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Research Paper

Sealing frequency of B104 cells declines exponentially with decreasing transection distance from the axon hillock

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ABSTRACT

Transection of nerve axons (axotomy) leads to rapid (Wallerian) degeneration of the distal portion of the severed axon whereas the proximal portion and the soma often survive. Clinicians and neuroscientists have known for decades that somal survival is less likely for cells transected nearer to the soma, compared to further from the soma. Calcium ion (Ca^{2+}) influx at the cut axonal end increases somal Ca^{2+} concentration, which subsequently activates apoptosis and other pathways that lead to cell death. The same Ca^{2+} influx activates parallel pathways that seal the plasmalemma, reduce Ca^{2+} influx, and thereby enable the soma to survive. In this study, we have examined the ability of transected B104 axons to seal, as measured by uptake or exclusion of fluorescent dye, and quantified the relationship between sealing frequency and transection distance from the axon hillock. We report that sealing frequency is maximal at about 150 μm (μm) from the axon hillock and decreases exponentially with decreasing transection distance with a space constant of about 40 μm. We also report that after Ca^{2+} influx is initiated, the curve of sealing frequency versus time is well-fit by a one-phase, rising exponential model having a time constant of several milliseconds that is longer nearer to, versus further from, the axon hillock. These results could account for the increased frequency of cell death for axotomies nearer to, versus farther from, the soma of many types of neurons.

1. Introduction

Neuroscientists and clinicians have known for many decades that transection of dendrites or axons can initiate a Ca^{2+} influx that leads to perikaryial death (Ramón y Cajal, 1928; Schlaepfer and Bunge, 1973; Lucas et al., 1985, 1990; Loewy and Schader, 1977; Yoo et al., 2004; Nguyen et al., 2005; Campbell, 2008; Wolfe et al., 2010; Moe et al., 2015). This influx increases somal Ca^{2+} concentrations, activating calpains and other proteases in enzymatic pathways that induce apoptosis or other mechanisms of cell death (Choi, 1988; Tymianski et al., 1993; Ziv and Spira, 1995; Yoo et al., 2004). Cell death occurs more frequently for cells transected nearer to, compared to further from, their cell body (Ramón y Cajal, 1928) due to increased concentrations of somal Ca^{2+} that probably disrupt somal protein synthesis (Yoo et al., 2004; Nguyen et al., 2005).

The same Ca^{2+} influx in all eukaryotic cells that initiates cell death (if left unchecked) also initiates plasmalemmal sealing, which reduces this continuing influx (Krause et al., 1994; Steinhardt et al., 1994; Spaeth et al., 2010, 2012a, 2012b, 2012c; Moe et al., 2015) and thus prevents somal Ca^{2+} concentrations from reaching levels that produce cell death. Ca^{2+} influx activates sealing through multiple parallel and redundant enzymatic pathways that induce vesicles to form and/or migrate to the lesion site where they create a vesicular plug that seals the membrane and reduces Ca^{2+} influx to levels seen in uninjured eukaryotic cells (Krause et al., 1994; Miyake and McNeil, 1995; Eddleman et al., 1997; Blanchette et al., 1999; Jimenez et al., 2014).

Plasmalemmal sealing is a gradual process, taking 10–30 min to complete depending in part on axon diameter, which is larger near the axon hillock, (Krause et al., 1994; Yoo et al., 2003; Spaeth et al., 2010) and on which enzymatic pathways are initiated (Spaeth et al., 2010, 2012a, 2012b, 2012c; Zuzek et al., 2013). Axon-like neurites transected less than 50 μm from their soma seal more slowly at lower frequencies, and are associated with a higher frequency of cell death than those transected greater than 50 μm from their somas (Yoo et al., 2004; Nguyen et al., 2005; Spaeth et al., 2010, 2012a, 2012b, 2012c). The longer sealing times, larger diameters, and shorter Ca^{2+} diffusion distances of cells transected nearer to their soma are associated with

Abbreviations: AIC, Akaike's Information Criterion; Ca^{2+}, calcium ion; CMH X^2, Cochran–Mantel–Haenszel Chi-Squared (test); DPBS, Dulbecco's phosphate buffered saline; DPBS+/+, Ca^{2+} and Mg^{+2} containing DPBS; DPBS−/−, Ca^{2+} and Mg^{2+} free DPBS; μm, micrometers; min, minutes; n-optimized, number of cells/bin optimized; PC times, post-Ca^{2+} addition times; R^2, coefficient of determination; λ, space constant; τ, time constant.

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their increased frequencies of cell death compared to cells transected farther from their soma (Yoo et al., 2004).

Although the mechanism(s) of plasmalemmal sealing and cell death after neurite transection and the consequences for transections qualitatively nearer vs. further from the perikaryon have been described in some detail (Spaeth et al., 2010, 2012a, 2012b, 2012c), the quantitative nature of the relationship between transection distance vs frequency of plasmalemmal sealing has not been determined. In this article, we have examined quantitative relationships for axon-like neurites of rat B104 cells transected from 2.5 μm to over 400 μm from the axon hillock at 2.5 min to 20 min post-Ca^{2+} addition times (PC times). We report that sealing frequency vs transection distance at any given PC time is best fit by a one phase exponential model that decreases with a space constant (λ) of about 40 μm from a maximum value about 150 μm from the axon hillock to near-zero at the axon hillock. The relationship between sealing frequency and PC time is best fit by a one phase rising exponential model with a time constant (τ) of about 3.6 min for transections <50 μm from the axon hillock and about 2.2 min for transections ≥50 μm from the axon hillock.

2. Methods

2.1. Cell culture

B104 cells, a rat hippocampal neuroblastoma line, were cultured as previously described (Spaeth et al., 2010, 2012a, 2012b, 2012c; Zuzek et al., 2013). Unlike PC-12 cells or other commonly used neuronal cell lines, B104 cells do not require the addition of nerve growth factor or other supplements to differentiate. B104 neurites are bipolar and have many properties typical of axons such as action potentials, smooth endoplasmic reticulum, and neurotransmitter release. After 24 h of differentiation, axons of B104 cells can vary from a few μm to over 500 μm in length, with most ranging from 20 μm to 150 μm long. B104 cells have often been used as an in vitro model system to study neuronal function (Bottenstein and Sato, 1979; Toda et al., 1999; Yoo et al., 2003, 2004; Nguyen et al., 2005; Miller et al., 2006).

B104 cells were grown in 75 cm^2 vented cap, tissue culture flasks containing 1:1 Dulbecco's Modified Eagle's Medium and Ham's F12 supplement with 10% fetal bovine serum in a humidified incubator that was maintained at 37 °C, 5% CO_2. This growth medium was replaced every two days until the flask reached 80% confluency. Cells were then either subcultured into a new flask or seeded at a density of 5000 to 15,000 cells/cm^2 on poly D-lysine coated (5 μg/ml) cell culture plates (60 × 15 mm, Falcon). The next day, plated cells were differentiated in serum-free 1:1 Dulbecco's Modified Eagle's Medium and Ham's F12 supplement for 24 h. Stock populations of B104 cells were subcultured at most 35 times to mitigate the effects of senescence.

2.2. Axon transection and microscopy

Glass knives were created by pulling 1 mm diameter microcapillary tubes with a micropipette puller (Sutter Instrument CO, USA), and breaking the pipettes at their tips. The glass knives were mounted on a micromanipulator (Narishige Instruments) attached to a Zeiss ICM-405 phase contrast microscope. Cell culture plates containing 100,000 to 300,000 differentiated B104 cells were gently rinsed twice and filled with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS −/−, Hyclone). To transect B104 axons, a cell culture plate was placed on the microscope stage and the glass knife was lowered onto the surface of the plate using the micromanipulator. The microscope stage was moved parallel to the knife's edge to transect some B104 axons, etching a visible "transection line" on the surface of the plate. Axons of 500–800 cells were transected on each plate within 10 min after adding the DPBS −/−. Note that most cells on a plate were *not* transected.

After transection, DPBS −/− medium was replaced with DPBS medium containing Ca^{2+} and Mg^{2+} (DPBS +/+) to initiate the sealing process, as previously reported [The addition of Ca^{2+} to the extracellular medium initiates plasmalemmal sealing in all eukaryotic cells (Krause et al., 1994; Blanchette et al., 1999; Detrait et al., 2000a, 2000b; Yoo et al., 2003; Spaeth et al., 2010)]. Cell culture plates containing transected B104 axons were returned to the CO_2 incubator and allowed to sit for either 2.5, 3.75, 5, 10, 15, or 20 min post DPBS +/+ addition, i.e., for 2.5–20 min post Ca^{2+} addition times (PC times: Spaeth et al., 2010). At each given PC time, the dye-free DPBS +/+ medium was replaced with DPBS +/+ medium containing 9 nM Dextran conjugated Texas Red (3 kDa, Molecular Probes, D3328). After 15 min, the DPBS +/+ containing Texas Red Dextran was washed out with dye-free DPBS +/+.

B104 cells were observed with a Zeiss Axio Vert.A1 microscope fitted with phase contrast and fluorescent optics, an excitation/emission filter of 595/615, a Zeiss AxioCam MRm camera and an eyepiece reticle (Klarmann Rulings, KR-407). Representative images of transected cells were acquired and their brightness and contrast were adjusted using Adobe Photoshop to enhance visibility. Images of the eyepiece reticle were also acquired using an iPhone 6S camera.

Individually-identified cells with axons that intersected a transection line or stopped near the transection line and aligned with an anuclear, distal axonal segment on the opposite side of the transection line were counted as transected. Cells transected more than once were not counted. The fluorescent state, a Yes/No value, of each uniquely-identified transected cell was recorded. The distance between the transection site and the axon hillock for each cell was recorded using the eyepiece reticle in 2.5 μm increments. We measured transection distance from the axon hillock because the rough endoplasmic reticulum in axons does not extend beyond this point, and other landmarks (e.g. nucleus) had varied locations in different cells. Curved axons or axons that extended outside the microscope field of view were measured in sequential segments, using membrane irregularities and cellular debris as markers. Typically, 60–120 cells could be counted on each plate in 30–40 min before photo-bleaching began to affect dye detection.

2.3. Analyses of sealing frequency versus transection distance

To determine sealing frequencies at any given PC time, cells were grouped by transection distance into different bins using various "binary" or "multinary" methods, and the frequency of cells that excluded Texas Red in each bin was calculated. For the binary groups, B104 cells were grouped into "transected <50 μm or ≥50 μm from the axon hillock. For the multinary groups, various methods (see Results) were used to generate a set of bin widths starting at ≥0 μm from the axon hillock. An outlier test was applied to both multinary and to binary data sets: Any cell culture plate with transected cells exhibiting a sealing frequency more than three standard deviations from the weighted mean for that given transection range and counting method was excluded from analysis. In total, 4 of 120 plates were excluded.

2.4. Statistical analyses

The relationship between transection distance (x axis) and sealing frequency (y axis) was characterized at each PC time by fitting a one-phase exponential model described by the equation $Y = Y_0 + Y_{max} * e^{-x/\lambda}$, where Y_0 is the Y-intercept, Y_{max} is the maximum sealing frequency for that PC curve, x is transection distance, and λ is the space constant (the distance required for the predicted sealing frequency to reach 63% of the plateau). Linear regressions were applied to the same data set as an alternative hypothesis. The F-test for regression and Akaike's Information Criterion (AIC) were used to compare the goodness of fit of different curves for exponential and linear models.

The effects of PC time on sealing frequency were identified by comparing data at each PC time in a pairwise fashion using two-tailed