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Ca²⁺/calmodulin-dependent protein kinase II and Dimethyl Sulfoxide affect the sealing frequencies of transected hippocampal neurons

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Abstract

Traumatic injury often results in axonal severance, initiating obligatory Wallerian degeneration of distal segments, whereas proximal segments often survive. Calcium ion (Ca²⁺) influx at severed proximal axonal ends activates pathways that can induce apoptosis. However, this same Ca²⁺-influx also activates multiple parallel pathways that seal the plasmalemma by inducing accumulation and fusion of vesicles at the lesion site that reduce Ca²⁺-influx and enhance survival. We examined whether various inhibitors of Ca²⁺/calmodulin-dependent protein kinases (CaMKs), and/or dimethyl sulfoxide (DMSO), a common solvent for biologically-active substances, affected the ability of a hippocampal-derived neuronal cell line (B104 cells) to seal membrane damage following axotomy. Axolemmal sealing frequencies were assessed at different transection distances from the axon hillock and at various times after Ca²⁺-influx (PC times) by observing whether transected cells took-up fluorescent dyes. Inhibition of CaMKII by tatCN21 and KN-93, but not inhibition of CaMKI and CaMKIV by STO-609, affected axonal sealing frequencies. That is, CaMKII is a component of previously reported parallel pathways that induce membrane sealing, whereas CaMKI and CaMKIV are not involved. The effects of these CaMKII inhibitors on plasmalemmal sealing depended on their mechanism of inhibition, transection distance, and PC time. DMSO at low concentrations (90 μ M – 28 mM or 0.00064% – 0.2% v/v) significantly increased membrane sealing frequencies at most PC times and transection distances, possibly by permeabilizing the plasmalemma to Ca²⁺. Inhibition of CaMKII, DMSO, PC time, and the transection distance significantly effect plasmalemmal sealing that is critical to somal survival in traumatic lesions.

Graphical Abstract

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Conflict of Interest Statement

All authors declare no competing interests.

Author Contributions

All authors had full access to all data collected in this study and take responsibility for the integrity of the data as well as the accuracy of the data analysis. All authors were also responsible for the critical review of this manuscript. Conceptualization and resource provision: ADP and SRBS. Methodology and data curation: ADP, SHM, and SRBS. Formal analysis, and visualization: ADP and SHM. Investigation and validation: ADP, EEK, PJD, SHM, SRBS, and ZSB. Writing of the original draft: ADP, GDB, PJD, SHM, SRBS, and ZSB. Project administration: ADP, GDB, SHM, and SRBS. Supervision and funding acquisition: GDB.

This paper examines substances that increase or decrease the probability that a B104 cell will seal (left) or will not seal (right) at various times after adding calcium, and at various transection distances (large arrowhead) from the axon hillock (delineated by small arrowheads).

Keywords

axotomy; axolemmal sealing; CaMKII; DMSO; membrane repair; RRID: CVCL_0154

Introduction

Traumatic injuries to the PNS and CNS, including blast injuries and concussions, often sever the axolemma of many neurons (He et al. 2012; Mortazzavi et al. 2015; Choe et al. 2016; Bittner et al. 2016). Traumatic plasmalemmal damage initiates a Ca^{2+} -influx that can lead to apoptosis or other mechanisms that result in the death of eukaryotic cells (Spaeth et al. 2010 and 2012a; Moe et al. 2015; Bittner et al. 2016), including nerve cells after dendritic or axonal transection (Ramón y Cajal 1928; Spaeth et al. 2012b). This same Ca^{2+} -influx also initiates plasmalemmal sealing by activating multiple parallel and redundant enzymatic pathways to form vesicles that travel to, fuse with, and seal the damaged membrane, thus reducing the Ca^{2+} -influx to levels measured in undamaged cells (Steinhardt et al. 1994; Krause et al. 1994; Bittner et al. 2016). The cellular/molecular mechanisms of plasmalemmal sealing are highly conserved, e.g., as reported for xenopus oocytes, drosophila embryos, invertebrate giant axons, mammalian epithelial cells, and mammalian PNS and CNS neurons (Bittner and Fishman 2000; Spaeth et al. 2010, 2012a,b; Zuzek et al. 2013; Moe et al. 2015; Bittner et al. 2016). Axons transected near their axon hillock seal more slowly at lower frequencies, and are associated with higher frequencies of cell death than those transected further from their axon hillock (Yoo et al. 2004; Nguyen et al. 2005; Spaeth et al. 2010, 2012a, b; McGill et al. 2016).

We examined at different times after adding Ca^{2+} (Post- Ca^{2+} influx time: PC time), whether the sealing of B104 cell axons, transected at different distances from the axon hillock, were affected by inhibitors of Ca^{2+} /calmodulin-dependent protein kinases (CaMK). Ca^{2+} /

calmodulin-dependent protein kinase II (CaMKII) is an extensively studied multifunctional enzyme that interacts with numerous proteins (Fink and Meyer 2002; Hudmon and Schulman 2002; Lisman et al. 2012; Takemoto-Kimura et al. 2017). These proteins include several Ca^{2+} -dependent proteins, such as synapsin (Czernik et al. 1987), syntaxin (Ohshima et al. 2002), and actin (Hoffman et al. 2013), that are associated with several sealing pathways (e.g., PKA, PLC), as previously reported (Spaeth et al. 2010, 2012a, b; Zuzek et al. 2013; Bittner et al. 2016).

We utilize three inhibitors (tatCN21, KN-93, STO-609) to alter the activity of CaM kinases and assess membrane sealing. TatCN21, a peptide fragment, is a specific inhibitor of the CaM-dependent and -independent ('autonomous') autophosphorylative activity of CaMKII (Buard et al. 2010; Vest et al. 2010; Pellicena and Schulman 2014). KN-93 also inhibits CaMKII but does not inhibit its autonomous activity (Sumi et al. 1991; Vest et al. 2010; Pellicena and Schulman, 2014). STO-609 directly inhibits Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK), a necessary activator of CaMKI and CaMKIV (Tokumitsu et al. 2002; Vest et al. 2010; Fujiwara et al. 2015; Takemoto-Kimura et al. 2017). We report that the inhibitors tatCN21 and KN-93, but not STO-609, affect plasmalemmal sealing. Furthermore, *with respect to saline controls*, tatCN21 inhibition of CaMKII promotes plasmalemmal sealing at early PC times (<5 mins), but suppresses sealing at later PC times (> 5 mins), KN-93 inhibition shows a similar effect to suppress sealing at later PC times, but does not increase sealing at earlier time points. These data show that CaMKII is a member of the membrane sealing pathway, but has multiple effects that are both dependent and independent of its autophosphorylative activity.

Dimethyl sulfoxide (DMSO) is commonly used as co-solvent to ensure the dissolution of hydrophobic reagents in saline (Kelava et al. 2011), such as the CaM kinase inhibitors employed in this study (e.g., KN-93, STO-609). We report that DMSO, at low concentrations of 90 μM (0.00064% v/v) and 28 mM (0.2% v/v), significantly increases plasmalemmal sealing frequencies at most transection distances from the soma, and at most PC times. Our results suggest that some (or all) of previously reported effects of some test substances (e.g., KN-93) dissolved in DMSO, may be due to DMSO rather than to the test substance. All of these variables that affect the rate and extent of plasmalemmal sealing (e.g., test substance, transection distance) can, in turn, affect the survival of a neuron with a transected axon and probably affect the survival of other cell types as well (Ramon y Cajal 1928; Yoo et al. 2004; Nguyen et al. 2005; Spaeth et al. 2010, 2012a, b; McGill et al. 2016).

Materials and Methods

Cell culture

B104 cells (Dr. Jane Bottenstein, San Diego, CA; ICLC Cat# ATL99008, RRID: CVCL_0154; Schubert et al. 1974), a rat hippocampal neuroblastoma line, typically extend two cytoplasmic processes with numerous axon-like properties (Bottenstein and Sato 1979; Spaeth et al. 2010). B104 cells were cultured at 37°C and 5% CO_2 in 75 cm^2 vented cap flasks (Celltreat, Pepperell, MA, US) with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F-12; HyClone, Logan, UT, US; or Corning, Manassas, VA, US) enriched with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT,

US; or Corning, Manassas, VA, US). This growth medium was refreshed every two days until cell confluency reached 80%. The cells were then subcultured in a new flask or plated at 5,000–15,000 cells cm^{-2} on cell culture dishes (60 $\times$ 15 mm; Corning, Manassas, VA, US) coated with poly-D-lysine (5 $\mu\text{g ml}^{-1}$; Sigma-Aldrich, St. Louis, MO, US). Plated cells were incubated in the growth medium for twenty-four hours and then differentiated in FBS-free DMEM/F-12 for another twenty-four hours. Stock B104 cells were subcultured at most 35 times to mitigate the consequences of senescence.

Axonal transection

Glass knives were made by pulling microcapillary tubes with an internal diameter of 1 mm (World Precision Instruments, Sarasota, FL, US) using a micropipette puller (Sutter Instrument Co., Novato, CA), and breaking their tips to produce a sharp edge (McGill et al. 2016). The glass knives were mounted on a micromanipulator (Narishige Instruments, Amityville, NY, US) attached to either a Zeiss ICM-405 or a Zeiss Axio Vert.A1 (Thornwood, NY, US) phase contrast microscope. Plates with differentiated B104 cells were rinsed twice, and then filled with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS $-/-$; Gibco, Gaithersburg, MD, US; or Hyclone, Logan, UT, US), with or without a test substance, and subsequently mounted on the microscope stage. Axons of B104 cells were transected by lowering the glass knife onto the plate and moving the stage parallel to the edge of the knife, etching an easily visualized "transection line" on the surface of the plate. Axons of 500–800 B104 cells were randomly transected in each plate within 7 minutes after adding DPBS $-/-$. Transecting axons in DPBS $-/-$ prevented Ca^{2+} -induced membrane sealing and allowed any test substances to enter the axoplasm via the transection site (Bittner et al. 2016; McGill et al. 2016).

To induce membrane sealing, Ca^{2+} was introduced to the cells (Krause et al. 1994; Detrait et al. 2000a, b; Spaeth et al. 2010) by replacing the DPBS $-/-$ medium (Ca^{2+} -free) with DPBS medium containing Ca^{2+} and Mg^{2+} (DPBS $+/+$; Gibco, Gaithersburg, MD, US; or Hyclone, Logan, UT, US), as previously described (Spaeth et al. 2010). DPBS $+/+$ initiated the post- Ca^{2+} addition time, or 'PC time' (Spaeth et al. 2010; McGill et al. 2016). At the end of a desired PC time of 2.5 min, 3.75 min, 5 min, 10 min, 15 min, or 20 min PC, DPBS $+/+$ was replaced with a DPBS $+/+$ solution containing 9 nM Dextran conjugated Texas Red (3 kDa; Molecular Probes, Eugene, OR, US) for 15 minutes. The dye-containing DPBS $+/+$ was washed out with dye-free DPBS $+/+$, and the plate was assessed for dye uptake by B104 cells.

Prior to performing any protocol using a test substance, a 'batch-control' experiment (5 min PC, no test substance) was always performed. This batch-control assessed the sealing frequencies for axons severed $<50 \mu\text{m}$ and $50 \mu\text{m}$ from the axon hillock to compare against established controls (McGill et al. 2016). If the batch control results did not fall within one standard deviation versus established controls, no additional experimental data were gathered from that batch. Batch control experiments that failed this test were still included for analysis with the appropriate control dataset.

Experimental test substances

All test substances were introduced into DPBS $-/-$ prior to axon transection. All cells were exposed to the test substance(s) in Ca^{2+} -free DPBS $-/-$ for 7 minutes, during which cells were transected, before adding Ca^{2+} -containing DPBS $+/+$ and no test substance(s). These test substances included tatCN21 (5 μM), tatSCR (5 μM), KN-93 (20 μM), KN-92 (20 μM) (EMD Millipore, Billerica, MA, US), STO-609 acetate (5 μM , Cayman Chemical, Ann Arbor, MI, US), and DMSO (90 μM and 28 mM, Fisher Scientific, Fair Lawn, NJ). To assist solvation, stock solutions of KN-93, KN-92 and STO-609 were dissolved in DMSO and diluted in DPBS $-/-$, resulting in a final DMSO concentration of 28 mM (0.2% v/v).

Assessing sealing frequencies and transection distances

Transected B104 cells (typically 40–200) were counted on each plate within 30 minutes after dye removal using a Zeiss Axio Vert.A1 phase contrast microscope equipped with an excitation/emission filter of 595/615 nm, and an eye piece reticle (Klarmann Rulings, Litchfield, NH, US). Representative images of cells were acquired with an iPhone 7 camera or Zeiss AxioCam MRm camera equipped with Zen software, and then had their brightness and contrast adjusted using Adobe Photoshop to enhance visibility. These individually identified B104 cells had an axon that intersected a transection line that often aligned with an anuclear, distal axonal segment on the opposite side of the transection line. Transected cells were counted as unsealed if they exhibited red fluorescence, and counted as sealed if they did not exhibit fluorescence. The distance between the transection line and the axon hillock (“transection distance”) was measured using the eye piece reticle. The distal end of the axon hillock was used as the starting point for these measurements as rough endoplasmic reticulum in axons does not extend beyond this point, and because other landmarks (e.g., nucleus) had varied locations in different cells. Cells transected more than once or cells that were transected through the soma, were not counted (McGill et al. 2016). A minimum of 300 cells from at least 3 plates were counted per experimental protocol, i.e., test substance(s) and PC time.

In order to identify outliers, the cells in each plate were sorted into two bins based upon their transection distance (<50 μm versus $\geq 50 \mu\text{m}$). The sealing frequency of both bins for each plate was identified, and then the weighted average sealing frequency of both bins across every plate in each condition was calculated. If a plate exhibited a bin that yielded a sealing frequency greater than three standard deviations away from the weighted average for the same condition, then that plate was excluded from all analyses. In total, 7 of the 306 plates tested failed this outlier test and were excluded.

Sealing frequencies at given PC times were calculated by placing data from each transected B104 cell into one of 12 different bins according to the axonal transection distance (McGill et al. 2016). These twelve bins starting from the axon hillock consisted of two bins each with a width of 5 μm , then five of 10 μm , two of 20 μm , two of 25 μm , and one bin containing all the remaining data of cells more distant from the axon hillock (>150 μm , see Figure 2e of McGill et al. 2016). In practice, no transection distances above 450 μm were measured. The sealing frequency for each bin was calculated as the percentage of transected B104 cells showing no fluorescence, i.e., no dye uptake, because the transected axon had sealed. These

data were plotted for a given PC time using GraphPad Prism 7 and fitted a one-phase exponential model to these plots, as described by Equation 1 (McGill et al. 2016, see Methods and corrigendum).

$$Y(x) = Y_{max} - (Y_{max} - Y_0)e^{-x/\lambda} \quad \text{Equation 1}$$

In Equation 1, x represents the transection distance, Y_0 is the sealing frequency at a transection distance of zero from the axon hillock, Y_{max} is the upper asymptote reached at an infinite transection distance, and λ represents the length constant, as described by Equation 2.

$$Y(\lambda) = (Y_{max} - Y_0)0.63 + Y_0 \quad \text{Equation 2}$$

Statistical comparisons

As previously determined (Detrait et al. 2000b; Yoo et al. 2004; Spaeth et al. 2010; Zuzek et al. 2013; McGill et al. 2016), the Cochran-Mantel-Haenszel Chi-Squared (CMH χ^2) test was the most suitable test of independence to compare sealing frequencies between an experimental treatment and a control, or between two experimental treatments. The CMH χ^2 test compiles multiple 2×2 χ^2 analyses that differ by a nominal replicate variable and determines significant differences between sealing frequencies. For this study, the independent and dependent variables were PC time and fluorescence (dye uptake), respectively. The replicate variable differentiating the comparisons was the transection distance. For plots of sealing frequencies versus PC times, the replicate variable was the average sealing frequency at each PC time.

Fisher's exact test, a χ^2 test for statistical significance, was used to analyze one specific case in which a sealing curve was significantly different than its control curve, but exhibited an inconsistent effect on sealing frequencies over all transection distances (Zuzek et al. 2013). In all cases, the Fisher exact test or the CMH χ^2 test featured only one degree of freedom. Of the twenty-eight different treatments, eleven were only experimental treatments and seven were only control treatments. The five 28 mM DMSO treatments and the five 90 μ M treatments were either control or experimental treatments, with their purpose determined by the specific comparison.

Results

B104 axons were transected for 7 minutes in Ca^{2+} -free saline, with or without a test substance and/or DMSO. The Ca^{2+} -free saline was then washed out and replaced with Ca^{2+} -containing saline, inducing a Ca^{2+} -influx at the unsealed open ends of transected axons that initiated axolemmal sealing in all cut cells simultaneously. This Ca^{2+} -influx initiated the post- Ca^{2+} (PC) time at 0 min PC (Spaeth et al. 2010). After a given PC time (e.g., 2.5 min PC, 15 min PC, etc.), Texas Red Dextran dye was added to the plate. Since Texas Red

Dextran is membrane impermeant, transected B104 cells that failed to seal after the PC time took up the dye; untransected cells and transected cells that successfully sealed, did not take up the dye. After a 15-minute exposure, the Texas Red Ca^{2+} -containing saline was washed out with dye-free Ca^{2+} -containing saline.

Transected B104 cells (Fig. 1a, d–g) were individually identified and assessed for the distance between the axonal transection point and the axon hillock (transection distance; Fig. 1a), as well for dye exclusion (sealed; Fig. 1e) or inclusion (not sealed; Fig. 1g).

Untransected B104 cells (Fig. 1b) were not counted and did not take up fluorescent dye (Fig. 1c). Previous studies have shown that dye inclusion of individually identified axons and cell bodies is stable for at least one hour (Spaeth et al. 2010; McGill et al. 2016). The transected B104 cells were sorted by transection distance into twelve consecutive bins (McGill et al. 2016), and the sealing frequency for each bin was plotted against the average transection distance for that bin. These sealing frequency versus transection distance plots (transection distance sealing curves) were fitted with a one-phase exponential model (Equation 1, see Methods; McGill et al. 2016, and corrigendum, 2017).

The bins of transection distance sealing curves were compared between conditions using the CMH χ^2 test for statistical significance (Figs. 2a–e, 3, 4a–e, 5, 6a–b; Table 1). Some figures (Figs. 2f, 4f, 6a–b, and 7) graph the average sealing frequencies for all transection distances at each given PC time (Zuzek et al. 2013; McGill et al. 2016). PC time sealing frequency curves for different protocols were also compared using the CMH χ^2 test (Supplemental Table 1–2; McGill et al. 2016).

TatCN21 inhibition of CaMKII affects plasmalemmal sealing frequency

TatCN21 is a cell-penetrating ‘tat’ peptide fused to the CN21 amino acid sequence that specifically inhibits CaM-dependent and autonomous autophosphorylative activity of CaMKII (Buard et al. 2010; Vest et al. 2010; Pellicena and Schulman, 2014). B104 cells were transected in Ca^{2+} -free saline containing 5 μM tatCN21. Figures 2a–b and Table 1 show that tatCN21 significantly increases axolemmal sealing frequencies at 2.5 and 3.75 min PC when compared against Ca^{2+} -free saline controls (CMH χ^2 $p < 0.001$). In contrast, tatCN21 significantly decreases membrane sealing frequencies at 5, 10, and 15 min PC, with its greatest effect occurring at 15 min PC (Fig. 2c–e, Table 1, CMH χ^2 $p < 0.001$).

To verify that the effects of tatCN21 on sealing frequencies were due to CaMKII inhibition rather than any non-specific effects, cells were similarly treated with Ca^{2+} -free saline containing tatSCR (5 μM). TatSCR is composed of a scrambled CN21 amino acid sequence fused to a ‘tat’ cell-penetrating peptide sequence and should not have any inherent effect on CaMKII activity (Vest et al. 2010). Figure 2e and Table 1 show that there are no significant differences (CMH χ^2 $p = 0.17$) in sealing frequencies at 15 min PC between tatSCR and the saline controls, and that tatSCR at 15 min PC produces significantly higher sealing frequencies (CMH χ^2 $p < 0.001$) compared to tatCN21. These data suggest that the effect of tatCN21 is primarily (perhaps entirely) due to its inhibition of CaMKII.

STO-609 inhibition of CaMKK does not affect plasmalemmal sealing

Since CaMKII influences membrane sealing, other members of the Ca^{2+} /calmodulin-dependent protein kinase (CaMK) family might also affect sealing. STO-609 is a membrane-permeable inhibitor of isoforms α and β of CaMKK (Tokumitsu et al. 2002; Fujiwara et al. 2015). CaMKK is necessary to activate CaMKI and CaMKIV (Takemoto-Kimura et al. 2017). To assess whether CaMKI and CaMKIV influence the sealing process, B104 cells were treated with STO-609 (5 μM) and examined for Ca^{2+} -induced membrane sealing at 15 min PC, the PC time at which Ca^{2+} -induced membrane sealing had its near-maximum effect (Figs. 2–5; Spaeth et al. 2010, 2012a, b; McGill et al. 2016). Note that STO-609 was dissolved in DMSO (28 mM).

Figure 3a and Table 1 show that there is no significant difference between the sealing frequencies of B104 cells treated with STO-609 dissolved in 28 mM DMSO versus cells treated with 28 mM DMSO alone at 15 min PC (Fig. 3a, Table 1, CMH $\chi^2 p = 0.36$). However, there is a significant effect on the sealing frequencies for 28 mM DMSO dissolved in Ca^{2+} -free saline when compared to the Ca^{2+} -free saline controls without DMSO at 15 min PC (Fig. 3f, Table 1, $p = 0.002$). These results suggest that inhibition of CaMKK does not influence membrane sealing, whereas DMSO seems to influence membrane sealing (see below).

28 mM DMSO significantly increases plasmalemmal sealing at most PC times

DMSO, at concentrations ranging from 90 μM (0.00064% v/v, Zuzek et al. 2013) to 11.3 M (80% v/v, Batista et al. 2014), is often used to dissolve hydrophobic test substances that are poorly soluble in water. However, DMSO can be cytotoxic or influence cellular processes, including membrane integrity (Howard et al. 1999, 70.4 – 704 mM or 0.5 – 5% v/v; de Ménorval et al. 2012, >1.41 M or >10% v/v; Galvao et al. 2014, 282 – 563 mM, 2 – 4%). We compared the sealing frequencies of B104 cells transected in Ca^{2+} -free saline containing 28 mM DMSO to cells transected in DMSO-free, Ca^{2+} -free saline controls at various PC times (Fig. 3b–e). Figure 3b–f shows that 28 mM DMSO significantly increases plasmalemmal sealing frequencies at 2.5 (CMH $\chi^2 p < 0.001$), 3.75 ($p < 0.001$), and 10 min PC ($p = 0.015$). At 15 min PC, there are significant differences between the 28 mM DMSO sealing curve and the saline control sealing curve ($p = 0.002$). However, the sign of the effect differs with transection distance. At 15 min PC, cells treated with 28 mM DMSO and transected <50 μm from their axon hillock sealed at significantly higher frequencies than saline controls ($p < 0.001$, Supplemental Table 3). In contrast, there was no significant difference between the sealing frequencies of cells treated with 28 mM DMSO and transected 50 μm from their axon hillock versus saline controls ($p = 0.49$, Supplemental Table 3). No significant effects of 28 mM DMSO on sealing frequencies were observed at 5 min PC (Fig. 3d, Table 1, CMH $\chi^2 p = 0.11$).

Effect of KN-93 inhibition of CaMKII on plasmalemmal sealing depends on PC time

KN-93 is a membrane-permeable competitive inhibitor of the CaM-dependent activity of CaMKII, but unlike tatCN21, does not inhibit its autonomous autophosphorylative activity (Sumi et al. 1991; Vest et al. 2010; Pellicena and Schulman, 2014). Additionally, KN-93 is known to inhibit CaMKI and CaMKIV (Takemura et al. 2009; Pellicena and Schulman,

2014), as well as both voltage gated K^+ (Rezazadeh et al. 2006) and voltage-gated Ca^{2+} channels (Gao et al. 2006; Vest et al. 2010). To assess the importance of CaMKII autonomous autophosphorylative activity for membrane sealing, we transected B104 cells in the presence of Ca^{2+} -free salines containing 20 μ M KN-93 dissolved in 28 mM DMSO at different PC times. We compared KN-93 sealing frequencies against cells transected in Ca^{2+} -free salines with 28 mM DMSO alone.

Figure 4 shows sealing frequencies of B104 cells treated with salines containing 20 μ M KN-93 dissolved in 28 mM DMSO versus those treated with salines containing only 28 mM DMSO at various PC times. Cells treated with KN-93 dissolved in DMSO show no significant difference in sealing frequencies at shorter PC times, i.e., 2.5 or 3.75 min PC, compared to cells treated with DMSO in saline alone (Fig. 4a–b, Table 1, CMH χ^2 p = 0.15 and 0.43 respectively). However, cells treated with KN-93 in DMSO at 5, 10, and 15 min PC show significantly lower sealing frequencies than cells treated with 28 mM DMSO (Fig. 4c–e, Table 1, CMH χ^2 p < 0.001).

To verify that some of the effects of **KN-93 in 28 mM DMSO** on sealing frequencies were due to KN-93 inhibition of the pathways outlined above rather than some non-specific effect, cells were treated with Ca^{2+} -free salines containing 20 μ M **KN-92** dissolved in 28 mM DMSO. KN-92 is a negative analog of KN-93 that does not inhibit CaMKII, but does inhibit voltage gated K^+ channels (Rezazadeh et al. 2006) and voltage-gated Ca^{2+} channels (Gao et al. 2006). Figure 4e shows that cells treated with **KN-92** in DMSO sealed at the same frequencies as did cells treated with DMSO (CMH χ^2 p = 0.85) and at significantly higher frequencies compared to cells treated with **KN-93** and DMSO (Fig. 4e, Table 1; CMH χ^2 p < 0.001). These data suggest that the inhibitory effects of KN-93 on CaMKII are responsible for at least some (and perhaps all) of the effects of KN-93 on sealing frequencies.

Effects of increasing transection distances and PC times on sealing frequencies

To better illustrate effects of PC time and transection distance on sealing frequencies, we plotted the sealing curves for each treatment (at 2.5, 3.75, 5, 10, and 15 min PC) on a single graph and compared the curves for each treatment to each other in a pairwise manner via CMH χ^2 (Fig. 5; Supplemental Tables 4–8).

Pairwise comparisons for cells treated with saline (Fig. 5a) show that for eight of the ten possible combinations, the cells assessed at the longer PC time have significantly greater sealing frequencies than cells assessed at the shorter PC time (p < 0.001, Supplemental Table 4). In the other two comparisons, no significant difference was found. In contrast, similar pairwise comparisons of cells treated with tatCN21 in saline (Fig. 5b) show **no** significant differences between any of the possible combinations (p = 0.053 in all cases, Supplemental Table 5).

The pairwise comparisons for 28 mM DMSO curves (Fig. 5c) reveal that four of the ten possible comparisons have significant differences (p < 0.05 in three cases, p < 0.01 in one, Supplemental Table 6). In all four circumstances, the higher PC time is associated with a higher sealing frequency. For pairwise comparisons of curves for saline containing KN-93 dissolved in 28 mM DMSO (Fig. 5d), four of ten possible comparisons yield significant

differences ($p < 0.001$ in three cases, $p < 0.05$ in one, Supplemental Table 7). All four of these comparisons that are significantly different compare 15 min PC with another PC time. For KN-93, the shorter PC time is associated with a significantly higher sealing frequency. A more detailed analysis of these curves shows that shorter PC times are associated with a higher sealing frequency at shorter transection distances, but not at the longest transection distances. We do not know why shorter PC times should be associated with higher sealing frequencies for curves of KN-93 dissolved in DMSO. We also note that both KN-93 and DMSO curves reach their maximum plateau sealing frequency ($Y_{\max}$) at short transection distances and currently have no explanation for this very-repeatable effect.

Examining the coefficients of the exponential model (Supplemental Table 9), we find that the $Y_{\max}$ generally increases with increased PC times from 3.75 to 10 min PC for control salines and salines containing DMSO. Other measures, such as λ (length constant) and Y_0 (sealing frequency at a zero-transection distance), show no correlation with PC time.

Effect of PC time on sealing frequencies at all transection distances for different test substances

To explore the effects of tatCN21, DMSO, and KN-93 on the rate of membrane sealing, we plotted the average sealing frequency of all transected B104 cells under each condition against their PC times. Figure 2f and Supplemental Table 1 compare the sealing frequencies of cells treated with 5 μ M tatCN21 in saline to cells treated with saline alone and show that the PC time sealing frequency curve for cells treated with tatCN21 is significantly different (CMH χ^2 , $p < 0.001$, Supplemental Table 1) from the saline control curve. Cells treated with tatCN21 reached maximum plateau sealing frequencies more rapidly than saline control cells, and exhibited a lower plateau sealing frequency (Supplemental Table 10).

Figure 4f compares the average sealing frequencies of cells treated with 20 μ M KN-93 dissolved in 28 mM DMSO and saline, 28 mM DMSO only in saline, or saline alone at different PC times. There was a significant difference between the PC time versus sealing frequency curves for cells treated with 28 mM DMSO in saline versus cells treated with saline alone (Supplemental Table 1, CMH χ^2 , $p < 0.001$). Cells treated with saline and cells treated with 28 mM DMSO have similar maximum plateaus, but cells treated with 28 mM DMSO reach their maximum plateaus at shorter PC times (Supplemental Table 10). Figure 4 and Supplemental Table 1 also show that there is a significant difference between the sealing frequency curves of cells treated with 20 μ M KN-93 dissolved in 28 mM DMSO and saline, and the curves for cells treated with 28 mM DMSO in saline (Supplemental Table 1, CMH χ^2 $p < 0.001$). Notably, the one-phase exponential model did not fit the KN-93 data. The average sealing frequency for cells treated with KN-93 remained consistent across all PC times, suggesting that the cells reach their plateau sealing frequency in less than 2.5 min, possibly even 1.25 min, after Ca^{2+} addition.

Effect of DMSO concentration on sealing frequencies

Our laboratory has previously published a few experiments that used DMSO in combination with other substances that affected B104 sealing frequencies. Zuzek et al. (2013) reported that 90 μ M DMSO had no significant effect on the average sealing frequencies of B104 cells

transected $>50\ \mu\text{m}$ from their axon hillock. To verify these results and to examine the effects of $90\ \mu\text{M}$ DMSO at all transection distances, we treated B104 cells with $90\ \mu\text{M}$ DMSO in Ca^{2+} -free saline and plotted the average sealing frequency of all cells transected $>50\ \mu\text{m}$ from their axon hillock at each PC-time. Figure 6a compares our current data against those reported by Zuzek et al. (2013), and shows no significant difference versus saline controls (Table 3, CMH $\chi^2\ p = 0.11$). However, Figure 6b shows that in our current data, $90\ \mu\text{M}$ DMSO significantly increases the sealing frequency of B104 cells transected $<50\ \mu\text{m}$ from their axon hillock versus saline controls (CMH $\chi^2\ p < 0.001$, Supplemental Table 2), a protocol not examined by Zuzek et al. (2013).

To better assess the effects of transection distance on sealing frequency of B104 cells treated with $90\ \mu\text{M}$ DMSO across all PC times, we plotted the sealing curves of all PC times onto a single graph (Fig. 6c). Pairwise comparisons of all curves show that 7 of the 10 pairs are significantly different (CMH $\chi^2\ p < 0.05$ in three cases, $p < 0.001$ in four, Supplemental Table 8). In each of these 7 pairs, the curve at the higher PC time was associated with a higher sealing frequency. The sealing curves of 2.5, 3.75, and 5 min PC have similar maximum plateau values (Supplemental Table 9). We also measured sealing frequencies at 1.25 min PC but found that these data have the greatest variability in sealing frequency between plates because a solution change requires 5–10 seconds to complete. Hence, the exact PC starting time cannot be precisely quantified and this uncertainty has a much greater effect on sealing frequency for shorter PC times than for longer PC times.

Considering the results of cells treated with $90\ \mu\text{M}$ DMSO, as well as those treated with 28 mM DMSO, we examined possible dose-dependent effects of DMSO on B104 sealing frequencies. We calculated the sealing frequencies of cells treated with 28 mM or $90\ \mu\text{M}$ DMSO at transection distances $<50\ \mu\text{m}$ or $>50\ \mu\text{m}$ from the axon hillock for each PC time, and plotted the PC time sealing frequency curves. B104 cells treated with $90\ \mu\text{M}$ DMSO do not have significantly different sealing frequencies compared to cells treated with 28 mM DMSO at $<50\ \mu\text{m}$ (Fig. 7a, Supplemental Table 2, CMH $\chi^2\ p = 0.68$), or $>50\ \mu\text{m}$ (Fig. 7b, Supplemental Table 2, CMH $\chi^2\ p = 0.37$) from their axon hillock.

Discussion

In this paper, we examined the effects of three CaM kinase inhibitors (tatCN21, KN-93, and STO-609) on plasmalemmal sealing of transected B104 cell axons. Our protocol includes the addition of these inhibitors before transecting axons, the removal of these inhibitors just prior to adding Ca^{2+} to initiate the sealing process, and the examination of sealing frequencies at various PC times and transection distances. We also examined the effect of DMSO on plasmalemmal sealing. We mitigated bias by having different researchers conduct the same experiments, combining the data prior to analysis, and testing to be certain no specific researcher consistently produced data with a given bias.

CaMKII and plasmalemmal sealing

Ca^{2+} -influx at sites of plasmalemmal trauma activates Ca^{2+} -sensitive proteins, including those in pathways that initiate membrane sealing, causing vesicles to migrate to the wound and form a vesicular plug (Bittner et al. 2016) or wound vesicle (Moe et al. 2015). This

Ca^{2+} -influx activates calmodulin (CaM) to form a Ca^{2+} /calmodulin substrate, which regulates a set of enzymes including the Ca^{2+} /calmodulin-dependent protein kinase family, namely CaMKII (Takemoto-Kimura et al. 2017). When activated, CaMKII enters an autophosphorylative state that persists after CaM dissociation, leading to CaM-independent ('autonomous') autophosphorylative activity (Lisman et al. 2012; Takemoto-Kimura et al. 2017).

CaMKII affects numerous targets (Fink and Meyer 2002; Hudmon and Schulman 2002) involved in vesicle interactions and membrane sealing (Steinhardt et al. 1994; Detrait et al. 2000a, b; Yoo et al. 2004; Spaeth et al. 2010, 2012a, b; Zuzek et al. 2013; Bittner et al. 2016). As one example, Synapsin I, a synaptic vesicle associated phosphoprotein that is phosphorylated on its domain D by CaMKII (Czernik et al. 1987), is involved in synaptic vesicle release and is implicated in membrane sealing (Steinhardt et al. 1994; Spaeth et al. 2010). As another example, Syntaxin I, which can bind to and be phosphorylated by CaMKII (Ohyama et al. 2002), is necessary for membrane sealing (Detrait et al. 2000a; Yoo et al. 2004). Both syntaxin and synapsin are substrates in PKA-dependent sealing pathways (Spaeth et al. 2010; Bittner et al. 2016). Moreover, CaMKII binds to F-actin while sequestering G-actin in the inactive state, but releases G-actin following CaM binding, leading to F-actin polymerization (Hoffman et al. 2013). F-actin has been implicated in membrane sealing in *Xenopus* oocytes (Mandato and Bement 2001) and *Drosophila* embryos (Abreu-Blanco et al. 2012). Other targets of CaMKII might also affect plasmalemmal sealing, such as microtubule-associated protein Tau (Yoshimura et al. 2003; Zhou et al. 2017), synaptotagmin (Hilfiker et al. 1999; Detrait et al. 2000a, b; Yoo et al. 2004) and synaptophysin (Rubenstein et al. 1993). CaMKII influences membrane sealing in fertilized and activated sea urchin embryos and 3T3 fibroblasts, but not in unfertilized eggs (Steinhardt et al. 1994).

Inhibition of CaM kinases

We report that the effects of inhibitors of CaMKII on plasmalemmal sealing depend on their mechanism of inhibition, PC time, and transection distance. TatCN21, which inhibits both CaMKII activation and autonomous autophosphorylative activity via T-site and kinase binding (Buard et al. 2010; Vest et al. 2010; Pellicena and Schulman 2014), increases sealing frequencies at shorter PC times (Fig. 2a–b, Table 1), but decreases sealing frequencies at longer PC times (Fig. 2c–e, Table 1), when compared to saline controls (Fig. 2a–e, Table 1) and tatSCR (negative analog) controls (Fig. 2e, Table 1). When tatCN21 sealing curves at different PC times are compared relative to each other (not to saline controls) against transection distance, no significant differences are found (Fig. 5b, Supplemental Table 5). These data suggest that B104 cells treated with tatCN21 reach a plateau more rapidly than we can confidently measure (Fig. 2f).

Unlike tatCN21, KN-93 inhibits CaMKII by competitively binding with its calmodulin binding site, leaving the T-site undisturbed, but does not inhibit the autonomous autophosphorylative activity of CaMKII (Sumi et al. 1991; Vest et al. 2010; Pellicena and Schulman 2014). We report that KN-93 inhibition of CaMKII significantly decreases sealing

frequencies at 5 min PC (Fig. 4a–e, Table 1) compared to DMSO controls (Fig. 4a–e, Table 1) and KN-92 (negative analog) controls (Fig. 4e, Table 1).

KN-93 is not as specific as tatCN21 in its effects. For example, KN-93 also inhibits CaMKI, CaMKIV (Takemura et al. 2009), voltage-gated Ca^{2+} channels (Gao et al. 2006) and voltage-gated K^{+} channels (Rezazadeh et al. 2006). STO-609 inhibition of CaMKK, and subsequently, CaMKI and CaMKIV (Tokumitsu et al. 2002; Vest et al. 2010; Takemoto-Kimura et al. 2017), shows no significant differences against DMSO controls (Fig. 3a, Table 1). These data suggest that the effects of KN-93 on sealing frequencies are probably not due to its inhibitory effects on targets other than CaMKII.

Both tatCN21 and KN-93 alter B104 sealing frequencies, but tatSCR, KN-92, and STO-609 do not alter sealing frequencies. Given the actions of each of these substances described above, all of these data suggest that CaMKII is in one or more sealing pathways, but that CaMKI and CaMKIV are not in any sealing pathways. Additionally, both tatCN21 and KN-93 show decreased sealing frequencies at longer PC times, relative to their respective controls. Yet, there is a very clear difference between the effects of tatCN21 and KN-93. Considering that only tatCN21 inhibits CaMKII autophosphorylation activity, these data suggest that tatCN21 inhibition of the autonomous state of CaMKII is responsible for the increase in sealing frequencies at early PC times, relative to respective controls. The differences between the effects of tatCN21 and KN-93 show that the role of CaMKII is multi-faceted, and that this protein likely performs multiple roles in the sealing process.

Low concentrations of DMSO usually enhance sealing frequency, possibly by increasing Ca^{2+} -influx

DMSO is widely used as a co-solvent to enhance solvation of hydrophobic substances in salines (Kelava et al. 2011). However, DMSO is not biologically inert and exhibits multiple effects on cell membranes and proteins. For example, Ca^{2+} -influx can be induced by DMSO concentrations of 282 – 563 mM (2 – 4% v/v, Galvao et al. 2014). At higher concentrations, ~10 mol % (4.3 M or 30.6% v/v), DMSO promotes the formation of water-filled membrane pores (de Ménorval et al. 2012). DMSO has also been shown to disrupt many proteins such as cation channels (0.1 – 2 M or 0.71 – 14.2% v/v, Nardid et al. 2013), cytoskeletal proteins (1.4 M or 10% v/v, Sanger et al. 1980), and albumins (2.82 M or 20% v/v, Batista et al. 2014). At yet-higher concentrations (5 M or 35% v/v), DMSO can act as a cell-cell fusogen (Akhong et al. 1975). DMSO enhances membrane sealing rates in guinea pig spinal cord axons at 704 mM (5% v/v, Shi et al. 2001) and sealing frequencies in rat dorsal root axons at 70.4 – 704 mM (0.5 – 5% v/v, Howard et al. 1999) in low extracellular $[\text{Ca}^{2+}]$ (200 μM ; physiological $[\text{Ca}^{2+}] = \sim 1 - 2 \text{ mM}$, Dulbecco and Vogt 1954; Howard et al. 1999), but not in zero extracellular $[\text{Ca}^{2+}]$.

Our data show that DMSO has effects on cellular processes at much lower concentrations than those previously reported. B104 cells treated with 28 mM (0.2% v/v) DMSO usually exhibit increased sealing frequencies and rates compared to DMSO-free saline controls (Fig. 3b–e, Table 1). Furthermore, cells treated with 90 μM (0.00064% v/v) DMSO and transected <50 μm from their axon hillock seal at higher frequencies than cells in DMSO-free saline (Fig. 6b, Supplemental Table 2). However, at 90 μM (0.00064% v/v), DMSO has no

significant effect on sealing of B104 axons transected $>50\ \mu\text{m}$ from their hillock (Zuzek et al. 2013), a result we confirm (Fig. 6a, Supplemental Table 2).

Following axotomy, Ca^{2+} -influx occurs primarily at the site of the injury to activate cell sealing pathways (Krause et al. 1994; Bittner and Fishman 2000). We hypothesize that exposure to DMSO during or after axotomy permeabilizes the membrane to Ca^{2+} along the entire length of the cell, thereby inducing more vesicles to form and migrate to the lesion site. This effect of DMSO should have a greater impact on sealing frequencies for transections nearer to the soma compared to further from the soma, a result consistent with our data. The hypothesis that DMSO permeabilizes the plasmalemma to Ca^{2+} is further supported by studies observing changes in cytoplasmic $[\text{Ca}^{2+}]$ using Ca^{2+} sensitive probes on other cell lines (14.1 mM – 563 mM or 0.1% – 4% v/v, He et al. 2012; $>1.41\ \text{M}$ or $>10\%$ v/v, de Ménorval et al. 2012). Alternatively, DMSO could decouple actin from spectrin embedded in the plasma membrane (Sanger et al. 1980; Howard et al. 1999) to decrease membrane tension, a phenomenon that usually precedes sealing (Togo et al. 2000).

Conclusion

Our results show that B104 cells serve as an effective model for examining sealing of axolemmal transections, and confirm that the effects of a particular substance (e.g., tatCN21, KN-93, DMSO) on sealing frequency often depend on transection distance and PC time (McGill et al. 2016). Furthermore, the effects of a particular test substance on sealing frequency depend on its mode of action, e.g., the inhibitions of CaMKII by tatCN21 versus KN-93. Our data also show that DMSO, at low concentrations, has significant effects on sealing frequency that need be considered and controlled for when DMSO is used as a solvent for test substances. These data further elucidate the role of CaMKII in sealing plasmalemmal damage, a process that is essential to repairing traumatic injury to the nervous system that commonly occurs in civilian accidents and military combat. Finally, our data herein, and other publications, consistently show that neurons with axonal transections near the axon hillock seal more slowly, with lower frequencies, and often have a higher frequency of cell death, compared to those with transections further from the axon hillock (Yoo et al. 2004; Nguyen et al. 2005; Spaeth et al. 2010, 2012a, b; McGill et al. 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Significance

Traumatic injuries to the PNS, spinal cord, and brain (including concussions) commonly produce axolemmal damage. Damaged axons undergo a Ca^{2+} -influx that initiates membrane sealing, and can prevent death of the perikaryon by Ca^{2+} -induced apoptosis. We report that CaMKII, but not CaMKI and CaMKIV, participates in several steps of the sealing process. We also report that DMSO predominantly increases sealing frequencies, likely by permeabilizing the membrane to Ca^{2+} . Increases or decreases in sealing probabilities by substances, like DMSO or inhibitors of CaMKII, increase or decrease the probability of cell survival after traumatic injury.

Figure 1. Phase contrast and fluorescent images of B104 cells

Images of a typical bipolar B104 cells under phase contrast (**a, b, d, f**) and fluorescence (**c, e, g**). B104 cells that were transected at a neurite (**a, d–g**) were measured for transection distance (**a**), or the distance between the transection site (arrow) and axon hillock (arrowheads). Transected B104 cells that sealed excluded dye (**e**), while cells that did not seal included fluorescent dye (**g**). Asterisks mark transection lines.

Figure 2. B104 cell sealing frequencies vs transection distance or PC times for Saline control, tatCN21 in saline, or tatSCR in saline

Sealing frequency (%) vs transection distance (μm) (a–e) for Saline (control; a, $n = 843$, $N = 10$; b, $n = 790$, $N = 10$; c, $n = 4,518$, $N = 78$; d, $n = 2,947$, $N = 10$; e, $n = 876$, $N = 13$), saline containing 5 μM tatCN21 (a, $n = 839$, $N = 7$; b, $n = 708$, $N = 5$; c, $n = 667$, $N = 7$; d, $n = 568$, $N = 5$; e, $n = 762$, $N = 8$), or saline containing 5 μM tatSCR (e, $n = 661$, $N = 7$), at 2.5 (a), 3.75 (b), 5 (c), 10 (d), or 15 min PC (e). Sealing frequency (%) vs PC time (min) (f) for solutions consisting of Saline (control, $n = 9,974$, $N = 152$), and tatCN21 dissolved in saline ($n = 3,544$, $N = 32$). Sample size (n) is represented in the number of transected B104 cells per condition. Replicate size (N) represents the number of plates of B104 cells treated under each condition. Horizontal bars show transection distance boundaries for each bin. Hollow circles (saline), filled squares (tatCN21), and X's (tatSCR) plot average sealing frequency and transection distance for each bin (a–e) or average sealing frequency for each PC time (f). Dashed (saline), solid (tatCN21), and dotted (tatSCR) lines show best-fit one-phase

exponential curves (a–f). For Figures 2–7, sealing frequency is represented in %, transection distance is represented in μm , and asterisks (*) show statistical significance according to CMH χ^2 : * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ for each bridged comparison. A bridged comparison with no asterisk is not statistically significant ($p > 0.05$).

Figure 3. Sealing frequencies vs transection distances for 28 mM DMSO, STO-609 in 28 mM DMSO, or Saline (control)

28 mM DMSO in saline (**a**, $n = 427$, $N = 6$; **b**, $n = 637$, $N = 7$; **c**, $n = 496$, $N = 6$; **d**, $n = 472$, $N = 7$; **e**, $n = 508$, $N = 8$; **f**, $n = 427$, $N = 6$), STO-609 in saline and 28 mM DMSO (**a**, $n = 452$, $N = 7$), or Saline (control) (**b**, $n = 843$, $N = 10$; **c**, $n = 790$, $N = 10$; **d**, $n = 4,518$, $N = 78$; **e**, $n = 2,947$, $N = 41$; **f**, $n = 876$, $N = 13$), at 2.5 (**b**), 3.75 (**c**), 5 (**d**), 10 (**e**), or 15 min PC (**a**, **f**). Sample size (n) is represented in the number of transected B104 cells per condition.

Replicate size (N) represents the number of plates of B104 cells treated under each condition. Horizontal bars show the boundary range for each transection distance bin. Solid triangles (STO-609), hollow squares (DMSO), and hollow circles (saline) plot average sealing frequency vs transection distance for each bin (**a–f**). The dashed line (**a**) or solid lines (**b–f**) show the best-fit one-phase exponential curves for 28 mM DMSO. The dashed lines (**b–f**) show the best-fit one-phase exponential curves for saline controls.

Figure 4. Sealing frequencies vs transection distances or PC times for KN-93, 28 mM DMSO, or KN-92, as stated in key of each panel

Sealing frequency vs transection distance (a–e) or vs PC time (f) for KN-93 (a, n = 758, N = 7; b, n = 591, N = 6; c, n = 452, N = 6; d, n = 598, N = 7; e, n = 691, N = 8; f, n = 3,638, N = 34), 28 mM DMSO (a, n = 637, N = 7; b, n = 496, N = 6; c, n = 472, N = 7; d, n = 508, N = 8; e, n = 427, N = 6; f, n = 3,003, N = 34), KN-92 (e, n = 552, N = 8), at 2.5 (a), 3.75 (b), 5 (c), 10 (d), or 15 min PC (e). Sample size (n) is represented in the number of transected B104 cells per condition. Horizontal bars show transection distance boundary for each bin. Replicate size (N) represents the number of plates of B104 cells treated under each condition. Filled circles (KN-93), hollow squares (DMSO), small dots (KN-92), or hollow circles (Saline) show average sealing frequency and transection distance for each bin (a–e) or average sealing frequency for each PC time (f). Solid lines (KN-93), dashed lines (DMSO), or dotted lines for KN-92 (e) or Saline (f) show best-fit one-phase exponential curves.

Figure 5. Sealing frequencies vs transection distances Saline (control), tatCN21, 28 mM DMSO, or KN-93 in 28 mM DMSO, as indicated as key to each panel

Vertical dashed lines represent a transection distance of 50 μm . Hollow triangles show the λ for each curve. The number associated with each line gives the PC time (min) for that curve.

Figure 6. Sealing frequencies vs PC times for cells transected in Saline or 90 μM DMSO >50 μm or <50 μm from their axon hillock

Cells transected >50 μm from their axon hillock (a) in 90 μM DMSO as reported herein ($n = 504$, $N = 20$) vs as reported by Zuzek et al, 2013 ($n = 513$, $N = 17$), and cells transected <50 μm from their axon hillock (b) in 90 μM DMSO ($n = 1,245$, $N = 20$) vs in Saline (control, $n = 6,056$, $N = 152$). Sample size (n) is represented in the number of transected B104 cells per condition. Replicate size (N) represents the number of plates of B104 cells treated under each condition. Sealing frequencies vs transection distances for cells transected in 90 μM DMSO at different PC times (c). Filled squares (Current 90 μM DMSO >50 μm), hollow circles (Zuzek 90 μM DMSO >50 μm), filled upward triangles (Current 90 μM DMSO <50 μm), and hollow squares (Current saline <50 μm) show average sealing frequency at each PC time. Solid lines show best-fit one-phase exponential curves for Current 90 μM DMSO >50 μm (a), Current saline <50 μm (b), and all 90 μM DMSO data (c). Dashed lines show best-fit one-phase exponential curves for Zuzek 90 μM DMSO >50 μm (a), and Current 90 μM DMSO <50 μm (b). Vertical dashed line marks 50 μm transection distance from axon hillock (c).

Figure 7. Sealing frequencies vs PC times for 28 mM DMSO vs 90 μM DMSO at <50 μm or >50 μm transection distances

Cells transected <50 μm from their axon hillock (**a**) in 28 mM DMSO ($n = 2,075$) vs 90 μM DMSO ($n = 1,245$), or for cells transected >50 μm from their axon hillock (**b**) in 28 mM DMSO ($n = 852$, $N = 34$) vs 90 μM DMSO ($n = 504$, $N = 20$). Sample size (n) is represented in the number of transected B104 cells per condition. Replicate size (N) represents the number of plates of B104 cells treated under each condition. Solid symbols show the average sealing frequency at each PC time, and solid lines show best-fit one-phase exponential curves for saline containing 28 mM DMSO. Hollow symbols show the average sealing frequency at each PC time, and dashed lines show the best-fit one-phase exponential curves for saline containing 90 μM DMSO.

Table 1

CMH χ^2 *p*-values for comparisons of conditions on B104 cell sealing frequency.

Comparison	2.5 min PC	3.75 min PC	5 min PC	10 min PC	15 min PC
tatCN21 vs Saline	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
DMSO vs Saline	< 0.001	< 0.001	0.109	0.015	0.002
KN-93 vs DMSO	0.153	0.427	< 0.001	< 0.001	< 0.001
tatCN21 vs tatSCR					< 0.001
tatSCR vs Saline					0.168
STO-609 vs DMSO					0.361
KN-93 vs KN-92					< 0.001
KN-92 vs DMSO					0.846

Bolded data represents statistical significance ($p < 0.05$).