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·Review·

Mechanisms of neuronal membrane sealing following mechanical trauma

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Membrane integrity is crucial for maintaining the intricate signaling and chemically-isolated intracellular environment of neurons; disruption risks deleterious effects, such as unregulated ionic flux, neuronal apoptosis, and oxidative radical damage as observed in spinal cord injury and traumatic brain injury. This paper, in addition to a discussion of the current understanding of cellular tactics to seal membranes, describes two major factors involved in membrane repair. These are line tension, the hydrophobic attractive force between two lipid free-edges, and membrane tension, the rigidity of the lipid bilayer with respect to the tethered cortical cytoskeleton. Ca^{2+}, a major mechanistic trigger for repair processes, increases following flux through a membrane injury site, and activates phospholipase enzymes, calpain-mediated cortical cytoskeletal proteolysis, protein kinase cascades, and lipid bilayer microdomain modification. The membrane tension appears to be largely modulated through vesicle dynamics, cytoskeletal organization, membrane curvature, and phospholipase manipulation. Dehydration of the phospholipid gap edge and modification of membrane packaging, as in temperature variation, experimentally impact line tension. Due to the time-sensitive nature of axonal sealing, increasing the efficacy of axolemmal sealing through therapeutic modification would be of great clinical value, to deter secondary neurodegenerative effects. Better therapeutic enhancement of membrane sealing requires a complete understanding of its intricate underlying neuronal mechanism.

Keywords: axolemmal sealing; membrane tension; line tension; phospholipase; calpain; poly-ethylene glycol; patch model

Introduction

The integrity of the plasma membrane is critical to the cell as it protects and maintains the functionality of the isolated intracellular environment. Situations involving trauma to the plasma membrane result in disrupted integrity and subsequent permeability to ions and molecules. Of principal interest is Ca^{2+} influx into the intracellular space, activating proteases, disrupting mitochondrial function, and activating apoptotic pathways[1, 2]. Thus, effective and efficient repair of plasma membrane integrity is essential for cell survival.

Mechanical disruption of the neuronal membrane has been extensively studied *in vitro*, *in vivo*, *ex vivo*, and after traumatic brain injury (TBI) or spinal cord injury (SCI)[3-6]. Membrane healing within the nervous system has been studied in a number of model organisms: sea slug (*Aplysia californica*)[7], cockroach (*Periplaneta americana*)[8], guinea pig[5], earthworm (*Lumbricus terrestris*)[9], squid (*Loligo pealei*)[10], rat[11], and snail (*Helisoma trivolvis*)[12]. Mechanical disruption of neurons has focused primarily on axonal transection, stretch, and compression[13-17]. Such injuries can produce a breach in membrane integrity from the moment of injury, and this is termed “mechanoporation”[3].

Experimental data on axonal trauma indicate that repair of the initial membrane breach is the necessary first step to allow growth-cone formation and subsequent axonal regeneration[18]. Thus, establishment of this membrane sealing is key to the successful recovery of the neuron, highlighting the value of understanding the sealing process. Well-established quantitative techniques in membrane integrity analysis include dye staining or fluorescent marker influx through a membrane breach[5,17,19,20], and de-staining of fluorescent markers localized intracellularly before the trauma[21]. The magnitude of the labeling through these techniques is a function of the applied mechanical load to the membrane[22].

While neurons differ from other cell types in morphology, environmental exposure, mitotic status, motility, and function, a consideration of different cell types is essential to better understand the healing of the neuronal membrane upon mechanical disruption. The cytoskeletal and structural distinctness between somal membrane and axolemma may cause differences in membrane sealing; however, the processes may have much in common due to the presence of cell-type specific enzymes involved in the repair process. One of the largest discrepancies in membrane sealing between neurons and other cell types is the time course, exemplified by neuronal membranes requiring minutes to hours[13] compared to Swiss 3T3 fibroblasts and sea urchin eggs requiring seconds to minutes[23, 24]. These discrepancies in physiological mechanisms could stem from an evolutionary lack of preparedness within the mechanically-protected neuron population compared to the relatively exposed fibroblast population. So, caution must be used when considering broad claims of applicability between these very different populations of cells.

Proposed Models of Membrane Sealing

Based on *in vitro* analysis of cellular and axolemmal membrane repair, several models have emerged to explain the cellular mechanics involved in membrane sealing. The factors of interest in this discussion are line tension and membrane tension, which are regulated by the dynamics of intracellular vesicles and cytoskeletal remodeling. Line tension refers to the thermodynamic force at the free phospholipid edge of a membrane lesion that favors hydrophobic interactions between adjacent phospholipids, thereby promoting spontaneous membrane sealing. The membrane tension is opposing membrane sealing based on the rigidity of the underlying tethered cortical cytoskeleton[25,26], which prevents the progression to a lower entropic state in which phospholipids interact between the lipid free edges during a membrane breach.

Considering the implications of tension, McNeil and Terasaki constructed a framework to illustrate the mechanism of membrane resealing[19]. Line tension has been proposed to dominate in situations of small disruptions less than 1 μm in diameter, thereby promoting membrane sealing[19] (Fig. 1C); above this diameter, membrane tension would overcome the energy for thermodynamic distortion of the free membrane edge, so it is necessary to reduce membrane tension to facilitate resealing[27] (Fig. 1A). Thus, a logical mechanism of sealing would involve reducing membrane tension to decrease the gap in the membrane to <1 μm, to facilitate association between adjacent phospholipids on each side of the gap (Fig. 1C). The reduction in membrane tension essential for sealing has been quantified using the laser tweezer method, and has been shown to correspond with vesicle exocytosis, which increases the membrane surface area and consequently decreases the tension[21]. For even larger membrane disruptions, Ca^{2+}-dependent intervesicular fusion forms a membrane patch to seal the larger gap more efficiently[24, 28, 29]. The cellular commitment to one of these two mechanisms of repair may be based on the magnitude of Ca^{2+} influx as determined by the disruption size, mediating the occurrence of vesicle-vesicle or vesicle-membrane fusion events based on the resulting level of intracellular Ca^{2+}. Soluble NSF attachment protein receptors (SNAREs) are implicated in this Ca^{2+}-dependent membrane repair process, as synaptotagamin, SNAP-25, synaptobrevin, and syntaxin mediate the Ca^{2+}-dependent vesicular fusion[10, 30]. An alternative idea to the membrane patch has recently been proposed, implicating Ca^{2+}-dependent transglutaminases in the cross-linking of intracellular proteins to form a proteinaceous clot that decreases the membrane permeability, similar to the principle of the membrane patch[31, 32]. In addition, recent studies with streptolysin O pores and electroporation have suggested that endocytosis helps remove lesions from

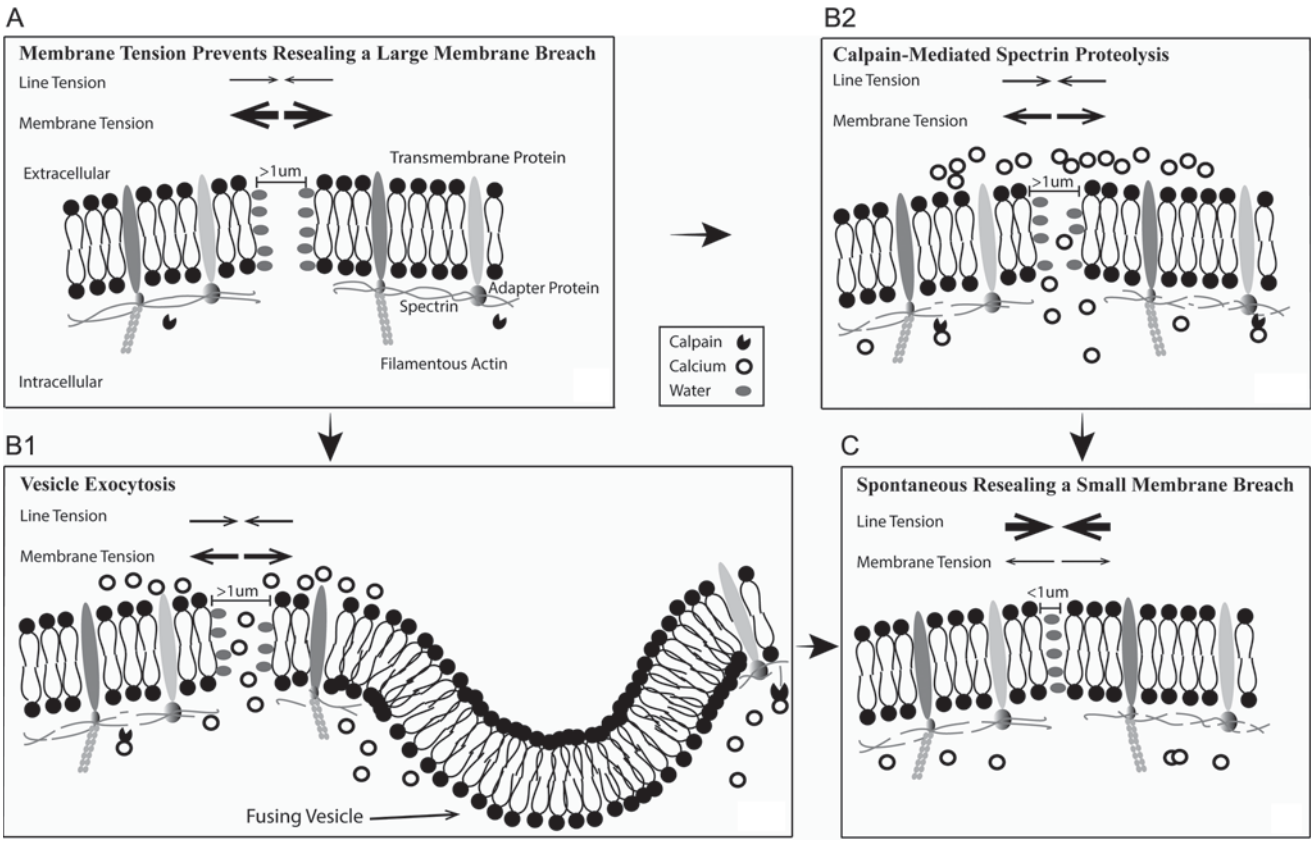

Fig. 1. Forces influencing membrane sealing. The membrane sealing process is governed by a balance between line tension and membrane tension at the axolemma. Line tension promotes membrane sealing through the attractive force between adjacent hydrophobic regions of the membrane, and membrane tension generated from tethers to the underlying cortical cytoskeleton hinders resealing. A: When the membrane breach is >1 μm, membrane tension is the dominant force and membrane sealing is prevented. However, when the breach is reduced by membrane-vesicle fusion (B1) or calpain-mediated cortical cytoskeletal proteolysis of targets such as spectrin (B2), line tension becomes the dominant force and spontaneous membrane sealing occurs (C). The magnitudes of these forces are represented by the sizes of the corresponding arrows in each diagram.

the plasma membrane, thereby facilitating membrane sealing[31,33,34]. Such a mechanism would first involve an exocytosis-dependent reduction in membrane tension, which is conducive to subsequent endocytosis of the membrane lesion.

Role of Line Tension in Axolemmal Sealing

Line tension, as described previously[35], promotes spontaneous membrane sealing through the thermodynamic force of attraction between hydrophobic phospholipid regions along the membrane site of disruption following injury. This force opposes the membrane tension; a predominance of line tension would result in spontaneous membrane sealing (Fig. 1C). Line tension relates largely to the packing ability within the membrane such that the efficient packing of lipid bilayer components results in decreased line tension[36]. Decreased packaging efficiency through cholesterol incorporation has been proposed to limit the free rotation of fatty acyl tails in the membrane, resulting in increased line tension[36]. Line tension is inversely proportional to the membrane disruption radius, as depicted by poration of liposomes and through the derived pore free energy equation[36].

Temperature Dependence of Line Tension

Hypothermic treatment following SCI and TBI has been implemented in clinical settings due to its proposed benefits of reducing intracranial pressure and providing neuroprotection. However, mechanistically, these benefits

have not been experimentally shown. The decreased temperature, based on elementary membrane dynamics, would induce decreased membrane fluidity and increased packing efficiency. As described above, increased packing efficiency would decrease line tension; thus, hypothermia would decrease the rate of membrane sealing through regulating thermodynamic potential energy alone. This proposition is supported by studies using the double sucrose-gap recording chamber, which showed that sealing efficiency *ex vivo* decreases dramatically at 25°C compared to that at 31°C and 37°C[13], indicating a need for reconsideration of the hypothermic treatment protocol. In addition, although there are no significant differences in membrane sealing between 31°C and 37°C[13], hypoxia-induced injury recovers significantly better at mildly hypothermic temperatures *in vitro*[37, 38]. Thus, the mild hypothermic condition of 31°C appears to be the most conducive for healing from mechanically-induced TBI or SCI.

Viral protein-mediated fusion, an experimental model for studying membrane fusion mechanics, has shown dependence on temperature, such that decreasing the temperature results in slowing of the fusion process[39]. Phospholipase C (PLC) is also temperature-dependent such that activation of PLC, vesicle aggregation, and vesicle fusion increase with increasing temperature[40]. Thus, the data further contraindicate the use of intense hypothermia in treating SCI and TBI during the early stages when membrane repair is the priority. Based on studies of axolemmal repair *in vitro*, the time course of sealing appears to be 45 min–1 h, using the presence of resting membrane potential and horseradish peroxidase exclusion as indicators of successful sealing[13]. More conclusive studies on the time course of mammalian neuronal membrane sealing *in vivo* are needed to further direct clinical care for patients with neurological trauma.

Role of Membrane Tension in Neuronal Membrane Sealing

Lowering the membrane tension following liposome pore formation is known to facilitate repair[36, 41]. Artificial decreases in membrane tension by the surfactants Pluronic F68 NF[27] and polyethylene glycol (PEG) also facilitate repair[17]. Further studies have shown that deposition of membrane vesicles at the lesion site decreases plasma membrane tension[21, 42] (Fig. 1B1). The implications for membrane tension in mammalian plasma membrane repair and the Ca^{2+}-dependent nature of the repair have been reported, using the laser-tweezer method of membrane tension quantification[43], within Swiss 3T3 fibroblasts[21]. Laser scissors or glass needle methods of injury (5 μm in diameter) revealed that the membrane repair process largely involves a quantifiable Ca^{2+}-dependent reduction in membrane tension following vesicle fusion to the plasma membrane[21]. Also, membrane tension increases proportionally to the square of the radius of the membrane pore[36], suggesting that reducing the pore radius is necessary to achieve a membrane seal. Moreover, using atomic force microscopy, Nehrt *et al.* found that PEG reduces the neuronal membrane tension[17]. Based on the small pore-diameter necessary for effective line tension-mediated sealing[19], a reduction in the pore diameter facilitated by a less rigid membrane would better facilitate repair of the injury (Fig. 1B2). Reduction in membrane tension has a complex group of potential causes that interplay simultaneously to produce the cytoskeletal and membrane changes necessary to facilitate repair.

Cytoskeletal Modification

The membrane tension of cells is largely derived from adhesion to the underlying cytoskeleton[44], implying the importance of cytoskeletal rearrangement in membrane sealing. Cytoskeletal modification is essential for membrane sealing in a number of cell types, including neurons, based on the finding that inhibition of the Ca^{2+}-activated cysteine protease calpain leads to incomplete membrane sealing[5,25,45]. Cytoskeletal modification is also critical for growth-cone formation following axolemmal sealing[7, 46]. Investigation into calpain has also linked its activation to cell mortality[47-49]. Thus, based on the apparently contradictory data, there must be an optimal level of activity to facilitate Ca^{2+}-dependent spontaneous membrane sealing without inducing the deleterious intracellular effects. It has been proposed that mechanisms of increasing membrane permeability can induce excessive calpain activation in the presence of high intracellular Ca^{2+} concentrations[2]. Therefore, calpain may effectively help membrane sealing in a threshold range of intracellular Ca^{2+} concentrations to facilitate optimal cytoskeletal reorganization.

The cysteine protease calpain has been implicated in cytoskeletal modification. Spectrin[50], part of the cortical cytoskeleton, has been suggested to act as a barrier against vesicle exocytosis, such that subsequent Ca^{2+}-dependent activation of calpain and proteolytic cleavage of the spectrin intracellular meshwork would facilitate vesicle exocytosis[51]. Calpain facilitates replenishment of the releasable vesicle pool in healthy neurons. This is hypothesized to be due to the mobilization of vesicles into a readily-releasable state[52], supporting the idea that calpain-mediated proteolysis also promotes vesicle exocytosis through its role in vesicle dynamics. Recent research into the cortical cytoskeleton of axons has shown the presence of a 180–190 nm periodic arrangement of circumferential actin rings, composed of short actin filaments, interconnected by spectrin tetramers[53]. This arrangement of the actin-spectrin network would give rigidity to the axolemma and following membrane injury may be the basis of the majority of membrane tension; as the spectrin recoils toward the circumferential actin, the membrane is pulled away from the injury site, thereby opposing axolemmal sealing.

Anchoring proteins in the cortical cytoskeleton participating in membrane-cytoskeletal interaction are also proposed to be calpain substrates. Protein 4.1R and G homologs identified in rat brain neurons play a modulatory role in spectrin and filamentous-actin (F-actin) association in the cortical cytoskeleton[54]. Data have shown the Ca^{2+}-dependent calpain cleavage of a protein 4.1A and B homolog present in erythrocytes[55, 56]; thus, calpain may mediate the cleavage of R and G homologs in neurons following Ca^{2+}-dependent activation. The actin cytoskeletal network also participates in this cortical skeleton[53] and is hypothesized to be a major modulator of microtubule polymerization[57]. This effect on cytoskeletal architecture by actin would thereby regulate the plasma membrane surface area in neurons[57]. Microtubule reorganization is an integral step in axolemmal sealing, facilitating subsequent growth-cone formation[51]. Microtubule-associated proteins (MAPs) are known targets of calpain-mediated proteolysis[48, 50]; by MAP cleavage, calpain participates in Ca^{2+}-dependent microtubule reorganization following axolemmal trauma, thereby altering cytoskeletal organization. Actin also has implications in membrane repair through its association with integrins, thereby forming focal adhesions *via* talin, which is a calpain substrate in fibroblasts[58]. Many of the examples of calpain-mediated cleavage of substrates described above can lead to a looser membrane association with the cortical cytoskeleton, thereby decreasing membrane surface tension (Fig. 1B2). It is also possible that the cleavage of talin and other focal adhesion mediators facilitates proximal axonal stump retraction, allowing for a greater plasma membrane surface area relative to the underlying cortical cytoskeleton, which further decreases the membrane tension and facilitates membrane sealing.

Exocytotic Vesicle Dynamics Relating to Membrane Sealing

Intracellular vesicle exocytosis, as well as vesicle formation *via* endocytosis, can largely impact membrane tension[59, 60]. The vesicle source for membrane repair has been reported to be Golgi-derived vesicles[51], lysosome vesicles[61, 62], vesicles formed by membrane endocytosis adjacent to the lesion[63], and myelin membrane delamination[9]. Kinesin and myosin-V through both filament systems have been implicated in the dynamics of the intracellular vesicle pool[64] and vesicle localization to the site of trauma, facilitating Ca^{2+}-dependent exocytosis and membrane resealing[65]. Multiple mechanisms exist for controlling the magnitude of the contribution of each of these vesicle sources to the membrane repair process.

Phospholipase enzymes following membrane trauma

The phospholipase enzymes act in pathways to modify the cytoskeleton, vesicle dynamics, and the phospholipid population. Phospholipases A2 (PLA2), D (PLD), and C (PLC) are involved in vesicle exocytosis[66] and thereby axolemmal sealing. PLA2 activity has been implicated in membrane sealing in some experimental systems: cockroach[8, 67], frog[68], mouse[69], and snail[18]. The influx of Ca^{2+} is a good candidate for the initiation of signal transduction in the phospholipase pathways (Figs. 2 and 3). In fact, Ca^{2+} has been hypothesized to mediate the membrane localization of specific isozymes of PLC and cytosolic PLA2 (cPLA2)[70-72]. PLCδ1, PLCη and cPLA2 present in neurons[73, 74] contain a C2 Ca^{2+}-sensing domain which facilitates Ca^{2+}-dependent phospholipase membrane localization[40, 70, 75-78] and subsequent recognition of substrates and catalytic activity. Specifically, PLA2 activity produces arachidonic acid and lysophophatidic acid (LPA)[75, 79].

All PLC isoforms contain C2 domains and EF-hands that classically function as Ca^{2+}-binding motifs, but their role in the broad Ca^{2+}-binding capacity among PLC isoforms needs to be clarified[80]. In contrast, the PLD C2 domains have only been identified in plant isozymes[81]; thus, the direct Ca^{2+}-dependent activation of PLD has not been implicated in mammalian models.

A subsequent step in the sealing pathway involves protein kinase C (PKC). PKC isozymes have been categorized into three major classes: conventional (Ca^{2+}/diacylglycerol (DAG)-activated), novel (DAG activated, Ca^{2+}-insensitive), and atypical (Ca^{2+}/DAG-insensitive)[75]. Arachidonic acid is a potent stimulator of novel PKC (nPKC) and synergistically enhances conventional PKC (cPKC) activity[75]. Thus, following mechanical membrane trauma, both cPKC and nPKC isoforms may be activated. cPKCα, cPKCβ, and nPKCδ would then activate PLD[82, 83]. Localization of nPKCδ in rat CNS neurons[84] and cPKCα and cPKCβ in rat sensory neurons[85] has been reported. Neuronal localization is suggestive of the potential relevance to axolemmal sealing. In addition, competitive inhibition studies have shown that the novel PKC subspecies nPKCη and nPKCθ are critical in sealing B104 cells[86, 87]. The downstream pathways for PKC isozymes are vast and have yet to be elucidated, but the sealing-relevant pathways have been identified.

PKC plays a role in the generation of a membrane source for repair processes in the intracellular Golgi-derived vesicle pool[27]. cPKC and nPKC are also implicated in the phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) which has been hypothesized to mask the interaction site for phosphatidylinositol 4,5-bisphosphate (PIP2), leading to the release of PIP2 microdomain clusters from the lipid bilayer[88]. In addition, phosphorylation of MARCKS by PKC is potentiated by arachidonic acid[75]. The proposed PKC activation (Fig. 2) initially occurs in response to both Ca^{2+} and arachidonic acid, hence the cycle of PKC activation is amplified following the production of DAG by PLC.

The model next includes the activity of PLD (Fig. 2), shown to be regulated by a Rho-family member, ADP-ribosylation factor (Arf) 1, and PKC[82]. The role of PLD in membrane sealing is hypothesized to involve the release of phosphatidic acid (PA) from phosphatidylcholine (PC) by cleavage[89]. PA stimulates phosphatidylinositol-4-phosphate 5-kinase (PI(4)P 5-kinase), thereby increasing PIP2 formation[90]. In addition, PA following cleavage by PA hydrolase forms DAG[90] which is not capable of enhancing membrane translocation of any PKC isoform[91]. Enhanced PA, through PLD, allows for PLA2 processing of PA to form LPA[90]. It has been noted that lysophospholipids have detergent-like properties[92], which may aid in sealing a membrane breach or dehydrating the lipid free edges to increase line tension. LPA, although not being well understood as a chemical messenger, has some hypothesized functions in the fusion and fission dynamics of vesicles[93]. LPA overexpression causes Rho-dependent cellular changes in focal adhesion, cell motility, the cytoskeleton, process retraction, and cell survival[94], implying its importance in cytoskeletal remodeling, which may reduce membrane tension. Moreover, it has been proposed that exogenous PLD acts through G-protein coupled LPA-receptors to activate Rho signaling pathways[95, 96]. These findings suggest that LPA activates Rho signaling.

Rho-family GTPase signaling has multiple roles in the membrane response to trauma. These include Rho-dependent cytoskeletal modification as described above, and inhibition of receptor-mediated endocytosis *in vivo* and clathrin-mediated endocytosis *in vitro* by Rho[97]. Therefore an increase in the intracellular vesicle stores would occur secondary to a continued Golgi-derived supply of vesicles. This is of importance to the membrane patch hypothesis due to the need for an enhanced presence of membrane vesicles to facilitate patch formation. A study in PC-12 and N1E-115 cells reported that Rho signaling results in neurite retraction following contraction of the cortical actomyosin cytoskeleton[98]. This corresponds well to the morphological response observed following axonal trauma[99] and would further reduce the membrane tension. This membrane retraction likely occurs with simultaneous endocytosis; thus, to fit temporally with the inhibition of endocytosis by Rho, it may occur as a later step of membrane repair following phospholipase activity. The Rho-family of GTPases has been implicated in PLCβ and PLCε activation, although the complete isozyme-specific pathways have yet to be elucidated[40, 80]. GTPase signaling has numerous possibilities for generating cellular changes, which need to be investigated in a cell-type isoform-specific manner

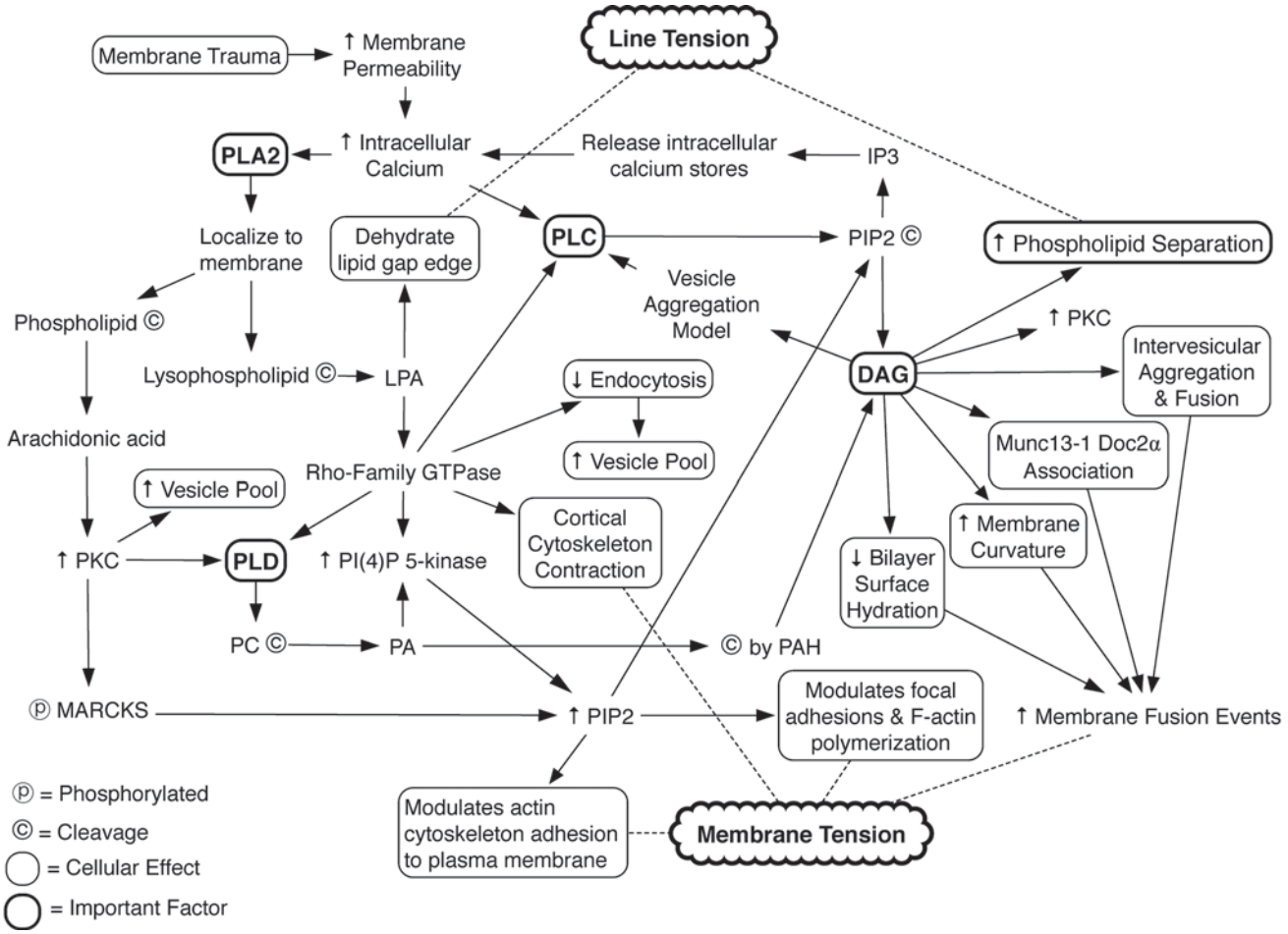

Fig. 2. Diagram of phospholipase enzyme activity and membrane sealing. Phospholipase enzyme activity in response to membrane trauma serves to amplify the magnitude of changes in cytoskeletal and membrane composition to promote membrane sealing. The major phospholipase enzymes involved are PLA2, PLC, and PLD. DAG, upon deposition primarily through PLC catalysis, amplifies membrane fusion events through proposed methods of membrane monolayer modification. DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PAH: phosphatidic acid hydrolase; PC, phosphatidylcholine; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C.

to ensure a correct hypothesis regarding its application to membrane sealing.

The activation of PLC is a key step in the phospholipase cascade to deposit DAG, which is known to modulate membrane characteristics. Activation of PLC may involve Ca^{2+}-dependent localization to the plasma membrane, or Rho-family signaling. An alternative hypothesis for activation of PLC is a vesicle aggregation model in which generation of DAG at a threshold level through PLC catalysis and subsequent vesicle aggregation induces full enzyme activity[40]. This mode has relevance considering the vesicle aggregation necessary in the patch hypothesis of membrane sealing[19]. PLC activation causes cleavage of PIP2 into inositol 1,4,5-trisphosphate (IP3) and DAG[40,75]. PLC through IP3 participates in Ca^{2+} signal amplification through the release of intracellular Ca^{2+} stores[40] (Fig. 2).

PLC cleavage of PIP2 is important in neurite membrane sealing through the production of DAG[87]. PIP2 also plays a role in cytoskeletal dynamics and cell signaling; it modulates the adhesion between the actin cortical cytoskeleton and the plasma membrane, and cleavage of PIP2 results in decreased adhesion energy[100]. The

decreased adhesion energy then decreases membrane tension, promoting membrane resealing. Further, PIP2 modifies actin-associated proteins such as profilin, gelsolin, α-actinin, and vinculin, causing alterations of the actin cytoskeleton[101-103]. Vinculin is modified by PIP2 such that dissociation of its head-tail configuration unmasks its talin and actin-binding sites[102]. Therefore, as PIP2 increases the cortical cytoskeletal association, cleavage of PIP2 by PLC would decrease protein component association and subsequently reduce membrane tension. However, as seen in the proposed phospholipase cascade (Fig. 2), Rho family GTPases act to stimulate the production of PIP2 by enhancing the activity of PI(4)P 5-kinase[102], and thus stimulate tighter cortical cytoskeletal adhesion. As vinculin is a key structural component within the cytoskeleton through talin- and actin-binding[104], exposure of its binding sites following PIP2 stimulation may allow for more effective calpain protease cleavage following axolemmal trauma. Vinculin cleavage by calpain within neurons has not been studied, but there is evidence for vinculin susceptibility to calpain-mediated proteolysis in other cell types[58,105]. Thus, early PIP2 deposition following membrane trauma may serve to increase the susceptibility to Ca^{2+}-dependent calpain cleavage in the cortical cytoskeleton through cytoskeletal conformational changes followed by cleavage of PIP2 after the full activation of PLC to mediate a decrease in membrane tension.

DAG is generated after PIP2 cleavage by PLC, and this has many implications in membrane sealing[40]. DAG decreases bilayer surface hydration and increases the separation between adjacent phospholipids[106]. This would increase membrane fluidity, thereby decreasing membrane tension, and the decreased packaging efficiency would increase line tension (Fig. 2). Certain membrane components can impart negative membrane curvature on a lipid monolayer based on their focal concentration; DAG has been shown to convey these properties on a monolayer[107]. DAG does not mix homogenously within the phospholipid bilayer resulting in islands of DAG-rich regions[106], and this has implications for imparting maximum membrane curvature within a localized region of the monolayer favoring membrane fusion. DAG has also been shown to promote intervesicular aggregation and vesicle fusion following production by PLC[106], a very relevant step for promoting the formation of a membrane patch and full PLC activation by the vesicle aggregation model. DAG is known to induce an association between Munc13-1 and Doc2α, a step involved in Ca^{2+}-dependent vesicle exocytosis[108]. DAG plays a well-established role in activation of the cPKC and nPKC subfamilies through DAG interaction with the C1 domain of PKC proteins[40, 75]. Overall, DAG is implicated in membrane dynamics directly by promoting vesicle fusion and indirectly through activation of other signaling cascades, both of which could promote axolemmal repair following trauma.

In sum, the complex interplay between the PLA2, PLC, and PLD families modulates plasma membrane and vesicle dynamics (Fig. 2), facilitating a reduction in membrane tension through increased fluidity and final membrane fusion. Full activation of the cascade would support the hypothesized membrane patch formation adjacent to the site of membrane trauma in response to Ca^{2+} influx.

SNARE protein involvement in exocytosis-mediated repair Ca^{2+}-dependent SNARE protein association participates in axolemmal sealing[10, 30] and membrane sealing in other invertebrate and mammalian cell types[10,109-111]. The interaction between Ca^{2+}-dependent vesicle-localized synaptotagamin and membrane-localized syntaxin, facilitated by C2A and C2B domains on synaptotagmin[112], mediates vesicle fusion[113-115]. Thus, Ca^{2+} influx at the site of membrane injury would facilitate vesicle-membrane fusion.

Synapsin, a synaptic vesicle-associated phosphoprotein, participates in vesicle dynamics and, following dissociation, enhances SNARE-mediated vesicle exocytosis through its role in vesicle-actin tethering[116-118]. Synapsin is also implicated in axonal regrowth and growth-cone dynamics based on the time course[119] and sites[120] of intracellular accumulation. Synapsin phosphorylation *via* protein kinase A (PKA) is hypothesized to regulate synapsin dissociation from the vesicle membrane, thereby trafficking the vesicle pool from the reserve to the readily releasable state[121]. Synapsin proteins enhance neurite outgrowth *via* cAMP-dependent PKA, hypothetically determining the rate of membrane insertion[122]. There are also phosphorylation sites on synapsin I for calcium/calmodulin-dependent protein kinase (CaMK)I, CaMKII, and CaMKVI[123, 124]. Data suggest that, following influx, Ca^{2+} activates CaMKII, which

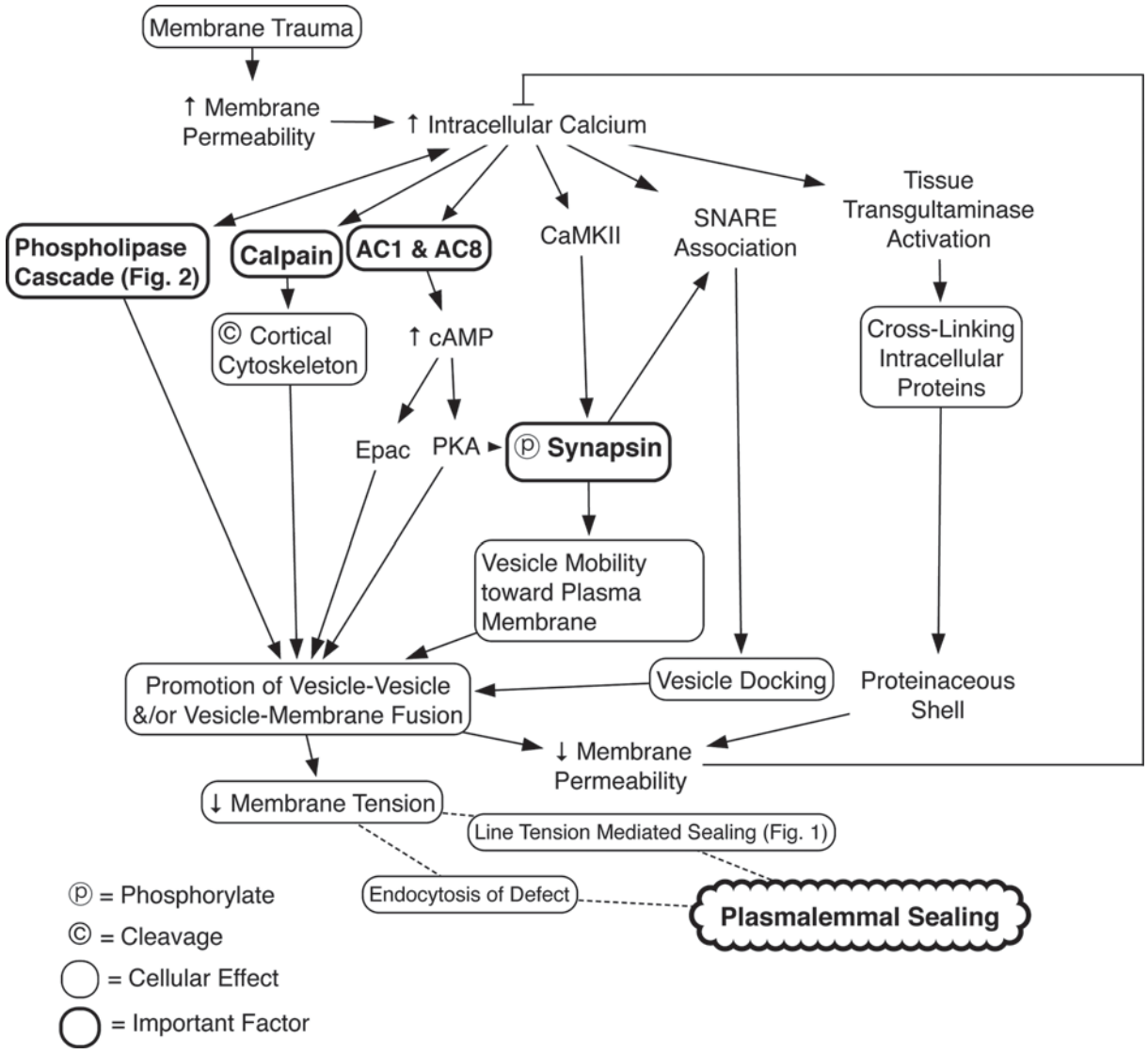

Fig. 3. Diagram of the overall neuronal membrane sealing mechanism. The major trigger for the signaling cascades that precipitate membrane sealing is the influx of Ca^{2+} through the membrane disruption following trauma. The major pathways involved in subsequent signaling are phospholipase enzymes, calpain proteases, the cAMP cascade, SNARE-mediated vesicle deposition, and the proposed formation of an intracellular proteinaceous shell. The resulting decrease in membrane permeability as the membrane seals serves as feedback on the signaling pathways to return to homeostasis following plasmalemmal sealing.

then phosphorylates synapsin I, thereby reducing its binding to vesicles[124], similar to the PKA-mediated phosphorylation noted above. CaMKII inhibition also reduces the slow and fast phases of vesicle recruitment during Ca^{2+}-mediated exocytosis[65]. These findings justify the inclusion of CaM kinases in the model of vesicle recruitment and fusion dynamics during membrane sealing (Fig. 3). In summary, a logical result of synapsin phosphorylation by CaMKII or cAMP-mediated PKA, which both increase as a result of Ca^{2+} influx (Fig. 3), would be an increase in vesicle exocytosis likely facilitated by SNARE proteins[116, 122].

Parallel cAMP axolemmal sealing pathways Ca^{2+}-dependent exocytosis has been reported to involve cAMP-dependent signaling pathways in pancreatic β-cells[125].

For neuron membrane sealing, the presence of adenylyl cyclase (AC)1 and AC8, the major Ca^{2+}-sensitive isoforms within neurons[126], could be the starting point for a cAMP-signaling cascade to generate vesicle exocytosis. These Ca^{2+}-dependent exocytotic events have been found in B104 cells[86]. cAMP mediates two parallel pathways of vesicle dynamics through interaction with PKA and exchange proteins activated by cAMP (Epac)[127-129], which has been suggested to be the mechanism of action in injured B104 cells investigated using small molecular inhibitors of PKA and Epac[86].

PKA has also been implicated in the potentiation of Ca^{2+}-dependent exocytosis and membrane resealing following an initial membrane injury in Swiss 3T3 fibroblasts[23]. Involvement of PKA in the signaling that promotes membrane sealing may be based on its regulation of synapsin, increasing the number of readily-releasable vesicles[122]. Overall, the cAMP pathway is another important Ca^{2+}-dependent mechanism to deposit membrane at the site of injury, and decrease the membrane gap and surface tension.

Membrane curvature and vesicle mechanics The spontaneous curvature imparted on the membrane by microdomains is a key characteristic in defining the fusibility of lipid membranes. Some agents that impart this negative spontaneous monolayer curvature are cholesterol, DAG, phosphatidylethanolamine[107], and α-tocopherol[130]. It is believed that agents imposing negative curvature have a small hydrophilic head group relative to a larger hydrophobic domain, leading to a concave structural conformation that imparts negative spontaneous curvature to the membrane[107]. Cholesterol-induced spontaneous membrane curvature has a threshold level for facilitating vesicle fusion[131], such that other substances must induce a comparable or more negative curvature to effectively induce fusion[107].

Following the current understanding of the stalk-pore model for membrane fusion, a point-like protrusion initially forms, followed by the stalk structure, then the transmonolayer contact forms as a hemifusion diaphragm, which decays into the fusion pore[39, 132, 133]. The energy barrier of these fusion intermediates is the rate-limiting step for membrane fusion[134]. The major energy barrier that the negative spontaneous membrane curvature aids in overcoming is the short-range hydration repulsion existent on the point-like protrusion structure between the fusing membranes[133] and development of the hemifusion diaphragm[134]. Such microdomains enriched in cholesterol, sphingomyelin, DAG, α-tocopherol, and phosphatidylethanolamine result in an increased capacity for membrane fusion by inducing membrane curvature[107, 131, 135]. The membrane components that convey spontaneous curvature also likely affect the local protein and lipid organization[107], altering enzymatic activity and binding specificity. In relation to membrane repair, deposition of negative curvature-inducing component DAG into local microdomains surrounding injured membrane following the phospholipase cascade (Fig. 2), could facilitate vesicle-vesicle and vesicle-membrane fusion events to restore plasma membrane integrity.

Endocytotic Vesicle Dynamics Relating to Membrane Sealing

Endocytosis is a mechanism of absorbing the membrane breach defect[31]. Application of the pore-forming protein streptolysin O and mechanical membrane disruption in NRK, HEK-293, and HeLa cells result in endosomes adjacent to the injury, representing endocytosed membrane[33]. During this process, actin cytoskeletal disruption is suggested to enhance endocytosis and thereby wound repair. In addition, exocytosis of adjacent lysosomal vesicles[61, 62], which is hypothesized to precede endocytosis of the membrane gap, would decrease membrane tension to a threshold level that facilitates subsequent endocytosis[31]. This is consistent with the phospholipase model (Fig. 2), in which inhibition of endocytosis by Rho-family GTPases occurs following membrane trauma. However, exocytosis would then serve to decrease the membrane tension, concurrent with decreasing Ca^{2+} influx as the membrane gap closes, which decreases Rho-GTPase signaling, both of which alleviate the inhibition of endocytosis. This would allow for a membrane breach to subsequently be absorbed into an endosomal vesicle and restore plasma membrane integrity, without the need for completion of a line tension-dependent sealing process (Fig. 1).

Endocytosis plays additional cellular roles during neuron injury. The retrograde axonal transport of endosomes has been implicated in a trauma-induced signaling mechanism to communicate injury signals to the

cell body[111]. Lastly, endocytosis is also a contributor to the vesicle supply for membrane sealing[63]. This emerging role of endocytosis in membrane repair is relatively recent and requires further studies to better understand the interplay between endocytosis and exocytosis, the two seemingly opposite vesicle processes in membrane sealing.

Role of Environmental Factors

Extracellular Ca^{2+} Concentration

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is essential for successful sealing of the axolemma following trauma[10, 12, 110, 136, 137]. The extracellular Ca^{2+} ($[Ca^{2+}]_o$) is also critical, in that the optimal efficiency of sealing occurs with 2 mmol/L $[Ca^{2+}]_o$, and an effective block on sealing occurs with $[Ca^{2+}]_o$ <0.5 mmol/L[5]. This may be due to the necessity of a steep gradient between the extracellular and intracellular $[Ca^{2+}]$ to promote influx and the subsequent activation of repair pathways (Figs. 2 and 3). This entry of Ca^{2+} would activate the pathways for membrane repair in the neuron, but the simultaneous uptake of Ca^{2+} into multiple damaged axons at the site of injury *in vivo* could focally deplete the extracellular gradient and prohibit sufficient influx of Ca^{2+} into the repairing axons. Such focal *in vivo* $[Ca^{2+}]_o$ depletion, of one to two orders of magnitude, has been found following mechanical injury and persists for several hours[137-139]. The lack of a corresponding $[Ca^{2+}]_i$ could prevent the effective activation of the sealing mechanism, leading to excessive axonal dieback or retrograde cell death[5].

Role of Reactive Oxidative Species in Membrane Sealing

Oxidative damage is a factor of interest concerning membrane sealing due to the lack of flux control at the membrane breach. With regard to studies of reactive oxidative species (ROS), highly noteworthy in the field of membrane repair has been the therapeutic application of the free-radical scavenger α-tocopherol[140], a physiologically-relevant form of vitamin E in humans, based on its lipophilic nature[141]. Experimental data have shown that when an oxidative challenge is presented to a resealing membrane, membrane repair is inhibited, but α-tocopherol blocks this[142]. However, this rescue only occurs in the presence of extracellular Ca^{2+}, so α-tocopherol enhances Ca^{2+}-dependent repair pathways and the antioxidant properties are necessary but not sufficient to promote repair[142]. These antioxidant properties may be effective in preventing the formation of the toxic metabolites of lipid peroxidation, such as acrolein, generated by lipid peroxidation and feed-forward ROS production[143].

Studies within vascular smooth muscle cells implicate α-tocopherol in the localization of protein phosphatase 2A to the membrane, and this facilitates PKCα dephosphorylation and subsequent inhibition[144,145]. This inhibition of PKC inhibits the formation of the membrane-bound enzyme complex NADPH-oxidase[146], thereby decreasing the amount of superoxide produced. However, in regard to membrane sealing, PKC inhibition appears counter-productive because according to the model proposed (Fig. 2) and as previous studies have shown[27,86,87], inhibition of PKC decreases plasma membrane sealing. Further studies are needed to better understand the cellular pathways that are altered during α-tocopherol application and the isozyme-specific PKC pathways involved after membrane trauma.

As discussed above, α-tocopherol is known to impart negative membrane curvature on phospholipid membranes, thereby enhancing the formation of fusion intermediates[107,130]. X-ray diffraction studies of α-tocopherol incorporation into plasma membrane do not show any direct binding of α-tocopherol to substrates[130], which would normally allude to the mechanism of action. It is hypothesized that the membrane curvature induced by the lipophilic domain of α-tocopherol causes stress in the membrane and distorts the lipid-protein interaction, thus, it may alter the protein conformation to a level sufficient to affect cellular activity[130]. This also illustrates similar effects that would be experienced by the membrane immediately following trauma, such that the membrane protein activity would be altered in response to the lipid bilayer conformation induced by mechanical trauma. Elucidating the mechanism of the impact of α-tocopherol on membrane sealing could prove clinically effective due to the feasibility of vitamin E supplementation in patients following trauma to enhance membrane repair.

Further studies of antioxidants in relation to membrane trauma have led to the postulation of another activation mechanism for sealing. Melatonin, a powerful antioxidant[147], has been tested for its impact on axolemmal sealing at high and low concentrations in B104 cells following axotomy[86]. The results showed that melatonin

application decreases sealing, probably due to the lack of activation of oxidative-induced membrane sealing mechanisms. This appears to contraindicate the application of anti-oxidants to enhance membrane sealing, which as described with α-tocopherol, was shown to promote sealing in otherwise sealing-incompetent cells[142]. In support of this oxidative trigger for membrane sealing, oxidative stress appears to give similar results through a novel repair protein present in skeletal muscle (MG53), which has been proposed to become activated and thereby promote membrane sealing through oxidation-induced mutation in the protein[148]. This protein is a member of the muscle-specific tripartite motif (TRIM)72 family and orthologous TRIM proteins have been identified in molluscan neurons[149]. This provides support for the potential involvement of TRIM family proteins in axolemmal sealing. This mechanism is important because it marks a pathway that may act upstream of Ca^{2+} to induce pathways for the promotion of membrane repair[148].

Mechanical Factors of Injury

During a study of mechanisms of membrane sealing, the model systems are often manipulated, for the sake of control, in a monolayer setting *in vitro*. While this controls variables during manipulation, it does not simulate the three-dimensional aspect of mechanical properties and the environment experienced by the system *in vivo*, pertaining to axon receding, sealing, growth-cone generation, and target localization. An effective balance between the *in vitro* and *in vivo* settings during experimentation is through the use of *ex vivo* experimental designs, such as the double sucrose-gap device[5, 13, 17, 150]. This device allows for the control of an *in vitro* setting while permitting the use of spinal cord segments that provide a three-dimensional native tissue environment conducive for monitoring the recovery of membrane potential in real time, which serves as a functional indicator of membrane resealing. These favorable conditions for tissue culture and reliable data acquisition support the pursuit of future *ex vivo* sealing experiments to further clarify the physiology of axolemmal sealing.

Axon caliber is also an important variable in successful membrane sealing, especially when considering a transection injury. It has been shown that axons with smaller diameters exhibit faster spontaneous resealing following transection[11]. Under conditions of therapeutic application of PEG[17] and warming from 25°C to 37°C[13] following axonal transection, faster sealing occurs in the smaller caliber axons. While the underlying mechanism remains unclear, it has been speculated that the sealing rates are based on the size of the axonal defect. The axon caliber prior to transection dictates the size of the corresponding membrane lesion following transection. A larger axolemmal defect will result in a lower line tension between the free lipid edges (see Section 3). Without the favorable effects of line tension to promote axolemmal sealing, a larger vesicle deposition will be required to close the membrane gap thereby allowing the thermodynamic force between the lipid free edges to facilitate sealing of the membrane or subsequent endocytosis of the membrane defect. Further exploration of the differential rates of sealing related to axonal caliber and effective manipulation of environmental conditions to promote resealing for all axon calibers has clinical significance, in that nervous system injuries involve axons ranging in caliber.

The method of injury must also be considered regarding the effectiveness of therapeutics and the timescale of resealing. It is evident from previous studies involving axonal transection that membrane disruptions exclude dye markers within 60 min post-injury[5, 13]. The time required to achieve competent membrane integrity, measured by dye exclusion, is much greater in compression injury, in that only 50% of the axons have measurable membrane integrity at the 60-min time point[151]. This contrast exemplifies the potential difference in cellular mechanisms between membrane sealing in a single plane, as in transection injury, and in a segmental out-of-plane region, as in compression injury. Also, compression injury may have the potential to generate multiple membrane breaches, requiring sealing of each to re-establish full integrity. Based on the high clinical prevalence of compression injuries, compared to transection injuries, further understanding into the membrane biophysics of compression injury is of great relevance to the clinical treatment of SCI and TBI.

Methods to Enhance Integrity and Repair of the Neuronal Membrane

Understanding the mechanism axons use to seal

membrane breaches, as well as capitalizing on novel pathways, will facilitate the discovery of agents that enhance natural membrane sealing. As described above, application of α-tocopherol enhances membrane sealing, likely on the basis of preventing lipid peroxidation and inducing spontaneous membrane curvature[142]. Temperature is implicated as a variable with potential therapeutic application through the evident clinical benefits of mild hypothermia[38]. A growing cohort of research has shown that beneficial therapeutic effects on axonal membrane sealing are achieved by application of the synthetic polymer PEG[6,17,151]. To date, there is no established mechanism of action for PEG-mediated membrane sealing. However, the characteristics of PEG-coated surfaces have been shown to affect membrane properties. These include a large excluded volume, high PEG chain mobility, a high degree of hydration, and low interfacial energy between PEG and water molecules[152,153]. Also, PEG *in vivo* has been shown to localize at sites of trauma[154,155] by an unknown mechanism, promoting its therapeutical use as a direct treatment or as a drug delivery vessel. This, in conjunction with the properties of PEG-coated surfaces, suggests that PEG acts like a chemical sponge at the site of trauma. So, PEG may be able to associate with the lipid-free edges because of the low interfacial energy between PEG and water molecules, facilitating a more favorable thermodynamic state of the lipid-PEG-water configuration. PEG may thereby be able to dehydrate the area surrounding the free lipid edge. By removing the water within the membrane gap, it may decrease the gap diameter thereby increasing the line tension[6]. This conceivably complex structural configuration necessary to associate with the lipid free edge is facilitated by the high chain mobility of PEG[152, 153]. PEG may also dehydrate the membrane bilayer surface hydration layer, similar to DAG, promoting the formation of vesicle fusion intermediates. This would enhance the membrane-sealing rate through increased efficiency of vesicle exocytosis to decrease membrane surface tension and promote vesicle-vesicle fusion as seen in the membrane patch model.

Experimental support for PEG shows a quantitative reduction in the membrane surface tension through measurements made by atomic force microscopy[17]. This effect has been reported in other surfactants, such as Pluronic F68 NF in Swiss 3T3 fibroblasts[27] and P-188 in muscle cell membranes[156], as well as in solvents such as dimethylsulfoxide[26, 157], which improves guinea-pig spinal cord axolemmal sealing[158]. Thus, surfactants such as PEG likely mediate enhanced membrane sealing through increasing line tension and decreasing membrane tension, thereby altering the membrane properties to promote membrane healing. Based on the proposed model (Fig. 3) centering on achieving the same results through cellular physiology, PEG appears to be a valuable therapeutic to supplement native membrane sealing mechanics.

Conclusion

In summary, many pathways are implicated in the complex cellular process of membrane sealing. To best understand the interplay between different proposed mechanisms, experimentation on membrane sealing must maintain a focus on each finding being a part of the whole process. The extent to which mechanisms of repair seen in fibroblasts, erythrocytes, myocytes, and even invertebrate giant axons overlap with those in the mammalian CNS is unclear. Despite the knowledge gap for the overlap between various experimental systems, investigation needs to continue into the mammalian neuronal sealing mechanism, with the current knowledge serving as a framework, to better understand recovery from neurological trauma and have additional implications for neurodegenerative disease.

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