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Diacylglycerol, novel protein kinase C isozymes η and θ, and other diacylglycerol activated proteins promote neuroprotective plasmalemmal sealing in B104 neurons *in vitro* and rat sciatic nerve axons *in vivo*

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Diacylglycerol, novel protein kinase C isozymes η and θ, and other diacylglycerol activated proteins promote neuroprotective plasmalemmal sealing in B104 neurons *in vitro* and rat sciatic nerve axons *in vivo*

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Dedication

To my wife, Michelle Helene Zuzek, thank you for all of the support and patience over the years, it was greatly appreciated.

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Diacylglycerol, novel protein kinase C isozymes η and θ, and other diacylglycerol activated proteins promote neuroprotective plasmalemmal sealing in B104 neurons *in vitro* and rat sciatic nerve axons *in vivo*

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To survive, neurons and other eukaryotic cells must rapidly repair (seal) plasmalemmal damage. Such repair occurs by an accumulation of intracellular vesicles at or near the plasmalemmal disruption. Diacylglycerol (DAG)-dependent and cAMP-dependent proteins are involved in many vesicle trafficking pathways. Although recent studies have implicated the signaling molecule cAMP in sealing, no study has investigated how DAG and DAG-dependent proteins affect sealing and, whether pharmacological inhibition of such proteins could promote immediate repair of damaged mammalian axons. To this end, I investigated the role of DAG, protein kinase C (PKC) and other DAG-activated proteins in plasmalemmal sealing in B104 neurons *in vitro* and rat sciatic nerves *in vivo*. Using dye exclusion to assess Ca^{2+}-dependent vesicle-mediated sealing of transected neurites of individually identifiable rat hippocampal B104 cells, I now report that, compared to non-treated controls, sealing probabilities and rates are increased by DAG and cAMP analogs that activate PKC and Munc13-1, and protein

kinase A (PKA). Sealing is decreased by inhibiting DAG-activated novel protein kinase C isozymes η (nPKCη) and θ (nPKCθ) and, Munc13-1, the PKC effector myristoylated alanine rich PKC substrate (MARCKS) or phospholipase C (PLC). DAG-increased sealing is prevented by inhibiting MARCKS or PKA. Sealing probability is further decreased by simultaneously inhibiting nPKCη, nPKCθ and PKA. Extracellular Ca^{2+}, DAG or cAMP analogs do not affect this decrease in sealing. I also report that applying inhibitors of nPKC and PKA to rat sciatic axons crush-severed *in vivo* under physiological calcium, do not promote immediate repair by polyethylene glycol (PEG), as assessed by compound action potential conduction and dye diffusion through crush sites. These and other data suggest that DAG increases sealing through MARCKS and that nPKCη, nPKCθ and PKA are all required to seal plasmalemmal damage in B104 neurons, and likely all eukaryotic cells.

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Chapter 1: General Introduction

IMPORTANCE OF PLASMALEMMAL SEALING

Cells that do not repair (seal) plasma membrane (plasmalemmal) damage, do not survive (McNeil, 1993, 2000; Nguyen et al., 2005). For victims of neurotrauma, such as peripheral nerve crush injury (PNCI) or stroke, loss of nerve cells (neurons) directly correlates with loss of functional behaviors and even paralysis. Furthermore, in mammals, regaining lost functional behaviors is dependent upon axonal regeneration; which occurs from a *surviving* proximal axonal end that slowly grows neuritic processes at 1-2mm/day that often do not re-establish original synapses (Britt et al., 2010). Thus, after PNCI or stroke, plasmalemmal sealing is a critical first step for neurons (Krause et al., 1994; Steinhardt et al., 1994; Bittner and Fishman, 2000; Detrait et al., 2000; Yoo et al., 2003; Spaeth et al., 2010), because only surviving neurons are capable of axonal regeneration, which correlates with increased functional behavior recovery (Britt et al., 2010; Bittner et al., 2012).

Thus, research that provides fundamental insights into the cellular mechanisms and pathways that normally promote sealing in eukaryotic cells should have direct and indirect implications on axonal repair and regeneration after traumatic central or peripheral nerve injury and, for treatment of stroke in which plasmalemmas of neurons are damaged.

PLASMALEMMAL SEALING

Vesicle-mediated repair theory states that sealing of plasmalemmal damage is a Ca^{2+}-induced, vesicle-mediated process that drives accumulation of vesicles at sites of damage (either small holes or complete transections) to produce a seal that restores ionic permeabilities to pre-lesion levels within 10-20 minutes (**Fig. 1**; Spaeth et al., 2010). However, little is known of the cellular mechanisms and pathways of sealing. Prior to 1994, textbooks (Kandel, *et al*., 1990) and research publications (Spira *et al*., 1993) assumed that minor plasmalemmal damage was sealed by the spreading of plasmalemmal lipids and complete cellular or axonal transection was sealed by collapse and fusion of plasmalemmal leaflets. Krause *et al*. (1994) showed that both lesion types in several invertebrate axons sealed by an accumulation of membrane-bound structures (mostly vesicles). Steinhardt *et al*. (1994) reported similar sealing mechanisms in sea urchin eggs and mammalian epithelial cells. These data were the first evidence that sealing is a vesicle-mediated process, similar to synaptic transmission and Golgi trafficking, in that vesicles fuse with undamaged plasmalemma or one another to form a seal at the damage site (**Fig. 1**).

More recent data (see Spaeth et al., 2010, for reference of sealing studies) using 19 different *in vivo, ex vivo* and *in vitro* preparations from many phyla from our lab (invertebrate giant or mammalian axons), and other labs, including plant cells (Schapire, et al., 2009), consistently show that endogenous, naturally-occurring plasmalemmal sealing requires various proteins, many of whose isomers are Ca^{2+}-dependent and involved in membrane trafficking/fusion in neurotransmission and Golgi apparatus

(Sudhof, 2007; Sudhof and Rothman, 2009), such as synaptobrevin, syntaxin, synaptophilin, synaptotagmin, calpains, protein kinase A (PKA), and protein kinase C (PKC) (Krause et al., 1994; Steinhardt et al., 1994; Ballinger et al., 1997; Eddleman et al., 2000; Bittner et al., 2000; Bittner and Fishman, 2000; Detrait et al., 2000; Reddy et al., 2001; Yoo et al., 2003; Togo, 2004; Nguyen et al., 2005; Spaeth et al., 2010). Studies on sealing have also shown that sealing can be enhanced by promoting the activity of endogenous sealing pathways. For instance, increasing cyclic adenosine monophosphate (cAMP) concentration, or specifically activating PKA or Epac via target-specific cAMP analogs, all produced similar increases in plasmalemmal sealing (Spaeth et al., 2010). However, no study to date has shown that inhibition of any single pathway of sealing abolished sealing in that preparation. That is, vesicle-mediated sealing likely involves redundant, parallel pathways initiated by Ca^{2+} influx. Lastly, data from many different preparations from many diverse phyla show that isomers of the same proteins, pathways and vesicle interactions are likely involved in plasmalemmal sealing of all eukaryotic cells, indicating that such proteins in eukaryotes evolved to repair plasmalemmal damage.

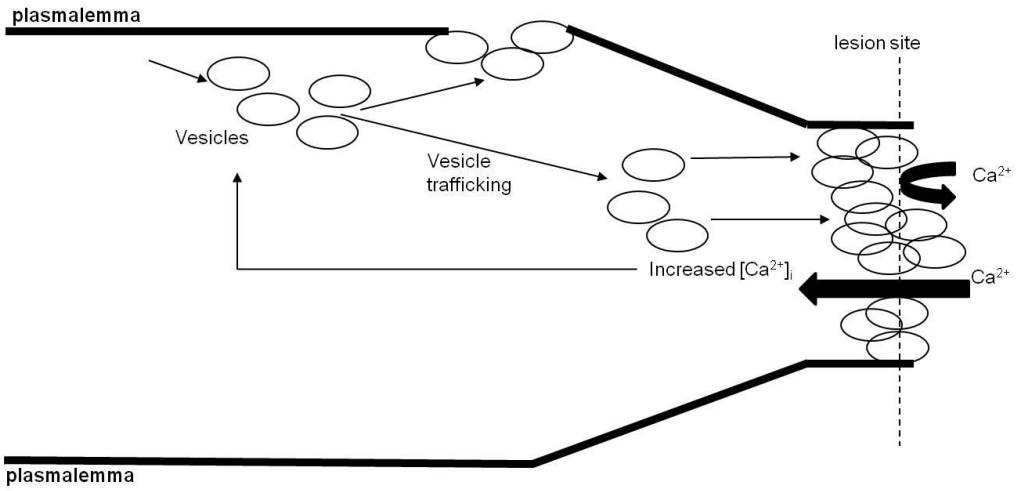

Figure 1: Schematic of endogenous, Ca^{2+}-dependent, vesicle-mediated sealing:

Sealing of an axon, in which vesicles form a plug at a *partly constricted* transection site (lesion site) and site of minor plasmalemmal damage (top of axon). Endogenous sealing in all eukaryotic cells likely occurs by this mechanism (Krause et al., 1994; Steinhardt et al., 1994; Ballinger et al., 1997; Eddleman et al., 2000; Bittner and Fishman, 2000; Detrait et al., 2000; Lichstein et al., 2000; Reddy et al., 2001; Yoo et al., 2003; Spaeth et al., 2010).

DIACYLGLYCEROL (DAG), PKC AND OTHER DAG-ACTIVATED PROTEINS IMPLICATED IN SEALING

DAG mediates many vesicle-mediated pathways (secretion, neurotransmission, trafficking of integral membrane proteins) typically by activating PKC isozymes. As previously mentioned, sealing occurs via vesicle (and other membrane) accumulation at sites of plasmalemmal damage. Togo et al. (2000, 2004, 2006) showed that inhibiting classical PKC (cPKC) isozymes with small-molecule inhibitors, Bisindoylmaleimide I (Bis I) and Gö6976, decreased sealing, suggesting for the first time that PKC promotes sealing in fibroblasts and possibly other eukaryotic cells. Recently, we demonstrated that

novel PKC isozyme η promotes sealing in B104 neurons *in vitro* (Spaeth et al., 2010). Taken together these data suggest that DAG and PKC isozymes are implicated in plasmalemmal sealing. Given these data, I investigated how DAG and which PKC isozymes influence endogenous plasmalemmal sealing in B104 cells.

Diacylglycerol

Diacylglycerol (DAG) and inositol trisphosphate (IP3) are two secondary messengers produced by hydrolysis of phosphatidyl inositol (4, 5) bisphosphate (PIP2) by phospholipase C (PLC) and both play important roles in PKC-dependent signaling. PLC is typically activated by G-protein coupled receptors, but can also be activated by cleavage by the Ca^{2+}-dependent cysteine protease, calpain (Glass et al., 2002; Ray et al., 2003). DAG remains in the plasmalemma and is necessary for both recruiting PKC isozymes to the plasmalemma and activating them. IP3 is soluble and diffuses through the cytoplasm. IP3 is bound by inositol trisphosphate ligand-gated Ca^{2+} channels of the endoplasmic reticulum (or sarcoplamsic reticulum in muscle cells), inducing release of Ca^{2+} from intracellular stores, thus further promoting PKC activity. Lastly, I have recently shown that nPKC isozyme η (requires only DAG for activation) promotes sealing in B104 neurons *in vitro* (Spaeth et al., 2010), suggesting that DAG influences plasmalemmal sealing in B104 neurons.

Protein kinase C (PKC)

Like PKA (which has been shown to promote sealing in B104 neurons *in vitro*, Spaeth et al., 2010, 2011), PKC isozymes are involved in many vesicle-mediated processes in neurons, such as turning of axonal growth cones, transport of beta-1

integrins and neurite/dendrite extension (Mikule et al., 2003; Sivasankaran et al., 2004; Gatlin et al., 2006; Siskova et al., 2006; Tsai et al., 2007), and are therefore likely involved in plasmalemmal sealing. PKCs are a family of serine/threonine kinases whose activity is dependent upon the plasmalemmal lipid, phosphoserine. The PKC family is divided into three groups: classical isozymes (cPKC α, β1, β2, γ) require both calcium and DAG for activation, novel isozymes (nPKC δ, ε, η, θ) require only DAG for activation and, atypical isozymes (aPKC ι/λ, ζ) require neither calcium nor DAG for activation and are thought to be regulated via phosphorylation by other PKC isozymes and kinases (Uberall et al., 1997; Larsson, 2006; Park et al., 2007). The PKC structure consists of two parts: a regulatory domain (pseudosubstrate region) that contains binding sites for the activators Ca^{2+} and DAG and a catalytic domain. Atypical PKC isozymes have a similar structure to cPKC and nPKC isozymes.

MARCKS and GAP43

PKC isozymes regulate many vesicle-mediated processes via phosphorylation of downstream targets (effectors). Because of their roles in axonal growth cone turning and dendrite formation some of the most-studied effectors of PKC are growth associated protein of 43kDa (GAP43) and myristoylated alanine rich protein kinase C substrate (MARCKS). Of these cytoskeleton associated proteins, GAP43 is exclusively found in neurons, whereas MARCKS is expressed in all tissues (Wiederkehr et al., 1997; Frey et al., 2000; Korshunova et al., 2006; Laux et al., 2000; Tsai et al., 2007). GAP43 and MARCKS are phosphorylated by various isozymes of PKC at their effector domain (ED) (Wiederkehr et al., 1997; Frey et al., 2000; Laux et al., 2000). GAP43 and MARCKS are

proposed to cause cytoskeleton rearrangements by altering the availability of PIP2 along the inner leaflet of the plasma membrane for hydrolysis by PLC (Frey et al., 2000; Laux et al., 2000).

As briefly mentioned above, PKC-dependent phosphorylation of MARCKS and GAP43 mediates axonal growth cone turning (Mikule et al., 2003), neurite extension (Kolkova et al., 2000; Sivasankaran et al., 2004; Tsai et al., 2007) and vesicle mediated transport of beta-1 Integrins to the plasma membrane (Siskova et al., 2006; Wiederkehr et al., 1997; Miranti, et al., 1999; Frey et al., 2000; Laux et al., 2000; Larsson, 2006). MARCKS and GA43 are abundantly expressed in growth cones (Kolkova et al., 2000; Gatlin et al., 2006; Korshunova et al., 2006). These data strongly suggest that PKC and PKC-dependent phosphorylation of GAP43 or MARCKS could influence vesicle-mediated plasmalemmal sealing.

Munc 13-1

Like cPKC and nPKC, Munc13-1 (a membrane fusion protein used in neurotransmission) contains the DAG binding C1 domain (Brose and Rosenmund, 2002). Binding of DAG by Munc13-1 increased the probability of Ca^{2+}-dependent and Ca^{2+}-independent vesicular fusion events of the readily releasable pool of vesicles (Basu, et al. 2007). Other membrane fusion proteins involved in synaptic transmission also promote sealing, such as synaptobrevin, syntaxin, synaptophilin, synaptotagmin. Taken together these data suggest that Munc13-1 may be involved in plasmalemmal sealing.

PURPOSE OF STUDYING PLASMALEMMAL SEALING WITH REGARDS TO PERIPHERAL NERVE CRUSH INJURY AND STROKE

A common traumatic peripheral nerve injury in humans is peripheral nerve crush injury (PNCI): typically caused by compression of surrounding tissues upon the nerve, sometimes completely severing axons within the nerve, resulting in significant behavioral deficits (Campbell, 2008). Stroke is the third leading cause of death in the USA (Roger, 2006; Janardhan and Qureshi, 2004). It is well documented that ischemia (oxygen glucose deprivation; OGD) and PNCI produce plasmalemmal damage (Rodriguez-Sinovas, 2005; Campbell, 2008). Improving the rate and extent of mammalian axonal regeneration has been a major research goal for many neuroscientists, because most current repair strategies rely on slow (sometimes ineffective) axonal outgrowths from surviving neurons to re-innervate target tissues. Such techniques have slightly improved the number and specificity of regenerating axons, but never the time course of behavioral recovery (Lago et al., 2007; Kalbermatten et al., 2009), or prevented Wallerian degeneration of severed distal axons (Lore et al., 1999; Marzullo et al., 2002; Stavisky et al., 2005; Britt et al., 2010).

Polyethylene glycol

Severed mammalian axons can be immediately repaired (within 1-2 minutes) using the membrane fusogen polyethylene glycol (PEG) (Krause et al., 1991; Stavisky et al., 2005; Britt et al., 2010; Bittner et al., 2012). Briefly, polyethylene glycol (PEG) has been used since the 1970s by cell biologists to fuse various cell types and to make cell hybrids (Ahkong, 1987). PEG is an amphipathic molecule able to remove waters of hydration from membrane-bound proteins allowing adjacent lipid membranes to flow into

one another. The membrane fusogenic properties of PEG have been advantageously used to rapidly and permanently repair peripheral nerve crush- and cut-severance in mammals, *in vivo* (Britt et al., 2010; Bittner et al., 2012).

Application of PEG to severed axons in hypotonic calcium free physiological saline restores axonal morphological continuity and conduction of action potentials through the lesion site of invertebrate and vertebrate (rat) axons *in vitro* and *in vivo* (Krause, 1991; Marzullo, 2002; Stavisky, 2005). Application of PEG to crush-severed sciatic axons significantly increased the rate of functional behavior recovery in crush injury animals that received PEG versus crush injury animals that received vehicle treatment *in vivo* (Britt et al., 2010). It is hypothesized that application of hypotonic calcium free saline immediately after crush-severance (prior to PEG), promotes PEG-fusion by impeding (inhibiting) endogenous sealing in crush-severed axonal ends. Thus, increasing the number of open closely apposed axonal ends for PEG-fusion.

In my proposal I originally hypothesized that application of compounds that inhibit endogenous sealing may be able to impede the effects of endogenous extracellular Ca^{2+} influx such that fewer crush-severed axonal halves seal and thus are available for PEG-fusion *in vivo.* Recently, we investigated whether compounds that decrease plasmalemmal sealing in B104 neurons *in vitro*, could be used to enhance PEG-fusion and promote even greater behavioral recovery in models of peripheral cut- and crush-severance injury in rats (Bittner et al., 2012). For example, the anti-oxidant methylene blue (MB) has been reported to decrease plasmalemmal sealing in B104 neurons *in vitro* (Spaeth et al., 2012) and slightly improve behavioral recovery following retinal/optical

damage (Zhang et al., 2006; Rojas et al., 2009). Application of MB in Ca^{2+}-free hypotonic physiological saline to crush- or cut-severed rat sciatic axons, prior to application of PEG, dramatically improved PEG-fusion induced behavioral recovery, *in vivo* (Bittner et al., 2012). These more recent data suggest that insights obtained from studies on endogenous sealing mechanisms in B104 neurons *in vitro*, can have very beneficial effects on immediate and long-term PEG-repair of peripheral nerve injury, and possibly stroke, in mammals *in vivo*.

STROKE: AS MODELED BY OXYGEN-GLUCOSE DEPRIVATION *IN VITRO*

To model ischemia (stroke) *in vitro*, cells are often incubated in an anaerobic (0% oxygen) chamber at 37°C in media lacking glucose (0% glucose) (Jones, 2004). Cells are then returned to normoxic, normoglycemic conditions – simulating reperfusion. Such OGD exposures increase Ca^{2+} influx, calpain activity, membrane permeability, membrane damage and cell death, as observed in tissue explants and models of stroke *in vivo* (Yoshioka, 2000; Cao, 2010).

It is well documented that ischemia (oxygen glucose deprivation; OGD) produces plasmalemmal damage (Rodriguez-Sinovas, 2005). OGD-induced plasmalemmal disruptions are easily observed as membrane blebs, *in vitro* (Neiminen, 1988). As an ischemic event progresses, the integrity of cell plasmalemmas worsen, blebs form, grow, coalesce and eventually burst, releasing cytoplasmic contents and causing cell death. Such plasmalemmal damage caused by stroke in humans, results in loss of functional behavior and is the third leading cause of death in the USA (Roger, 2011; Janardhan and Qureshi, 2004). However, increasing cells endogenous ability to repair (seal) their

damaged plasmalemma can decrease the severity of tissue damage and increase functional behavior recovery (Rojas et al., 2009; Nehrt, 2010; Britt, 2010; Bittner et al., 2012).

SUMMARY OF RESULTS

The goals of my dissertation research were as follows: **1**) determine which PKC isozymes and their effectors, and whether DAG and other DAG-activated proteins are involved in plasmalemmal sealing (Chapter 2), **2**) investigate whether inhibiting/enhancing PKC- and PKA-dependent sealing promotes PEG-repair of PNCI in a rat model (Chapter 3) and **3**) determine what role PKCs may have in sealing of ischemia-induced plasmalemmal damage in an *in vitro* model of stroke in B104 neurons (Chapter 4).

Chapter 2 Summary

To investigate whether DAG, PKC isozymes, MARCKS, Munc13-1 influence sealing, we transected one neurite of individually-identified B104 hippocampal cells, in a DAG-analog or inhibitors of PKC, MARCKS, Munc13-1 or phospholipase C (PLC). We assessed plasmalemmal repair by formation of a barrier to extracellular dye (Krause et al. 1994; Steinhardt et al. 1994; Spaeth et al. 2010). I report, for the first time a role for DAG and DAG-activated proteins in sealing. Specifically, data show that exogenously increasing DAG concentration increased sealing, and decreasing endogenous DAG production via PLC or MARCKS inhibition, or inhibition of DAG-activated targets, such as nPKC η and θ and Munc-13, but not cPKCs, decreased sealing. Additionally,

simultaneous inhibition of nPKC η, θ and PKA nearly eliminated sealing, suggesting that DAG-dependent sealing pathways interact with previously reported cAMP-dependent pathways of sealing (Spaeth et al 2010, 2011). These data and other demonstrate a role for DAG-dependent proteins that act in parallel with previously identified cAMP-dependent proteins during plasmalemmal sealing of B104 and other eukaryotic cells.

Chapter 3

I investigated if application of inhibitors of PKC and PKA to crush-severed axons *in vivo*, immediately following crush-severance in *physiological calcium concentration*, could promote PEG-fusion. We used dye exclusion (as previously described in Chapter 2) to assess the effect of PKC and PKA inhibitors singly, or in combinations with PEG on endogenous sealing in B104 cells *in vitro*. We assessed restoration of electrophysiological and morphological continuity of crush-severed axons by measuring conduction of compound action potentials and dye diffusion across crush-lesion sites *in vivo* and *in vitro*, respectively (Britt et al., 2010).

I report that only simultaneous inhibition of nPKC η and θ, and PKI (ηPSF+θPSF+PKI) was capable of decreasing plasmalemmal sealing when neurites of B104 cells were transected in calcium *in vitro*. B104 cells with transected neurites always sealed when PEG was applied, even in inhibitors of nPKCs and PKA (individually or in combination), and in Ca^{2+}-free saline *in vitro*. Applying PEG in ηPSF+θPSF+PKI proved to be a cytotoxic combination for both intact and damaged (neurite transected) B104 cells.

All crush-severed sciatic nerves receiving ηPSF+θPSF+PKI treatment to their crush-lesion site, following crush-severance in Ca^{2+}-free or Ca^{2+}-containing physiological salines, had dye diffuse through their lesion site. Dye did not diffuse across the lesion site of sciatic nerves receiving only crush treatment in Ca^{2+}-free or Ca^{2+}-containing physiological salines. As expected, compound action potential conduction was not restored after ηPSF+θPSF+PKI was applied to crush-severed sciatic axons, following crush in Ca^{2+}-free or Ca^{2+}-containing physiological salines *in vivo*. PEG application restored morphological continuity and electrophysiological conductivity of crush-severed sciatic axons only in treatment groups in which the crush was performed in Ca^{2+}-free hypotonic physiological saline. Taken together, our B104 cell and sciatic nerve data suggest that nearly-complete inhibition of endogenous axonal sealing via ηPSF+θPSF+PKI, has no beneficial effect on PEG-fusion, i.e., this treatment does not promote PEG-fusion following crush-severance in Ca^{2+}-containing saline. Lastly, these data strongly suggest that endogenous axonal sealing is neuroprotective and that acutely inhibiting sealing at the time of injury may have long-lasting effects on axonal viability and therefore, regeneration after such treatments.

Chapter 4

I performed a preliminary study that investigated whether the same PKC isozymes involved in sealing transected B104 neurites, also promote sealing of oxygen-glucose deprivation (OGD)-induced plasmalemmal damage in B104 neurons. I report that, unlike sealing of transected B104 neurites, the presence of Ca^{2+} following immediate OGD exposures of 1, 3, 5 or 10 minutes, did not promote sealing probability

in B104 cells compared to cells not receiving Ca^{2+} following similar OGD exposures. However, cells exposed to peptide inhibitors of nPKC η or θ, during OGD exposures of 1, 3, 5 or 10 minutes had significantly decreased sealing probability compared to normoxic (no treatment) controls and, cells exposed to only OGD. These data suggest that nPKC η and θ promote sealing of OGD-induced plasmalemmal damage in B104 cells, as they do for sealing of transected neurites of B104 cells.

CONCLUSION

The data presented here in this dissertation suggest that enhancing PKC-dependent endogenous sealing following PNCI or stroke, should directly promote neuron survival and indirectly, axonal regeneration. As demonstrated in Chapter 3, near-complete inhibition of endogenous sealing did not promote PEG-fusion of severed axons crushed in physiologically relevant Ca^{2+} concentrations. Suggesting that following any form of neurotrauma, promotion of neuroprotective endogenous sealing should be a primary goal because, cells that do not seal, do not survive (McNeil et al., 1993; Nguyen et al., 2005), much less regenerate axons.

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Chapter 2: Sealing of transected neurites of rat B104 cells requires a diacylglycerol PKC-dependent pathway and a PKA-dependent pathway

SUMMARY

To survive, neurons and other eukaryotic cells must rapidly repair (seal) plasmalemmal damage. Such repair occurs by an accumulation of intracellular vesicles at or near the plasmalemmal disruption. Diacylglycerol (DAG)-dependent and cAMP-dependent proteins are involved in many vesicle trafficking pathways. Although recent studies have implicated the signaling molecule cAMP in sealing, no study has investigated how DAG and DAG-dependent proteins affect sealing. Using dye exclusion to assess Ca^{2+}-dependent vesicle-mediated sealing of transected neurites of individually identifiable rat hippocampal B104 cells, we now report that, compared to non-treated controls, sealing probabilities and rates are increased by DAG and cAMP analogs that activate PKC and Munc13-1, and PKA. Sealing is decreased by inhibiting DAG-activated novel protein kinase C isozymes η (nPKCη) and θ (nPKCθ) and Munc13-1, the PKC effector myristoylated alanine rich PKC substrate (MARCKS) or phospholipase C (PLC). DAG-increased sealing is prevented by inhibiting MARCKS or protein kinase A (PKA). Sealing probability is further decreased by simultaneously inhibiting nPKCη, nPKCθ and PKA. Extracellular Ca^{2+}, DAG or cAMP analogs do not affect this decrease in sealing. These and other data suggest that DAG increases sealing through MARCKS and that nPKCη, nPKCθ and PKA are all required to seal plasmalemmal damage in B104, and likely all eukaryotic cells.

INTRODUCTION

Ca^{2+} influx at sites of plasmalemmal damage activates competing pathways, one leading to cell death by Ca^{2+}-dependent proteases (Schlepfer et al. 1973; Nguyen et al. 2005) and the other to vesicle-mediated plasmalemmal repair (sealing) (Krause et al., 1994; Steinhardt, et al. 1994; Eddleman, et al. 1998; Detrait et al. 2000a,b; McNeil et al. 2001; Nguyen et al. 2005). To survive, all eukaryotic cells (including neurons) must rapidly repair (seal) plasmalemmal damage (Fishman and Bittner, 2003; Spaeth et al. 2010). Vesicles formed by endocytosis of nearby undamaged membrane (Eddleman et al. 1997, 1998), lysosomes (Reddy et al. 2001), and/or myelin delaminations (Ballinger et al. 1997) migrate, accumulate, and pack tightly at the damage site to reduce the influx of extracellular Ca^{2+} and other ions. This vesicle-mediated repair of plasmalemmal damage is increased by calpain (Godell et al. 1997; Yoo et al. 2003), proteins involved in vesicle trafficking and membrane fusion, such as SNAP-25, NSF (Spaeth, et al. 2011), synaptobrevin (Yoo, et al. 2003), cAMP effectors PKA and Epac, calpain (Godell et al. 1997; Yoo et al. 2003) and novel protein kinase C isozyme η (nPKCη) (Spaeth et al. 2010). However, little is known about how PKCs and other diacylglycerol (DAG) - activated proteins mediate sealing or if DAG-dependent sealing is part of, or parallel to, cAMP-dependent sealing pathways (Spaeth et al. 2010, 2012).

The PKC family is divided into three subgroups based on allosteric activators that bind to its regulatory domain (pseudosubstrate region; Larsson, 2006). The classical isozymes (cPKC α, β1, β2, γ) require both calcium and diacylglycerol (DAG), a product of phosphatidyl inositol (4,5) bisphosphate (PIP2) hydrolysis by phospholipase C (PLC).

Novel isozymes (nPKC δ, ε, η, θ) require only DAG for activation. Both cPKC and nPKC isozymes contain the DAG binding C1 domain (Brose and Rosenmund 2002). In contrast, atypical isozymes (aPKC ι/λ, ζ) require neither calcium nor DAG (Larsson, 2006). Active PKC isozymes regulate many vesicle-mediated pathways. Specifically, axonal growth cone turning, transport of beta-1 integrins and neurite extension require cPKC and nPKC-dependent phosphorylation of myristoylated alanine rich C kinase substrate (MARCKS: Sivasankaran et al. 2004; Gatlin et al. 2006; Siskova et al.2006; Tsai et al. 2007). DAG also activates Munc13-1, a vesicle priming protein during neurotransmitter release (Mochida et al. 1998), which increases the probability of Ca^{2+} dependent and Ca^{2+} independent vesicular fusion events (Basu et al. 2007). Hence, DAG activation of cPKC and nPKC isozymes, MARCKS and/or Munc13-1 might influence vesicle-mediated plasmalemmal sealing.

To investigate whether DAG, PKC isozymes, MARCKS, and/or Munc13-1 influence sealing, we transected one neurite of individually-identified B104 hippocampal cells, in a DAG-analog or inhibitors of PKC, MARCKS, Munc13-1 or phospholipase C (PLC). We assessed plasmalemmal repair by formation of a barrier to extracellular dye (Krause et al. 1994; Steinhardt et al. 1994; Spaeth et al. 2010). We report a role for DAG and DAG-activated proteins in sealing. Specifically, our data show that exogenously increasing DAG concentration increased sealing, and decreasing endogenous DAG production via inhibition of PLC or MARCKS, or inhibition of DAG-activated targets, such as nPKC η and θ and Munc-13, but not cPKCs, decreased sealing. Additionally, simultaneous inhibition of nPKC η, θ and PKA nearly eliminated sealing, suggesting that

DAG-dependent sealing pathways interact with previously reported cAMP-dependent sealing pathways (Spaeth et al 2010, 2011). These and other data suggest that DAG-dependent proteins act in parallel with previously identified cAMP-dependent proteins during plasmalemmal sealing of B104 and other eukaryotic cells.

RESULTS

Experimental conditions for assessing sealing

The plasmalemma of B104 cells was damaged by transecting a single neurite with a glass micropipette (Yoo et al., 2004), always >50μm from the soma in a Petri dish containing Ca^{2+}-free saline (Spaeth et al., 2010; 2011), fluorescein (green) dye, and when applicable, treatment substances. Typically, 30 – 100 cells were transected per dish for each treatment during a 10 min period (see Online Resource 3). After transection, the Ca^{2+}-free saline was replaced with Ca^{2+}-containing saline (referred to as Ca^{2+} addition) to simultaneously induce sealing in all transected cells. At various times Post-Ca^{2+} addition (PC time), a second extracellular dye, Texas red dextran, was added for 10 minutes to the Ca^{2+}-containing saline. The Texas red dextran was then removed by rinsing with Ca^{2+}-containing saline.

Transected cells were uniquely identified by uptake of fluorescein (green) and the bisection of a neurite by the score mark made by the micropipette. Plasmalemmal sealing was assessed by uptake or exclusion of Texas red dextran from each individually-identifiable fluorescein-labeled cell (Nguyen et al., 2005). For example, transected cells that did not seal at a given PC time emitted both green and red (fluorescein and Texas red

dextran) fluorescence. Sealing probability was calculated as the percent of cells that sealed (i.e. excluded Texas red dextran) divided by the total number of cells damaged per condition at any given PC time. Control sealing data describe cells transected in Ca^{2+}-free saline without any treatment, and then exposed to Ca^{2+}-containing saline at various PC times. Control sealing data obtained in this study were not significantly different (CMH χ^2, $p > 0.05$) compared to control sealing data in three previous studies (Spaeth et al., 2010, 2011a,b) at the same PC times by different researchers performing the same protocol. Hence, we use the same control sealing curve (gray dashed-line) in all figures.

Because some compounds (Bis I, Gö6976, U73122, U73343) used in this study were dissolved in DMSO, as a further control we tested whether DMSO influenced plasmalemmal sealing. DMSO (90 μM) had no effect on rate ($p > 0.05$, FZT) or probability ($p > 0.05$, CMH χ^2) of sealing at any PC time compared to control sealing (gray dashed line in **Fig. 2A** and all subsequent figures).

As described previously, the Cochran-Mantel-Haenszel χ^2 (CMH χ^2) test for independence was used to determine statistically significant differences between sealing probabilities of treatment versus control sealing or between two treatments (Agresti, 1996; Yoo et al. 2004; Nguyen et al. 2005; Spaeth et al. 2010, 2011a,b,c). Significant differences between treatment versus control sealing or between two treatment groups are indicated on graphs with no symbol ($p > 0.05$) or by asterisks ($p \leq 0.05$ by *, $p \leq 0.01$ by ** and $p \leq 0.001$ by ***).

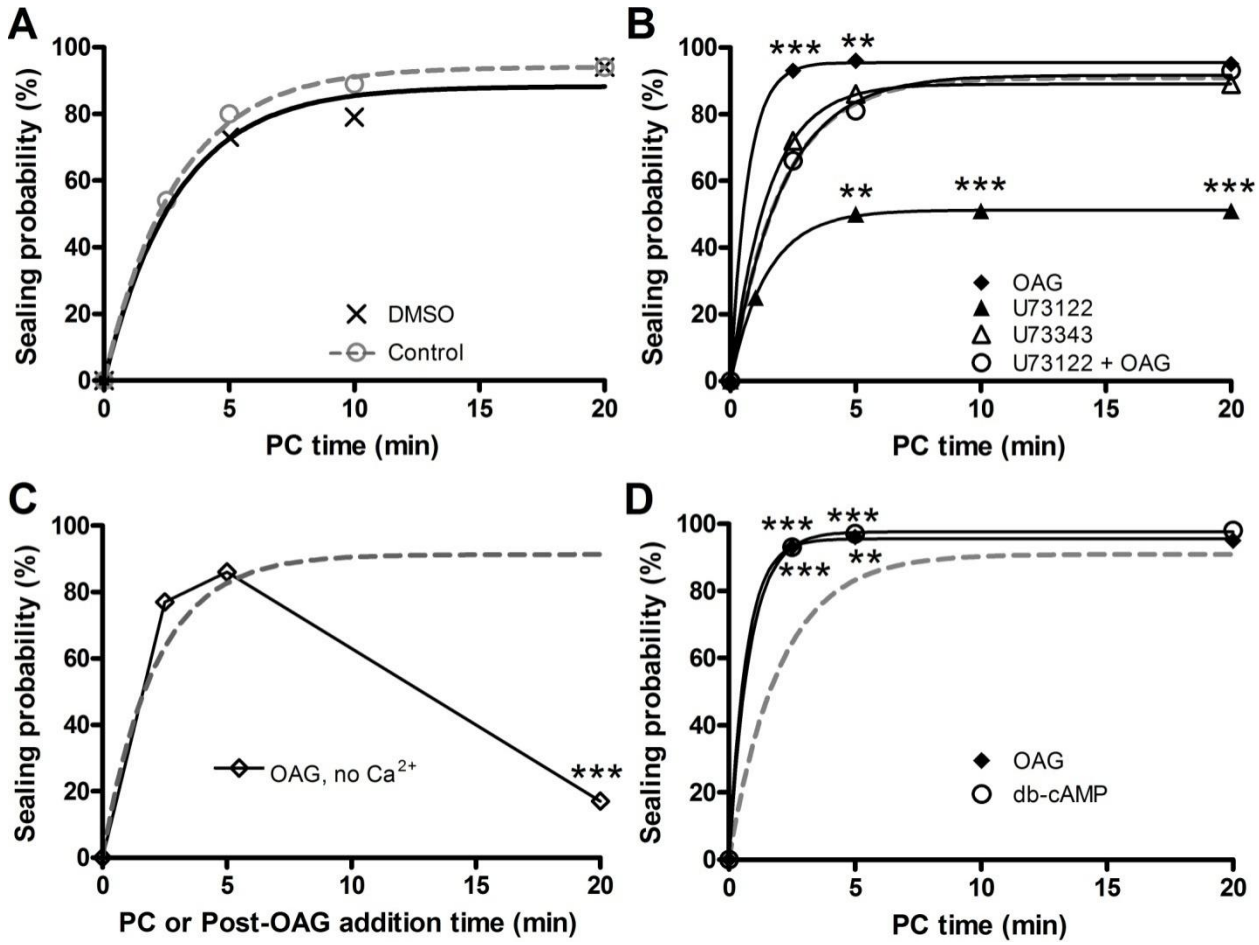


Figure 2: Sealing probability (%) vs. PC time (min):

(A) Effect of DMSO vehicle (90 μM) on sealing compared to control sealing (gray dashed line in this and all other graphs).
(B) Effect of 100μM OAG (an exogenous DAG analog), 16μM U73122, 16μM U73343 (inactive analog of U73122), or combination of 16μM U73122+100μM OAG on sealing.
(C) Effect of 100μM OAG addition, post-transection (Post-OAG addition time), on sealing (i.e., cells never exposed to exogenous Ca^{2+}).
(D) Effect of 100 μM OAG or 1mM db-cAMP (analog of cAMP and enhancer of sealing: Spaeth et al. 2010) on sealing.
Significance levels between a treatment and control sealing for this and all other figures and tables are indicated by no symbol (asterisk) = $p > 0.05$, * =, $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

B104 cells express PKA, cPKC α, and β_1, and nPKC η, and θ

As reported previously, PKA is expressed in B104 cells (Spaeth et al. 2011). To examine whether nPKC and cPKC isozymes are expressed in B104 cells, cytosolic fractions collected from B104 cells were separated on 4 to 20% SDS PAGE and transferred to nitrocellulose membranes that were probed with monoclonal PKCα or PKCθ or, polyclonal PKCβ_1 or PKCη antibodies. We observed expression of cPKC α and β_1 and, nPKC η and θ isozymes (**Fig. 3**).

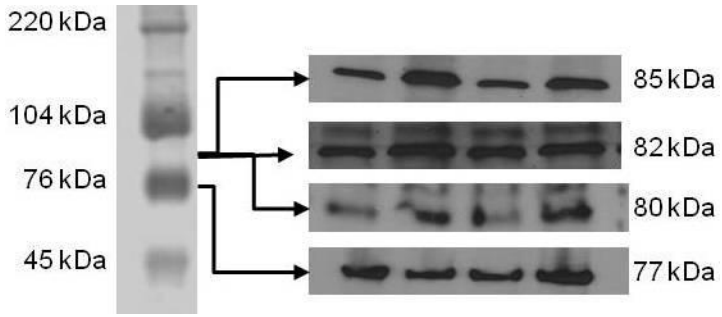


Figure 3: PKC isozyme expression in B104 cells:

Western blots confirmed expression of cPKCα (77 kDa), cPKCβ_1 (80 kDa), nPKCη (82 kDa), and nPKCθ (85 kDa). Molecular weight marker: Blue Ranger, Pierce (see Methods).

The DAG analog OAG increases sealing

As previously reported, inhibition of nPKCη by 7μM nPKCη pseudosubstrate fragment (ηPSF) significantly decreases the rate and probability of sealing at all PC times compared to control sealing (Spaeth et al. 2010). Because nPKC isozymes such as nPKCη require DAG for activation, we hypothesized that a DAG analog would increase

sealing of transected neurites of B104 cells. We exogenously added the membrane-permeable DAG analog 1-Oleoyl-2-acetyl-sn-glycerol (OAG: Doherty et al. 1995; Wang and Oram, 2007; Ju et al. 2010), to increase the intracellular DAG concentration. B104 cells transected in 100μM OAG sealed at a significantly increased probability at 2.5 and 5 minutes PC ($p \leq 0.001$ and $p \leq 0.01$, CMH χ^2, respectively) and rate of sealing ($p \leq 0.01$, FZT) compared to control sealing (**Fig. 2B; Table 1**). These data suggest that OAG increases sealing of B104 cells.

OAG does not substitute for Ca^{2+}

Because OAG treatment enhanced Ca^{2+}-dependent sealing, we investigated whether OAG could substitute for Ca^{2+} during sealing. We transected B104 cell neurites in Ca^{2+}-free saline, and instead of replacing Ca^{2+}-free saline with Ca^{2+}-containing saline following transections, we added Ca^{2+}-free saline containing 100μM OAG to transected cells. We assessed dye exclusion at various post-OAG times, instead of PC time (**Fig. 2C, Table 1**). Damaged B104 cells initially sealed at a probability and rate similar to control sealing but, exhibited a significant ($p \leq 0.001$) decline in sealing probability at 20 minutes PC compared to control sealing. The observed decrease in sealing may be due to cell death, the breakdown of a previously formed plasmalemmal seal, or both. These data suggest that DAG can initially bypass Ca^{2+} requirements for sealing, but longer-term cell viability requires Ca^{2+}-dependent processes (Spaeth et al. 2011).

Inhibiting PLC decreases sealing

Because PLC hydrolyzes PIP2 into DAG (and IP3), we hypothesized that inhibition of PLC with 16μM U73122, a potent and specific PLC inhibitor (Bleasdale et al. 1990; Hildebrandt et al. 1997; Tanaka et al. 2003) would decrease plasmalemmal sealing, possibly by limiting DAG production. B104 cells transected in 16μM U73122 sealed at a significantly decreased probability ($p \leq 0.001$, CMH χ^2) at 5, 10 and 20 minutes PC, but not different rate ($p > 0.05$, FZT), compared to control sealing (**Fig. 2B; Table 1**). As an additional control, we transected B104 cells in 16μM U73343, an inactive analog of U73122 (Bleasdale et al. 1990; Hildebrandt et al. 1997; Tanaka et al. 2003) and assessed dye exclusion at various PC times. Cells transected in U73343 did not seal at a significantly different rate ($p > 0.05$, FZT) or probability ($p > 0.05$, CMH χ^2) at any PC time compared to control sealing (**Fig. 2B; Table 1**). These results suggest that PLC activity promotes sealing of transected neurites of B104 cells.

The DAG analog OAG promotes sealing when PLC is inhibited

Because the DAG analog OAG enhanced sealing (**Fig. 2B**), and DAG acts downstream from PLC, we hypothesized that OAG would overcome decreased sealing due to PLC inhibition. Cells transected in U73122+100μM OAG sealed at a significantly faster rate ($p > 0.05$, FZT) and higher probability ($p > 0.05$, CMH χ^2) at all PC times compared to cells transected in U73122 alone (**Fig. 2B**). This observed increase in sealing probability and rate was not significantly different from control

sealing. That is, the DAG analog OAG returned sealing to control levels in the presence of PLC inhibition, suggesting that DAG promotes sealing downstream of PLC.

Inhibition of cPKC α or β_1 does not decrease sealing

Because plasmalemmal sealing is a Ca^{2+}-dependent vesicle-mediated process (Krause et al. 1994; Fishman and Bittner, 2003), we hypothesized that inhibition of Ca^{2+}-dependent cPKC α and β_1 by adding cPKC PSF 20-28 (Jungnickel et al. 2007) would decrease plasmalemmal sealing. However, B104 cells transected in 8μM PSF20-28 did not seal at a significantly different probability ($p > 0.05$, CMH χ^2) or rate ($p > 0.05$, FZT) of sealing compared to control sealing (**Fig. 4A; Table 1**). These data suggest that cPKCs do not influence sealing or that PSF20-28 does not inhibit cPKCs in B104 cells.

To investigate whether cPKCs influence sealing, we used two small-molecule inhibitors of cPKCs, Bisindoylmaleimide I (Bis I) and Gö6976, at concentrations reported to affect only cPKCs and decrease sealing in fibroblasts in vitro (Togo et al. 2004). B104 cells transected in 1μM Gö6976, 100nM Bis 1, or 1μM Bis 1, did not seal at a significantly different probability ($p \geq 0.05$, CMH χ^2) or rate ($p > 0.05$, FZT) compared to control sealing (**Fig. 4A; Table 1**). These results suggest that DAG-activated nPKC θ and η (Spaeth et al. 2010), but not Ca^{2+}-dependent cPKC α or β_1, promote sealing of transected B104 cells.

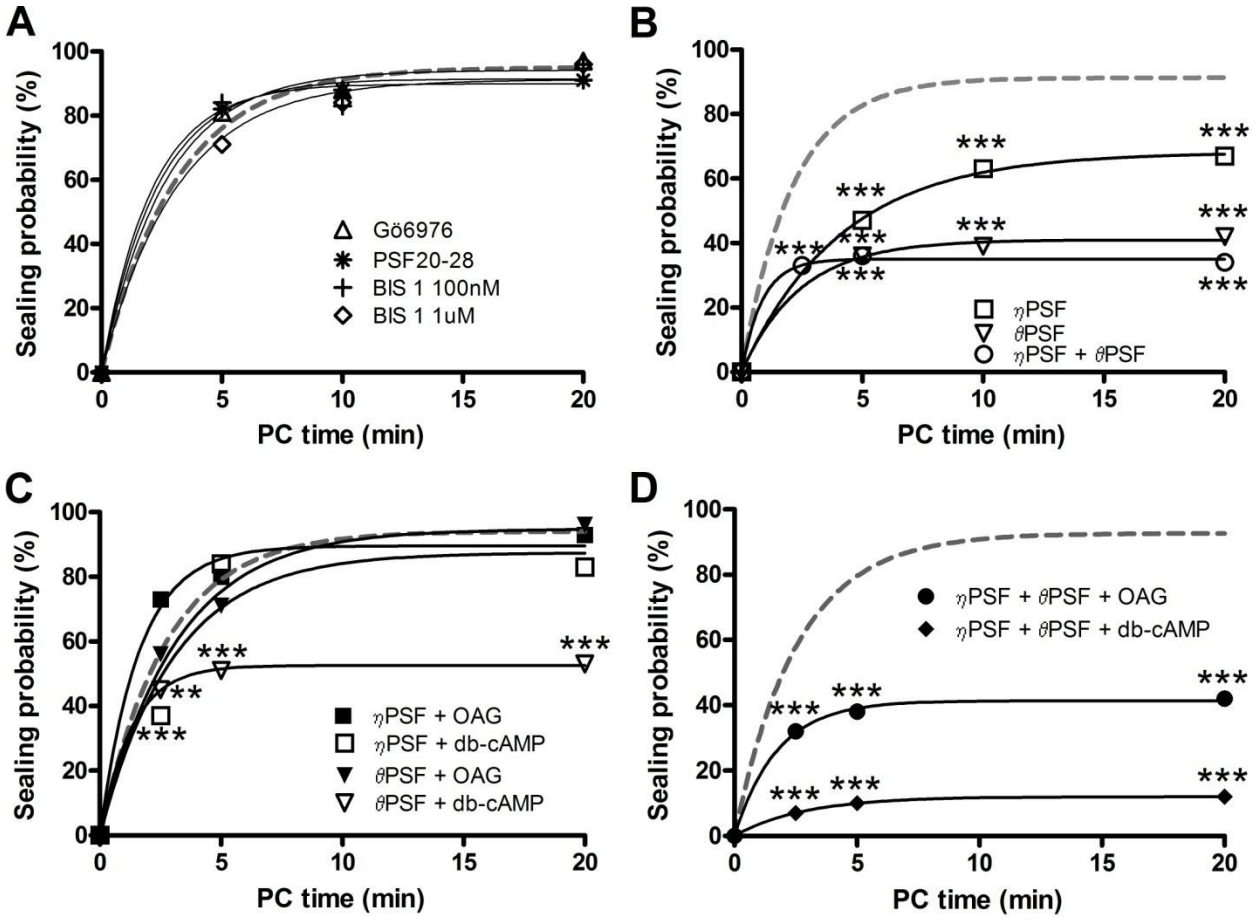


Figure 4: Sealing Probability (%) vs. PC time (min):

(A) Effect of Bis 1 (100nM, 1μM), 1μM Gö6976, or 8μM PSF20-28 on sealing.
(B) Effect of 7μM ηPSF, 9μM θPSF, or combination of 7μM ηPSF+9μM θPSF on sealing. For additional data on nPKC PSFs, see Online Resources 1 and 2.
(C) Effect of 100 μM OAG or 1mM db-cAMP in 7μM ηPSF or 9μM θPSF on sealing.
(D) Effect of 100 μM OAG or1mM db-cAMP in 7μM ηPSF+9μM θPSF on sealing.

Inhibition of nPKCη or θ decreases sealing

Since nPKC isozymes are activated by DAG, and inhibition of nPKCη by 7μM nPKCη pseudosubstrate fragment (ηPSF) has been reported to significantly decrease the

rate and probability of sealing at all PC times compared to control sealing (Spaeth et al. 2010), we hypothesized that inhibition of other PKC isozymes would decrease sealing.

To inhibit PKC isozymes, we used peptide pseudosubstrate fragments (PSFs) that bind and inhibit active catalytic domains of PKC. [PSFs typically have increased target specificity and reduced off-target effects compared to small-molecule inhibitors (Davies et al. 2000; Churchill et al. 2009).] B104 cells transected in 9μM θPSF (Staal et al. 2008; Barros et al. 2009) sealed at a significantly decreased probability ($p \leq 0.001$, CMH χ^2) at all PC times, but not rate ($p > 0.05$, FZT), compared to control sealing (**Fig. 4B; Table 1**). These results suggest that nPKCθ promotes sealing in B104 cells.

Table 1: Effect of compounds on sealing rate (k) and time (τ) constants

Treatment	> 50 μm k (min^{-1})	> 50 μm τ (min)	> 50 μm R^2	Sealing probability at 20 min PC
Control	0.49	2.03	0.9991	94
DMSO	0.30	3.30	0.9868	94
η PSF	0.15	6.58 **	0.9618	67
θ PSF	0.41	2.45	0.9975	42
η PSF+θ PSF	1.17 *	0.85	0.9975	34
20-28 PSF	0.48	2.09	0.9995	91
Go6976	0.38	2.66	0.9788	97
Bis1 100 nM	0.50	2.00	0.9866	96
Bis1 1 μM	0.26	3.79	0.9962	96
U73122	0.68	1.48	0.9998	51
U73343	0.66	1.51	0.9998	89
OAG	1.46 **	0.69	0.9999	95
OAG no Ca^{2+}	N/A	N/A	N/A	17
U73122+OAG	0.49	2.06	0.9979	93
MARCKSpi	0.20	5.05 *	0.9993	51
MARCKSpi+OAG	0.40	2.48	0.9995	67
NEM	0.29	3.42	0.9989	46
NEM+OAG	0.44	2.25	0.9970	82
PKI	0.23	4.35	1.0000	42
NEM+PKI	0.40	2.49	0.9224	33
PKI+OAG	0.31	3.21	0.9952	37
θ PSF+OAG	0.53	1.90	0.9924	96

η PSF+OAG	0.94	1.07	0.9908	93
η PSF+θ PSF+OAG	0.57	1.75	0.9985	42
db-cAMP	1.22 *	0.82	1.0000	98
θ PSF+db-cAMP	0.76	1.31	0.9998	53
η PSF+db-cAMP	0.33	3.06	0.9300	83
η PSF+θ PSF+db-cAMP	0.35	2.84	1.0000	12
η PSF+θ PSF+PKI (PBS^{2+})	N/A	N/A	N/A	3
η PSF+θ PSF+PKI	0.18	5.45	0.9439	2
η PSF+θ PSF+PKI+OAG (PBS^{2+})	N/A	N/A	N/A	7
η PSF+θ PSF+PKI+OAG	0.44	2.28	1.0000	3
η PSF+θ PSF+PKI+db-cAMP (PBS^{2+})	N/A	N/A	N/A	1
η PSF+θ PSF+PKI+db-cAMP	14.99 ***	0.07	1.0000	1
Bref-A	0.58	1.73	0.9255	52
Bref-A+OAG	0.42	2.39	1.0000	97
Muncpi	4.14 **	0.74	1.0000	28
Muncpi+OAG	0.37	2.71	0.9972	94
Muncpi+db-cAMP	0.35	2.82	0.9933	93
Muncpi scramble	0.31	3.20	1.0000	92
Muncpi scramble+OAG	1.82 **	0.55	1.0000	95
Muncpi scramble+db-cAMP	1.53 **	0.65	0.9997	97

Table 1: Statistical comparisons of sealing rate (k, min^{-1}) or exponential time constants (τ, min) for transected neurites of B104 cells. R^2 >50μm represents goodness of fit values obtained from a single exponential model fitted to sealing probability at all PC times for control sealing or a given treatment.

Simultaneous inhibition of nPKC η and θ does not further decrease sealing

Because 7μM ηPSF or 9μM θPSF (inhibitors of nPKC η and θ, respectively) individually decreased, but did not completely eliminate sealing, we hypothesized that simultaneous inhibition of nPKCη+nPKCθ, with 7μM ηPSF+9μM θPSF would further decrease plasmalemmal sealing compared to either inhibitor alone. We first confirmed that B104 cells transected in 7μM ηPSF sealed at a decreased rate ($p > 0.05$ FZT) and probability ($p \leq 0.001$, CMH χ^2) at all PC times, compared to control sealing (**Fig. 4B; Table 1**), as reported by Spaeth et al (2010). We next observed that B104 cells transected in 7μM ηPSF+9μM θPSF sealed at a significantly ($p \leq 0.001$, CMH χ^2) decreased

probability at 2.5, 5 and 20 minutes PC and significantly ($p \leq 0.05$, FZT) increased rate compared to control sealing (**Fig. 4B; Table 1)**. However, cells transected in 7μM ηPSF+9μM θPSF sealed at a significantly ($p \leq 0.001$, CMH χ^2) decreased probability at 20 minutes PC compared to cells transected in 7μM ηPSF, but *not* other PC times (**Fig. 4B**). Furthermore, cells transected in 7μM ηPSF+9μM θPSF sealed at a significantly ($p \leq 0.05$, FZT) faster rate of sealing compared to cells transected in either 7μM ηPSF or 9μM θPSF (**Table 1**). That is, sealing was *not* further decreased by simultaneous inhibition of nPKC η and θ compared to either inhibitor acting alone, suggesting that nPKC η and θ likely target the same substances in sealing pathways.

nPKC η and/or θ act downstream of DAG

Since DAG-dependent sealing requires nPKC η and θ, we hypothesized that the decrease in sealing due to inhibition of nPKCs singly, or in combination, would not be affected by addition of OAG if nPKC η and/or θ act downstream of DAG. B104 cells transected in ηPSF+100μM OAG or, 9μM θPSF+100μM OAG did not seal at a significantly different rate ($p > 0.05$, FZT) or probability ($p > 0.05$ CMH χ^2) at any PC time compared to control sealing (**Fig. 4C; Table 1**). B104 cells transected in 7μM ηPSF+9μM θPSF+100μM OAG sealed at a significantly decreased probability ($p > 0.05$ CMH χ^2), but not rate ($p > 0.05$, FZT) at all PC times compared to control sealing (**Fig. 4D; Table 1**). Furthermore, 7μM ηPSF+9μM θPSF+100μM OAG had no effect on rate ($p > 0.05$, FZT) or probability ($p > 0.05$ CMH χ^2) of sealing compared to 7μM ηPSF+9μM θPSF (compare open circles, **Fig. 4B** to filled circles, **Fig. 4D**). These data

suggest that nPKC η and θ act downstream to DAG in the DAG-dependent sealing pathway.

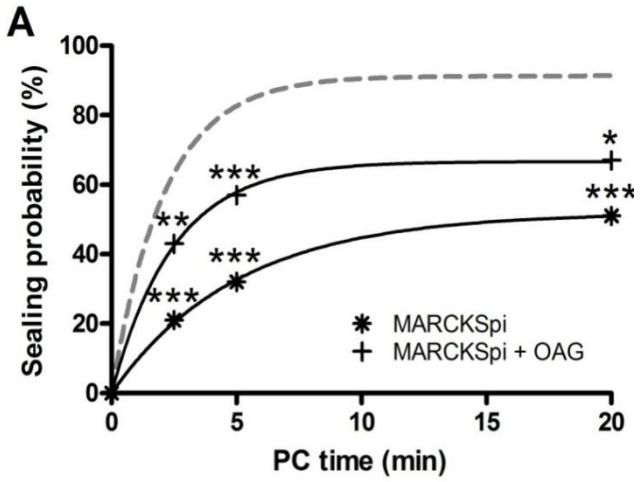
Inhibition of other DAG-dependent proteins decrease sealing

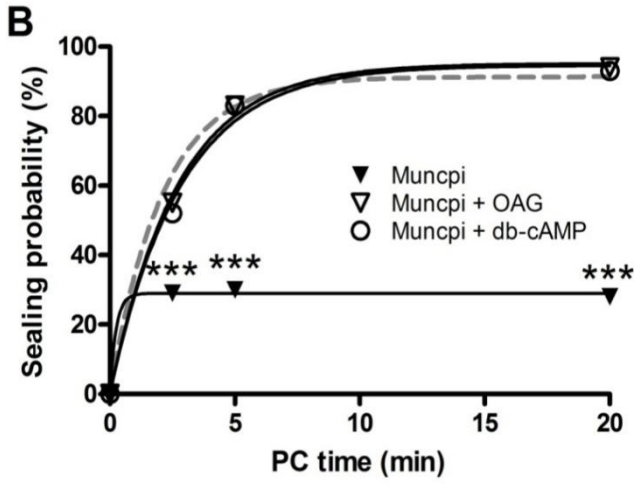
Inhibition of MARCKS decreases sealing

In neurons, cPKC- and nPKC-dependent phosphorylation of MARCKS increases PIP2 availability for hydrolysis by PLC, thus increasing DAG concentration (Laux et al. 2000). Therefore, we hypothesized that inhibiting MARCKS with 100μM MARCKS peptide inhibitor (MARCKSpi; Agrawal et al. 2007), would decrease plasmalemmal sealing. B104 cells transected in 100μM MARCKSpi sealed at a significantly decreased probability ($p \leq 0.001$, CMH χ^2) at 2.5, 5 and 20 minutes PC, but not rate ($p > 0.05$, FZT), compared to control sealing (**Fig. 5A; Table 1**). These data suggest that MARCKS promotes sealing of transected B104 cells and acts upstream of DAG.

Inhibition of Munc13-1 decreases sealing

Munc13-1, a synaptic fusion protein (Basu et al. 2007), requires DAG for activation (Brose and Rosenmund, 2002). Because a DAG analog and DAG-activated nPKC η and θ promote plasmalemmal sealing (**Figs. 2B, 4B**), we hypothesized that inhibiting Munc13-1 with a peptide (Mochida et al. 1998; referred to as, Muncpi) would decrease plasmalemmal sealing. B104 cells transected in 10 μM Muncpi sealed at a significantly decreased probability at 2.5, 5, and 20 minutes PC ($p \leq 0.001$, CMH χ^2), but not rate of sealing ($p > 0.05$, FZT), compared to control sealing (**Fig. 5B; Table 1)**.





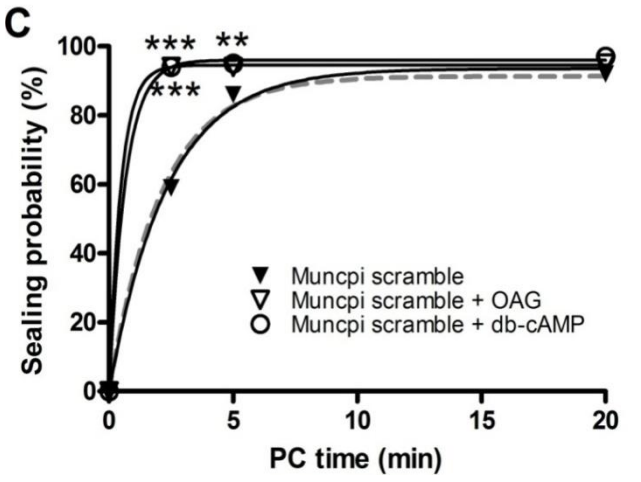


Figure 5: Sealing Probability (%) vs. PC time (min):

(A) Effect of 100μM MARCKSpi; 100μM MARCKSpi+100μM OAG on sealing.
(B) Effect of 10μM Muncpi, 10μM Muncpi+100μM OAG, 10μM Muncpi+1mM db-cAMP on sealing.
(C) Effect of 10μM Muncpi scramble, 10μM Muncpi scramble+100μM OAG, 10μM Muncpi scramble+1mM db-cAMP on sealing.

As a control for non-specific effects of Muncpi, we transected B104 cell neurites in 10 μM Muncpi with amino acids in a different order (termed "Muncpi scramble") (Mochida et al. 1998), and assessed dye exclusion at various PC times. B104 cells transected in 10 μM Muncpi scramble did not seal at a significantly different rate ($p > 0.05$, FZT) orprobability ($p > 0.05$, CMH χ^2) compared to control sealing (**Fig. 5C; Table 1)**. These data suggest that Munc13-1 promotes sealing of transected B104 cells.

OAG only partially reverses inhibition of MARCKS

We hypothesized that MARCKS acts upstream of DAG during plasmalemmal sealing, and thus increasing DAG would only partly overcome the effects of MARCKS inhibition. To test this hypothesis, we transected cells in 100μM MARCKSpi+100μM OAG. These cells sealed at a significantly increased probability at 2.5, 5 and 20 minutes PC ($p \leq 0.001$, $p \leq 0.001$ and $p \leq 0.05$ CMH χ^2), but not rate ($p > 0.05$, FZT), compared to 100μM MARCKSpi alone (**Fig. 5A; Table 1**). However, B104 cells transected in 100μM MARCKSpi+100μM OAG sealed at a significantly decreased probability at 2.5, 5 and 20 minutes PC ($p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.05$ CMH χ^2), but not rate ($p > 0.05$, FZT), compared to control sealing (**Fig. 5A; Table 1**). These data demonstrate that OAG cannot restore sealing to control levels when MARCKS is inhibited, suggesting that MARCKS acts upstream of DAG.

OAG restores sealing to control levels when Munc-13 is inhibited

Since Munc13-1 is one of many redundant fusion proteins used for DAG-dependent plasmalemmal sealing, we hypothesized that exogenously increasing DAG would increase sealing to control levels when Munc-13 is inhibited by 10 μM Muncpi. We observed no significant difference in rate ($p > 0.05$, FZT) or probability ($p > 0.05$, CMH χ^2) of sealing at any PC time compared to control sealing when B104 cells were transected in 10 μM Muncpi+100μM OAG (**Fig. 5B)**. An experimental control, we transected B104 cells transected in 10 μM Muncpi scramble+100μM OAG and observed that they had a significantly increased rate ($p \leq 0.01$, FZT) and probability of sealing at 2.5 and 5 min PC ($p \leq 0.001$, $p \leq 0.01$ CMH χ^2) compared to control sealing. These data suggest that Munc13-1 acts downstream of DAG and is one of many membrane-fusion proteins, such as syntaxin, SNAP-25, synaptobrevin, used by DAG-PKC-dependent sealing pathways to form a plasmalemmal seal (Spaeth et al. 2010, 2011).

OAG does not overcome inhibition of PKA

Inhibition of PKA by 50μM PKI decreases sealing in B104 cells (Spaeth, et al. 2010) and inhibiting DAG decreases sealing (**Figs.** 2**B, 5A**). To determine if PKA- and DAG-dependent sealing pathways are independent, we investigated if exogenously increasing DAG concentration could overcome PKI inhibition and return sealing to control levels. We first confirmed that B104 cells transected in 50μM PKI sealed at a significantly decreased probability ($p \leq 0.001$, CMH χ^2) at all PC times, but not rate ($p > 0.05$ FZT) of sealing, compared to control sealing (**Fig. 6A; Table 1**), as previously reported (Spaeth, et al. 2010), We then determined that B104 cells transected in 50μM

PKI+100μM OAG also sealed at a significantly decreased probability ($p \leq 0.001$, CMH χ^2) at all PC times, but not rate ($p > 0.05$ FZT), compared to control sealing (**Fig. 6A; Table 1**). However, cells transected in 50μM PKI+100μM OAG (open triangles in **Fig. 6A**) did not seal at a significantly different probability ($p > 0.05$, CMH χ^2) at any PC time, or rate or rate ($p > 0.05$ FZT) compared to cells transected only in 50μM PKI (**Fig. 6A**, open circles). These data suggest PKA acts downstream to DAG-PKC-dependent effects on sealing.

cAMP-dependent sealing is not independent of nPKC-dependent sealing

Another second messenger, cAMP, in addition to Ca^{2+}, also increases sealing of transected B104 neurites (Spaeth et al. 2010, 2011a,b) We hypothesized that db-cAMP, a cAMP analog, would increase plasmalemmal sealing to control levels in the presence of inhibition of nPKC. As an experimental control, we transected B104 cells in 1mM db-cAMP, and observed that they sealed at a significantly increased probability ($p \leq 0.001$, CMH χ^2) at 2.5 and 5 min PC, and rate ($p \leq 0.05$ FZT) compared to control sealing (**Fig. 2D; Table 1**). [These data correlate well with previously published data on the effects of 1mM db-cAMP on sealing in B104 cells (Spaeth et al. 2010).]

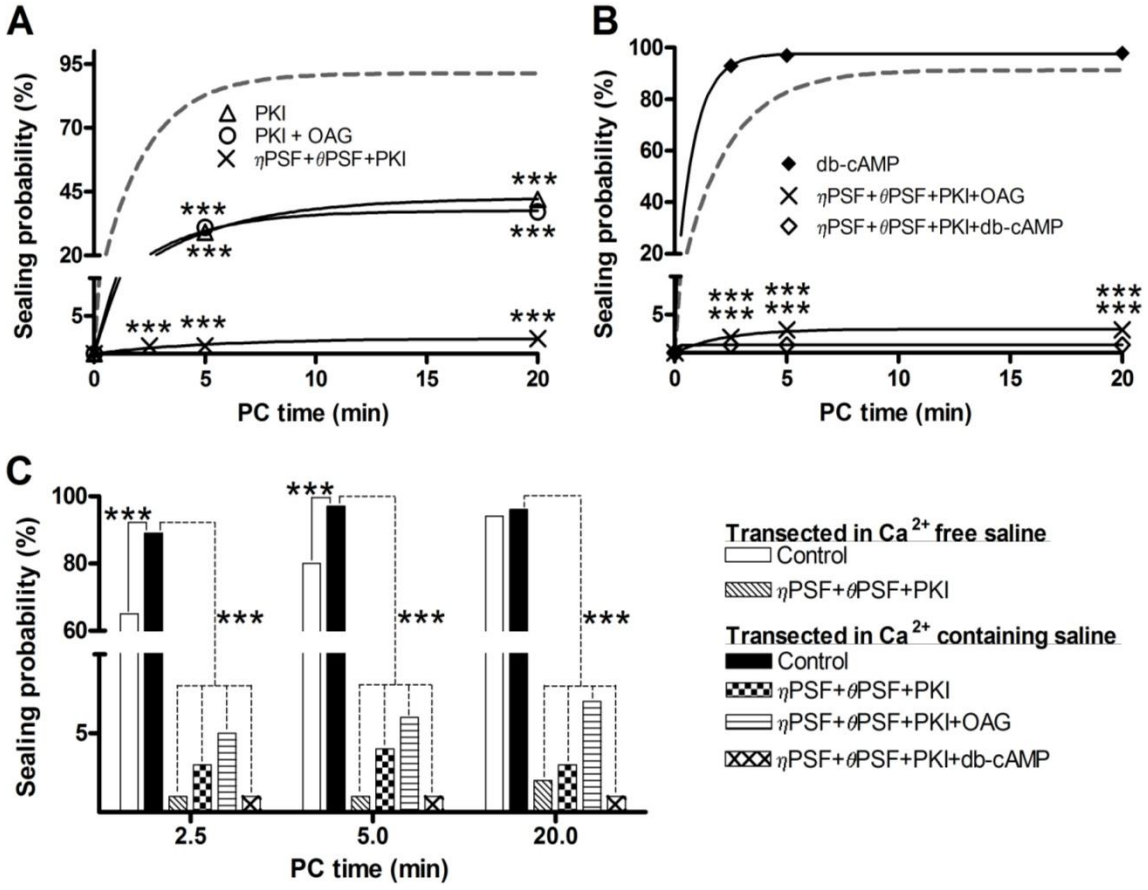


Figure 6: Sealing Probability (%) vs. PC time (min):

(A) Effect of 50μM PKI, 100μM OAG in 50μM PKI or, 7μM ηPSF +9μM θPSF+ 50μM PKI on sealing.
(B) Effect of 100μM OAG or 1mM db-cAMP in 7μM ηPSF + 9μM θPSF + 50μM PKI on sealing.
(C) Effect of performing transections in Ca^{2+}-containing saline in 7μM ηPSF + 9μM θPSF + 50μM PKI, 7μM ηPSF + 9μM θPSF + 50μM PKI +100μM OAG or 7μM ηPSF + 9μM θPSF + 50μM PKI + 1mM db-cAMP on sealing.
A-C: y-axes modified to better display low sealing probabilities.

However, B104 cells transected in 7μM ηPSF+1mM db-cAMP sealed at a significantly decreased probability ($p \leq 0.001$, CMH χ^2) at 2.5 min PC, but at any other point compared to control sealing.; the rate of sealing was no significantly different ($p > 0.05$ FZT) compared to control sealing (**Fig. 4C; Table 1**). B104 cells transected in 9μM θPSF+1mM db-cAMP sealed at a significantly decreased probability at 2.5, 5 and 20 min PC ($p \leq 0.01$, $p \leq 0.001$, $p \leq 0.001$ CMH χ^2, respectively) but, not rate ($p > 0.05$ FZT), compared to control sealing (**Fig. 4C; Table 1**). B104 cells transected in 7μM ηPSF+9μM θPSF+1mM db-cAMP had dramatically decreased ($p \leq 0.001$, CMH χ^2) sealing probability (≤ 12%) at all PC times, but not rate ($p > 0.05$ FZT) of sealing, compared to control sealing (**Fig. 4D**; Table 1). Furthermore, 7μM ηPSF+9μM θPSF+1mM db-cAMP dramatically decreased ($p \leq 0.001$, CMH χ^2) sealing probability at all PC times compared to 7μM ηPSF+9μM θPSF (compare open circles **Fig. 4B** to filled diamonds **Fig. 4D**). These data suggest that cAMP and nPKC affect at least some of the same pathways, i.e. cAMP-dependent sealing is not independent of nPKC-dependent sealing.

db-cAMP promotes sealing to control levels when Munc-13 is inhibited

If Munc13-1 is only one of many redundant fusion proteins used for plasmalemmal sealing, we hypothesized that exogenously increasing cAMP would increase sealing to control levels if Munch 13-1 were inhibited by 10 μM Muncpi. B104 cells transected in 10 μM Muncpi+1mM db-cAMP had no difference in rate ($p > 0.05$, FZT) or probability ($p > 0.05$, CMH χ^2) of sealing at any PC time compared to control

sealing (**Fig. 5B)**. For experimental controls, we performed the same protocols in 10 μM Muncpi scramble, instead of 10 μM Muncpi. B104 cells transected in 10 μM Muncpi scramble+1mM db-cAMP sealed at a significantly increased rate ($p \leq 0.01$, FZT) and probability of sealing at 2.5 and 5 min PC ($p \leq 0.001$, $p \leq 0.01$ CMH χ^2) compared to control sealing. These data suggest that Munc13-1 is one of many membrane-fusion proteins, such as syntaxin, or SNAP-25, used by cAMP-PKA-dependent sealing pathways to form a plasmalemmal seal (Spaeth et al. 2010, 2011).

Sealing requires nPKC η and θ, and PKA

Sealing persists following inhibition of nPKC η and/or θ (**Fig. 4A**) or PKA (**Fig. 6A**), providing additional evidence that sealing is promoted by redundant, parallel pathways, as previously suggested (Spaeth et al. 2010, 2011). To examine the possibility that DAG-PKC and PKA sealing pathways are redundant, we investigated if sealing would be further inhibited when simultaneously inhibiting both pathways compared to inhibiting only one. B104 cells transected in 7μM ηPSF+9μM θPSF+50μM PKI to inhibit both pathways sealed at a dramatically decreased ($p \leq 0.001$, CMH χ^2) probability (≤ 2%) at all PC times, but not rate ($p > 0.05$ FZT), compared to control sealing (**Fig. 6A; Table 1**). As a control, we verified that 7μM ηPSF+9μM θPSF+50μM PKI had no cytotoxic effects on intact, non-transected cells, i.e., intact cells did not include dye or show signs of membrane blebbing (data not shown). These data suggest that sealing requires nPKC η and θ, and PKA.

To further investigate whether sealing requires nPKC η and θ, and PKA.**,** we examined whether enhancers of sealing, OAG or db-cAMP (**Fig. 2B, D**), could promote sealing when nPKCη and θ and PKA were simultaneously inhibited to inhibit both pathways. B104 cells transected in 7μM ηPSF+9μM θPSF+50μM PKI+100μM OAG or, 7μM ηPSF+9μM θPSF+50μM PKI+1mM db-cAMP sealed at a dramatically decreased ($p \leq 0.001$, CMH χ^2) probability (≤ 5%) at all PC times compared to control sealing. Furthermore, B104 cells transected in 7μM ηPSF+9μM θPSF+50μM PKI+1mM db-cAMP sealed at a dramatically increased rate ($p \leq 0.001$ FZT) compared to control sealing (**Fig. 6B; Table 1**). B104 cells transected in 7μM ηPSF+9μM θPSF+50μM PKI+100μM OAG sealed at a rate ($p > 0.05$ FZT) not significantly different from control sealing (**Fig. 6B; Table 1**).

Because 7μM ηPSF+9μM θPSF+50μM PKI decreased sealing so dramatically, even in OAG or db-cAMP, we hypothesized that 7μM ηPSF+9μM θPSF+50μM PKI might inhibit sealing even when B104 cells were transected in Ca^{2+}-containing saline (PBS^{2+}) and 100μM OAG or 1mM db-cAMP. **Figure 5C** shows that sealing was immediately induced when B104 cells were transected in Ca^{2+}-containing saline. B104 cells transected in and PBS^{2+} containing either 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF, or 50μM PKI did not seal at a significantly different probability at 5 min PC compared to control sealing (see Online Resource 4). However, B104 cells transected in PBS^{2+} containing 7μM ηPSF+9μM θPSF+50μM PKI or,100μM OAG or 1mM db-cAMP, sealed at a significantly decreased ($p \leq 0.001$, CMH χ^2) probability (≤ 7%) at all PC times compared to control sealing (**Fig. 6C; Table 1**). Taken together, these data are

consistent with the hypothesis that nPKC η and θ and PKA are all normally required for sealing of B104 cells

OAG restores sealing to control levels when Golgi trafficking is inhibited

Inhibition of Golgi trafficking with Brefeldin A (Bref A) decreases plasmalemmal sealing in fibroblasts (Togo et al. 2003), rabbit epithelial corneal cells (Shen and Steinhardt, 2005) and B104 cells (Spaeth et al. 2011). Such decreased sealing can be partially overcome by activation of PKA (Spaeth et al. 2011). We hypothesized that inhibiting Golgi traffic would also inhibit PKC-DAG-dependent plasmalemmal sealing. That is, decreased sealing produced by Bref A inhibition of Golgi trafficking could be reversed by increased DAG concentrations. We first confirmed (Spaeth et al. 2010) that B104 cells transected in 10μM Bref-A to reduce Golgi trafficking had significantly decreased sealing probability ($p \leq 0.001$, CMH χ^2) at all PC times, but not rate ($p > 0.05$, FZT) of sealing, compared to control sealing (**Fig. 7A, Table 1**). In contrast, B104 cells transected in 10μM Bref-A+100μM OAG did not seal at a significantly different rate ($p > 0.05$, FZT) or probability ($p > 0.05$, CMH χ^2) at all PC times compared to control sealing (**Fig. 7A, Table 1**). These data suggest that OAG can overcome inhibition of Golgi-trafficking.

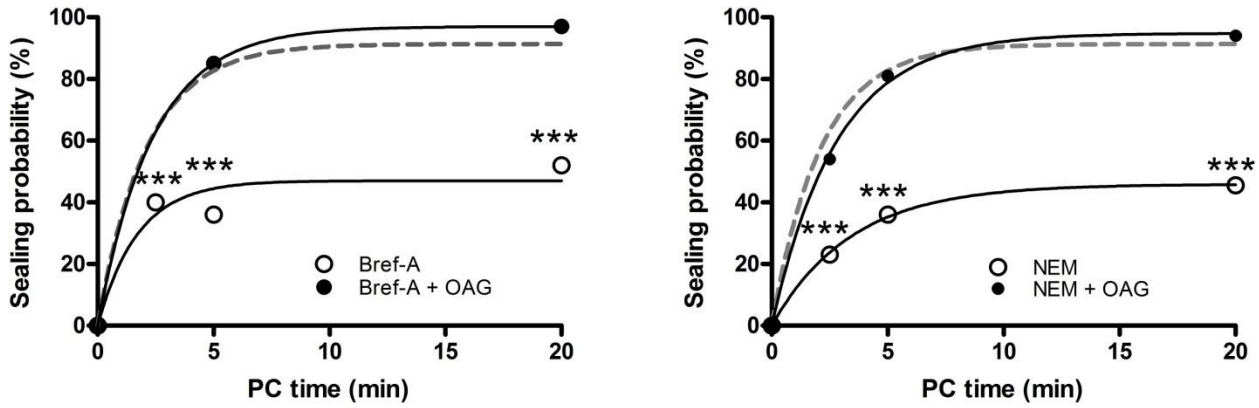


Figure 7: Sealing Probability (%) vs. PC time (min):

(A) Effect of 10μM Bref-A or 10μM Bref-A + 100μM OAG on sealing.
(B) Effect of 1mM NEM or 1mM NEM + 100μM OAG on sealing.

OAG restores sealing to control levels in presence of NSF inhibition

Because NSF inhibition cannot be overcome by cAMP-dependent activation of PKA, we reason that NSF is downstream of PKA activated substances (Spaeth et al. 2011). We hypothesized that PKC-DAG-dependent sealing would also require NSF similar to cAMP-dependent sealing, and therefore increased DAG concentration would not restore sealing when NSF was inhibited by NEM. We transected neurites in 1mM NEM (NSF inhibitor: Rodriguez et al. 1994) or, 1mM NEM+100μM OAG. B104 cells transected in 1mM NEM sealed at a significantly decreased probability ($p \leq 0.001$, CMH χ^2) at all PC times, but not rate ($p > 0.05$, FZT) of sealing, compared to control sealing (**Fig. 7B, Table 1**). In contrast, B104 cells transected in 1mM NEM+100μM OAG had no difference in rate ($p > 0.05$, FZT) or probability ($p > 0.05$, CMH χ^2) of sealing at any

PC time compared to control sealing(**Fig. 7B, Table 1**). These data suggest that DAG, unlike cAMP, acts downstream of NSF.

DISCUSSION

Summary of Results

Our present and previously reported data from B104 cells and data from many other eukaryotic preparations (see Supplementary Reference list in Spaeth et al. 2010) suggest that vesicle-mediated plasmalemmal sealing in eukaryotic cells, is an evolutionarily conserved process. This sealing is induced by Ca^{2+} influx at plasmalemmal damage sites (Fishman and Bittner, 2003) and enhanced by increased cAMP concentration (Spaeth et al. 2010) and DAG concentration and utilizes multiple vesicle-mediated pathways and membrane sources (Ballinger et al. 1997; Eddleman et al. 1997, 1998; Reddy et al. 2001; Spaeth et al. 2010). These pathways utilize both Ca^{2+}-dependent and Ca^{2+}-independent membrane fusion proteins, such as synaptobrevin, syntaxin, synaptophisin, synaptotagmin (Steinhardt et al. 1994; Detrait et al. 2000a,b; Yoo et al. 2003), Munc13-1 and, enzymes, such as PKA (Spaeth et al. 2010), nPKC η and θ for formation and maintenance of a plasmalemmal seal (**Fig. 8A, B**).

In this study we transected neurites of B104 cells in the presence of specific peptide inhibitors of PKC isozymes PKA, MARCKS and Munc13-1 and small-molecule inhibitors of PKC, PLC, NSF and Golgi trafficking --sometimes in the presence of analogs of DAG or cAMP. Our data suggest that DAG increases sealing by activating MARCKS, and Munc 13-1, and that nPKCη, nPKCθ and PKA are all required to seal plasmalemmal damage (**Fig. 8**).

nPKCθ promotes sealing

B104 cells express cPKC α and β_1 and, nPKC η and θ isozymes (**Fig. 3**). DAG-activated nPKCθ, but not Ca^{2+}-activated cPKC isozymes, promotes plasmalemmal sealing of transected neurites (**Fig. 4A, B**). Our data also suggest that nPKCθ-dependent mechanisms influence sealing more than nPKCη-dependent mechanisms (Spaeth et al., 2010). For example: **1**) nPKCθ inhibition alone, cause a greater decrease in sealing compared to nPKCη inhibition (**Fig. 4B**), (**2**) combining nPKC θ and η inhibitors further decreases sealing compared to nPKCη inhibition alone, at 20 min PC, (**Fig. 4B**) and (**3**) combining nPKC θ and η inhibitors does *not* further decrease sealing compared to nPKCθ inhibition alone (**Fig. 4B**).

Endogenous DAG production is important for sealing

DAG is produced when PLC hydrolyzes PIP2. PLC is typically activated by G-protein coupled receptors but can also be activated by Ca^{2+}-induced calpain cleavage (Glass et al. 2002; Ray et al. 2003; **Fig. 8A**). Our data show that exogenously adding the membrane permeant DAG analog OAG (Ju et al. 2010) increases sealing probability and rate in a Ca^{2+}-dependent manner, but DAG cannot substitute for Ca^{2+} (**Fig. 2B, C**). Conversely, decreasing endogenous DAG production with the PLC inhibitor U73122 (Tanaka et al. 2003) decreases sealing probability (**Fig. 2B**). These data are consistent with previous reports that many vesicle-mediated processes such as axonal growth cone turning, transport of beta-1 integrins and neurite extension (Gatlin et al. 2006; Sivasankaran et al. 2004; Gatlin et al. 2006; Siskova et al. 2006; Tsai et al. 2007) require

c/nPKC isozymes (all of which require DAG for activation) and acute rises in local $[Ca^{2+}]_i$.

MARCKS is required for an increase in sealing produced by DAG

Previous studies suggest that nPKC-dependent phosphorylation of MARCKS unmasks PIP2 micro-domains, making PIP2 available for hydrolysis by PLC (Frey et al. 2000; Laux et al. 2000). We observe that inhibition of MARCKS by a peptide-specific inhibitor (that blocks PKC phosphorylation site) significantly decreases sealing, even in exogenously increased DAG concentration (**Fig. 5A**). These data suggest that MARCKS-PIP2 micro-domains are a crucial source of DAG that may promote and reinforce DAG-dependent plasmalemmal sealing in a feed-back loop. DAG production at a site of plasmalemmal damage might activate nPKC, which then phosphorylates MARCKS, freeing more PIP2 for PLC-dependent hydrolysis. Increased PIP2 would then increase intracellular [DAG], thereby further activating nPKC or other DAG-activated proteins, like Munc13-1 (**Fig. 8A**).

Previous studies also suggest that PKC-dependent phosphorylation of MARCKS increases DAG concentration along plasmalemmal leaflets, acting as a chemoattractant to direct DAG-activated proteins, such as PKC isozymes and PKC-associated vesicle traffic (Frey et al. 2000; Laux et al. 2000) to sites of increased DAG concentration. Hence, increased DAG concentration caused by PKC-dependent phosphorylation of MARCKS might direct PKC-dependent vesicles, such as those required for axonal growth cone turning, transport of beta-1 Integrins and neurite extension (Frey et al. 2000; Laux et al.

2000; Kolkova et al. 2000; Gatlin et al. 2006; Siskova et al. 2006; Sivasankaran et al. 2004; Tsai et al. 2007) to plasmalemmal damage sites in B104 cells via DAG-chemoattraction of nPKC isozymes (**Fig. 8B**).

DAG-activated fusion protein, Munc13-1, promotes sealing in B104 cells (Fig. 5B, C)

Inhibition of Munc13-1 decreases sealing.that can be overcome by exogenously increasing DAG or cAMP concentration (**Fig. 5B**). These data suggest that Munc13-1 promotes sealing in B104 cells, and that DAG-PKC and cAMP-PKA sealing pathways converge at Munc13-1. DAG-activation of Munc13-1 increases both Ca^{2+}-dependent and Ca^{2+}–independent membrane fusion events (Basu et al. 2007). Thus, DAG could influence the initial formation of a plasmalemmal seal by activating fusion proteins such as Munc13-1 to promote vesicle-vesicle, vesicle-membrane interactions as $[Ca^{2+}]$ increases at the site of plasmalemmal damage and those vesicle interactions could also help maintain the seal. Munc13-1 could also promote sealing through Doc2α binding region interactions with SNAREs, such as syntaxin (Mochida et al. 1998).

nPKCη, nPKCθ and PKA are all required for sealing (Figs. 4C-E; 6A-C)

Previously published data show that sealing is promoted by a cAMP-PKA dependent pathway (Spaeth et al. 2010). Our data show that exogenously increasing cAMP concentration increases sealing that can be decreased by inhibitors of nPKCη (**Fig. 4C**), but not nPKCθ (**Fig. 4C**) or nPKCη+nPKCθ (**Fig. 4D**). Sealing probability is further decreased (compared to nPKCη+nPKCθ) by exogenously increasing cAMP

concentration, as in PKCη+nPKCθ (**Fig. 4D**). Together, these data (**Fig. 4C, D**) suggest that DAG-PKC and cAMP-PKA sealing pathways may interact in a feedback loop. That is, a decrease in nPKC in increased cAMP has negative feedback on PKA-dependent sealing pathways (**Fig. 8A, B**). This negative feedback loop would decrease the activity of sealing pathways not blocked by nPKC inhibitors, such as PKA, and would further decrease sealing compared to ηPSF+θPSF alone (compare open circles **Fig. 4B** to filled diamonds **Fig. 4D**).

Exogenously increasing DAG concentration in nPKCη+nPKCθ (**Fig. 4D**) or PKA (**Fig. 6A**) inhibition does not increase sealing. Furthermore, simultaneous inhibition of nPKCη, nPKCθ and PKA greatly reduces sealing probability to ≤7% at all PC times (**Fig. 6A**), even when cells are transected in Ca^{2+} (to induce sealing immediately after neurite transection) and the sealing enhancers OAG or db-cAMP (Spaeth et al. 2010) (**Fig. 6B, C**). Taken together, these data (**Fig. 6A-C**) strongly suggest that sealing in B104 cells requires nPKCη, nPKCθ and PKA. These data (**Fig. 6A-C**) suggest that the combination of ηPSF+θPSF+PKI very likely limits nearly all pathways of sealing, such as endocytosis and trafficking of vesicles to the damage site, and that the remaining sealing pathways are DAG and cAMP independent.

DAG-dependent sealing influenced by Golgi-derived vesicle traffic, but not NSF

Exogenously increasing DAG concentration promotes sealing to control, but not OAG-enhanced levels in the presence of Brefeldin-A (**Fig. 7A**), a potent inhibitor of Golgi-derived vesicle traffic. These data suggest that, like cAMP-PKA dependent

sealing (Spaeth et al. 2010, 2011), DAG-dependent sealing is enhanced by Golgi-dependent traffic, and that OAG can partially overcome inhibitory effects of Brefeldin A by recruiting vesicles from nPKC- and MARCKS-dependent sources. Alternatively, OAG may also increase the ability of a more limited supply of vesicles to fuse, thereby enhancing sealing in Bref A. Since Brefeldin A may also inhibit activity of the cAMP activated guanine exchange factor Epac (Spaeth et al. 2011), these data also suggest that sealing initiated by DAG does not strongly depend on Epac.

Exogenously increasing DAG concentration promotes sealing to control levels in the presence of NEM (**Fig. 7B**), a potent inhibitor of NSF. These data suggest that, unlike cAMP-PKA dependent sealing (Spaeth et al. 2010, 2011), DAG-PKC dependent sealing is not greatly influenced by NSF and that components of DAG-PKC dependent sealing work downstream of NSF. For example, these data suggest that DAG-activated fusion proteins, such as Munc13-1, which primes vesicles and is involved in membrane fusion (Basu et al. 2007), likely acts downstream of NSF.

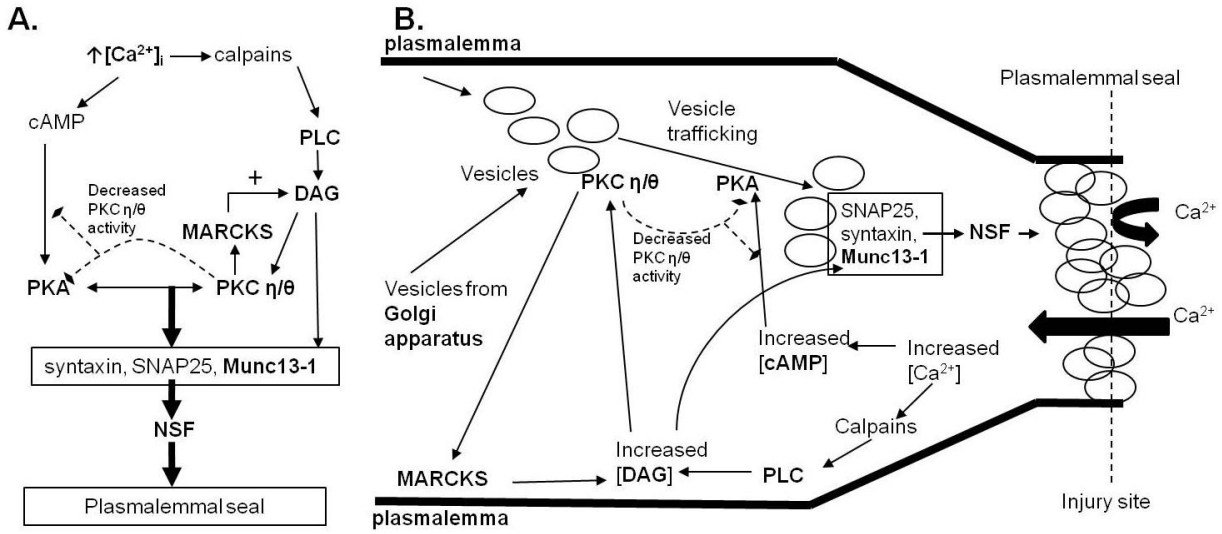


Figure 8: Plasmalemmal sealing, an evolutionarily conserved process:

(A) Flow chart of Ca^{2+} induced plasmalemmal sealing including PLC, nPKCs, MARCKS, DAG and Munc13-1, cAMP, PKA and membrane-fusion proteins that have been shown to promote sealing.
(B) Model of Ca^{2+} induced plasmalemmal sealing including PLC, nPKCs, MARCKS, DAG and Munc13-1 (See in References: Steinhardt et al. 1994; Godell et al. 1997; Detrait et al. 2000a,b; McNeil and Terasaki, 2001; Shen and Steinhardt, 2005; Togo et al. 2003, 2004, 2006; Spaeth et al. 2010).

SIGNIFICANCE

In this study, we describe what percentage of cells seal at a given PC time (sealing probability) and how quickly (sealing rate) the population of transected cells progresses to a maximum sealing probability for a given treatment. The high R^2 Goodness of Fit values in Table 1 suggest that various treatments can affect both probability and rate of sealing. Our data are consistent with many mechanisms that could affect rate and probability of sealing, e.g. substrate limitation or allosteric changes in proteins.

Our data show that PKCs and DAG influence plasmalemmal sealing in neurons *in vitro*. Such insights have direct and indirect implications for axonal regeneration after traumatic injury (Bozkurt 2008; Bittner et al, 2012) and for stroke in which neuronal membranes are damaged (Rodriguez-Sinovas et al. 2005). For example, cells that do not seal plasmalemmal damage, do not survive (Nguyen et al. 2005). Therefore, compounds that enhance endogenous sealing (OAG or db-cAMP) may be capable of rapidly promoting sealing of neurons damaged by traumatic injury or stroke, thereby greatly diminishing loss of behavioral function correlated with neurotrauma and stroke. As a second example, compounds that inhibit endogenous sealing (nPKC or PKA peptide inhibitors) may be capable of enhancing polyethylene glycol (PEG) induced fusion of plasmalemmas, thereby greatly increasing the already profound effects of PEG on recovery of physiological and behavioral functions of crushed and/or severed peripheral nerves (Bittner et al. 2012). Thus, a greater knowledge of proteins and substrates that influence plasmalemmal repair (sealing) *in vitro*, should provide targets and substrates to improve neuroprotective treatment modalities.

MATERIALS AND METHODS

B104 cells:

B104 cells derived from a CNS neuroblastoma (Bottenstein and Sato, 1979) have often been used as a model system to study neuronal function in vitro (Yoo et al. 2003; Nguyen et al. 2005; Miller et al. 2006; Spaeth et al. 2010). B104 cells extend neurites that have properties typical of nerve axons such as generation of action potentials, smooth (but not rough) endoplasmic reticulum, release of neurotransmitter, and regeneration of severed neurites. These cells have easily identifiable cell bodies and neurites, allowing precise identification of individually transected cells at their injury site (Detrait et al. 2000b; Yoo et al. 2003, 2004; Spaeth et al. 2010). Unlike some other model neuronal cell lines (e.g., PC12 cells), B104 cells do not require growth factor supplements of fetal bovine serum to proliferate (Detrait et al. 2000b; Yoo et al. 2003; Spaeth et al. 2010). Data on sealing of B104 cells are consistent with similar data on sealing from at least 20 other preparations from many phyla and different cell types (see supplemental reference list in Spaeth et al. 2010).

Cell culture:

As described previously (Nguyen et al. 2005; Spaeth et al. 2010), B104 cells were grown in 75 cm^2 vented cap flasks (BD Falcon; BD Biosciences) in a humidified incubator at 37°C in 5% CO2 and in 4 ml of cell growth medium: consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM:F-12, HyClone) supplemented for growth with 10% heat-inactivated fetal bovine serum (FBS; HyClone)

and 1% antibiotics (10,000 U of penicillin/ml and 10 mg/ml streptomycin; Sigma-Aldrich). The cell growth medium was changed every 2 d. Cultures were passaged at 80% confluency, and cells were then either subcultured in a vented cap flask or seeded at ~2000 cells/cm ^{2} in cell growth media on Petri dishes coated with poly-D-lysine (Sigma-Aldrich) to prevent cells from detaching during solution changes and/or neurite transection. After 24 h, the growth media was replaced with serum-free DMEM:F12 (Hyclone) to allow the B104 cells to differentiate. B104 neurites were transected 24–48 h after replacing the cell growth media with serum-free DMEM:F12.

Transection of neurites of B104 cells:

Before transecting neurites, the solution (DMEM:F12) in the Petri dish was washed out twice with a Ca^{2+}-free phosphate-buffered saline (referred to as "Ca^{2+}-free saline," PBS=; HyClone). All neurites were transected in Ca^{2+} -free saline at a distance >50 μm from the soma using a sharpened, pulled glass, microcapillary tube ("micro-knife") that was placed on a micromanipulator (Narishige Instruments) and quickly drawn across the surface of the Petri dish, etching a score line that showed the path of the micro-knife. In all experiments, 3 kDa fluorescein dextran (Invitrogen) was added to the Ca^{2+}-free saline during transection of B104 cell neurites to label cells transected by the microknife. As described previously (Yoo et al. 2004), only B104 cells with transected neurites uptake fluorescein dye, therefore we were able to uniquely and individually identify each B104 cell with a transected neurite by fluorescein dextran fluorescence and the relation of the transected cell/neurite to the score mark on the plate.

Microscopic observations of dye exclusion:

For all experiments, B104 cells were observed under an inverted Zeiss ICM-405 fluorescent microscope with a 40X long focal distance lens. Cells with neurites obviously transected >50 μm from the soma were observed for fluorescein dextran uptake and Texas Red dextran in/exclusion. No further observations were made on transected cells that did not uptake fluorescein dextran.

Assessment of plasmalemmal sealing:

As described previously (Spaeth et al. 2010), we used the most reliable measure of plasmalemmal repair (dye exclusion) to assay plasmalemmal sealing (Blanchette et al. 1999; Detrait et al. 2000a,b). To assess plasmalemmal repair, we first transected 10–130 cells in Ca^{2+}-free saline containing fluorescein dye within 10 min. The Ca^{2+}-free saline was then replaced with a phosphate-buffered saline containing 1 mM Ca^{2+} ("Ca^{2+}-saline," PBS^{2+}; HyClone) to initiate the sealing process (Detrait et al. 2000b; Yoo et al. 2003; Spaeth et al. 2010). The time elapsed after exposing cells to Ca^{2+}-saline is defined as the "post- Ca^{2+} addition time" (PC time). Thus, all cells on a given Petri dish have the same PC time. At various PC times (0–20 min PC), 3 kDa Texas Red dextran (Invitrogen) was added to the Ca^{2+}-saline. After a 10 min exposure, Texas Red dextran was thoroughly washed out with Ca^{2+}-saline, and then sealing was assessed. Transected cells (damaged neurite, green fluorescence) that excluded Texas Red dextran were counted as "sealed". Cells that did not exclude Texas Red dextran were counted as "not sealed". Nearby undamaged cells never took up Texas Red dextran**.** As stated previously (Spaeth et al.

2010), we consistently used 3 kDa Texas Red dextran to assess sealing in all experiments to decrease variability in dye exclusion assessments because, fluorescent dyes of higher molecular weight are excluded faster than lower molecular weight dyes (Detrait et al. 2000a; Yoo et al. 2003), which are excluded faster than ions having even lower molecular weights than the smallest dye (Krause et al. 1994; Eddleman et al. 2000).

Pharmacological reagents:

We used pharmacological agents to quickly inhibit or activate a target protein to avoid complications of compensatory pathways that may result from gene knock-outs or chronic applications of pharmacological inhibitors (Steinberg et al. 2008). All pharmacological reagents were typically dissolved in Ca^{2+}-free saline and added to cells immediately before neurites were transected to backload cells with each reagent during transection. The following isozyme specific peptide inhibitors of PKC were purchased from EMD Chemicals, Gibbstown, NJ: myristoylated cPKC pseudosubstrate fragment 20-28 (8μM, 1.2 kDa) to inhibit cPKCα/β_1 (Liu et al. 2006; Jungnickel et al. 2007; Ratz and Miner, 2009), myristoylated nPKCη pseudosubstrate fragment (7 μM, 2 kDa) was used to inhibit nPKCη (Liu et al. 2006; Barros et al. 2009; Spaeth et al. 2010), myristoylated nPKCθ pseudosubstrate fragment (9 μM, 2.3 kDa) to inhibit nPKCθ (Staal et al. 2008; Barros et al. 2009). Bisindoylmaleimide I (100nM, 1μM and 10μM, 0.4 kDa; Sigma Aldrich) and Gö6976 (1μM, 0.3 kDa; Sigma Aldrich) were used to inhibit cPKC isozymes (Togo, 2004). We used U73122 (16 μM, 0.4 kDa; Sigma Aldrich) to inhibit phospholipase C (PLC) activity and for a negative control, we used the inactive analog of

U73122, U73343 (16 μM, 0.4 kDa; Sigma Aldrich) (Bleasdale et al. 1990; Hildebrandt et al. 1997; Tanaka et al. 2003). To increase diacylglycerol (DAG) activity, we exogenously applied the DAG analog, 1-Oleoyl-2-acetyl-sn-glycerol (100 μM; Cayman Chemicals, Ann Arbor, MI), referred to as OAG (Doherty et al. 1995; Wang and Oram, 2007; Ju et al. 2009). We used Mid-peptide (10 μM, 2.9 kDa, Genscript, Piscataway, NJ) to inhibit Munc13-1 and scrambled Mid-peptide (10 μM, 2.9 kDa) as a control (Mochida et al. 1998). We used BIOMARCKS-11006 peptide inhibitor (100 μM; a gracious gift from Dr. Indu Parikh of BioMARCKS Pharmaceuticals, Durham, NC) to inhibit MARCKS (Agrawal et al. 2007; Damera et al. 2010). PKI (50 μM, 1.8 kDa; Sigma-Aldrich; a generous gift from Drs. Michael Markham and Harold Zakon, University of Texas, Austin, TX) was used to specifically inhibit PKA (Spaeth et al. 2010). Dibutyryl-cAMP (db-cAMP, 1 mM, 0.4 kDa; Sigma-Aldrich) was used to increase intracellular cAMP activity. We used N-ethylmaleimide (NEM, 1mM; Thermoscientific, Rockford, IL) to inhibit NSF (Rodriguez et al. 1994) and Brefeldin-A (Bref-A, 10 μM; Biomol/Enzo Lifesciences, Farmingdale, NY) to inhibit Golgi-derived vesicle traffic (Togo et al. 2003; Shen and Steinhardt, 2005).

Western Blotting:

As described previously (Spaeth et al. 2010), once cells in vented cap flasks reached 80% confluency, they were removed from the surface of the flask using 1% Trypsin (Sigma), and collected in a pellet by gentle (4000 rpm) centrifugation. The pellet was re-suspended in 500 μL M-Per mammalian protein extraction buffer (Fisher). A broad

protease cocktail inhibitor was added to prevent protein degradation (Fisher). Cells were gently shaken on a rotating table at 4˚ C for 30 minutes, and then vortexed for 1-2 minutes. Samples were denatured using Running Buffer/Lane marker (Fisher) and run on 4% stacking, 20% separating gels (Promega). Proteins were electro-transferred onto nitrocellulose membranes (Fisher). The membranes were pre-blotted with 5% BSA (Fisher) and probed with monoclonal PKCα or PKCθ or, polyclonal PKCβ_1 or PKCη primary antibodies (SantaCruz Biotechnologies). (AbCam). The blots were washed in TBST (0.1% Tween 20 in TBS) and incubated in goat anti-rabbit IgG-HRP at a dilution of 1:10,000. The blots were washed and developed with Super Signal (Pierce). Blue Ranger (Pierce) molecular weight standard was run on every gel in lanes 1 and 10.

Statistical analyses:

As described previously (Spaeth, 2010), we obtained data from individual, uniquely identified B104 cells that all received similar, well-specified, neurite transections as plasmalemmal injuries in a tightly controlled environment. For each treatment group, at a given PC time the data were pooled for all cells (n) from all Petri dishes (N). See **Appendix Table B**, for n and N values for each data set at each PC time point in all figures presented in this paper. "Sealing probability" is defined as the percentage of a set of individually-transected and uniquely identified cells that exclude 3 kDa Texas Red dye (sealed) at a given PC time. As described previously (Detrait et al. 2000b; Yoo et al. 2003, 2004; Spaeth et al. 2010), the Cochran-Mantel-Haenszel χ^2 (CMH χ^2) test for independence was used as the most appropriate statistical test to determine whether

differences between sealing probabilities at a given PC time for different experimental treatments were statistically significant ($p<0.05$). Briefly, the CMH χ^2 test for independence is used to compare data separated into two-by-two contingency tables (sealed or not sealed, and control vs. experimental data sets). Furthermore, the CMH χ^2 test requires binary outputs, which applies to our sealing assay (sealing is treated as a yes or no event at a given PC time). Because sealing of a given cell and not each Petri dish (sometimes containing over 100 transected cells) are independent events, measures of variance (such as SE or SD) are not applicable.

GraphPad Prism4 was used to curve-fit a one-phase exponential equation (Equation 1 below) to sealing probability data at all PC times for a given control or treatment (dashed and solid lines on all graphs, respectively) to obtain a "sealing rate". Sealing rate is the time it takes for a population of transected cells to reach the sealing maximum for a given treatment.

$$y(t) = Y_{max} * (1 - e^{-kt}), \qquad \text{(Equation 1)}$$

Equation 1: y(t) is the sealing probability (%) of cells examined at a given PC time (t), Y_{max} is the maximum plateau sealing probability (%) of cells for that control or treatment , e is Euler's constant, t is a given PC time (min), and k is the rate constant (min^{-1}) of a given exponential equation. The rate constant k is the reciprocal of the time constant (τ, min) defined as the elapsed PC time needed to achieve 63.2% (reciprocal of Euler's constant) of the observed maximum (plateau) sealing. The time constants were calculated according to Eq. 1 where $\tau = 1/k$. The maximum sealing probability (plateau) was always reached within 20 min PC. Sealing time constants or rate constants for

controls and treatments were normalized and compared by using Fisher's Z transformation (FZT). Two sealing constants were considered significantly different if p was < 0.05, using a Z table. Goodness of fit (R^2) values were obtained from GraphPad Prism4 to determine how closely the exponential equations modeled the observed data (see **Table 1**).

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Chapter 3: Application of nPKC and PKA inhibitors to crush-severed sciatic axons, immediately following crush in physiological calcium concentration, do not increase probability of PEG-fusion (immediate axonal repair)

INTRODUCTION

One of the most common traumatic peripheral nerve injuries in humans is peripheral nerve crush injury (**Fig. 9**). Typically caused by compression (**Fig. 9C**) of surrounding tissues upon the nerve, that sometimes completely severs axons (**Fig. 9D**) within the nerve, resulting in significant behavioral deficits (Campbell, 2008). Severed distal axonal segments in mammals undergo Wallerian degeneration within 1-3 days post-injury, compounding loss of functional behaviors. Recovery of functional behavior for denervated target tissues is a function of axonal regeneration. Outgrowths from surviving proximal axonal stumps (**Fig. 9D**) grow at 1-2mm/day, often only partially and non-specifically re-innervating distal target tissues. At this slow regeneration rate, it can take weeks-to-years before regenerating motor axons reach denervated and usually, atrophied, target tissues in large mammals, post-operatively (Ramon y Cajal, 1928; Bozkurt et al. 2008; Campbell, 2008; Lago et al. 2007; Kalbermatten et al. 2009).

Improving the rate and extent of mammalian axonal regeneration has been a major research goal for many neuroscientists. All repair strategies to date, including nerve grafts, nerve growth guides, and microsutures, have relied upon axonal outgrowths to re-innervate target tissues. Such techniques have improved the number and specificity of regenerating axons slightly, but never the time course of behavioral recovery (Lago et

al. 2007; Kalbermatten et al. 2009). Likewise, it has failed to prevent Wallerian degeneration of severed distal axons (Lore et al. 1999; Marzullo et al. 2002; Stavisky et al. 2005; Britt et al. 2010).

Following plasmalemmal damage, mammalian axons that fail to seal do not survive (Schlaepfer and Bunge, 1973; Detrait et al. 2000; Yoo et al. 2003; Nguyen et al. 2005). That is, sealing of plasmalemmal damage can be neuroprotective. Plasmalemmal sealing of small holes, as well as complete axonal transections in mammalian neurons and other eukaryotic cells is normally produced by a Ca^{2+}-induced accumulation of membrane-bound structures (mostly vesicles). These vesicles accumulate at damage sites, fusing to each other and nearby undamaged plasmalemma, by various protein isomers, many of which are Ca^{2+}-dependent and involved in membrane fusion at synapses or the Golgi apparatus, to seal the damage site (see Chapter 2, Introduction on Sealing; also reviewed in Spaeth et al. 2010).

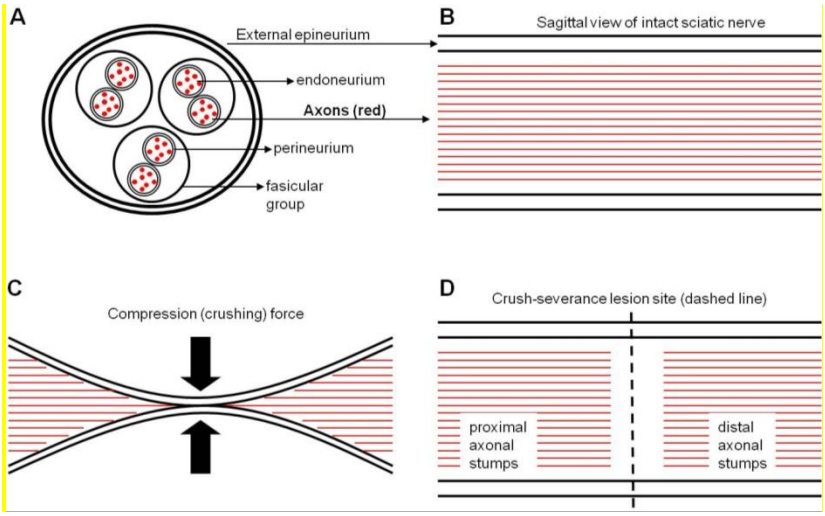

Figure 9: Simplified schematic of peripheral nerve crush injury.

(A) Cross section of sciatic nerve (peripheral nerve of leg in humans) showing general anatomy of nerve.
(B) Sagittal (lengthwise) section of sciatic nerve, simplifying axonal paths as perfectly parallel within fascicles.
(C) Depiction of force compressing external epineurium upon sciatic axons, crush-severing axons.
(D) Result of such a compression force is complete crush-severance of sciatic axons resulting in loss of axonal continuity, loss of conduction of action potentials and loss of functional behavior in target tissues.

In contrast to the slow and incomplete restoration of functional behavior of severed mammalian axons, many invertebrates demonstrate complete restoration of behavioral function within days after axonal severance. This is promoted by fusion of proximal outgrowths with surviving distal stumps or activation of distal stumps by closely apposed proximal outgrowths (Hoy et al. 1967; Bittner et al. 2000; Bittner and Fishman, 2000). Severed mammalian axons can be repaired within 1-2 minutes following injury using the membrane fusogen, polyethylene glycol (PEG) (Krause et al.

1991; Stavisky et al. 2005; Britt et al. 2010; Bittner et al. 2012). Indeed, polyethylene glycol (PEG) has been used since the 1970s by cell biologists to fuse various cell types and to make cell hybrids (Ahkong, 1987). PEG is an amphipathic molecule able to remove waters of hydration from membrane-bound proteins allowing adjacent lipid membranes to flow into one another and is thus used for rapid and permanent *in vivo* repair of severed peripheral nerves in mammals (Britt et al. 2010; Bittner et al. 2012).

Previous studies reported successful PEG-fusion of axons, especially those using mammalian nerve preparations, obtained by bathing the lesion site in hypotonic, Ca^{2+}-free physiological saline prior to PEG application (**Fig. 10B**). This is performed to reduce or blow out accumulations of vesicles directed to sites of axolemmal disruption and transection via Ca^{2+}-induced vesicle-mediated repair (**Fig. 10A, B**; Bittner et al. 2012; also see Chapter 2 for details on vesicle-mediated repair/sealing). Such leads to the hypothesis that, by reducing vesicle-mediated repair prior to PEG application, more closely apposed, severed axonal ends are rendered available for PEG-fusion, increasing the number of PEG-fused axons compared to application of PEG to already sealed or nearly-sealed axonal ends (**Fig. 10**). Following PEG application, isotonic, Ca^{2+}-containing physiological saline is applied to the lesion site to induce endogenous sealing mechanisms to "plug" any remaining disruptions in axolemmas (**Fig. 10D).** Such PEG-induced fusions restored morphological continuity and conduction of action potentials through the lesion site of invertebrate and vertebrate (rat) axons *in vitro* and *in vivo* (Krause, 1991; Marzullo, 2002; Stavisky, 2005). Application of PEG to crush-severed sciatic axons significantly increased the rate of functional behavior recovery in crush

injury animals that received PEG *versus* crush injury animals that received vehicle treatment *in vivo* (Britt et al. 2010).

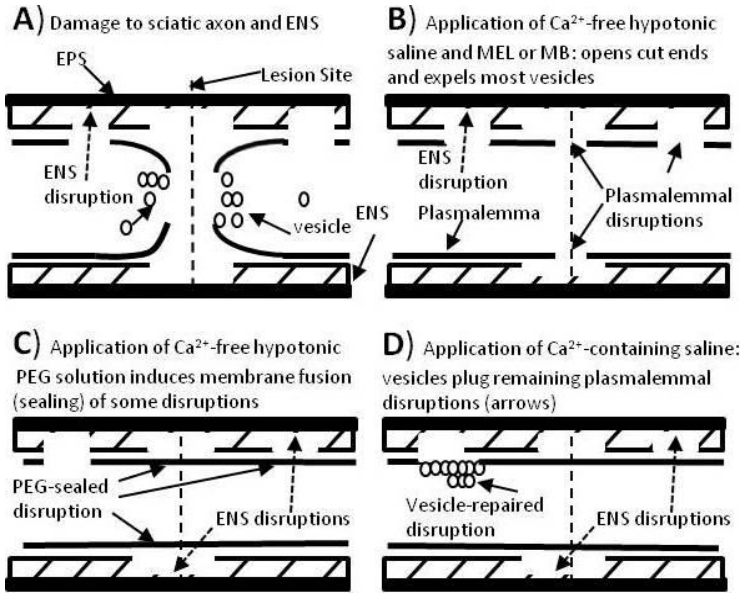

Figure 10: PEG-fusion protocol for crush-severance repair using Ca^{2+}-free hypotonic saline.

Hypothesized effects of each solution used in sequence to produce PEG-fusion of crush-severed *axons*. Crush-severance completely disrupts the axolemma and the endoneurial sheath (ENS) at the lesion site, but leaves the epineurial sheath (EPS) intact, ensuring that crush-severed ends remain in close apposition. MEL: melatonin, MB: methylene blue, PEG: polyethylene glycol. Concentrations: 2mM MEL, 100µM MB, 500mM 2kDa PEG (Stavisky et al. 2005, Britt et al. 2010; Spaeth et al. 2011b; Bittner et al. 2012).

Recently, we began to test whether or not compounds that retard *in vitro* plasmalemmal sealing in B104 cells, can be used to enhance PEG-fusion (using the protocol in Figure 3.2) and to promote greater behavioral recovery in models of peripheral nerve cut- and crush-severance injury in rats (Bittner et al. 2012). The antioxidant methylene blue (MB) has been reported to decrease plasmalemmal sealing in B104 cells *in vitro* (Spaeth et al. 2012) and to improve behavioral recovery slightly

following optical (retinal) trauma (Zhang et al. 2006; Rojas et al. 2009). MB application in Ca^{2+}-free hypotonic physiological saline to crush- or cut-severed rat sciatic axons, prior to application of PEG, dramatically improved PEG-fusion induced behavioral recovery, *in vivo* (**Fig. 10B**; Bittner et al. 2012).

The aforementioned treatments (PEG and MB+PEG) produced restoration of morphological continuity, electrophysiological conduction, and behavioral function following crush- or cut-severance of invertebrate and vertebrate axons when axonal damage was performed in hypotonic, Ca^{2+}-free physiological saline *in vitro*, *ex vivo* and *in vivo*. However, it has yet to be tested whether any treatment in conjunction with PEG is capable of increasing the probability of PEG-fusion following crush-severance in physiological calcium concentration, as occurs to human neurotrauma patients, whose tissues and interstitial fluids are neither hypotonic nor void of calcium at time of injury.

Thus, we hypothesize that application of compounds or combinations of compounds that greatly reduce plasmalemmal sealing in B104 cells *in vitro* (such as, ηPSF+θPSF+PKI from Chapter 2) to crush-severed axons *in vivo*, should be capable of increasing the probability of PEG-fusion following *crush-severance in physiological calcium concentration*. Dye exclusion (as previously described in Chapter 2) was used to assess the effect of combinations of compounds and PEG on endogenous sealing in B104 cells *in vitro*. We studied restoration of electrophysiological and morphological continuity of crush-severed axons by measuring conduction of compound action potentials and dye diffusion across crush-lesion sites *in vivo* and *in vitro*, respectively (Britt et al. 2010).

In this chapter, I report that only the inhibitor combination of ηPSF+θPSF+PKI was capable of decreasing plasmalemmal sealing of B104 cells transected in calcium *in vitro*. One-minute PEG application always sealed transected B104 cells, even in inhibitors of nPKCs and PKA (individually or in combination) or in Ca^{2+}-free saline *in vitro*. However, ηPSF+θPSF+PKI+PEG treatment proved to be cytotoxic for both intact and damaged (neurite transected) B104 cells.

As predicted, PEG application restored morphological continuity and electro-physiological conductivity to crush-severed sciatic axons only in treatment groups in which the crush was performed in Ca^{2+}-free hypotonic physiological saline. Dye diffused throughout crush-severed sciatic nerves receiving ηPSF+θPSF+PKI (with or without PEG) treatment at the crush-lesion site, subsequent to crush-severance in Ca^{2+}-containing physiological saline. Suggesting such treatment was capable of inhibiting endogenous sealing in crush-severed sciatic axons *in vivo*. However, no treatment was capable of increasing the probability of PEG-fusion of sciatic axons crush-severed in Ca^{2+}-containing physiological saline *in vivo*. The weight of our B104 cell and sciatic nerve data suggest that nearly-complete inhibition of endogenous axonal sealing via ηPSF+θPSF+PKI does not increase the probability of PEG-fusion of sciatic axons crush-severed in Ca^{2+}-containing physiological saline *in vivo*. Moreover, these data strongly suggest that endogenous axonal sealing is neuroprotective and that almost completely inhibiting sealing at the time of injury may have long-lasting negative effects on axonal viability and therefore, regeneration after such treatments.

RESULTS

Effect of inhibitors of nPKC and PKA on sealing of transected B104 cell neurites *in vitro*

To determine if application of PKC and PKA inhibitors following crush in physiological Ca^{2+} increases the probability of *in vivo* PEG-fusion of crush-severed sciatic axons, we first determined the effects of these compounds, sometimes in combination with PEG, on plasmalemmal sealing of B104 cell neurites transected in Ca^{2+}-free or Ca^{2+}-containing (1mM) saline, *in vitro*. Neurites of individually identifiable B104 cells were transected >50 μM from their soma in Petri dishes *in vitro* (see Methods and Spaeth et al. 2012). (see Methods). Sealing probabilities, defined in Methods as the percent of B104 cells that excluded 3kDa Texas Red dextran for different treatments were compared using a Cochran-Mantel-Haenszel χ^2 test (CMH χ^2) (Spaeth et al. 2010, 2012). [We used 10mM PEG for all B104 cell *in vitro* experiments because this concentration was previously determined as the lowest effective concentration that provided maximal PEG-sealing (Spaeth et al. 2012).]

Only simultaneous inhibition of nPKC η and θ and PKA decreases sealing of neurites transected in Ca^{2+}

For experimental controls we demonstrated, again, that inhibition of nPKC η or θ, or PKA decreases endogenous plasmalemmal sealing of transected B104 cells (**Fig. 11A**). B104 cells transected in 7μM nPKCη pseudosubstrate fragment (ηPSF), 9μM θPSF, 7μM ηPSF+9μM θPSF, 50μM PKI, 7μM ηPSF+9μM θPSF+50μM PKI sealed at a

significantly ($p \leq 0.001$) decreased sealing probability at 5 min PC compared to control sealing (**Fig. 11A**).

To determine if these compounds immediately inhibit sealing, we repeated the above experiments with the exception that we transected neurites of individually identifiable B104 cells in Ca^{2+}-containing saline (to i induce immediate sealing following transection) and assessed sealing at 5 min PC (**Fig. 11B**). B104 cells transected in Ca^{2+} or Ca^{2+} and either 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF or 50μM PKI sealed at a significantly ($p \leq 0.001$, CMH χ^2) increased sealing probability compared to Ca^{2+}-free, but not Ca^{2+}-containing control sealing (**Fig. 11B**). B104 cells transected in Ca^{2+} and 7μM ηPSF+9μM θPSF+50μM PKI sealed at a significantly ($p \leq 0.001$, CMH χ^2) decreased sealing probability compared to Ca^{2+}-free and Ca^{2+}-containing control sealing (**Fig. 11B**). These data suggest that simultaneous inhibition alone of nPKC η and θ and PKA decreases endogenous sealing when B104 cell neurites are transected in calcium.

In combination with PEG, simultaneous inhibition of nPKC η, θ and PKA is cytotoxic to B104 cells in vitro

In order to determine if inhibitors of nPKC η, θ and PKA could increase the probability of PEG-fusion following crush in calcium *in vivo*, we investigated if inhibition of nPKC η, θ and PKA had any effect on PEG-induced sealing of B104 cell neurites transected in Ca^{2+}-containing saline, *in vitro*. First, we noted the effect of PEG alone on endogenous plasmalemmal sealing by transecting B104 cell neurites in Ca^{2+}-free saline in 10mM PEG and assessed sealing at 5 min PC. To mimic our *in vivo* PEG-

fusion protocol, 10mM PEG was applied to cells for only one minute, i.e. PEG was applied to cells in the last minute of transection (Spaeth et al. 2012). B104 cells transected in 10mM PEG sealed with a significantly ($p \leq 0.001$, CMH χ^2) increased sealing probability compared to Ca^{2+}-free control sealing (**Fig. 11A, C**).

We next investigated the effect of nPKC and PKA inhibitors on PEG-induced sealing. B104 cells transected in 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF, 50μM PKI and 10mM PEG sealed at a significantly ($p \leq 0.001$, CMH χ^2) increased sealing probability compared to Ca^{2+}-free control sealing (**Fig. 11C**). Unexpectedly, all B104 cells (intact and damaged) exposed to 7μM ηPSF+9μM θPSF+50μM PKI and 10mM PEG included dye. These data suggest that nPKC and PKA inhibitors do not inhibit PEG-sealing in Ca^{2+}-free saline. However, nearly-complete inhibition of endogenous sealing with 7μM ηPSF+9μM θPSF+50μM PKI in combination with PEG proved to be cytotoxic.

We then assayed the effect of Ca^{2+} and, nPKC and PKA inhibitors on PEG-induced sealing by transecting B104 cell neurites in Ca^{2+}-containing saline and the presence of such inhibitors, followed by one minute exposure to 10mM PEG (**Fig. 11D**). B104 cells damaged in Ca^{2+} and the presence of 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF, 50μM PKI and 10mM PEG had significantly ($p \leq 0.001$, CMH χ^2) increased sealing probability compared to Ca^{2+}-free, but not Ca^{2+}-containing control sealing (**Fig. 11D**). Again, all B104 cells (intact or damaged) exposed to 7μM ηPSF+9μM θPSF+50μM PKI and 10mM PEG included dye (**Fig. 11D**).

All transection data (**Fig. 11C, D**) suggest the following, to wit:

1. PEG can rapidly seal (PEG-seal) plasmalemmal damage in Ca^{2+}-free saline, and does not require endogenous sealing mechanisms (nPKC η, θ or PKA) to do so (**Fig. 11C**).
2. PEG application does not enhance endogenous sealing of neurites transected in Ca^{2+}-containing saline (compare Ca^{2+}-containing control to PEG in **Fig. 11B, D**)
3. Most importantly, simultaneous inhibition of nPKCs and PKA in conjunction with PEG is cytotoxic to intact and transected cells in Ca^{2+}-free and –containing salines (**Fig. 11B, D**). These last data (**Fig. 11B, D**) suggest that such treatment (nPKC+PKA inhibitors + PEG) to crush-severed sciatic axons *in vivo*, is unlikely to increase the probability of PEG-fusion following crush in calcium.

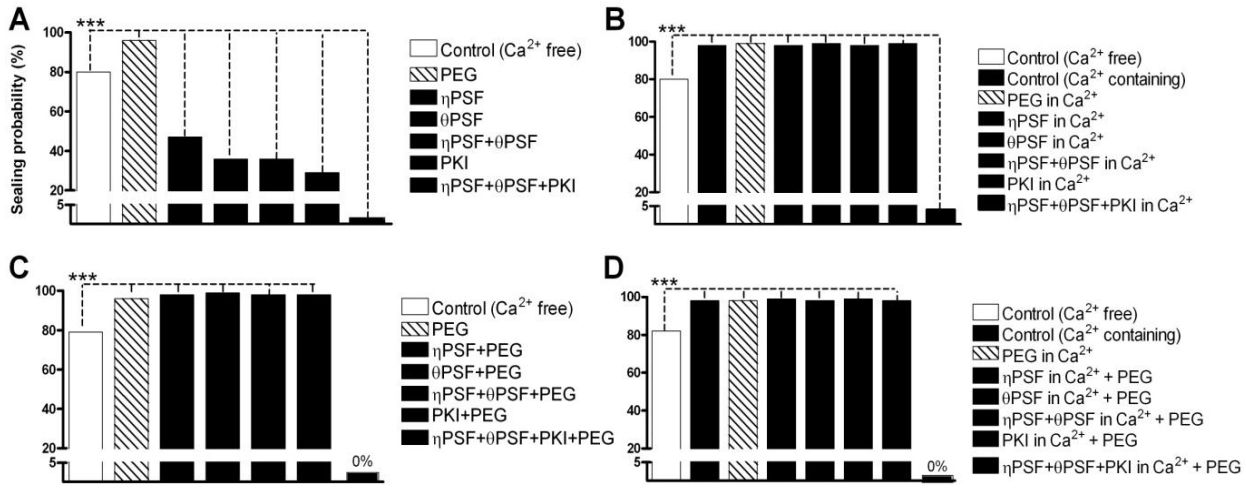

Figure 11: Sealing Probability vs. PC time (5 min)

B104 cells transected in the presence of compounds in Ca^{2+}-free (**A, C**) or Ca^{2+}-containing (**B, D**) saline for 10 minutes, rinsed with and then exposed to fresh (compound free) Ca^{2+}-containing saline for 5 minutes. Texas red dye (3kDa) was added at 5 minutes PC. Note, 10mM PEG was applied for only one minute (i.e. the final minute) of 10 minute transection period (Spaeth et al. 2012). Statistical significance (CMH $\chi 2$) indicated as asterisks on graph: ***, $p \leq 0.001$. See Table 4 in Methods for number of Petri dishes used and cells observed in panels **A-D**.
(A) Effect of 10mM PEG, 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF, 50μM PKI or 7μM ηPSF+9μM θPSF+50μM PKI on sealing. Ca^{2+}-free Control sealing (no treatment): white column in this and all other panels.
(B) Effect of 10mM PEG, 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF, 50μM PKI or 7μM ηPSF+9μM θPSF+50μM PKI on instantaneously induced sealing of neurites transected in Ca^{2+}-containing saline.
(C) Effect of 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF, 50μM PKI or 7μM ηPSF+9μM θPSF+50μM PKI on PEG-induced sealing..
(D) Effect of Ca^{2+}, 7μM ηPSF, 9μM θPSF, 7μM ηPSF+9μM θPSF, 50μM PKI or 7μM ηPSF+9μM θPSF+50μM PKI on PEG-induced sealing.

Effect of inhibitors of nPKC and PKA applied to crush-severed sciatic axons *in vivo*

We have recently demonstrated that application of methylene blue (MB), an inhibitor of sealing in B104 cells (Spaeth et al. 2012), to crush- and cut-severed rat sciatic axons prior to PEG treatment (MB+PEG) significantly improved behavioral recovery

from 72 hrs through 3 weeks post-operation, *in vivo*, compared to PEG treatment only (Bittner et al. 2012). As previously mentioned in the introduction, such successful treatments (PEG alone or MB+PEG) depend upon bathing the nerve in Ca^{2+}-free physiological saline before cut- or crush-severance injury, then in hypotonic Ca^{2+}-free physiological saline immediately after injury and during administration of treatments *in vivo* (**Fig. 10**; Britt et al. 2010; Bittner et al. 2012). Here, we test whether application of inhibitors of nPKCs and PKA (recall these inhibitors decrease sealing in B104 cells, like MB) prior to PEG application can increase the probability of PEG-fusion following crush-severance in calcium, *in vivo*.

First, we assessed if such treatments could restore electrophysiological continuity as assessed by measurements of compound action potential (CAP) conduction across the crush (lesion) site *in vivo*. Second, we assessed restoration of morphological (cytoplasmic) continuity by fluorescence microscopy assessments of intra-axonal diffusion of Texas Red dye through the lesion site *in vitro* (Britt et al. 2010; Bittner et al. 2012; Spaeth et al. 2012). [For details on surgical procedures, electrophysiological recordings of CAPs and dye diffusion apparatus, see Methods.]

Application of inhibitors of nPKC η, θ and PKA to crush-severed sciatic axons immediately following crush in physiological Ca^{2+} environment did not increase probability of PEG-fusion as assessed by CAP conduction across the lesion site

Because simultaneously inhibiting nPKC η, θ and PKA dramatically decreased sealing in B104 cells transected in calcium (**Fig. 11A, B**), we hypothesized that such treatment applied to crush-severed sciatic axons immediately following a crush injury in

calcium could increase the probability of PEG-fusion, and restore electrophysiological continuity, *in vivo*.

To assess if application of inhibitors of nPKC η, θ and PKA, prior to PEG, could restore electrophysiological continuity of crush-severed axons crushed in calcium, we extracellularly stimulated and recorded the ability of sciatic axons to conduct CAPs *in vivo*. We used two pairs of hook electrodes, one pair to stimulate and the other to record CAPs on an oscilloscope (Britt et al. 2010). All crush-severance lesions were made between the two electrodes, abolishing CAP conduction (Crush CAP data not shown). Tables **2, 3** give CAP amplitude data recorded *in vivo* from crush-severed sciatic nerves before (Initial CAP) crush-severance, after PEG application (PEG-fusion CAP), and percent restoration of initial CAPs by PEG-fusion. Following CAP recordings, nerves were excised and used for *in vitro* dye diffusion assessments.

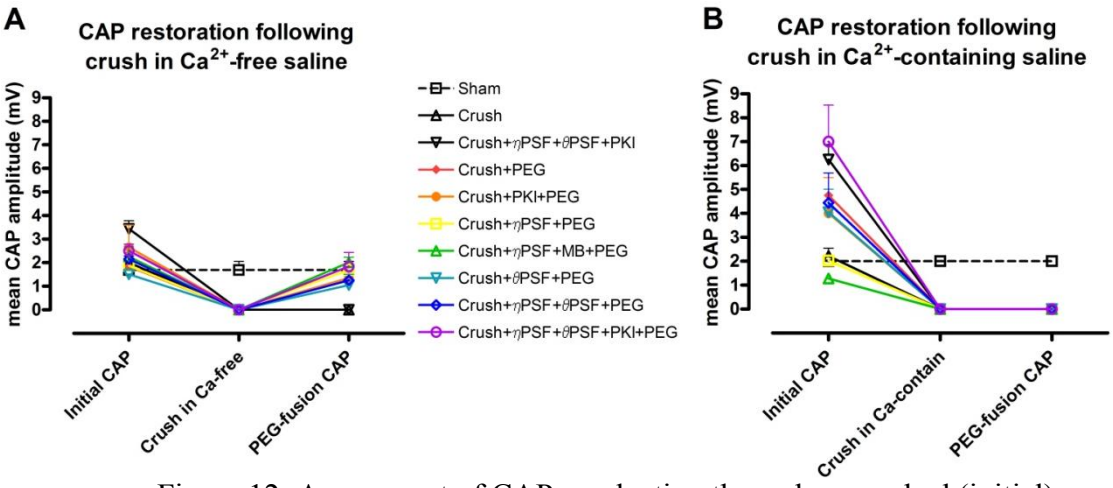

Figure 12: Assessment of CAP conduction through uncrushed (initial), crushed and PEG-fused sciatic axons

A: Crush in hypotonic, Ca^{2+}-free physiological saline: CAP conductivity was restored only after PEG treatment.
B: Crush in isotonic, Ca^{2+}-containing physiological saline: CAP conductivity was not restored by PEG or any other treatment.

CAPs were *always* detected in all intact sciatic nerves and *never* detected across the lesion site immediately after crushing the sciatic nerve (for n values, see **Tables 2, 3**). CAPs were *only* detected post-crush only for Sham (no crush or treatment), and all Crush+PEG treatment groups, in which crush, and PEG application were performed in Ca^{2+}-free and hypotonic, Ca^{2+}-free physiological salines respectively (**Fig. 12A, Table 2**). No treatment (with or without PEG) was capable of restoring CAP conductivity following crush in Ca^{2+}-containing physiological saline; not even Methylene blue (MB) in addition to ηPSF (**Fig. 12B, Table 3**).

We statistically analyzed CAP amplitudes using One-Way ANOVA with Tukey's *post-hoc* analysis (Bittner et al. 2012). Initial CAPs from Ca^{2+}-free and Ca^{2+}-containing treatment groups were not statistically different ($p > 0.05$) compared to their respective

Shams (**Tables 2, 3**). PEG-fusion CAPs (**Table 2**) from treatment groups including nPKC and/or PKA inhibitors and PEG were not statistically ($p > 0.05$) different compared to Crush+PEG alone (**Table 2**). Lastly, there was no statistical ($p > 0.05$) difference in CAP amplitude between Initial and PEG-fusion CAPs for any treatment with successful PEG-fusion (**Table 2**). Together, these electrophysiological data suggest that inhibitors of nPKC and PKA are not capable of promoting PEG-fusion of crush-severed sciatic axons crushed in physiological calcium concentration.

Application of inhibitors of nPKC η, θ and PKA to crush-severed sciatic axons immediately following crush in physiological Ca^{2+} environment did not increase probability of PEG-fusion as assessed by dye diffusion across the lesion site

To assess if application of inhibitors of nPKC η, θ and PKA, prior to PEG, could restore morphological continuity of crush-severed sciatic axons crushed in calcium, we assessed diffusion of Texas red dye (3 kDa) through crush lesion sites *in vitro*, using conventional fluorescence microscopy. We excised 3-4 cm lengths (with lesion site roughly in middle of excised segment) of sciatic nerve from rats previously used in our *in vivo* assessments of electrophysiological continuity. The most proximal excised end was placed in a watertight Vaseline well containing dye (Texas Red dextran) dissolved in Ca^{2+}-free saline (see Dye diffusion in Methods). The lesion site was outside the well and maintained in Ca^{2+}-containing isotonic saline.

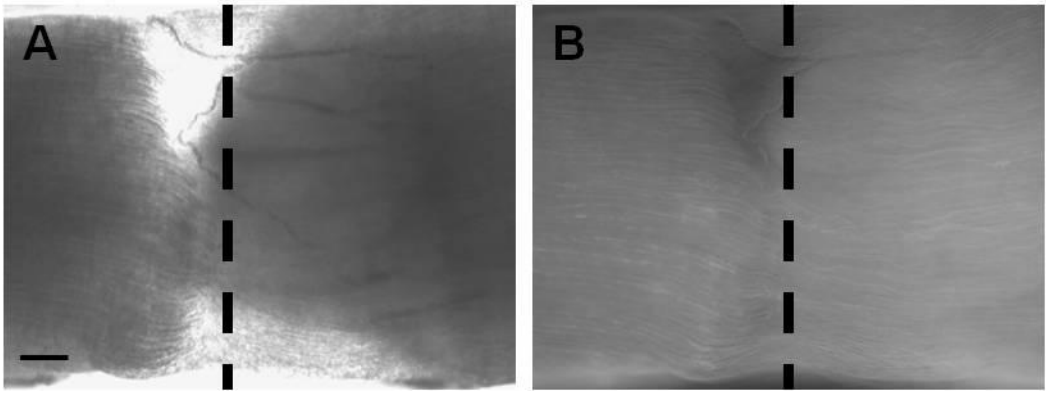

Figure 13: Dye diffusion through a representative PEG-fused crush-severed nerve

A, B: Light (**A**) and fluorescence (**B**) images of a crush-severed rat sciatic nerve, crushed in hypotonic, Ca^{2+}-free physiological saline. Dashed line indicates crush lesion site in **A** and **B**. Proximal portion of nerve left of crush lesion, distal portion of nerve right of lesion. (**B**) Dye diffused through lesion site. Scale bar 100 μm at 10x magnification. For additional dye diffusion images in rat sciatic nerves see, Spaeth et al. 2012.

Dye *always* diffused (**Fig. 13**) through the lesion site for Sham (no crush or treatment), and all Crush+PEG treatment groups, in which crush, and PEG application were performed in Ca^{2+}-free and hypotonic, Ca^{2+}-free physiological salines respectively (**Table 2**). Dye *also* diffused through the lesion site of Crush+7μM ηPSF+9μM θPSF+50μM PKI treated nerves. This group did not receive PEG, but dye did diffuse through the lesion site (**Table 2**). Dye *never* diffused through the lesion site of Ca^{2+}-containing crush treatment groups, except for sham-operated (no crush or treatment), crush+7μM ηPSF+9μM θPSF+50μM PKI and crush+7μM ηPSF+9μM θPSF+50μM PKI+PEG (note for the latter two treatments, nor was CAP conduction restored, even with PEG application) (**Table 3**).

These dye diffusion data suggest that no treatment (nPKC or PKA inhibitors and PEG), following crush-severance in Ca^{2+}-containing saline, is capable of restoring morphological continuity of crush-severed axons. As expected, dye diffused through the lesion site of all PEG-fused nerves (i.e. crush-severance and PEG application was performed in Ca^{2+}-free and hypotonic, Ca^{2+}-free saline respectively). These PEG-fusion data correlate with our previously published results on restoration of morphological continuity to those crush-severed axons, that received crush-severance injury and PEG application in Ca^{2+}-free and hypotonic, Ca^{2+}-free saline respectively (Britt et al. 2010; Bittner et al. 2012). Moreover, these dye diffusion data suggest that simultaneous application of inhibitors of nPKCη, θ and PKA can decrease sealing in severed axons, even those crush-severed in calcium, as evidenced by dye diffusion through the lesion site of Crush+7μM ηPSF+9μM θPSF+50μM PKI treated nerves; whether or not calcium was present during crush or, PEG was applied to the lesion site (**Tables 2, 3**).

Table 2: in vivo crush in Ca^{2+} free

Treatment	N	Initial CAP ± SE (mV)	PEG-fusion	PEG-fusion CAP ± SE (mV)	%PEG-fusion CAP of Initial	Dye Diffusion
Sham	3	1.7±0.4	n/a	n/a	100	Yes
Crush	5	2.0±0.3	n/a	n/a	100	No
Crush+PEG	2	1.8±0.6	Yes	1.3±0.1	72	Yes
Crush+ηPSF+θPSF+PKI	4	3.4±0.3	n/a	n/a	n/a	Yes
Crush+PKI+PEG	2	2.7±1.0	Yes	1.4±0.3	52	Yes
Crush+ηPSF+PEG	6	1.8±0.3	Yes	2.0±0.3	111	Yes
Crush+ηPSF+MB+PEG	6	2.3±0.4	Yes	2.0±0.2	87	Yes
Crush+θPSF+PEG	2	1.5±0.2	Yes	1.1±0.5	73	Yes
Crush+ηPSF+θPSF+PEG	2	2.2±0.2	Yes	1.3±0.1	59	Yes
Crush+ηPSF+θPSF+PKI+PEG	4	2.5±0.3	Yes	1.8±0.6	72	Yes

Table 2: CAP and dye diffusion data for treatment groups in which crush was performed Ca^{2+}-free saline and subsequently bathed in hypotonic Ca^{2+}-free physiological saline. Immediately following crush injury, inhibitors of nPKCs or PKA (dissolved in hypotonic Ca^{2+}-free physiological saline) were applied to crush site. (Sham group: no crush, no treatment)

Tables 2 and 3:
CAPs: Prior to injury, CAP conduction was assessed in intact nerves (Initial). Crush-severance was determined as complete when CAP conduction was abolished, i.e., CAP = 0mV. Hence, "crush" CAPs are not indicated in table for any treatment group. Immediately following crush injury, inhibitors of nPKCs or PKA (dissolved in hypotonic Ca^{2+}-free physiological saline) were applied to crush site. CAPs were measured post-inhibitor application to assess whether inhibitor treatment alone, produced any restoration of CAP conduction. Such treatments did not restore CAP conduction (data not shown). Following inhibitor treatments, PEG was applied to lesion and PEG-fusions were assessed by measuring CAP conduction through lesion site (PEG-fusion).
%PEG-fusion CAP of Initial calculated using following equation: (PEG-fusion CAP / Initial CAP) x 100%. **n/a** = assessment not applicable for treatment.

Dye diffusion: To assess restoration of morphological continuity of crush-severed axons within the lesion site, *in vitro* (Britt et al. 2010; Spaeth et al. 2012), all nerves that received treatment and electrophysiological assessments *in vivo* (including Sham treated nerves) were excised and placed in dye diffusion apparatus (see Methods). **Yes -** dye diffused through lesion site; **No –** dye did not diffuse through lesion site.

Table 3 in vivo crush in Ca^{2+} containing

Treatment	N	Initial CAP ± SE (mV)	PEG-fusion	PEG-fusion CAP ± SE (mV)	%PEG-fusion CAP of Initial	Dye Diffusion
Sham	2	2.0 ± 0.0	n/a	n/a	n/a	Yes
Crush	4	2.2 ± 0.3	n/a	n/a	n/a	No
Crush+PEG	4	4.8 ± 0.1	No	0	0	No
Crush+ηPSF+θPSF+PKI	4	6.3 ± 0.6	n/a	n/a	n/a	Yes
Crush+PKI+PEG	2	4.0 ± 1.5	No	0	0	No
Crush+ηPSF+PEG	6	2.0 ± 0.1	No	0	0	No
Crush+ηPSF+MB+PEG	6	1.3 ± 0.1	No	0	0	No
Crush+θPSF+PEG	6	2.4 ± 0.6	No	0	0	No
Crush+ηPSF+θPSF+PEG	2	4.5 ± 1.3	No	0	0	No
Crush+ηPSF+θPSF+PKI+PEG	4	7.0 ± 1.5	No	0	0	Yes

Table 3: CAP and dye diffusion data for treatment groups in which crush was performed with nerve bathed in *Ca^{2+}-containing* physiological saline. Immediately following crush injury, inhibitors of nPKCs or PKA (dissolved in hypotonic Ca^{2+}-free physiological saline) were applied to crush site. (Sham group: no crush, no treatment)

DISCUSSION

Summary of Results

In this study we used an *in vitro* cell-based dye exclusion assay to determine if compounds that inhibit Ca^{2+}-induced plasmalemmal sealing in neuronal B104 cells *in vitro* (**Fig. 11A**) could increase the probability of PEG-fusion of sciatic axons *in vivo*, following crush-severance in physiological calcium concentration (to realistically mimic peripheral nerve crush injury) (**Table 3**). Inhibitors of nPKC η, θ and PKA decreased endogenous Ca^{2+}-induced plasmalemmal sealing in B104 cells *in vitro* (**Fig. 11A**). Simultaneously inhibiting nPKC η, θ and PKA, dramatically decreased sealing, even when cells were transected in calcium *in vitro* (**Fig. 11A, B**). Inhibitors of nPKC and PKA do not inhibit PEG-induced sealing *in vitro* (**Fig. 11C, D**). However, when PEG was applied to cells transected in the combined presence of nPKC η, θ and PKA inhibitors, whether or not calcium was present during transection, all cells (intact and damaged) included Texas red dye suggesting that such treatment was cytotoxic *in vitro* (**Fig. 11C, D**).

Given these *in vitro* data, we investigated whether nPKC η, θ and PKA inhibitor treatments could increase the probability of PEG-fusion following crush-severance in physiological calcium concentration. We here report that applying inhibitors of nPKC and PKA (individually or in combination) to lesion sites immediately after crush-severance in Ca^{2+}-containing physiological saline, but prior to PEG application, do not promote PEG-fusion as assessed by CAP conduction and dye diffusion through lesion

sites (**Table 3**). That is, we had 0% PEG-fusion success rate when PEG-fusion was attempted following crush in Ca^{2+}-containing physiological saline.

We also report that PEG-fusions were always observed for all treatment groups in which PEG was applied to lesion sites immediately after crush-severance in hypotonic Ca^{2+}-free physiological saline, as assessed by CAP conduction and dye diffusion through the lesion site (**Table 2; Figs. 12, 13**). That is, in this study we had 100% PEG-fusion success rate when PEG-fusion was performed following the protocol set forth in Figure 3.2 and as previously reported in Stavisky et al. (2005), Britt et al. (2010) and Bittner et al. (2012).

Translation of PEG-fusion from laboratory to clinic

We performed B104 cell *in vitro* and sciatic nerve *in vivo* experiments to determine if our current PEG-fusion protocol (**Fig. 10**) could be modified to successfully PEG-fuse axons crush-severed in physiological calcium concentration (~2 mM). A strength of our cell-based assay is that we can quickly screen for proteins (or substrates) involved in endogenous sealing, that may be therapeutic targets for improving PEG-fusion of severed axons. A weakness of this assay is that the maximum effective concentrations of compounds used *in vitro*, such as those presented in this report for nPKC and PKA inhibitors, may not provide similar results *in vivo*, and may also have unforeseen cytotoxic effects on other cell types within the nerve and surrounding muscle.

Although we are capable of obtaining nearly 100% PEG-fusion using our PEG-fusion protocol, *ex vivo* and *in vivo* (**Fig. 10**; Britt et al. 2010; Bittner et al. 2012), it is by

no means a procedure that could be translated readily to emergency wards and operating theatres to provide immediate repair of traumatically crush-severed human peripheral nerves for a few very important reasons:

1) Peripheral nerve crush injury is the most prevalent nerve injury (Taylor et al. 2008) commonly incurred in motor vehicle accidents (Campbell, 2008).

2) Ambulance response times, specifically to motor vehicle accidents, are ≥ 8 minutes (Trowbridge et al. 2009).

3) Ca^{2+} influx at plasmalemmal disruptions of endogenous sealing is immediately induced and a complete seal formswithin 10 minutes (Yoo et al. 2003; Spaeth et al. 2010 and supplemental reference, 2012; Chapter 2).

These statistics (**1 – 3**), highlight the obstacles impeding successful implementation of our PEG-fusion protocol (**Fig. 10**).

Conclusion

Our data suggest that much research and development are needed to translate our current PEG-fusion protocol into a successful neurotrauma treatment. Specifically, the major obstacle to successful PEG-fusion of peripheral nerve crush or cut injuries seems to be, calcium-induced endogenous sealing. To be able to reverse or slow endogenous

sealing, minutes after it has begun, could allow first responders (EMT) or surgeons the ability to apply PEG, minutes after traumatic injury, to closely apposed, re-opened (unsealed) axonal ends, to PEG-fuse severed axons. We suggest that future studies of PEG-fusion and its feasibility as a neurotrauma therapeutic modality continue to implement cell-based screens because they can rapidly detect probable therapeutic targets. Such assays in the future should investigate the effects of compounds that could reverse/weaken a formed plasmalemmal seal, such as Triton X-100 (a detergent), which is typically used in microbiology to permeabilize plasmalemmas. Such a compound may be capable of weakening or reversing a seal, re-opening previously sealed axonal ends, providing a window of opportunity to PEG-fuse closely apposed severed axonal ends.

MATERIALS AND METHODS

IN VITRO B104 CELL PREPARATIONS

B104 cells:

B104 cells derived from a CNS neuroblastoma (Bottenstein and Sato, 1979) have often been used as a model system to study neuronal function in vitro (Yoo et al. 2003; Nguyen et al. 2005; Miller et al. 2006; Spaeth et al. 2010). B104 cells extend neurites that have properties typical of nerve axons such as generation of action potentials, smooth (but not rough) endoplasmic reticulum, release of neurotransmitter, and regeneration of severed neurites. These cells have easily identifiable cell bodies and neurites, allowing precise identification of individually damaged cells at their injury site (Detrait et al. 2000b; Yoo et al. 2003, 2004; Spaeth et al. 2010). Unlike some other model neuronal cell lines (e.g., PC12 cells), B104 cells do not require growth factor supplements of fetal bovine serum to proliferate (Detrait et al. 2000b; Yoo et al. 2003; Spaeth et al. 2010). Data on sealing of B104 cells are consistent with similar data on sealing from at least 20 other preparations from many phyla and different cell types (see supplemental reference list in Spaeth et al. 2010).

Cell culture:

As described by Nguyen et al. (2005) and Spaeth et al. (2010), B104 cells were grown in 75 cm^2 vented cap flasks (BD Falcon; BD Biosciences) in a humidified incubator at 37°C in 5% CO2 and in 4 ml of cell growth medium: consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM:F-12, HyClone) supplemented for

growth with 10% heat-inactivated fetal bovine serum (FBS; HyClone) and 1% antibiotics (10,000 U of penicillin/ml and 10 mg/ml streptomycin; Sigma-Aldrich). The cell growth medium was changed every 2 d. Cultures were passaged at 80% confluency, and cells were then either subcultured in a vented cap flask or seeded at ~2000 cells/cm ^{2} in cell growth media on Petri dishes coated with poly-D-lysine (Sigma-Aldrich) to prevent cells from detaching during solution changes and/or neurite transection. After 24 h, the growth medium was replaced with serum-free DMEM:F12 (Hyclone) to allow the B104 cells to differentiate. B104 neurites were transected 24–48 h after replacing the cell growth medium with serum-free DMEM:F12.

Transection of neurites of B104 cells:

Before transecting neurites, the solution (DMEM:F12) in the Petri dish was washed twice with a Ca^{2+}-free phosphate-buffered saline (referred to as "Ca^{2+}-free saline," PBS=; HyClone). All neurites were transected in Ca^{2+} -free saline at a distance >50 μm from the soma using a sharpened, pulled glass, micro capillary tube ("micro-knife") that was placed on a micromanipulator (Narishige Instruments) and quickly drawn across the surface of the Petri dish, etching a score line that showed the path of the micro-knife. In all experiments, 3 kDa fluorescein dextran (Invitrogen) was added to the Ca^{2+}-free saline during transection of B104 cell neurites to label cells damaged by the micro-knife. As described previously (Yoo et al. 2004), only B104 cells with transected neurites uptake fluorescein dye, therefore we were able to uniquely and individually identify each B104

cell with a transected neurite by fluorescein dextran fluorescence and the relation of the damaged cell/neurite to the score mark on the plate.

Microscopic observations of dye exclusion:
As previously described in Spaeth et al. (2010, 2012) for all experiments, B104 cells were observed under an inverted Zeiss ICM-405 fluorescent microscope with a 40X long focal distance lens. Cells with neurites transected >50 μm from the soma were observed for fluorescein dextran uptake and Texas Red dextran in/exclusion. No further observations were made of transected cells that failed to uptake fluorescein dextran.

Assessment of plasmalemmal sealing:
As Spaeth et al. (2010) described, the most reliable measure of plasmalemmal repair, dye exclusion, was used to assay plasmalemmal sealing (Blanchette et al. 1999; Detrait et al. 2000a,b). To assess plasmalemmal repair, we transected 10–130 cells in Ca^{2+}-free saline containing fluorescein dye within 10 min. The Ca^{2+}-free saline was then replaced with a phosphate-buffered saline containing 1 mM Ca^{2+} ("Ca^{2+}-saline," PBS^{2+}; HyClone) to initiate the sealing process (Detrait et al. 2000b; Yoo et al. 2003; Spaeth et al. 2010, 2012). The time elapsed after exposing cells to Ca^{2+}-saline is defined as the "post- Ca^{2+} addition time" (PC time), ensuring that all cells on a given Petri dish have the same PC time. After 5 minutes PC time, 3 kDa Texas Red dextran (Invitrogen) was added to the Ca^{2+}-saline. After a 10 min exposure, Texas Red dextran was thoroughly washed out with Ca^{2+}-saline, and then sealing was assessed. Transected cells (damaged neurite,

green fluorescence) that excluded Texas Red dextran were counted as "sealed". Cells that did not exclude Texas Red dextran were counted as "not sealed". Proximate undamaged cells never took up Texas Red dextran**.** As stated previously (Spaeth et al. 2010), fluorescent dyes of higher molecular weight are excluded faster than lower molecular weight dyes (Detrait et al. 2000a; Yoo et al. 2003) at the same PC time. Thus, to decrease variability in assessment of sealing probabilities for various treatments, based on dye exclusion, we consistently used 3 kDa Texas Red dextran in all experiments.

Pharmacological reagents:

We used pharmacological agents to inhibit or activate target proteins to avoid complications of compensatory pathways that may result from gene knock-outs or chronic applications of pharmacological inhibitors (Steinberg et al. 2008). All pharmacological reagents were typically dissolved in Ca^{2+}-free saline and added to cells immediately before neurites were transected to backload cells with each reagent during transection. We used the following isozyme specific peptide inhibitors of PKC, purchased from EMD Chemicals, Gibbstown, NJ: myristoylated nPKCη pseudosubstrate fragment (7 μM, 2 kDa) was used to inhibit nPKCη (Liu et al. 2006; Barros et al. 2009; Spaeth et al. 2010), myristoylated nPKCθ pseudosubstrate fragment (9 μM, 2.3 kDa) to inhibit nPKCθ (Staal et al. 2008; Barros et al. 2009). PKI (50 μM, 1.8 kDa; Sigma-Aldrich; a generous gift from Drs. Michael Markham and Harold Zakon, University of Texas, Austin, TX) was used to specifically inhibit PKA (Spaeth et al. 2010). A 50% stock solution of polyethylene glycol 2000 (PEG 2kDa; Fluka, Sigma Aldrich) was made

in double-distilled H_2O (5 g PEG 2000 in 5 mL of ddH_2O). In-dish concentration of PEG was 10 mM (Spaeth et al. 2012).

Statistical analyses:

As described previously (Spaeth, 2010), we obtained data from individual, uniquely identified B104 cells that all received similar, well-specified, neurite transections as plasmalemmal injuries in a tightly controlled environment. For each treatment group, at a given PC time the data were pooled for all cells (n) from all Petri dishes (N). See **Table 4** below, for n and N values for each data set at 5 min PC time point in Figure 11. "Sealing probability" is defined as the percentage of a set of individually-transected and uniquely identified cells that exclude 3 kDa Texas Red dye (sealed) at a given PC time. As described previously (Detrait et al. 2000b; Yoo et al. 2003, 2004; Spaeth et al. 2010, 2012), the Cochran-Mantel-Haenszel χ^2 (CMH χ^2) test for independence was used to determine differences among sealing probabilities at a given PC time for different experimental treatments were statistically significant ($p < 0.05$), as previously described (Agresti, 1996; Detrait et al. 2000; Yoo et al. 2004; Spaeth et al. 2010, 2012). The CMH χ^2 test for independence is used to compare data separated into two-by-two contingency tables (sealed or not sealed, and control vs. experimental data sets). This test entails binary outputs, applied to our sealing assay (sealing is treated as a yes or no event at a given PC time). Because sealing of a given cell and not each Petri dish (sometimes containing over 100 transected cells) are independent events, measures of variance (such as SE or SD) are not applicable.

Table 4: Number of plates (N) and transected B104 cells (n) per treatment in Figure 11

Treatment	Ca^{2+}-free	Ca^{2+}-containing
Control (Ca^{2+}-free)	(4, 362)	
Control (Ca^{2+}-containing)		(4, 481)
PEG	(2, 164)	(2, 172)
η PSF	(3, 108)	(2, 153)
θ PSF	(2, 185)	(2, 162)
η PSF + θ PSF	(2, 140)	(2, 246)
PKI	(3, 113)	(2, 157)
η PSF + θ PSF + PKI	(2, 116)	(2, 134)
η PSF + PEG	(2, 201)	(2, 130)
θ PSF + PEG	(2, 171)	(2, 137)
η PSF + θ PSF + PEG	(2, 171)	(2, 141)
PKI + PEG	(2, 130)	(2, 145)
η PSF + θ PSF + PKI + PEG	#	#

Table 4: The numbers in parentheses (N, n) represent the number of Petri dishes (N) used to obtain sealing data from (n) transected B104 cells. The total number of cells (n) listed per condition were obtained by combining the sum of all cells counted (sealed or not sealed) from each dish per treatment at 5 min PC time. Blank space: no experiments performed for condition at designated PC time. **"#"** indicates cells were transected for these treatments however, treatments proved cytotoxic; thus no cells were counted as sealed or not sealed following dye wash-out.

IN VIVO OR *IN VITRO* PREPARATIONS OF RAT SCIATIC NERVES

All procedures were approved by the University of Texas at Austin's Institutional Animal Care and Use Committee (Protocol no. AUP-2010-00183). All animals were housed in groups of three in polycarbonate cages with sawdust bedding, maintained on a 12:12 dark/light cycle, and given food and water *ad libitum*.

Surgical procedures:

As previously described in Bittner et al. (2012), Sprague Dawley rats were anesthetized with intra-peritoneal injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). The sciatic nerve was exposed by an incision about 1.5 cm long in posterior-thigh muscles of the hind limb. Exposed sciatic nerves were cleaned of connective tissue and bathed with hypotonic Ca^{2+}-free (**Table 2**) or Ca^{2+}-containing (**Table 3**) Krebs physiological saline perfused from a Pasteur pipette. One liter of hypotonic Ca^{2+}-free saline contained in mM: 0.5 EGTA, 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 26 $NaHCO_3$, 10 Na ascorbate, 10 dextrose, 2 $MgCl_2$, pH 7.35, 295 mOsm dissolved in double distilled H_20 (ddH_20). One liter of isotonic Ca^{2+}-containing saline contained in mM: 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 26 $NaHCO_3$, 10 Na ascorbate, 10 dextrose, 2 $CaCl_2$, pH 7.35, 345 mOsm dissolved in ddH_20. We confirmed in anesthetized animals that exposed, intact sciatic nerves in hypotonic Ca^{2+}-free or Ca^{2+}-containing saline conducted action potentials across the site of any intended lesion (see CAPs below). Animals in the Sham group received no nerve injury following exposure of the sciatic nerve *via* a skin incision. Throughout all procedures, the nerve was moistened with the appropriate saline, e.g. nerves in Table 2 received hypotonic Ca^{2+}-free saline during exposure of nerve and crush injury, but then received Ca^{2+}-containing saline after PEG application; nerves in Table 2 received Ca^{2+}-containing saline during exposure of nerve, during crush injury and, after PEG application

Sciatic *crush-severance* injuries were made with Dumont #5 forceps, applying enough force to sever every axon. The crush-severance site about 1 mm long was much

less opaque than adjacent uninjured sites viewed at 20-50x through a dissecting microscope. After crush injury, the sciatic nerve was again assessed for CAP conduction across the lesion site. If a CAP was detected, the nerve was crushed again. To apply treatment solutions containing nPKC and PKA inhibitors or PEG in hypotonic Ca^{2+}-free saline , or isotonic Ca^{2+}-containing saline to crush-severed sciatic axons, the epineurial sheaths of crush-severed sciatic nerves were nicked at the lesion site with microdissection scissors to allow better access of solutions to the crush-severed axons.

For animals treated with 7 μM ηPSF (EMD-Calbiochem), 9 μM θPSF (EMD-Calbiochem) or 50 μM PKI (Sigma-Aldrich), compounds were dissolved in 1 mL of hypotonic Ca^{2+}-free saline and applied from a micropipette positioned 1-5 mm above the nick in the nerve's epineurial sheath so that the solution flowed in a narrow stream (about 1 mm wide) for 1-3 min over the crushed axons at the lesion site. Treatments of 500 mM PEG (2 kDa PEG, Fluka, SigmaAldrich) dissolved in ddH_2O were then applied to the lesion site *via* a similar micropipette so that the denser PEG-containing solution flowed for 1.5-2 min over the lesion site. After such treatments, a second pipette was used to perfuse the sciatic nerve with isotonic Ca^{2+}-containing saline.

500 mM (50% w/v) Polyethylene glycol (PEG) solution:
5 g of PEG 2000 dissolved in 5 mL of ddH_20 (Britt et al. 2010; Bittner et al. 2012).

Treatment groups and their rationale:

Crush-severed rat sciatic nerves were assayed *in vivo* (CAP conduction) or *ex vivo* (Dye Diffusion). Some were left untreated (Crush), some did not receive PEG following compound treatment (Crush+ ηPSF+ θPSF+PKI). Other crush-severed nerves were treated with PEG (Crush+PEG) or with nPKC and PKA inhibitors, individually or in combination, prior to PEG as indicated: 7 μM ηPSF (Crush+ ηPSF+PEG), 9 μM θPSF (Crush+ θPSF+PEG), 7 μM ηPSF and 9 μM θPSF (Crush+ ηPSF+ θPSF+PEG), 50 μM PKI (Crush+PKI+PEG), or 7 μM ηPSF and 9 μM θPSF and 50 μM PKI (Crush+ ηPSF+ θPSF+PKI+PEG). Crush and Crush+PEG treatment groups were used as experimental controls for qualitative comparisons of CAP conduction and dye diffusion assessments. All treated groups were compared to the Crush treatment group to assess possible PEG-fusion success.

Electrophysiological recording of CAPs:

Electrophysiological recordings of CAPs conducting across a lesion site *in vivo* were made by two pairs of nickel-tipped hook electrodes placed on or beneath the sciatic nerve to stimulate and record CAPs, which were displayed on an analog (Tektronix 502A, Lore et al. 1999; Marzullo et al. 2002; Britt et al. 2010; Bittner et al. 2012) or digital (AD Instruments PowerLab 4/35 with Lab Chart Pro analysis software) oscilloscope. All lesions were made between the two stimulating and recording hook electrodes. Complete crush-severance of all axons was confirmed by an inability to record a detectable CAP conducted through the lesion site. The sciatic nerve was always stimulated after any

treatment to determine if CAPs conducted through the lesion site. During pre-operative and post-operative CAP recordings *in vivo*, the nerve was continuously moistened with appropriate physiological saline.

To correlate a measure of electrophysiological continuity with a measure of morphological continuity we recorded CAPs from nerves, then following treatments, excised these same nerves (n = 48) to assess morphological continuity with an intra-axonal dye diffusion assay (see description in following section).

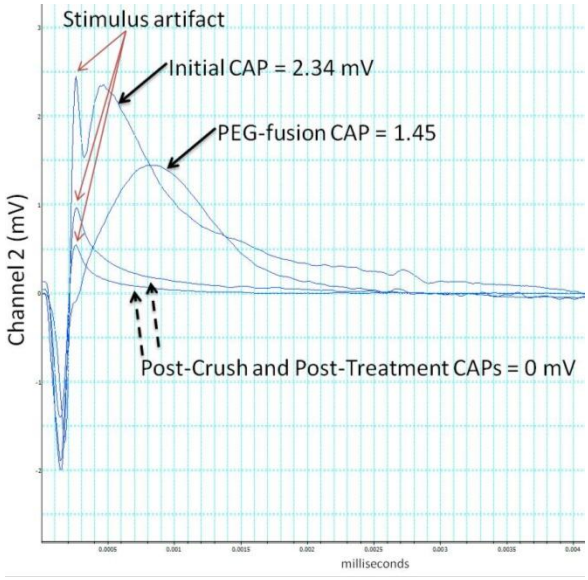

Figure 14: CAP recordings as visualized on AD instruments Lab Chart Pro analysis software.

Intra-axonal dye diffusion across a lesion site:

After recording CAPs, we correlated *in vivo* CAP conduction with *ex vivo* intra-axonal dye diffusion through the lesion site. This was obtained by excising a 2-4 cm length of the sciatic nerve (including lesion sites) from animals (Lore et al. 1999; Britt et al. 2010; Spaeth et al. 2012). The proximal end of the sciatic nerve segment was placedin a petroleum jelly (Vaseline) ring containing Ca^{2+}-free saline and 100 µL of hydrophilic dye

(2kDa Texas red dextran, Molecular Probes). The remainder of the nerve, including any crush-severance site, was bathed in Ca^{2+}-containing saline. The Petri dish containing the nerve in the Vaseline well was refrigerated for 14 hours at 4˚C. Nerves were examined for intra-axonal diffusion of fluorescent dye beyond the crush lesion site using a Zeiss ICM-405 inverted fluorescence microscope. Some nerves were imaged using a Nikon TS100F inverted fluorescent microscope (Nikon Instruments Inc., Melville, NY) equipped with a rhodamine filter and DS Fi-2 fluorescence color camera.

Statistical Analyses:

As previously described (Britt et al. 2010; Bittner et al. 2012), Student't T-test with Bonferroni's correction for multiple comparisons was used to compare statistical ($p < 0.05$) differences of CAPs among treatment groups.

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Chapter 4: Preliminary Study: Role of nPKC η and θ in neuroprotective sealing of ischemia-induced plasmalemmal damage

SUMMARY

Oxygen-glucose deprivation (OGD) is often used *in vitro* to mimic ischemic stroke. OGD-induced plasmalemmal damage has been extensively described in many eukaryotic cell types in early studies of stroke. However, no study has investigated the cellular mechanisms that promote repair (sealing) of OGD-induced plasmalemmal damage. We exposed cells to normoxia (atmospheric O_2; 0mM glucose) or, immediate OGD (1, 3, 5, 10 min) in Ca^{2+}-free phosphate buffered saline ($PBS^{=}$). Following OGD exposures, we assessed sealing as the ability of cells to exclude extracellular dye. We now report that cells maintained in phosphate buffered saline containing Ca^{2+} (PBS^{2+}), following 1, 3, 5 or 10 minutes of OGD exposure, did not have significantly different sealing probabilities compared to cells maintained in $PBS^{=}$ following similar OGD exposures. Cells exposed to 10 minutes of immediate OGD only, had significantly ($p<0.001$; CMH χ^2) decreased sealing probability compared to normoxic controls (74% vs. 98%, respectively). Lastly, cells exposed to 1, 3, 5 or 10 minutes of OGD in the presence of nPKC η or θ peptide inhibitors had significantly decreased sealing probabilities compared to normoxic controls ($p<0.001$ for 1, 3, 10 min; $p<0.01$ for 5 min; CMH χ^2) and, cells exposed to OGD only. These data show that extent of OGD-induced plasmalemmal damage in B104 cells is dependent upon duration of OGD exposure and, that sealing of OGD-induced plasmalemmal damage is promoted by nPKC η and θ – similar to sealing of transected neurites of B104 cells.

INTRODUCTION

Stroke is the third leading cause of death in the USA (Roger, 2006; Janardhan and Qureshi, 2004). Clinical data show that 87% of all documented strokes in the USA are ischemic (NHLBI, 2006). It is well documented that ischemia (oxygen glucose deprivation; OGD) produces plasmalemmal damage (Hoffstein, 1975; Chien, 1978; Farber, 1981; Herman, 1988; Neiminen, 1988; Zahrebelski, 1995; Wang, 1996; Nishimura, 2001; Rodriguez-Sinovas, 2005). OGD-induced plasmalemmal disruptions are easily observed as membrane blebs, *in vitro* (Neiminen, 1988). As an ischemic event progresses, the integrity of cell plasmalemmas worsen, blebs form, grow, coalesce and eventually burst, releasing cytoplasmic contents. Such plasmalemmal damage occurs often in humans (see above), resulting in loss of functional behavior. However, increasing cells ability to repair (seal) their damaged plasmalemma can decrease the severity of tissue damage and increase functional behavior recovery (Nehrt, 2010; Britt, 2010).

To model ischemia *in vitro*, cells are often incubated in an anaerobic (0% oxygen) chamber at 37°C in media lacking glucose (0% glucose) (Jones, 2004). Cells are then returned to normoxic conditions – simulating reperfusion. Such OGD exposures increase Ca^{2+} influx, calpain activity, membrane permeability, membrane damage and cell death (Yoshioka, 2000; Cao, 2010). Several studies have previously demonstrated that fluorescent dyes label cells with OGD-induced plasmalemmal damage (Hoffstein, 1975; Chien, 1978; Farber, 1981; Herman, 1988; Neiminen, 1988; Zahrebelski, 1995; Wang, 1996; Nishimura, 2001; Rodriguez-Sinovas, 2005; Cao, 2010).

Similarly, studies on plasmalemmal sealing have shown, by use of fluorescent dye exclusion assays, that Ca^{2+} influx (Eddleman, 1998; Detrait, 2000a,b; McNeil, 2001) through plasmalemmal disruptions induces plasmalemmal sealing by vesicle-mediated repair (Steinhardt, 1994): vesicles from nearby undamaged membrane (Eddleman, 1997, 1998), lysosomes (Reddy, 2001), and/or myelin delaminations (Ballinger, 1997) migrate, accumulate, and pack tightly (likely through the interactions of SNAREs) at the damage site to reduce the influx of extracellular Ca^{2+} and other ions until plasmalemmal continuity is restored. We have also reported that vesicle-mediated repair of plasmalemmal damage is promoted by calpain (Godell, 1997), novel protein kinase C isozyme η (nPKC η), protein kinase A (PKA), and cAMP (Spaeth, 2010), as well as, nPKCθ, DAG, MARCKS and Munc13-1 (Chapter 2).

We have recently used the murine hippocampal B104 cell line to investigate proteins and substrates that influence plasmalemmal sealing, *in vitro*, by transecting a single neurite (axon-like projection) of a B104 cell and assessing the cell's ability to exclude an extracellular dye (Yoo, 2003, 2004; Nguyen, 2005; Spaeth, 2010, 2011). We have now translated our work on sealing of transected neurites to test the hypothesis that, the same proteins and substrates involved in sealing damaged (transected) B104 neurites should also promote sealing of OGD-induced plasmalemmal damage in B104 neurons. We now report that, unlike sealing of transected B104 neurites, the presence of Ca^{2+} following immediate OGD exposures of 1, 3, 5 or 10 minutes, did not promote sealing probability in B104 cells compared to cells not receiving Ca^{2+} following similar OGD exposures. However, cells exposed to peptide inhibitors of nPKC η or θ, during OGD

exposures of 1, 3, 5 or 10 minutes had significantly decreased sealing probability compared to normoxic (no treatment) controls and, cells exposed to OGD only. These data suggest that nPKC η and θ promote sealing of OGD-induced plasmalemmal damage in B104 cells, as they do for sealing of transected neurites of B104 cells.

RESULTS

For all plasmalemmal sealing data (sealing probability) shown, B104 cells were exposed to 1, 3, 5 or 10 minutes of OGD or normoxia in phosphate buffered saline without Ca^{2+} ($PBS^{=}$) and sometimes, in the presence of previously identified sealing inhibitors or enhancers (Spaeth, 2010; Chapter 2) dissolved in $PBS^{=}$. Following such exposures, cells were returned to normoxia, typically in normoglycemic phosphate buffered saline with Ca^{2+} (PBS^{2+}) or, sometimes in normoglycemic $PBS^{=}$. Five minutes after return to normoxia, Texas Red dextran dye (neutral, 3kDa) was added to each dish. Cells were exposed to dye for no longer than 10 minutes; dye was rinsed from dishes using respective normoxic saline. We assessed sealing as the ability of viable B104 cells (i.e., the cell must have an intact plasmalemma) to exclude extracellular dye. The total number of viable cells (*n*), sealed and unsealed, were pooled from (*N*) number of dishes used for each condition (**Table 5**). From the total, pooled number of cells per treatment, we calculated sealing probability as the percent of viable cells excluding dye (sealed).

Table 5: Sealing probabilities and total number of plates and cells (*N*, *n*) observed per OGD condition.

Treatments	Duration of exposure (minutes) to *Treatments* **1**	**3**	**5**	**10**
Normoxic control ($PBS^{=} \rightarrow PBS^{2+}$)	99% (4, 439; **Fig. 16**) 100% (12, 1346; **Fig. 17**)	99% (5, 532; **Fig. 16**) 99% (13, 1379; **Fig. 17**)	99% (7, 765; **Fig. 16**) 99% (5, 469; **Fig. 17**)	98% (4, 404; **Fig. 16**) 98% (4, 400; **Fig. 17**)
OGD ($PBS^{=} \rightarrow PBS^{2+}$)	99% (2, 257; **Fig. 16**) 100% (4, 436; **Fig. 17**)	98% (4, 408; **Fig. 16**) 96% (8, 810; **Fig. 17**)	99% (2, 197; **Fig. 16**) 99% (2, 200; **Fig. 17**)	74% (4, 456; **Fig. 16**) 74% (4, 403; **Fig. 17**)
Following data shown in Figure 2 only				
Normoxia ($PBS^{=} \rightarrow PBS^{=}$)	99% (2, 181)	100% (2, 206)	98% (2, 235)	88% (4, 396)
OGD ($PBS^{=} \rightarrow PBS^{=}$)	99% (2, 200)	100% (2, 204)	98% (2, 200)	82% (4, 407)
Following data shown in Figure 3 only				
Normoxia ($PBS^{=} + \eta PSF \rightarrow PBS^{2+}$)	98% (4, 441)	98% (4, 427)	99% (4, 412)	96% (4, 411)
Normoxia ($PBS^{=} + \theta PSF \rightarrow PBS^{2+}$)	100% (4 ,429)	98% (4, 436)	97% (4, 413)	97% (4, 429)
OGD ($PBS^{=} + \eta PSF \rightarrow PBS^{2+}$)	86% (4, 417)	91% (4, 405)	64% (7, 589)	52% (4, 422)
OGD ($PBS^{=} + \theta PSF \rightarrow PBS^{2+}$)	87% (2, 317)	88% (3, 299)	81% (6, 541)	45% (4, 411)
OGD ($PBS^{=} + \eta PSF + \theta PSF \rightarrow PBS^{2+}$)	94% (4, 416)	85% (4, 409)	DNC	DNC

*DNC = Did Not Count plates; treatment consistently caused cell death on all dishes (at least 4 plates per OGD exposure).

Statistically significant differences between normoxic control (no treatment) and experimental treatments or, between experimental treatments were calculated using Cochran-Mantel-Haenszel χ^2 (CMH χ^2; Spaeth, 2010, 2011; Chapter 2) test for independence (**Table 6**). Statistically significant differences between normoxic control and experimental treatments, only, are indicated on graphs with asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Lastly, for all abbreviations of control and experimental treatments in the results, phrases preceding a solid arrow (→) indicate the experimental or control treatment. The arrow always indicates return to normoxia and, phrases

following the arrow indicate the type of phosphate buffered saline used during return to normoxia. For example, OGD ($PBS^{=} \rightarrow PBS^{=}$), indicates that cells were exposed to OGD in $PBS^{=}$ and then, returned to normoxia in $PBS^{=}$.

Table 6: Post-OGD sealing probability comparisons using CMH χ^2 statistical analysis

	Comparison of sealing probabilities after duration of exposure (minutes) to *Treatments*			
Treatment comparisons for data shown in Figure 2	**1**	**3**	**5**	**10**
Normoxic control ($PBS^{=} \rightarrow PBS^{2+}$) *vs.* Normoxia ($PBS^{=} \rightarrow PBS^{=}$)	99% *vs.* 99% NS	99% *vs.* 100% NS	99% *vs.* 98% NS	98% *vs.* 88% $p<0.05$
Normoxic control *vs.* OGD ($PBS^{=} \rightarrow PBS^{2+}$)	99% *vs.* 99% NS	99% *vs.* 98% NS	99% *vs.* 99% NS	98% *vs.* 74% $p<0.001$
Normoxia ($PBS^{=} \rightarrow PBS^{=}$) *vs.* OGD ($PBS^{=} \rightarrow PBS^{=}$)	99% *vs.* 99% NS	100% *vs.*100% NS	98% *vs.* 98% NS	88% *vs.* 82% NS
OGD ($PBS^{=} \rightarrow PBS^{2+}$) *vs.* OGD ($PBS^{=} \rightarrow PBS^{=}$)	99% *vs.* 99% NS	98% *vs.* 100% NS	99% *vs.* 98% NS	74% *vs.* 82% NS
Treatment comparisons for data shown in Figure 3				
Normoxia ($PBS^{=} + \eta PSF \rightarrow PBS^{2+}$) *vs.* Normoxic control	98% *vs.* 100% NS	99% *vs.* 99% NS	99% *vs.* 99% NS	96% *vs.* 98% NS
OGD ($PBS^{=} + \eta PSF \rightarrow PBS^{2+}$) *vs.* Normoxic control	86% *vs.* 100% $p<0.001$	91% *vs.* 99% $p<0.01$	64% *vs.* 99% $p<0.001$	52% *vs.* 98% $p<0.001$
OGD ($PBS^{=} + \eta PSF \rightarrow PBS^{2+}$) *vs.* OGD ($PBS^{=} \rightarrow PBS^{2+}$)	86% *vs.* 100% $p<0.01$	91% *vs.* 96% NS	64% *vs.* 99% $p<0.001$	52% *vs.* 74% $p<0.01$
Normoxia ($PBS^{=} + \theta PSF \rightarrow PBS^{2+}$) *vs.* Normoxic control	100% *vs.* 100% NS	98% *vs.* 99% NS	97% *vs.* 99% NS	97% *vs.* 98% NS
OGD ($PBS^{=} + \theta PSF \rightarrow PBS^{2+}$) *vs.* Normoxic control	87% *vs.* 100% $p<0.001$	88% *vs.* 99% $p<0.01$	81% *vs.* 99% $p<0.001$	45% *vs.* 98% $p<0.001$
OGD ($PBS^{=} + \theta PSF \rightarrow PBS^{2+}$) *vs.* OGD ($PBS^{=} \rightarrow PBS^{2+}$)	87% *vs.* 100% $p<0.001$	88% *vs.* 96% NS	81% *vs.* 99% $p<0.001$	45% *vs.* 74% $p<0.001$
OGD ($PBS^{=} + \eta PSF \rightarrow PBS^{2+}$) *vs.* OGD ($PBS^{=} + \theta PSF \rightarrow PBS^{2+}$)	86% *vs.* 87% NS	91% *vs.* 88% NS	64% *vs.* 81% $p<0.05$	52% *vs.* 45% NS
OGD ($PBS^{=} + \eta PSF + \theta PSF \rightarrow PBS^{2+}$) *vs.* Normoxic control	94% *vs.* 100% $p<0.05$	85% *vs.* 99% $p<0.001$	NC	NC
OGD ($PBS^{=} + \eta PSF + \theta PSF \rightarrow PBS^{2+}$) *vs.* OGD ($PBS^{=} \rightarrow PBS^{2+}$)	94% *vs.* 100% $p<0.05$	85% *vs.* 96% $p<0.05$	NC	NC
OGD ($PBS^{=} + \eta PSF + \theta PSF \rightarrow PBS^{2+}$) *vs.* OGD ($PBS^{=} + \eta PSF \rightarrow PBS^{2+}$)	94% *vs.* 86% $p<0.05$	85% *vs.* 91% NS	NC	NC
OGD ($PBS^{=} + \eta PSF + \theta PSF \rightarrow PBS^{2+}$) *vs.* OGD ($PBS^{=} + \theta PSF \rightarrow PBS^{2+}$)	94% *vs.* 87% $p<0.05$	85% *vs.* 88% NS	NC	NC

Table 6: Statistically significant differences between normoxic control and experimental treatments are indicated on graphs with asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). NS = No statistical significance. NC = No comparison made because treatment [OGD ($PBS^{=} + \eta PSF + \theta PSF \rightarrow PBS^{2+}$)] caused extensive cell death.

OGD exposures did not adversely affect cell morphology

We first assessed if decreased sealing probability (i.e., increased dye inclusion) caused by increased OGD exposures were attributable to extensive plasmalemmal damage resulting in blebbing and loss of cell morphology – typical signs of non-viable cells. We compared cells prior to OGD exposure (**Fig. 15A**), to cells after 1, 3, 5 or 10 minutes of OGD exposure (**Fig. 15B-E**) and did not observe any difference in cell morphology between cells prior to or after OGD exposure. Lastly, viable cells including dye (i.e., did not seal) did not show signs of blebbing or morphological changes compared to viable, sealed cells (**Fig. 15H, I**).

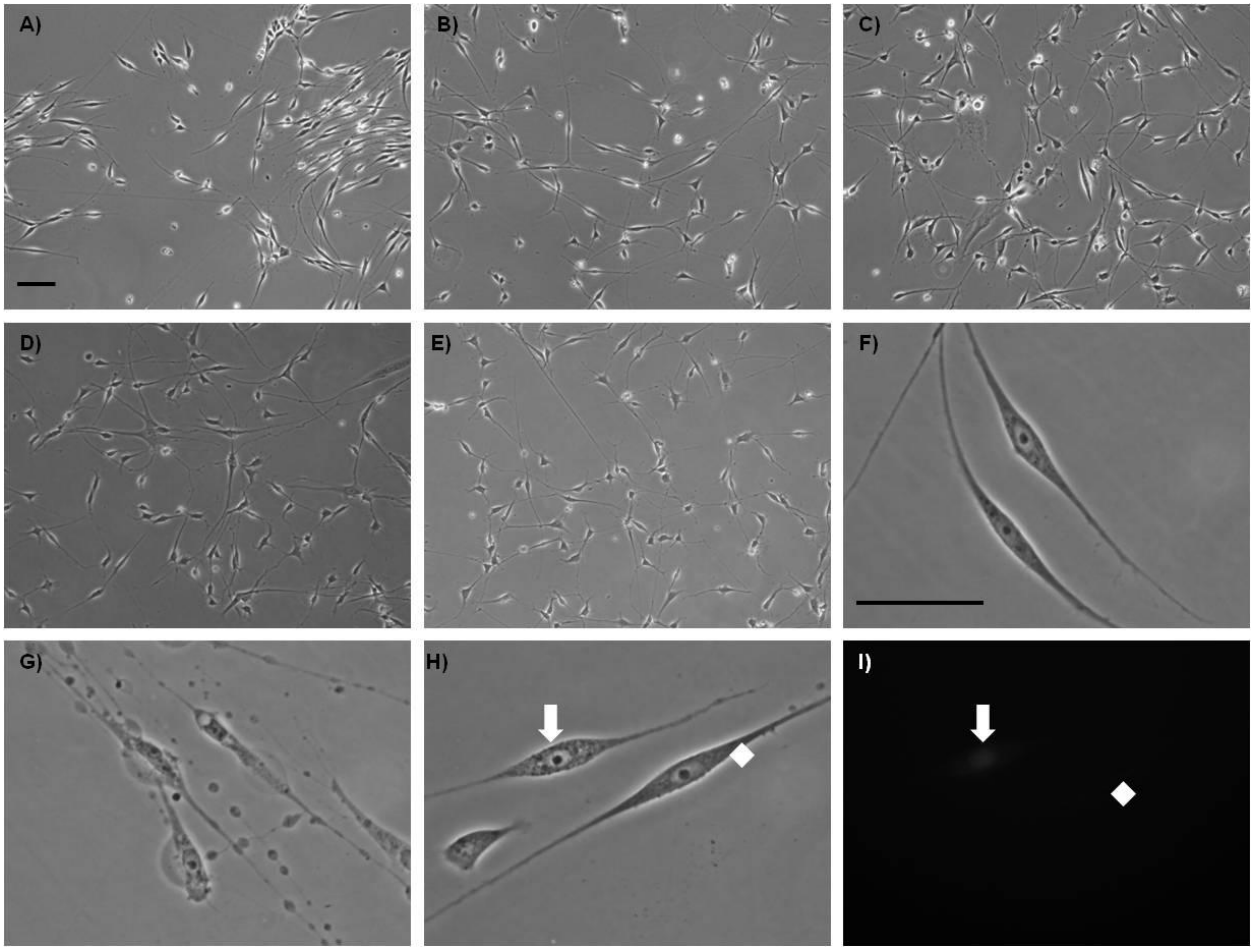

Figure 15: Images of B104 cells before and after OGD exposure

A-E: Taken before (**A**) or, after 1 minute (**B**), 3 minutes (**C**), 5 minutes (**D**) or, 10 minutes (**E**) of OGD exposure. Scale bar 100 μm at 10x magnification.
F, G: Images of cells that have retained (viable, **F**) or lost (non-viable, **G**) morphology following ischemic insult. **F-I:** Scale bar 50 μm at 60x magnification.
H-I: Light (**H**) and fluorescence (**I**) images of adjacent cells that sealed (white diamond) or did not seal (white arrow) following 10 minutes of OGD exposure

Presence of Ca^{2+} following OGD did not increase sealing of OGD-induced plasmalemmal damage

Studies of plasmalemmal sealing in various vertebrate and invertebrate preparations (Krause, 1994; Eddelman, 1997; Steinhardt, 1994; Togo, 2003), including B104 cells (Nguyen, 2005; Spaeth, 2010; Chapter 2), have all concluded that Ca^{2+} influx

at plasmalemmal damage sites is required to promote sealing at such damage sites. Since OGD causes plasmalemmal damage, we determined if Ca^{2+} promoted sealing of OGD-induced plasmalemmal damage in B104 cells. We first determined the effect of the presence or absence of Ca^{2+} on normoxic controls. B104 cells were exposed to normoxia (0% glucose, atmospheric O_2) in $PBS^{=}$ for 1, 3, 5 or 10 minutes, following such exposures, $PBS^{=}$ was exchanged with $PBS^{=}$ or PBS^{2+}, cells were maintained under normoxia, and dye was added 5 minutes after media exchange. B104 cells receiving Ca^{2+} after 10 min exposure to normoxia in $PBS^{=}$ (Normoxia $PBS^{=} \rightarrow PBS^{2+}$) had significantly ($p<0.05$, CMH χ^2) better sealing probability (98% *vs.* 88%) than cells not receiving Ca^{2+} (Normoxia $PBS^{=} \rightarrow PBS^{=}$) after such $PBS^{=}$ exposure (**Table 6**; **Fig. 16**, compare unfilled, gray circles to, unfilled, black circles). No statistically significant difference ($p>0.05$, CMH χ^2) in sealing probability was observed between B104 cells receiving or not receiving Ca^{2+} after 1, 3 or 5 minutes exposure to normoxia in $PBS^{=}$ (**Table 6**; **Fig. 16**). From here on, all treatments are compared to the normoxic control receiving Ca^{2+} following exposure to normoxia in $PBS^{=}$; referred to as "normoxic control" sealing and abbreviated as Normoxia ($PBS^{=} \rightarrow PBS^{2+}$) in all graphs and, Normoxic control in all tables.

We next determined if Ca^{2+} addition, following exposure to OGD in $PBS^{=}$, promoted sealing of OGD-induced plasmalemmal damage. B104 cells receiving Ca^{2+} after 10 min exposure to OGD in $PBS^{=}$ (OGD $PBS^{=} \rightarrow PBS^{2+}$) had significantly ($p<0.001$, CMH χ^2) decreased sealing probability (74% *vs.* 98%) compared to normoxic control sealing (**Table 6**; **Fig. 16**). B104 cells not receiving Ca^{2+} after 10 min exposure to

OGD in $PBS^{=}$ (OGD $PBS^{=}$ → $PBS^{=}$) had significantly ($p<0.001$, CMH χ^2) decreased sealing probability (82% *vs.* 98%) compared to normoxic control sealing (**Table 6**; **Fig. 16**). Lastly, no statistically significant difference ($p>0.05$, CMH χ^2) in sealing probabilities was observed between B104 cells receiving or not receiving Ca^{2+} after 1, 3, 5 or 10 minutes exposure to OGD in $PBS^{=}$ (**Fig. 16,** compare filled to unfilled diamonds).

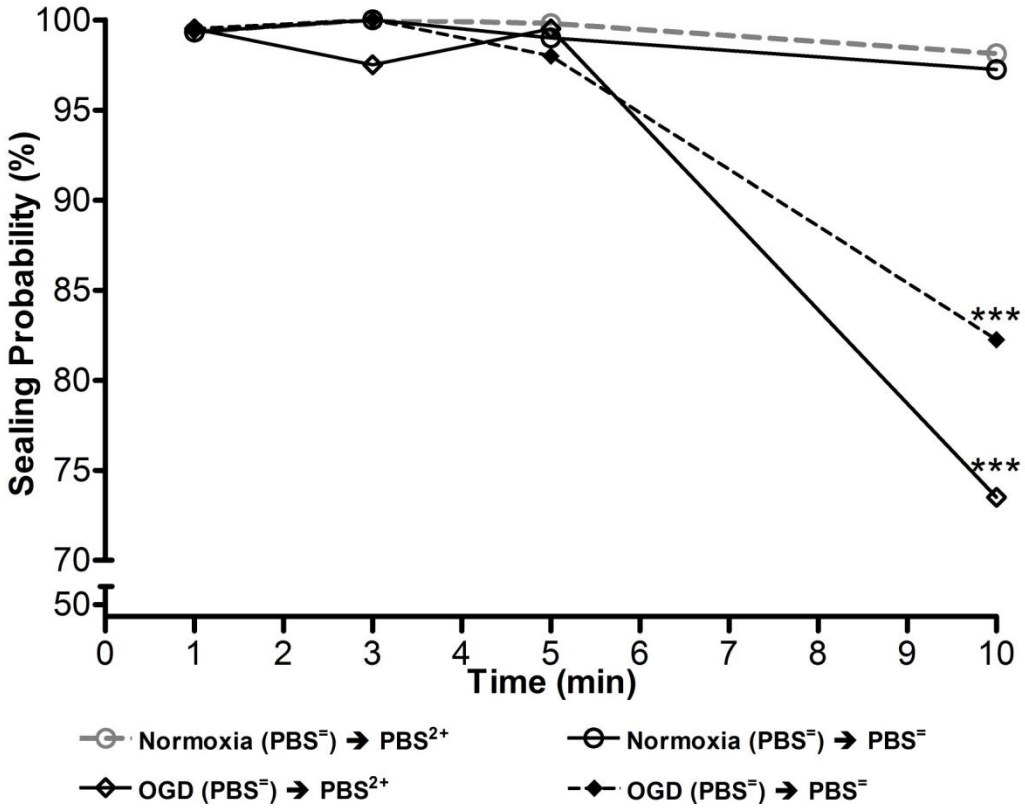

Figure 16: Sealing probability (%) *vs.* Normoxia or OGD exposure (min).

Effect of Ca^{2+} addition following normoxia or OGD in Ca^{2+}-free phosphate buffered saline. Normoxic control sealing (gray, unfilled circles and dashed line) on this and all other graphs.

Inhibition of η or θ PKC during oxygen-glucose deprivation decreased post-ischemic plasmalemmal repair

We have recently shown that inhibition of nPKC η (Spaeth, 2010) or nPKCθ (Chapter 2) decreased sealing of transected neurites of B104 cells – suggesting these nPKC isozymes promote plasmalemmal sealing. To this end, we determined if nPKC η or θ promotes sealing of OGD-induced plasmalemmal damage in B104 cells. We first determined the effect of nPKC η or θ pseudosubstrate fragments (ηPSF, Spaeth, 2010; θPSF, Staal, 2008; Barros, 2009) on B104 cells under normoxia. B104 cells were exposed to normoxic conditions (0% glucose, atmospheric O_2) in $PBS^{=}$ for 1, 3, 5 or 10 minutes in the presence of ηPSF or θPSF, following such exposures, $PBS^{=}$ was exchanged with PBS^{2+}, cells were maintained under normoxia, and dye was added 5 minutes after media exchange. B104 cells exposed to ηPSF or θPSF for 1, 3, 5 or 10 minutes under normoxia had no statistically significant ($p>0.05$, CMH χ^2) difference in sealing probabilities compared to normoxic sealing (**Table 6**; **Fig. 17,** compare unfilled triangle and unfilled, black circle to, unfilled, gray circle).

We determined if the presence of ηPSF during OGD in $PBS^{=}$, inhibited sealing of OGD-induced plasmalemmal damage. B104 cells exposed to ηPSF during OGD in $PBS^{=}$ (OGD $PBS^{=}$ + ηPSF → PBS^{2+}) had significantly decreased sealing probability after 1, 3, 5 and 10 minutes ($p<0.01$,$p<0.01$, $p<0.001$, and $p<0.001$, respectively, CMH χ^2) of exposure compared to normoxic control sealing (**Table 6**; **Fig. 17,** compare filled triangles to, unfilled, gray circles). Furthermore, exposure to ηPSF during OGD significantly decreased sealing probability after 1, 5 and 10 minutes ($p<0.01$, $p<0.001$

and $p<0.01$, respectively, CMH χ^2) of exposure compared to cells exposed to OGD only (**Table 6**; **Fig. 17,** compare filled triangles to, unfilled diamonds).

Next, we determined if the presence of θPSF during OGD in $PBS^{=}$, inhibited sealing of OGD-induced plasmalemmal damage. B104 cells exposed to θPSF during OGD in $PBS^{=}$ (OGD $PBS^{=}$ + θPSF → PBS^{2+}) had significantly decreased sealing probability after 1, 3, 5 and 10 minutes ($p<0.001$, $p<0.01$, $p<0.001$, and $p<0.001$, respectively, CMH χ^2) of exposure compared to normoxic control sealing (**Table 6**; **Fig. 17,** compare filled circles to, unfilled, gray circles). Furthermore, exposure to θPSF during OGD significantly ($p<0.001$, CMH χ^2) decreased sealing probability after 1, 5 and 10 minutes of exposure compared to cells exposed to OGD only (**Table 6**; **Fig. 17,** compare filled triangles to, unfilled diamonds). No statistically significant difference ($p>0.05$, CMH χ^2) in sealing probabilities was observed, when comparing B104 cells exposed to OGD in $PBS^{=}$ in the presence of ηPSF, to cells exposed to OGD in $PBS^{=}$ in the presence of θPSF (**Table 6**; **Fig. 17,** compare filled circles to filled triangles), except at 5 minutes ($p<0.05$, CMH χ^2).

Lastly, we determined if the presence of ηPSF and θPSF during OGD in $PBS^{=}$, would produce even greater inhibition on sealing of OGD-induced plasmalemmal damage. B104 cells exposed to ηPSF + θPSF during OGD in $PBS^{=}$ (OGD $PBS^{=}$ + ηPSF + θPSF → PBS^{2+}) had significantly decreased sealing probability after 1 and 3 minutes ($p<0.05$ and, $p<0.01$ respectively, CMH χ^2) of exposure compared to normoxic control sealing (**Table 6**; **Fig. 17,** compare X to, unfilled, gray circles). Furthermore, exposure to ηPSF + θPSF during OGD significantly ($p<0.05$, CMH χ^2) decreased sealing probability

after 1 and 3 minutes of exposure compared to cells exposed to OGD only (**Table 6**; **Fig. 17,** compare X to, unfilled diamonds). However, all cells exposed to OGD and ηPSF + θPSF for 5 minutes died, suggesting plasmalemmal integrity was significantly effected by this treatment.

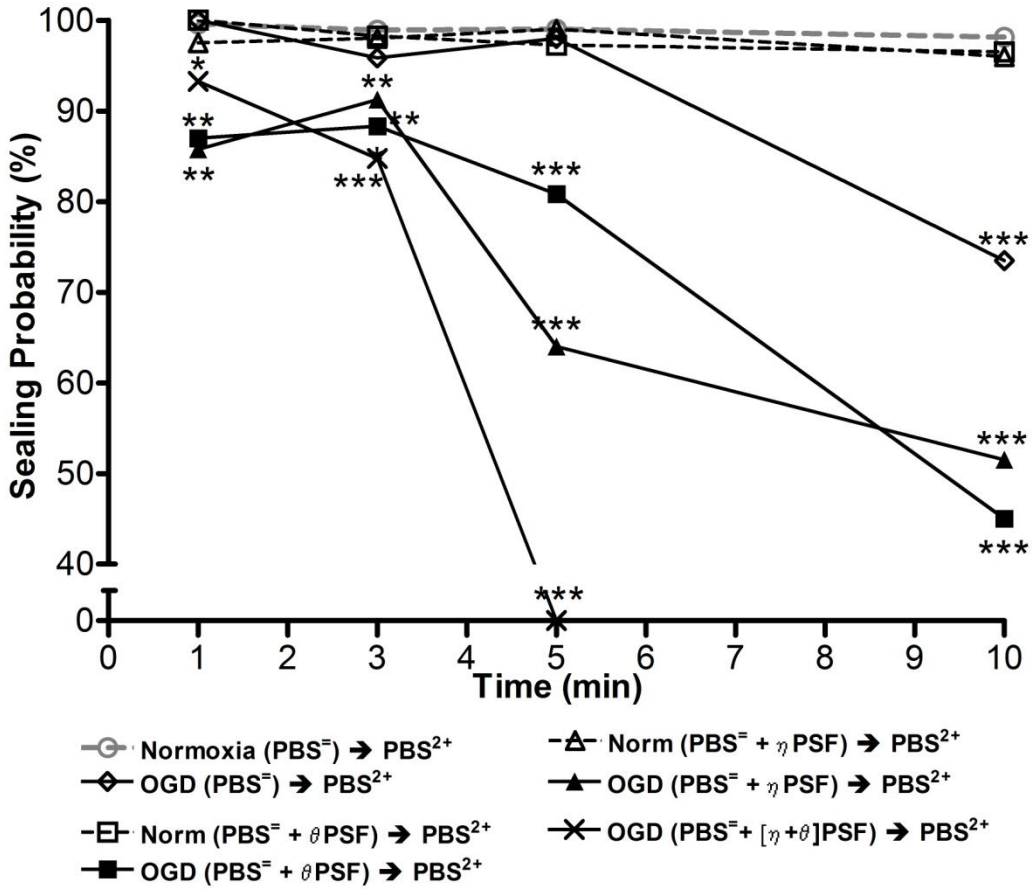

Figure 17: Sealing probability (%) *vs.* Normoxia or OGD exposure (min) in PKC inhibitors.

Effect of ηPSF or θPSF during OGD in $PBS^{=}$, on post-ischemic plasmalemmal sealing.

DISCUSSION

In this study we induced plasmalemmal damage in B104 cells by exposure to ischemic (oxygen and glucose deprivation; OGD) insult, sometimes in the presence of specific peptide inhibitors of nPKC isozymes η and θ, to determine the role of such nPKC isozymes in sealing of OGD-induced plasmalemmal damage. We now report that the presence of nPKC η or θ peptide inhibitors, during all OGD exposures (1, 3, 5 or 10 min), decreased sealing probabilities compared to both normoxic control and cells exposed to OGD only (**Table 6**; **Fig. 17**). Furthermore, exposing cells to OGD in the presence of both ηPSF + θPSF further decreased sealing probabilities compared to either inhibitor alone. These data suggest that nPKC η and θ promote sealing of OGD-induced plasmalemmal damage in B104 cells. Furthermore, our data suggest that sealing of plasmalemmal damage in B104 cells, whether transection- (Spaeth, 2010; Chapter 2) or OGD-induced, occurs via a universal mechanism that requires nPKC η and θ.

OGD exposure: Viable *vs.* Non-viable cells

Ischemia (OGD) is well known to produce easily observable plasmalemmal damage such as, membrane blebs, loss of cell morphology and extracellular dye inclusion (Hoffstein, 1975; Chien, 1978; Farber, 1981; Herman, 1988; Neiminen, 1988; Zahrebelski, 1995; Wang, 1996; Nishimura, 2001; Rodriguez-Sinovas, 2005). However, following acute OGD exposures, viable B104 cells did not exhibit blebbing or loss of cell morphology (**Fig. 15A-E**), even in cells including extracellular dye (**Fig. 15H, I**). Furthermore, only non-viable B104 cells (not used in this study) demonstrated blebbing,

loss of cell morphology or cytoplasmic contents -- typically after 10 min OGD exposure (images not shown). These data suggest that most cells were capable of sealing (repairing) plasmalemmal damage caused by such OGD-exposures. And, that decreased sealing probability (increased dye inclusion) caused by increased OGD exposure and presence of nPKC η or θ peptide inhibitors during OGD exposure, was likely caused by an increase in the number of plasmalemmal damage sites and inhibition of sealing, not loss of viability -- as demonstrated by maintenance of cell morphology and lack of blebbing in cells including dye after OGD exposures (**Fig. 15H, I**).

With or without Ca^{2+}: B104 cells seal OGD-induced plasmalemmal damage

As stated previously, plasmalemmal sealing in vertebrate and invertebrate preparations (Krause, 1994; Eddelman, 1997; Steinhardt, 1994; Detrait, 2000b: Togo, 2003), including B104 cells (Nguyen, 2005; Spaeth, 2010; Chapter 2), is induced by Ca^{2+} influx at plasmalemmal damage sites. We expected that sealing of OGD-induced plasmalemmal damage would increase when cells exposed to OGD were returned to normoxia in the presence of Ca^{2+}. However, data in **Figure 16** show that B104 cells have similar sealing probabilities (**Table 6**) of OGD-induced plasmalemmal damage when returned to normoxia in the presence or absence of Ca^{2+}. A plausible explanation for this unexpected result may be that 1, 3, 5 or 10 min exposures to OGD create many, small plasmalemmal damage sites (large enough for 3 kDa Texas Red dextran dye to enter) that require few vesicles and little to no increase in local $[Ca^{2+}]_i$ to be sealed. As reported previously, it is possible to promote sealing in the absence of Ca^{2+}: addition of a DAG-

(Chapter 2) or cAMP- (Spaeth, 2010) analog to cell media following neurite transection promoted sealing in the absence of Ca^{2+}. However, in both cases, neither increased DAG nor cAMP concentrations could substitute for Ca^{2+}, which is required to maintain cell viability.

Thus, it is plausible that OGD-induced plasmalemmal damage activates a Ca^{2+}-independent mechanism that increases DAG- or cAMP-dependent sealing pathways (Spaeth, et al., 2010), such as cytosolic oxidation (Spaeth et al., 2011). Cytosolic oxidation may enhance DAG-dependent proteins such as nPKC η and θ and, Munc13-1 (Chapter 2). As reported in Spaeth et al. (2011) cytosolic oxidation does enhance cAMP-dependent sealing via proteins such as Epac and PKA (Spaeth, 2010). Therefore, cytosolic oxidation may promote sealing of OGD-induced plasmalemmal damage without requiring large increases in $[Ca^{2+}]_I$ to induce sealing. Testing of longer exposures to OGD and normoxia in calcium-free saline with oxidizing and reducing agents (Spaeth et al., 2011) should elucidate whether cytosolic oxidation has neuroprotective effects on OGD-induced plasmalemmal sealing.

Conclusion

In conclusion, the most disconcerting fact of current stroke treatment modalities is that none are capable of preventing massive loss of cells following reperfusion. Our current preliminary study suggests that repair (sealing) of ischemia (OGD) induced plasmalemmal damage in murine B104 cells is similar to repair of transected neurites of B104 cells; which is similar to sealing in many invertebrate and vertebrate preparations (Spaeth, 2010). These data suggest that sealing may be a universal mechanism regardless

of mode of plasmalemmal damage. Thus, promoting endogenous plasmalemma repair factors (such as nPKC η and θ, PKA and Epac) before, during or after repurfusion may reduce loss of cells. Continued research on proteins, such as nPKC η and θ (Chapter 2), PKA and Epac (Spaeth, 2010) and, molecular messengers, such as DAG (Chapter 2) or cAMP (Spaeth, 2010) that promote plasmalemmal sealing *in vitro*, under normoxic and hypoxic environments, should greatly and rapidly improve neuroprotective treatment modalities. Such improvements should seriously diminish loss of cells and correlated behavioral functions, and improve overall recovery following a stroke.

MATERIALS AND METHODS

B104 cells:

B104 cells derived from a CNS neuroblastoma (Bottenstein and Sato, 1979) have often been used as a model system to study neuronal function *in vitro* (Toda et al., 1999; Tan et al., 2003; Yoo et al., 2003, 2004; Nguyen et al., 2005; Miller et al., 2006; Spaeth et al., 2010). B104 cells extend neurites that have properties typical of nerve axons such as generation of action potentials, smooth (but not rough) endoplasmic reticulum, release of neurotransmitter, and regeneration of severed neurites. Unlike some other model neuronal cell lines (e.g., PC12 cells), B104 cells do not require growth factor supplements of fetal bovine serum to proliferate (Detrait et al., 2000b; Yoo et al., 2003; Spaeth et al., 2010). Data on sealing of B104 cells are consistent with similar data on sealing from at least 19 other preparations from many phyla and different cell types.

Cell culture:

As described previously (Nguyen et al., 2005; Spaeth et al., 2010), B104 cells were grown in 75 cm^2 vented cap flasks (BD Falcon; BD Biosciences) in a humidified incubator at 37°C in 5% CO2 and in 4 ml of cell growth medium: consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM:F-12, HyClone) supplemented for growth with 10% heat-inactivated fetal bovine serum (FBS; HyClone) and 1% antibiotics (10,000 U of penicillin/ml and 10 mg/ml streptomycin; Sigma-Aldrich). The cell growth medium was changed every 2 d. Cultures were passaged at 80% confluency, and cells were then either subcultured in a vented cap flask or seeded at ~2000 cells/cm^2 in cell growth media on Petri dishes coated with poly-D-lysine (Sigma-Aldrich) to prevent cells from detaching during solution changes and/or neurite transection. After 24 h, the growth media was replaced with serum-free DMEM:F12 (Hyclone) to allow the B104 cells to differentiate. B104 cells were exposed to oxygen and/or glucose deprivation 24–48 h after replacing the cell growth media with serum-free DMEM:F12.

Oxygen-Glucose Deprivation (OGD):

For immediate OGD experiments, Petri dishes containing normoglycemic media (DMEM:F12) were placed inside an anaerobic vinyl-glove-box (Coy Laboratory Products, Grass Lake, MI) kept at 37°C. Petri dishes were quickly rinsed twice with anoxic, hypoglycemic $PBS^{=}$ (Hyclone). [Note: To ensure solutions were anoxic at start of OGD exposures, $PBS^{=}$ and PKC peptide inhibitor solutions were maintained or created in

anaerobic vinyl-glove-box at least 24 h prior to experimentation.] Immediately following rinses, cells were exposed to OGD for 1, 3, 5 or 10 minutes in $PBS^{=}$. Following OGD exposures, $PBS^{=}$ in Petri dishes was typically replaced with normoglycemic and normoxic Ca^{2+}-containing PBS (PBS^{2+}) or, sometimes with normoglycemic and normoxic PBS=, and all dishes were returned to a normoxic 37°C incubator.

Assessment of dye exclusion:

At 5 min after return to normoxic conditions we added Texas Red dextran (Molecular Probes, Eugene, OR; 3 kDa), to the PBS^{2+} or, sometimes $PBS^{=}$, to assess the formation of a plasmalemmal seal using a Nikon TS100F inverted fluorescent microscope (Nikon Instruments Inc., Melville, NY) equipped with a rhodamine filter, at 60x magnification. Prior to observation, plates were rinsed twice with respective PBS solution. Viable B104 cells (i.e., the cell must have an intact plasmalemma) exposed to OGD that exclude Texas Red dextran are counted as "sealed" and cells that do not exclude Texas Red dextran are counted as "not sealed" (**Fig. 15F,G**). We consistently use a 3 kDa dye to standardize our comparisons of sealing between experimental and control treatments because higher MW dyes are excluded faster than lower MW dyes (Yoo, *et al*., 2003; Spaeth et al., 2010).

Reagents:

Pharmacological agents (treatments) were dissolved in $PBS^{=}$ (0 mM glucose), maintained in anaerobic chamber for at least 24 h prior to experimentation and, added to Petri dishes

to start OGD. To inhibit nPKC isozymes we used myristoylated nPKCη pseudosubstrate fragment (ηPSF; Spaeth, 2010), myristoylated nPKCθ pseudosubstrate fragment (θPSF; Staal et al., 2008; Barros, 2009), purchased from EMD Chemicals, Gibbstown, NJ.

Statistical Analyses:

We obtained dye exclusion data from many, individually-identifiable B104 cells that all received similar treatment (Normoxia or OGD) in a tightly controlled environment. To obtain sealing probabilities (%) for each treatment group, after a given exposure time, cell count data were pooled for all cells (*n*) from all Petri dishes (*N*). See **Table 5**, for *n* and *N* values for each data set at each exposure time for all figures presented in this paper. "Sealing probability" is defined as the percentage of cells that excluded 3 kDa Texas Red dextran dye (sealed) after a given exposure time. As described previously (Detrait, 2000b; Yoo , 2003, 2004; Spaeth, 2010; Chapter 2 and 3), the Cochran-Mantel-Haenszel (CMH) χ^2 test for independence was used to determine statistically significant differences between sealing probabilities of a given treatment exposure to normoxic control or, another treatment (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). GraphPad Prism4 was used to graph all data.

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Chapter 5: General Conclusions and Future Studies

IMPORTANCE OF PLASMALEMMAL SEALING

Cells that do not repair (seal) plasma membrane (plasmalemmal) damage, do not survive (McNeil, 1993, 2000; Nguyen et al. 2005). For victims of neurotrauma, such as peripheral nerve crush injury (PNCI) or stroke (plasmalemmal damage is produced by oxygen-glucose deprivation), loss of nerve cells (neurons) directly correlates with loss of functional behaviors and even paralysis. Furthermore, in mammals, regaining lost functional behaviors is dependent upon axonal regeneration; which occurs from a *surviving* proximal axonal end that slowly grows neuritic processes at 1-2mm/day that often do not re-establish original synapses (Britt et al. 2010). Thus, after PNCI or stroke, plasmalemmal sealing is a critical first step for neurons (Krause et al. 1994; Steinhardt et al. 1994; Bittner and Fishman, 2000; Detrait et al. 2000; Yoo et al. 2003; Spaeth et al. 2010), because *only surviving neurons are capable of axonal regeneration*, which correlates with increased functional behavior recovery (Britt et al. 2010; Bittner et al. 2012).

GENERAL SUMMARY OF DISSERTATION RESULTS

The goals of my dissertation research were as follows: (**1**) determine which PKC isozymes and their effectors, and whether DAG and other DAG-activated proteins are involved in plasmalemmal sealing (Chapter 2), (**2**) investigate whether inhibiting PKC- and PKA-dependent sealing could increase the probability of PEG-repair of PNCI following injury in physiologically relevant conditions, in a rat model (Chapter 3) and (**3**)

determine what role PKCs may have in sealing of ischemia-induced plasmalemmal damage in an *in vitro* model of stroke in B104 neurons (Chapter 4). In the following paragraphs, I will briefly summarize the important results and conclusions presented in Chapters 2 – 4. I will then briefly discuss future studies, some utilizing techniques other than dye exclusion, which should provide further insight on protein-protein interactions during sealing to better determine sealing pathways.

Chapter 2 – Role of DAG, nPKC, MARCKS and Munc13-1 in sealing

The first goal of my dissertation research was to determine which PKC isozymes and their effectors, and whether DAG and other DAG-activated proteins were involved in plasmalemmal sealing in mammalian neurons (B104 cells). To determine how these proteins and substrates influenced sealing, I transected neurites of B104 cells in the presence of specific peptide inhibitors of PKC isozymes PKA, MARCKS and Munc13-1 and small-molecule inhibitors of PKC, PLC, NSF and Golgi trafficking --sometimes in the presence of analogs of DAG or cAMP. Data presented in Chapter 2 suggest that DAG increases sealing by activating MARCKS, and Munc 13-1, and that nPKCη, nPKCθ and PKA are all required to seal plasmalemmal damage (**Fig. 8**).

Furthermore, data presented in Chapter 2 (also see, Zuzek et al. 2012), and previously reported data from B104 cells and data from many other eukaryotic preparations (see Supplementary Reference list in Spaeth et al. 2010) suggest that vesicle-mediated plasmalemmal sealing in eukaryotic cells, is an evolutionarily conserved process. This sealing is induced by Ca^{2+} influx at plasmalemmal damage sites (Fishman and Bittner, 2003) and enhanced by increased cAMP concentration (Spaeth et

al. 2010) and DAG concentration and utilizes multiple vesicle-mediated pathways and membrane sources (Ballinger et al. 1997; Eddleman et al. 1997, 1998; Reddy et al. 2001; Spaeth et al. 2010). These pathways utilize both Ca^{2+}-dependent and Ca^{2+}-independent membrane fusion proteins, such as synaptobrevin, syntaxin, synaptophisin, synaptotagmin (Steinhardt et al. 1994; Detrait et al. 2000a,b; Yoo et al. 2003), Munc13-1 (Zuzek et al. 2012) and, enzymes, such as PKA (Spaeth et al. 2010), nPKC η and θ (Zuzek et al. 2012) for formation and maintenance of a plasmalemmal seal (**Fig. 8A, B**).

Data presented in Chapter 2 provide evidence of how PKCs and DAG influence plasmalemmal sealing in neurons *in vitro*. Such insights have direct and indirect implications for axonal regeneration after traumatic injury (Bozkurt 2008; Bittner et al, 2012) and for stroke in which neuronal membranes are damaged (Rodriguez-Sinovas et al. 2005). For example, cells that do not seal plasmalemmal damage, do not survive (Nguyen et al. 2005). Therefore, compounds that enhance endogenous sealing (OAG or db-cAMP) may be capable of rapidly promoting sealing of neurons damaged by traumatic injury or stroke, thereby greatly diminishing loss of behavioral function correlated with neurotrauma and stroke. As a second example, compounds that inhibit endogenous sealing (nPKC or PKA peptide inhibitors) may be capable of enhancing polyethylene glycol (PEG) induced fusion of plasmalemmas, thereby greatly increasing the already profound effects of PEG on recovery of physiological and behavioral functions of crushed and/or severed peripheral nerves (Bittner et al. 2012). Thus, a greater knowledge of proteins and substrates that influence plasmalemmal repair (sealing)

in vitro, should provide targets and substrates to improve neuroprotective treatment modalities.

Chapter 3 – Trial and Error: Inhibiting nPKC's and PKA do not increase the probability of PEG-fusing rat sciatic axons crushed in physiological calcium

The second goal of my dissertation was to determine whether inhibiting PKC- and PKA-dependent sealing could increase the probability of PEG-repair of PNCI, following injury in physiologically relevant calcium concentration, in a rat model (Chapter 3). Our current PEG-fusion protocol shown in Figure 3.2 relies on bathing sciatic nerves in hypotonic, calcium free physiological saline prior to and during crush, for successful PEG-fusions (Stavisky et al. 2005; Britt et al. 2010; Bittner et al. 2012). In Chapter 3, I used an *in vitro* cell-based dye exclusion assay to determine whether compounds that inhibit Ca^{2+}-induced plasmalemmal sealing in neuronal B104 cells *in vitro* (**Fig. 11A**) could be used to promote PEG-fusion of sciatic axons *in vivo*, following crush-severance in physiological calcium concentration (to realistically mimic peripheral nerve crush injury) (**Table 3**). Only simultaneous inhibition of nPKC η, θ and PKA, dramatically decreased sealing when cells were transected in calcium *in vitro* (**Fig. 11A, B**). Inhibitors of nPKC and PKA did not inhibit PEG-induced sealing *in vitro* (**Fig. 11C, D**). However, when PEG was applied to cells transected in the combined presence of nPKC η, θ and PKA inhibitors, whether or not calcium was present during transection, all cells (intact and damaged) included Texas red dye suggesting that such treatment was cytotoxic *in vitro* (**Fig. 11C, D**).

Given these *in vitro* data, I carried on investigating whether nPKC η, θ and PKA inhibitor treatments could increase the probability of PEG-fusion following crush-severance in physiological calcium concentration. Data presented in Chapter 3 conclusively show that applying inhibitors of nPKC and PKA (individually or in combination) to lesion sites immediately after crush-severance in Ca^{2+}-containing physiological saline, but prior to PEG application, do not promote PEG-fusion as assessed by CAP conduction and dye diffusion through lesion sites (**Table 3**).

As expected, PEG-fusions were always observed for all treatment groups in which PEG was applied to lesion sites immediately after crush-severance in hypotonic Ca^{2+}-free physiological saline, as assessed by CAP conduction and dye diffusion through the lesion site (**Table 2; Fig. 13**). That is, this study confirmed that successful PEG-fusions require that crushed axons are bathed in hypotonic Ca^{2+}-free physiological saline, as previously reported in Stavisky et al. (2005), Britt et al. (2010) and Bittner et al. (2012).

Chapter 4 – Preliminary Study: Role of nPKC η and θ in neuroprotective sealing of ischemia-induced plasmalemmal damage

The third goal of my dissertation was to determine the roles of nPKC η and θ in sealing of ischemia-induced plasmalemmal damage. I induced plasmalemmal damage in B104 cells by exposure to ischemic (oxygen and glucose deprivation; OGD) insult, sometimes in the presence of specific peptide inhibitors of nPKC isozymes η and θ, to determine the role of such nPKC isozymes in sealing of OGD-induced plasmalemmal damage. Data presented in Chapter 4 show that inhibition of nPKC η or θ, during all

OGD exposures (1, 3, 5 or 10 min), decreased sealing probabilities compared to both normoxic control and cells exposed to OGD only (**Table 6**; **Fig. 17**). Furthermore, exposing cells to OGD in the presence of both ηPSF + θPSF further decreased sealing probabilities compared to either inhibitor alone [but proved toxic after only 5 minutes of exposure to OGD and ηPSF + θPSF]. These data suggest that nPKC η and θ promote sealing of OGD-induced plasmalemmal damage in B104 cells. Furthermore, our data suggest that sealing of plasmalemmal damage in B104 cells, whether transection- (Spaeth, 2010; Chapter 2) or OGD-induced, occurs via a universal mechanism that requires nPKC η and θ.

General Conclusion on Dissertation Results

In conclusion, continued research on proteins, such as nPKC η and θ (Chapter 2), PKA and Epac (Spaeth, 2010) and, molecular messengers, such as DAG (Chapter 2) or cAMP (Spaeth, 2010) that promote plasmalemmal sealing *in vitro*, under normoxic (neurite transection) and hypoxic (stroke) environments, should greatly and rapidly improve neuroprotective treatment modalities. Such improvements should seriously diminish loss of cells and correlated behavioral functions, and improve overall recovery following a stroke.

Future studies on plasmalemmal sealing should focus on determining protein-protein interactions to better resolve sealing pathways, and provide more accurate targets for therapeutic treatment modalities.

FUTURE STUDIES

Testing different [cAMP] in ηPSF+θPSF to begin to determine if nPKC negatively feedbacks on PKA in B104 cells

In Chapter 2, Figure 4D, we unexpectedly observed that addition of db-cAMP to ηPSF+θPSF significantly decreased sealing compared to ηPSF+θPSF alone. These data suggest that DAG-PKC and cAMP-PKA sealing pathways not only converge, but may interact in a feedback loop. That is, a decrease in nPKC activity (produced by nPKC inhibitors) in the presence of increased cAMP during plasmalemmal sealing, initiates negative feedback on PKA-dependent sealing pathways. Such a feedback loop would decrease the activity of those sealing pathways not blocked by nPKC inhibitors, such as PKA, and would further decrease sealing compared to ηPSF+θPSF alone, as seen in Figure 4D.

A preliminary study to determine whether such a feedback loop exists could be performed by using our current dye exclusion protocol on transected B104 cells: cells would be transected in ηPSF+θPSF, and differing concentrations of db-cAMP (1mM was used in Chapter 2, and Zuzek et al. 2012). If the aforementioned feedback loop does exist, decreasing [cAMP] in ηPSF+θPSF should promote sealing to levels similar to ηPSF+θPSF alone; however, increasing [cAMP] even more (>1mM) in ηPSF+θPSF should further decrease sealing compared to ηPSF+θPSF alone by increasing negative feedback on PKA. If feedback loop does *not* exist, increasing [cAMP] greater than 1mM in ηPSF+θPSF should increase sealing, suggesting that the previously used [cAMP] was not high enough to overcome ηPSF+θPSF. Possible pitfalls include the following: increasing [cAMP] higher than 1mM may be cytotoxic and, increasing [cAMP] may have

no affect at all, suggesting the current concentration is the maximum effective concentration. Taken together, performing a db-cAMP dose response curve on sealing in B104 cells would be advantageous in determining whether or not increased or decreased cAMP concentrations have differing effects on sealing compared to 1mM db-cAMP.

Protein Kinase D and Ras guanyl nucleotide releasing protein1

Like nPKC isozymes and Munc13-1, protein kinase D (PKD) and Ras guanyl nucleotide releasing protein1 (RasGRP) also contain the DAG binding C1 domain (Brose and Rosenmund, 2002). Like most of the targets studied in this dissertation, PKD and RasGRP are involved in constitutive vesicle trafficking, and interact with nPKCη and nPKCθ, respectively (Carrasco and Merida, 2004; Diaz-Anel and Malhotra, 2005).

Firstly, western blotting should be performed to determine whether PKD and RasGRP are expressed by B104 cells. Secondly, to determine if PKD or RasGRP influence sealing, B104 cells would be transected in inhibitors of PKD or RasGRP, and dye exclusion would be assessed at various PC times to determine the effect of such inhibitors on sealing probability and rate (as was done in Chapter 2 for inhibitors of nPKC, PLC, Munc13-1, MARCKS, etc). Inhibitors of PKD and RasGRP should decrease sealing, suggesting that PKD and RasGRP promote sealing in B104 cells, and likely all other eukaryotic cells.

Considering the known interactions between PKD and RasGRP with nPKCη and nPKCθ (Carrasco and Merida, 2004; Diaz-Anel and Malhotra, 2005), such data should be a catalyst to employ techniques that would better resolve protein-protein interactions in

B104 and other eukaryotic cells during sealing. Because, our current protocol of dye exclusion cannot explicitly distinguish protein interactions.

Fluorescence Resonance Energy Transmission (FRET): A microscopy method for confirming protein interactions from past and future sealing studies

Our current protocol, dye exclusion, does not provide evidence of protein interactions. To better resolve sealing pathways, imaging techniques, such as FRET, should be used to determine whether hypothetical interactions exist in future sealing studies; and retroactively to confirm previously hypothesized interactions as well. FRET is a fluorescence imaging technique that relies on energy transfer from one excited fluorophore (donor) to a second (different) fluorophore (acceptor) in close proximity (10 nm, or 100 angstroms). FRET does require that a cell be stably transfected and express fluorophore conjugated proteins. Thus, if the proteins conjugated to the donor and acceptor fluorophores do interact, the typical result is quenching of the donor fluorophore and increased emission from the acceptor fluorophore. An alternative FRET approach is loss of FRET upon interaction, typically caused by phosphorylation of a target protein (causing allosteric changes in protein structure) that undergoes FRET in its unphosphorylated state (Redden et al. 2012). This latter approach to FRET could be very useful for determining PKC and PKA interactions in sealing pathways.

As mentioned above, current dye exclusion data suggest that nPKC and PKA may interact in a negative feedback loop (**Fig. 4D**, Chapter 2). Both PKA and PKC interact with A-kinase anchoring protein (AKAP), and AKAP7 binds both active and inactive

PKC isozymes (Redden et al. 2012). Some AKAPs are inhibitory, that is, binding of a catalytically active enzyme to such an AKAP, like AKAP12, decreases its activity (Redden et al. 2012). Of interest for future studies would be to conjugate nPKC η and θ, and PKA to respective fluorophores (e.g. PKCs = donors; and PKA acceptor), and observe FRET during sealing. Furthermore, such studies should also include observing FRET in the presence or absence of nPKC or PKA specific peptide inhibitors, and exogenously increased cAMP. Such conditions would provide further evidence of interaction between nPKCs and PKA, and whether decreased nPKC activity negatively feedbacks on PKA, likely through AKAP.

Of particular interest for sealing studies employing FRET is the use of an alternative FRET approach: loss of FRET in target protein upon phosphorylation by an active kinase. One such method is the use of CKAR, a target peptide for active PKC and thus, a reporter of PKC activity (Redden et al. 2012). In Redden et al. (2012), CKAR was conjugated to AKAP7 to determine whether PKC interacted with AKAP7; loss of FRET in CKAR was observed when PKC was stimulated. A similar approach could be employed with regards to PKA and nPKC η and θ: here only CKAR and similar peptide target for PKA would be conjugated to an AKAP (likely AKAP7). Under such conditions, loss of FRET from CKAR or PKA's target peptide conjugated to an AKAP would be evidence of direct interaction between nPKC η and θ or PKA at an AKAP. Furthermore, analogous experiments could employ truncated versions of AKAPs (AKAP fragments), which can be used as selective peptide inhibitors to inhibit binding of PKCs and PKA to AKAP (Redden et al. 2012). Data from these analogous experiments should

provide further evidence of which AKAP both nPKC η and θ, and PKA interact with (i.e., in the presence of AKAP fragments, CKAR FRET should be expected, even when PKC is stimulated).. Taken together, data collected from FRET studies *during sealing* would provide evidence of whether nPKCs and PKA interact at specific AKAPs, and whether decreased nPKC activity negatively feedbacks on PKA, through interaction at AKAP.

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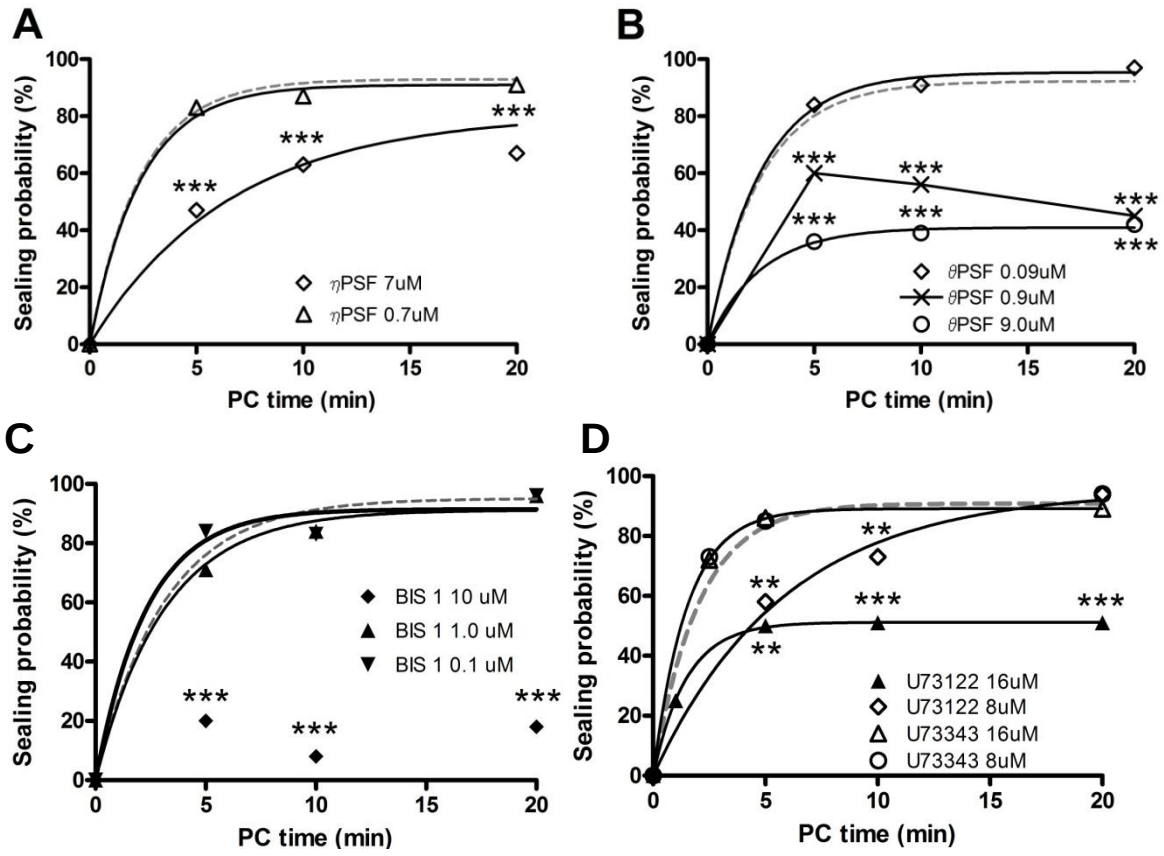
APPENDIX

Appendix Figure A1: Effect of differing concentrations of treatments on sealing probability (%)



Appendix Figure A1:
(**A**) Effect of 0.7 or 7.0μM ηPSF on sealing.
(**B**) Effect of 0.09, 0.9 or 9.0μM θPSF on sealing.
(**C**) Effect of 0.1, 1.0 or 10.0μM Bisindoylmaleimide 1 (Bis1) on sealing.
(**D**) Effect of 8 or 16μM U73122 or, inactive analog of U73122, U73343, on sealing.
Significance levels for this figure are indicated with asterisks: no symbol, $p>0.95$; ***, $p\leq0.001$.

We tested the following compounds to insure that we were using appropriate concentrations to affect sealing in B104 cells: nPKC pseudosubstrate fragments η and θ PSF, reported use of such PSFs in micromolar concentrations (ηPSF, Spaeth et al. 2010;

θPSF, Staal et al. 2008; Barros et al. 2009); Bisindoylmaleimide (Bis 1), reported use in nanomolar concentrations to inhibit PKCs (Togo, 2004); U73122 in micromolar concentrations to inhibit phospholipase C (PLC) activity and for a negative control, we used the inactive analog of U73122, U73343 in similar concentrations (Bleasdale et al. 1990; Hildebrandt et al. 1997; Tanaka et al. 2003). [The Methods section, Pharmacological Reagents, states the concentrations used and the primary references for the use of these concentrations.] To determine the best concentration for inhibition of plasmalemmal sealing, we employed two assays:

1) A dye exclusion assay (see Methods) to assess sealing in cells with transected neurites (data shown, see Results and Online Resource 1) exposed to differing concentrations of compounds. As stated above, this Figure can be moved to the main text.

2) A cytotoxicity assay (data not shown) in which intact B104 cells were exposed to differing concentrations of a compound for 10 minutes in Ca^{2+}-free saline (to mimic exposure time during transection), meanwhile we looked for changes in morphology of intact cells, dye inclusion in intact cells, or cell death. If a specific concentration of a compound caused cytotoxic effects, that concentration was not used in dye exclusion experiments. The following compounds, db-cAMP, PKA, Brefeldin A and NEM, have all been previously reported to effect sealing in B104 cells (Spaeth et al. 2010, 2011 a, b).

Appendix Table A: Concentration of compounds used based on Primary references

Compounds	Concentration used in our study	Primary Reference(s) for compounds used	Concentration used in primary reference(s)
nPKC inhibitors			
η PSF	7 μM	Liu, 2006; Barros, 2009	2 and 20 μM
θ PSF	9 μM	Staal, 2008; Barros, 2009	2 and 10 μM
cPKC inhibitors			
20-28 PSF	8 μM	Liu, 2006; Jungnickel, 2007; Ratz and Miner, 2009	10 and 20 μM
Gö6976	1 μM	Togo, 1999 and 2006	1 μM
Bis1	0.1, 1 and 10 μM	Togo, 1999	0.5 μM
PLC inhibitors			
U73122	16 μM	Bleasdale, 1990: Hildebrandt, 1997; Tanaka, 2003	10 and 20 μM
U73343 (inactive analog of U73122)	16 μM	Bleasdale, 1990; Hildebrandt, 1997; Tanaka, 2003	10 and 20 μM
DAG analog			
OAG	100 μM	Doherty, 1995; Wang and Oram, 2007; Ju, 2010	10, 50, 100 μM
PKA inhibitor			
PKI	50 μM	Spaeth, 2010	50 μM
PKA activator			
db-cAMP	1 mM	Spaeth 2010	1 mM
MARCKS inhibitor			
BIO-11006 (peptide)	100 μM	Agrawal, 2007; Damera, 2010	100 μM
Munc13-1 inhibitor			
Mid-protein	100 μM	Mochida, 1998	125 μM
NSF inhibitor			
NEM	1 mM	Rodriguez, 1994; Spaeth 2011	1 or 3 mM
Golgi traffic inhibitor			
Brefeldin A	10 μM	Togo and Steinhardt, 2003; Spaeth 2011	50 μM

PSF = pseudosubstrate fragment.

Appendix Table B: Number of plates and transected B104 cells per PC time point

Treatment	PC time (min)				
	1	2.5	5	10	20
Control	(2, 99)	(2, 108)	(4, 258)	(5, 64)	(6, 286)
DMSO			(8, 209)	(3, 102)	(6, 202)
η PSF			(3, 108)	(3, 53)	(3, 99)
θ PSF			(2, 85)	(3, 125)	(3, 115)
η PSF + θ PSF		(2, 174)	(2, 246)		(2, 167)
20-28 PSF			(2, 130)	(3, 81)	(3, 113)
Go6976			(3, 109)	(5, 102)	(3, 88)
Bis1 100 nM			(3, 101)	(3, 90)	(3, 95)
Bis1 1 μM			(3, 126)	(3, 78)	(3, 154)
U73122	(2, 139)		(2, 157)	(3, 136)	(2, 118)
U73343		(2, 99)	(3, 134)	(2, 96)	(3, 124)
OAG		(2, 122)	(2, 172)		(2, 241)
OAG no Ca^{2+}		(2, 145)	(4, 263)		(1, 89)
U73122 + OAG		(2, 122)	(2, 182)		(2, 120)
MARCKSpi		(2, 121)	(2, 168)		(2, 208)
MARCKSpi + OAG		(2, 88)	(2, 182)		(2, 208)
NEM		(3, 134)	(3, 180)		(3, 128)
NEM + OAG		(2, 140)	(4, 318)		(2, 134)
PKI		(2, 112)	(3, 113)		(3, 121)
NEM + PKI		(2, 273)	(2, 169)		(2, 204)
PKI + OAG		(2, 235)	(2, 141)		(2, 112)
θ PSF + OAG		(2, 163)	(2, 136)		(2, 143)
η PSF + OAG		(2, 211)	(2, 286)		(2, 148)
η PSF + θ PSF + OAG		(2, 176)	(2, 182)		(2, 214)
db-cAMP		(2, 107)	(2, 112)		(2, 120)
θ PSF + db-cAMP		(2, 109)	(2, 130)		(1, 98)
η PSF + db-cAMP		(2, 108)	(2, 102)		(1, 127)
η PSF + θ PSF + db-cAMP		(2, 357)	(2, 225)		(1, 115)
η PSF + θ PSF + PKI (PBS^{2+})		(2, 147)	(2, 134)		(2, 114)
η PSF + θ PSF + PKI		(2, 137)	(2, 116)		(2, 144)
η PSF + θ PSF + PKI + OAG (PBS^{2+})		(2, 114)	(2, 134)		(2, 122)
η PSF + θ PSF + PKI + OAG		(2, 107)	(2, 116)		(2, 116)
η PSF + θ PSF + PKI + db-cAMP (PBS^{2+})		(2, 121)	(2, 143)		(2, 182)
η PSF + θ PSF + PKI + db-cAMP		(2, 137)	(2, 118)		(2, 144)
Bref-A		(1, 75)	(2, 161)		(2, 159)
Bref-A + OAG		(2, 160)	(4, 504)		(3, 354)
Munc-pi		(2, 202)	(2, 194)		(2, 225)
Munc-pi + OAG		(2, 191)	(2, 138)		(2, 227)
Munc-pi + db-cAMP		(2, 180)	(2, 168)		(2, 245)
Munc-pi scramble		(2, 241)	(2, 222)		(2, 236)
Munc-pi scramble + OAG		(2, 202)	(2, 106)		(2, 206)
Munc-pi scramble + db-cAMP		(2, 194)	(2, 208)		(2, 159)

Table B: The numbers in parentheses (N, n) represent the number of Petri dishes (N) used to obtain sealing data from (n) transected B104 cells. The total number of cells (n) listed per condition was obtained by combining the sum of all cells counted (sealed or not sealed) from each dish per condition at a given PC time. Blank space: no experiments performed for condition at designated PC time. (PBS^{2+}) indicates neurites were transected in Ca^{2+}-containing saline for that condition.

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Vita

A native of Texas, I was interested in the natural world from a very early age. Nose down in the grass, digging in the dirt, or eyes gazing across the horizon are some of the ways you will often find me while hiking in Austin, or at any one of the amazing State and National Parks in this country.

After living overseas in London, England with my parents for 8 years (ages 9 – 17), I moved back to Texas to attend The University of Texas at Austin. I obtained two bachelor's degrees in Biology (BA and BS) and, participated in research as an undergraduate assistant in Dr. George D. Bittner's lab.

I have a keen interest in the cellular mechanisms of plasmalemmal sealing and repair and, how these mechanisms affect cell viability, regeneration and recovery of functional behaviors. I have recently become very interested in stroke-related research regarding mechanisms of plasmalemmal sealing and repair. As it seems, promotion of such mechanisms would prove highly beneficial following a stroke, to prevent further cell death and loss of functional behaviors. I am looking forward to my career in research and what 'Science' has waiting for me beyond my doctoral research!

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