase in $[\text{Ca}^{2+}]_i$. Subsequent addition of $10 \mu\text{M}$ CPA (solid bar) results in a very small increase in Ca^{2+} entry and no detectable increase in Ca^{2+} release. The results depicted in (**a**) and (**b**) were typical of responses in type-1 astrocytes, type-2 astrocytes and oligodendrocytes.

additive (Figure 2a). Essentially identical results were also obtained with type-1 astrocytes and oligodendrocytes (not shown). Similarly, CPA ($10 \mu\text{M}$) was found to have little additional effect after 4–6 min thapsigargin ($1 \mu\text{M}$) treatment, either in Ca^{2+} -free or normal Ca^{2+} -containing medium (Figure 2b). In the experiments in oligodendrocytes shown in Figure 2(b), thapsigargin ($1 \mu\text{M}$) evokes a substantial $[\text{Ca}^{2+}]_i$ response in normal Ca^{2+} and a very small response in nominally Ca^{2+} -free medium, as previously shown in Figure 1(b). Addition of CPA ($10 \mu\text{M}$) during the continued presence of thapsigargin evokes a small elevation of $[\text{Ca}^{2+}]_i$ in normal Ca^{2+} -containing medium, and no additional response in nominally Ca^{2+} -free medium (Figure 2b). Essentially identical results were also obtained with type-1 and type-2 astrocytes (not shown). It appears therefore that the majority of the effects on $[\text{Ca}^{2+}]_i$ evoked by these agents in rat cortical glia occur as the result of actions on the same Ca^{2+} store(s).

Thapsigargin evokes Ca^{2+} waves in glial cells

To investigate the nature of thapsigargin-evoked responses in glia in greater detail, we incubated glial cells with fluo 3 and measured the Ca^{2+} responses in individual cells with high spatio-temporal resolution. Addition of $1 \mu\text{M}$ thapsigargin to type-1 astrocytes was found to evoke a Ca^{2+} wave in both Ca^{2+} -free (Figures 3 and 4) ($n = 3$) and normal Ca^{2+} (not shown) ($n = 2$) conditions. In Figure 3, waves can be seen to be initiated at different time points in four cells within the field (see arrows), and these waves are then slowly propagated through closely connected neighbouring cells. Responses are initiated in two cells in panel 2 (arrows a, b), and waves travel through these cells and into neighbouring cells in panels 3–6. A third wave begins in a

Figure 3 Thapsigargin-evoked Ca^{2+} responses are Ca^{2+} waves that travel intercellularly in astrocytes

Pseudocolour images of thapsigargin-evoked Ca^{2+} responses measured in fluo 3-loaded astrocytes in Ca^{2+} -free medium. Images shown were acquired 0.4 s apart, starting at the top left (panel 1), which shows resting Ca^{2+} levels. Responses are initiated in two cells in panel 2 (arrows a, b), and waves travel through these cells and into neighbouring cells in panels 3–6. A third wave begins in a cell at the bottom in panel 3 (arrow c), and this is propagated into its immediate neighbour above in panels 6–8. A wave begins in a cell on the extreme left in panel 4 (arrow d), and this wave is propagated through its neighbours in panels 5–11. Waves thus appear to travel from cell to cell within closely connected groups. Responses begin to decline in some cells by panel 13, under the Ca^{2+} -free conditions.

cell at the bottom in panel 3 (arrow c), and this is propagated into its immediate neighbour above in panels 6–8. A fourth wave begins in a cell in panel 4 (arrow d), and this wave is propagated slowly through several nearby cells in panels 5–11. The waves rapidly decline, consistent with the lack of sustained responses in the Ca^{2+} -free medium as illustrated above. Previous work has shown that InsP_3 -generated Ca^{2+} signals are propagated through astrocytic networks in culture via gap-junctional connections [27].

We measured the local kinetics of Ca^{2+} release (peak amplitude and rate of rise of response) in defined 0.83 or 1.6 μm -wide x, y sections along the longitudinal axis of cells as described in the

Experimental section (see also [8,9]). Marked variations in both the amplitude of the response and the rate of rise were found along the length of a cell during a propagating Ca^{2+} wave induced by thapsigargin (Figure 4a). In the cell shown in Figure 4, both these parameters of Ca^{2+} release were significantly higher in several specific loci than in surrounding regions (Figure 4a, asterisks). The pattern of the local Ca^{2+} -release amplitudes and the rates of increase in the response along the length of the cell were compared using cross-correlation analysis. This analysis revealed that a marked spatial correlation existed between the peak amplitude and rate of increase in response (Figure 4c). Consolidated data from a number of such experiments are shown

Figure 4 Characteristics of thapsigargin-evoked Ca^{2+} responses in astrocytes and oligodendrocytes

$[\text{Ca}^{2+}]$ responses were measured with high spatiotemporal resolution in fluo 3-loaded glial cells. Addition of thapsigargin to type-1 astrocytes (**a, b, c**), type-2 astrocytes (not shown) or oligodendrocytes (**d, e, f**) in Ca^{2+} -free medium was found to evoke responses that propagated as waves. Waves were analysed by measuring Ca^{2+} in serial slices through cells using previously described techniques (see the Experimental section [8]). (**a, d**) Example plots of the amplitude and rate of rise of the $[\text{Ca}^{2+}]$ response evoked along the length of the cell in astrocytes (**a**) and oligodendrocytes (**d**). Thapsigargin is found to evoke $[\text{Ca}^{2+}]$ changes in most regions of these cells, including cell body and processes. The amplitude of the response varies markedly along the cell axis, as does the rate of increase in response. These two indices of release kinetics were found to be closely correlated with each other (see **e, f**) along the cell length. Notably, several regions possessing particularly high release kinetics (i.e. elevated peak amplitude compared with neighbouring regions *and* high rate of increase in response, asterisks) were found in both cell types. Peak and rate of rise were calculated as described previously [8] and represent $\Delta F/F$ and $\Delta F/F$ per s respectively (see the Experimental section). (**b, e**) Analysis of the onset of the $[\text{Ca}^{2+}]$ response along the length of the same cells shown in (**a**) and (**d**). Plots represent the time (in seconds) between the initiation of fluorescence recording and the time at which the response reaches 50% of its peak level. These plots demonstrate that thapsigargin evokes Ca^{2+} waves which are initiated (i.e. reach 50% of maximum at an earlier time than neighbouring regions) in several distinct cell regions (asterisks), and are subsequently propagated throughout the cell. (**c, f**) Cross-correlation analysis of amplitude with rate of increase in response. A high correlation was observed between these release characteristics for thapsigargin-evoked Ca^{2+} waves, the highest correlation being close to phase (0 μm). ●, Peak amplitude versus rate of rise comparison. The auto-correlation plots for thapsigargin peak amplitudes (—) are included for comparison. It can be seen that the cross-correlation of peak with slope is similar to the auto-correlation function.

in Table 1. This is consistent with the expression of specialized loci in glia which display elevated levels of Ca^{2+} flux, as we have previously reported for InsP_3 -evoked responses [8–10].

Waves are initiated in several distinct cellular domains within an astrocyte, which are readily identifiable as ‘minima’ in the plot of the time delay to 50% of maximum amplitude against cell length (Figure 4b). Responses are initiated at these sites, which are spatially distinct from the sites of high- Ca^{2+} release kinetics, then travel throughout the cell (Figure 4b) and through nearby cells (see also Figure 3). Apparently, then, different specialized sites exist for wave initiation (Figure 4b) and wave amplification (Figure 4a). These results show that a Ca^{2+} wave with complex

spatiotemporal kinetics can be evoked in type-1 astrocytes in the absence of InsP_3 generation. Thapsigargin-evoked waves often travelled at a relatively low rate compared with InsP_3 -mediated waves in these cells (Figures 3 and 4b) [8,28]. This is consistent with the fact that the mechanism of thapsigargin-generated waves is different from that for waves mediated by InsP_3 , despite the fact that thapsigargin-evoked Ca^{2+} mobilization appears to occur because of Ca^{2+} passing through InsP_3R channels [29].

Parallel experiments in type-2 astrocytes and in oligodendrocytes yielded comparable results. In the presence of extracellular Ca^{2+} , a large sustained thapsigargin-evoked Ca^{2+} response was found in these cells. In most experiments, waves

Table 1 Comparison of InsP_3 - and thapsigargin-evoked Ca^{2+} waves in oligodendrocytes and type-1 astrocytes

Cross-correlation values are calculated as described in the Experimental section. Location of peak corresponds to the distance from phase correlation [i.e. maximum amplitude of auto-correlation ($0\ \mu\text{m}$), see Figure 4] at which the peak cross-correlation is found. Peak = maximum amplitude of response; slope = rate of increase in response; Thaps = thapsigargin; Nor = noradrenaline.

Comparison	Peak correlation	Location of peak (μm)	<i>n</i>
Oligodendrocytes			
Thaps wave peak versus MCh wave peak	0.76 ± 0.15	8.03 ± 7.70	3
Thaps wave slope versus MCh wave slope	0.33 ± 0.17	0.00 ± 0.00	3
Thaps wave peak versus Thaps wave slope	0.38 ± 0.26	0.72 ± 1.57	7
Astrocytes			
Thaps wave peak versus Nor wave peak	0.43 ± 0.13	6.00 ± 1.89	4
Thaps wave slope versus Nor wave slope	0.54 ± 0.20	2.00 ± 1.51	4
Thaps wave peak versus Thaps wave slope	0.37 ± 0.23	4.28 ± 2.25	6

travelled through the cell bodies of type-2 astrocytes ($n = 2$ type-2 astrocytes, not shown) and oligodendrocytes ($n = 7$ oligodendrocytes, Figures 4d, 4e and 4f), and out along their processes. Both ER [9] and SERCA pumps (see Figure 5) are expressed throughout the arborization of glial cell processes. The waves in the two cell types of the O-2A lineage resembled those found in type-1 astrocytes, being initiated at several distinct cellular regions in both cell bodies and processes (Figure 4e, asterisks). Wave propagation was found to be supported by subcellular microdomains with markedly elevated local Ca^{2+} -release kinetics at several sites along the length of the cell (Figure 4d, asterisks). The pattern of local amplitudes of Ca^{2+} release correlated with the pattern of release rate measured in the same areas (Figure 4f). Compiled data from a number of experiments are shown in Table 1.

In another series of experiments, cells were initially stimulated with an InsP_3 -producing agonist [noradrenaline (200 nM) for type-1 astrocytes, MCh (100 μM) for oligodendrocytes], left for more than 15 min to recover, and then exposed to 1 μM thapsigargin. The spatial characteristics of the Ca^{2+} waves evoked by each treatment were measured and the local Ca^{2+} -release kinetics were compared by cross-correlation analysis. A marked correlation was found between cellular regions with higher Ca^{2+} -release kinetics during waves generated by InsP_3 and by thapsigargin in the same cell (Table 1). This result suggested that wave propagation in the two cases may depend on similar cellular mechanisms.

Distribution of SERCA pumps in cultured glial cells

In order to determine the sites of SERCA expression in cultured glial cells, cells were incubated with a fluorescently tagged form of thapsigargin, BODIPY FL thapsigargin. Digital confocal analysis showed SERCA distribution throughout the cell body region of type-1 astrocytes (Figure 5a), oligodendrocytes (Figure 5b) and type-2 astrocytes (Figure 5c), with only the nucleus remaining unstained. A low level of fluorescence was seen throughout type-1 astrocytes, within which distinct sites of high-intensity labelling ('hot-spots') were scattered (Figure 5a). These hot-spots appeared at random orientation to each other and were highly punctate. In oligodendrocytes (Figure 5b) and type-2 astrocytes (Figure 5c), thapsigargin labelling was particularly concentrated in multiple clusters which were unequally spaced

Figure 5 SERCA pumps are expressed in a variegated pattern in cultured glia

Type-1 astrocytes (a), oligodendrocytes (b) and type-2 astrocytes (c) were incubated with BODIPY FL thapsigargin (1 μM , 1 min), and fluorescence images were acquired using digital confocal microscopy. A single optical section from a stack of images acquired every 0.25 μm in the *z* dimension is shown for each cell type. SERCA pumps are expressed at high levels in the cell body region of all three glial cell types. In type-1 astrocytes (a), a number of circular 'hot-spots' are found in the cell body and along the processes. In oligodendrocyte processes (b) and type-2 astrocytes (c) expression is highly varied, such that certain sites along the processes are found to possess markedly higher levels of SERCA than surrounding regions. All scale bars = 20 μm .

along processes. Significant labelling was also observed in the cell body region (Figures 5b and 5c).

We have previously shown that mitochondria and high levels of a number of ER proteins involved in Ca^{2+} regulation are expressed together in specialized domains along oligodendrocyte processes and in type-1 astrocytes [9,10,30,31]. In particular, we have previously reported that, in oligodendrocytes, mitochondria play an important role in the propagation of Ca^{2+} waves, apparently by modulation of ER Ca^{2+} -release kinetics at wave-amplification sites [9]. Mitochondria also appear to be important in local Ca^{2+} regulation in type-1 astrocytes, and are closely localized to wave-amplification sites in these cells as well ([32]; P. B. Simpson, S. Mehotra, D. Langley, C. A. Sheppard and J. T. Russell, unpublished work). Such ER/mitochondria specializations are likely to provide the regenerative Ca^{2+} release necessary to support wave propagation [9,10]. One possibility is that mitochondrial Ca^{2+} -uptake processes may regulate Ca^{2+} levels around nearby InsP_3 Rs, thus controlling their activity and inhibiting receptor inactivation. SERCA pumps would be expected to largely outcompete the mitochondrial Ca^{2+} uniporter for Ca^{2+} uptake, because of their higher affinity for Ca^{2+} [33]. Thus high-expression levels of SERCA pumps might be needed

Figure 6 Comparison of the distribution of SERCA pumps and mitochondria in glia

(a) MitoTracker fluorescence in a type-1 astrocyte. A type-1 astrocyte was labelled with BODIPY FL thapsigargin then with MitoTracker CMXRos, and digital confocal images were acquired (every $0.25 \mu\text{m}$ in the z dimension). Single optical sections are shown in (a) and (b), while (c) shows volume views made using all the images acquired in the z dimension. Scale bar in (b) = $15 \mu\text{m}$. Many mitochondria are found throughout the cell, particularly heavily in the cell body region, except in the immediate vicinity of the nucleus (see b). In the processes, their distribution appears to be varied. (b) BODIPY FL thapsigargin fluorescence in the same cell. SERCA is expressed throughout the cell body and process regions of astrocytes. A single large 'hot-spot' is found in this cell near several smaller ones, near the nucleus (see the box). (c) Enhanced contrast volume views of the section indicated by the box in (b). The region shown by the box in (b) is shown in pseudocolour where MitoTracker staining is coloured red, with BODIPY FL thapsigargin staining in green. Left, view from above shows that the large hot-spot of BODIPY FL thapsigargin fluorescence is not closely co-localized with mitochondria. Right, a side-on view shows that the SERCA hot-spot passes throughout the stack of images, without mitochondria on top or bottom, or close by in any plane. (d) MitoTracker fluorescence in an oligodendrocyte process. An oligodendrocyte process was labelled with BODIPY FL thapsigargin and MitoTracker CMXRos, and digital confocal images were acquired (every $0.09 \mu\text{m}$ in the z dimension). Individual optical sections are shown in (d) and (e). (f) shows volume views rendered from all the z sections. Scale bar in (e) = $25 \mu\text{m}$. Mitochondria are found singly or in groups in a variegated pattern, with several groups being found like beads along the processes. (e) BODIPY FL thapsigargin fluorescence in the same cell. SERCA expression is highly heterogeneous along this process, with large beads of fluorescence found at several sites. (f) Volume view of the image within the box in (e) is shown in pseudocolour in two views. MitoTracker staining is coloured red and BODIPY FL fluorescence is coloured green. Left, view from above reveals that convoluted mitochondria are surrounded by high levels of SERCA fluorescence in this region. Right, a side-on view shows that several mitochondria are central to the bead of SERCA fluorescence, which surrounds the mitochondrial group on all sides.

at wave-amplification sites to maintain sufficiently high Ca^{2+} levels within the ER for large release responses, or to assist mitochondria in regulation of cytosolic Ca^{2+} -dependent InsP_3R activity.

We therefore examined whether either the 'hot-spots' of SERCA in type-1 astrocytes or the patches of SERCA expression along oligodendrocyte processes were associated with mitochondria. Living cells were incubated with the mitochondrial dye MitoTracker CMXRos, then with BODIPY FL thapsigargin, and the fluorescence patterns of the two dyes compared using digital confocal microscopy. In the type-1 astrocyte shown in Figure 6, mitochondria were found to be distributed throughout the cytosol in all regions except the nucleus (Figure 6b). SERCA expression was also found throughout the cell (Figure 6b), with some high-intensity hot-spots of staining. In this cell (Figure 6b, box), one large 'hot-spot' of SERCA fluorescence was found near the nucleus. Figure 6(c) shows that the SERCA 'hot-spot' (green) was in a region without notably high mitochondrial (red) expression levels. Mitochondria were not near this hot-spot in either the x, y (left panel) or z (right panel) dimensions. Thus, unlike in oligodendrocyte processes, in the type-1 astrocyte cell bodies with a more flat morphology, high-intensity SERCA labelling occurred in the absence of mitochondria.

In oligodendrocyte processes, mitochondria stained with MitoTracker were found in a highly punctate pattern singly or in groups (Figure 6d), as we have previously reported using other mitochondrial dyes [9]. Large beads of SERCA staining were also found along processes (Figure 6e), and these were always found in close association with convoluted groups of mitochondria (Figure 6f). In the process shown, every mitochondrion appears to be surrounded by dense coils of ER expressing high levels of SERCA pumps. Such high-density SERCA expression at these sites would be expected to participate in the regulation of Ca^{2+} release through InsP_3R channels in these cellular microdomains [9,30]. This finding of heterogeneous SERCA distribution in astrocytes and oligodendrocytes may also provide a partial explanation for the heterogeneous spatial responses to SERCA inhibition found in the present study.

DISCUSSION

The results presented here show that inhibition of SERCA pumps by thapsigargin or CPA in glia derived from neonatal rat cerebral cortex evokes significant Ca^{2+} release from intracellular stores, and Ca^{2+} entry after store depletion. In addition we show that these agents activate propagating Ca^{2+} waves, which like

InsP₃-mediated waves are supported by specialized microdomains with amplified Ca²⁺-release kinetics. In oligodendrocytes, high levels of SERCA pumps were expressed in these specialized cellular sites, in addition to other structural specializations including the presence of mitochondria. Our results show that, in glia, CPA and thapsigargin treatment both result in an increase in [Ca²⁺]_i throughout the length of the cells, and that both agents empty essentially the same Ca²⁺ store. In cultured mouse astrocytes, two distinct Ca²⁺ stores, only one of which is sensitive to CPA, have previously been reported [19].

It has previously been argued that the capacitative entry mechanism does not operate in adult cultured astrocytes in response to nucleotide receptor activation or thapsigargin treatment, such that the refilling of Ca²⁺ stores is primarily determined by reuptake of cytosolic Ca²⁺ [26]. Although it is entirely possible that mechanisms present in cultured neonatal astrocytes may be absent from cultured adult astrocytes, it should be pointed out that the above conclusion was based largely on the results of Mn²⁺ quench experiments in thapsigargin-treated cells. We and others have previously shown that Mn²⁺ quench of fura 2 fluorescence cannot be relied on to detect the activation of store-operated channels [25,34]. Also, although the thapsigargin- and CPA-evoked Ca²⁺-entry responses are significant in neonatal astrocytes, their onset is delayed and the rise time is slow, such that the brief treatment periods and measurement used in the previous study may have precluded detection of the [Ca²⁺]_i elevation evoked by store depletion. Clearly the present results demonstrate that, at least in neonatal astrocytes, store-operated channels appear to be activated by SERCA inhibition to evoke significant Ca²⁺ entry and presumably rapid store refilling (see also [19]). Time-dependent reproducibility of agonist-evoked responses in neonatal astrocytes [28] thus appears to depend more on recovery from receptor desensitization [35] than on store refilling. Store-operated Ca²⁺ entry may underlie the recently reported extracellular Ca²⁺-dependent ability of prolonged stimulation with carbachol to activate gene expression in glia, via phosphorylation of the transcription factor cAMP-dependent response-element-binding protein [36].

In either normal or nominally Ca²⁺-free conditions, thapsigargin-evoked Ca²⁺ release initially begins at specific cellular sites in glial cells and subsequently travels as waves from these points of origin. In each glial cell type, multiple sites of wave initiation were found. The reason why thapsigargin evokes Ca²⁺ waves rather than simply a uniform Ca²⁺ leakage from stores throughout the cell is not known, but it could be due to differences in the threshold of activation of the InsP₃R channels in different regions of the cells. The differences in threshold could result from a number of factors, including variable distribution of InsP₃Rs [10,30,31], expression of different types of InsP₃Rs [1,35,37] and localized differences in Ca²⁺ buffering at rest [9,30]. Previous studies from our laboratory have suggested that waves are initiated at sites along astrocytes that have high resting Ca²⁺ levels, which may result in higher sensitivity of nearby InsP₃Rs [8,10,31].

During wave propagation, the local amplitudes of Ca²⁺ release and the rate of release were significantly elevated in several subcellular microdomains (which we shall refer to as wave-amplification sites [8,10,31]), these sites being well correlated with similar regions in InsP₃-mediated responses in the same cells. We have previously reported that multiple sites with elevated Ca²⁺-flux kinetics can underlie regenerative saltatory propagation of InsP₃-evoked Ca²⁺ waves over long distances [8–10]. Thus despite the apparently different mechanisms of action, the characteristics of the thapsigargin response bear certain important resemblances to InsP₃-mediated waves. Thapsigargin-evoked inter- and intra-

cellular Ca²⁺ waves exhibited these complex spatiotemporal kinetics in the absence of InsP₃ generation. This finding argues that wave-initiation and -amplification sites in glia are unlikely to be determined by spatial variations in InsP₃ production, consistent with initiation sites being related to variations in resting Ca²⁺ levels [8,10,31], and amplification sites being related to structural specializations in the Ca²⁺-release machinery of the cell [9,10]. The latter appears to include variations in distribution of InsP₃Rs [31,37], calreticulin [36], mitochondria [9,32] and, as shown here, SERCA pumps.

We have previously shown that caffeine-evoked Ca²⁺-response characteristics correlate highly with InsP₃-mediated waves in glia [9], and that thapsigargin or CPA pretreatment abolishes responses to both InsP₃-coupled agonists and caffeine in glia [28,37]. Thus InsP₃Rs, ryanodine receptors and SERCA pumps sensitive to both thapsigargin and CPA may all be expressed on a single functional pool in rat cortical glia. Consistent with this hypothesis, fluorescently tagged thapsigargin labels sites throughout the cell body and process arborization of all three glial cell types examined. It is notable that expression of SERCA pumps is heterogeneous over the cell body and processes in glial cells with distinct clusters of labelled sites. High-intensity sites of SERCA clustering may play a key role in local variations in intraluminal [Ca²⁺], which are thought to be important for ER-mediated wave propagation [1,10,38]. Whereas in oligodendrocyte processes, SERCA clusters were found to be in close association with mitochondria, in other cells with long thin processes, mitochondria appear to be preferentially localized near the plasma membrane, rather than near the ER [39]. This arrangement appears to allow such mitochondria to primarily buffer Ca²⁺ entry rather than Ca²⁺ release [39,40]. In contrast, colocalization of mitochondria with ER appears to facilitate interactions between these two organelles in Ca²⁺ signalling [2,39]. The specialized arrangement of organelles found in the present study, i.e. high-density expression of SERCA pumps on ER membranes entangled around clusters of mitochondria in oligodendrocyte processes, is suggestive of a role for SERCA pumps in functional ER-mitochondrial interactions. One possibility is a role for SERCAs in local regulation of Ca²⁺-signal kinetics in glia by enabling locally high Ca²⁺ uptake into stores. This may regulate both the amount and rate of Ca²⁺ releasable by stimuli [1,2,38], and modulate the dependence of InsP₃Rs on cytosolic Ca²⁺ [1]. We hypothesize that, whereas mitochondria are needed at wave-amplification sites to regulate activity of InsP₃Rs located in close apposition to mitochondria [9,30], the principal role of high SERCA levels may be to sequester cytosolic Ca²⁺ which might otherwise be buffered by the nearby mitochondria, thus maintaining locally high levels of Ca²⁺ within the ER and thereby contributing to elevated Ca²⁺ flux at wave-amplification sites. Interestingly, in type-1 astrocytes, mitochondria were not found close to the hot-spots of SERCA labelling, suggesting that a high level of SERCA expression is not a characteristic of wave-amplification sites in all cells.

In summary, we show here that in rat cortical glia, inhibition of SERCA pumps expressed on a single Ca²⁺ pool initiates both Ca²⁺-wave propagation and store-operated channel activation. These waves resemble InsP₃-mediated waves in many respects and are supported by specialized regions of elevated Ca²⁺ release. SERCA expression is markedly heterogeneous in these cells, and regions with elevated SERCA expression levels in oligodendrocytes correlate well with wave-amplification sites. These findings are consistent with the suggestion that multiple specializations may underlie discrete regions of enhanced Ca²⁺ release which support long-distance propagation of Ca²⁺ signals. Microdomains of high Ca²⁺-release kinetics within oligo-

dendrocytes appear to be due to a multiplicity of cellular specializations, including the presence of mitochondria [9], calreticulin [30], InsP_3Rs [30] and SERCA pumps. Although the precise mechanisms and kinetics of the interactions between these proteins within and on ER membranes and mitochondria are currently uncertain, this study highlights the complex and heterogeneous relationship between InsP_3Rs , calreticulin, SERCAs and mitochondria which may govern many aspects of Ca^{2+} signalling in glia and other cell types.

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