Vesicle-Mediated Restoration of a Plasmalemmal Barrier in Severed Axons

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Ca\(^{2+}\)-induced endocytic vesicles undergo protein-mediated interactions to restore a selectively permeable barrier and propagated action potentials in severed invertebrate giant axons. Similar barrier-restoration phenomena observed in cultured mammalian cells with transected neurites suggest that cellular/molecular mechanisms that repair plasmalemmal damage are phylogenetically conserved.

When axons in a nerve fiber tract are severed, a presumed prerequisite for restoring communication via that nerve is the restoration of the selective barrier function of the axolemma in the proximal segment of some of the severed axons so that they can survive the injury and eventually regenerate. (When the injury is minor compared with a complete severance, communication may be restored by forming a barrier that reinstates action potential propagation through the repaired plasmalemmal region rather than by regeneration and reconnection.) Despite over a century of extensive studies of axon regrowth after injury (11), the processes that restore a selective barrier to severed axons were not examined until about a decade ago. In the initial studies, images of the cut ends of individual, isolated squid giant axons (GAs) in confocal and electron microscopes showed an accumulated mass of membranous, injury-induced vesicles that appeared to occlude the open, cut ends (8, 13). Measurements of the injury current density (\(I_1\)) at cut ends showed a substantial inwardly directed \(I_1\) that continued for >2 h posttransection (PT) (9, 13). That is, the accumulated mass (plug) of vesicles did not restore an ionic seal at cut ends of isolated squid GAs.

Like squid GAs, an accumulated mass of membranous, injury-induced vesicles appeared to occlude the open, cut ends of isolated earthworm and crayfish GAs (5, 14). In contrast to squid GAs, \(I_1\) at the cut end of earthworm (13) and crayfish (5, 9) medial GAs at 1 h PT decayed to the value of \(I_1\) measured in uninjured, intact axons before transection. The restoration of a selective barrier function in these crayfish GAs was confirmed by the recovery of nominal resting potentials (reflecting the return of normal transmembrane ion distributions of permeant ions) and the recovery of propagating action potentials (6). Furthermore, when fluorescent, hydrophilic dyes were placed outside and inside a crayfish GA, the boundaries of fluorescence intensity at the cut ends for both dyes were sharp and at the same location, indicating that the barriers to entry of the outside dye and to exit of the inside dye were coincident within the resolution (~1 \(\mu\)m) of the confocal microscope (Fig. 1). These dye barriers formed amid an accumulation of injury-induced vesicles at a time consistent with the formation of an electrical seal (5, 14). These data were the first to suggest that a plug of vesicles somehow sealed a severed axon. Restored barriers amid accumulated vesicles were also observed at other types of axolemmal lesions, such as small holes in crayfish medial GAs (4).

These observations of sealing in earthworm and crayfish GAs were inconsistent with the then-prevailing explanation of sealing, which assumed that a completely severed axon was repaired at the cut ends by collapse and fusion of the axolemma (12). In contrast, the open, cut ends did not completely collapse in any of the GAs examined, including squid, precluding fusion along the disrupted plasmalemmal membrane boundary. Furthermore, the structure that appeared to seal the open, cut ends was a tightly packed plug of vesicles rather than an intact, continuous membrane sheet. Scanning electron micrographs (7) of the cut end of a crayfish medial GA fixed at the time of a restored electrical seal (Fig. 2, A and B) support this vesicular-plug model of plasmalemmal sealing.

In the magnified image of the plug of vesicles (Fig. 2B), a wide distribution of vesicle sizes is apparent. Such a distribution is consistent with tight packing of vesicles, producing an ionic seal (a high electrical resistance) at the cut, open ends of the axon.

Dye molecules of different sizes, used to measure barrier permeation during the formation of a plasmalemmal seal (6), also provide data to support the vesicular plug model of axonal repair. Fluorescent, hydrophilic dyes of different molecular sizes were added to the extracellular solution of crayfish medial GAs at various times PT (Fig. 3). Large dye molecules were excluded at short times PT. At progressively longer times PT, barrier permeability continuously decreased as smaller dye molecules were excluded until, finally, the barrier became an ionic seal (i.e., \(I_1\) decayed to control levels in intact, uninjured axons). These continuous and gradual changes in the permeability properties of a forming barrier following axonal transection are not consistent with a sudden collapse and fusion of the axolemma but are consistent with the assembly, interaction, and fusion of vesicles of different sizes with each other and with the transected axolemma to plug (i.e., electrically seal) open, cut ends.

The accumulated tightly packed vesicles appear to be a temporary structure that initially occludes cut ends, which allows recovery processes (e.g., ion pumps, exchangers, and...
channels) to restore plasmalemmal functions and, eventually, axolemmal continuity. A transition period from this temporary vesicular plug to an eventual continuous axolemma was indeed observed in transected earthworm medial GAs (14). That is, at 1 h PT, vesicles accumulated and excluded hydrophilic dye at axonal cut ends, but at 24 h PT, these vesicles disappeared and the axolemma was continuous. Axonal outgrowth was observed only after the restoration of axolemmal continuity at cut ends, suggesting that axolemmal continuity must be reestablished before regeneration can occur.

Plasmalemmal sealing involves Ca²⁺-initiated processes

Measurements of \( I_i \) at the cut end of severed axons showed that \( I_i \) is carried primarily by the inward movement of Na⁺ and Ca²⁺ (see Fig. 2 in Ref. 1). A large increase in the internal free calcium concentration ([Ca²⁺]) of >1 μM is well known to initiate degenerative processes in various cell types (see Ref. 1). Although Na⁺, the other major cationic carrier of \( I_i \), is not thought to have a direct role in cell death after injury, an increased intracellular Na⁺ concentration to millimolar levels after injury may indirectly induce cellular degeneration by increasing [Ca²⁺], via reversal of the plasmalemmal Na⁺/Ca²⁺ exchanger (17).

In contrast to its destructive role of triggering degeneration, increased [Ca²⁺] also has a constructive role in restoring a plasmalemmal barrier after injury of cells from many different species (see Ref. 1). The first of many constructive roles of Ca²⁺ in the restoration of a selective barrier in GAs is the initiation of vesiculation, i.e., vesiculation does not occur in severed GAs without Ca²⁺ in the extracellular solution (Fig. 2C) (1, 4, 8, 13). Furthermore, vesicle formation is induced after increasing axoplasmic [Ca²⁺], to >100 μM in internally microdialyzed squid GAs with intact plasma membranes bathed in solution lacking Ca²⁺ (4) and in intact crayfish medial GAs microinjected with Ca²⁺ (see Fig. 8 in Ref. 1). Ca²⁺ also has other constructive roles (described below) in barrier restoration, such as the activation of cytosolic proteases and mem-

FIGURE 1. Confocal fluorescence images of the cut end of a crayfish medial giant axon (GA) transected in physiological solution with one hydrophilic, fluorescent dye placed extracellularly (A) and another placed intracellularly (B) showing the abrupt inner and outer dye boundaries of fluorescence intensity amid a vesicle accumulation, imaged with a membrane-incorporating styryl dye placed extracellularly (C), and the differential interference contrast image (D). A and B: long-axis fluorescence images obtained at the same midsection 90 min posttransection (PT). A: image of 0.1% Texas red-dextran added to physiological saline 60 min PT. B: image of 0.1% FITC-dextran in intracellular saline injected into GA before transection. C: fluorescence image at the same midsection as A and B, acquired at 120 min PT, of the GA following addition of FM1-43 to the bath at 95 min PT. D: DIC image taken simultaneously with C. Bar in D = 20 μm. Modified from Eddleman et al. with permission (5).

FIGURE 2. Scanning electron micrographs (SEMs) of the cut end of a crayfish medial GA, fixed at the time (30 min PT) of a restored electrical seal, support the vesicular-plug model of selective barrier restoration. Top: close apposition of the discontinuous axonal boundary along a “seam” marking the cut end. Notice the vesicular mass that fills and protrudes from the residual opening. Middle: magnified image of boxed region in top SEM shows a dense mass of vesicles varying in size, which are consistent with a plug-like seal. Bottom: SEM of another crayfish medial GA fixed in Ca²⁺-free solution. Modified from Eddleman et al. with permission (7).
formation, Ca\textsuperscript{2+} activates proteases and/or proteins that are malemmal sealing (see Ref. 1). In addition to initiating vesicle late and become densely packed at the disrupted portion of axolemma (Fig. 2 and Ref. 5).

Squid GAs (1), can seal a disrupted plasmalemma. The origin and formation of Ca\textsuperscript{2+}-induced vesicles

The axolemma is the source of Ca\textsuperscript{2+}-induced vesicles in crayfish GAs (4, 5). The axolemmal origin was determined by extracellularly labeling the axon membrane with a lipid-incorporating, membrane-impermeant fluorescent (styril) dye and subsequently observing ring-shaped structures in the axoplasm in confocal images. These ringed, fluorescent structures were observed only after axonal injury or elevation of [Ca\textsuperscript{2+}], without injury. Furthermore, the lack of glialemmal membrane evagation with invaginating axolemmal membrane indicated that the origin of the ring-shaped structures in the axoplasm was axonal rather than glial. Moreover, time-lapse confocal imaging directly confirmed that vesicles were formed by endocytosis of the axolemma. However, in other neuronal and nonneuronal cells, the source of vesicles that seal damaged plasma membranes can vary. For example, myelin delaminations contribute significantly to vesicles that seal transected earthworm medial GAs (see Ref. 1). In some nonneuronal cells, preformed vesicles, such as cortical and yolk granules in oocytes (16, 18) and lysosomes in fibroblasts (15), can seal a disrupted plasmalemma.

Axolemmal sealing involves Ca\textsuperscript{2+}-activated proteases and proteins

Previous studies in both neural and nonneural cells from invertebrates (GAs and oocytes) and vertebrates (neurons and fibroblasts, cardiac and skeletal muscle, endothelial and epithelial cells) have shown that Ca\textsuperscript{2+} is necessary for plasmalemmal sealing (see Ref. 1). In addition to initiating vesicle formation, Ca\textsuperscript{2+} activates proteases and/or proteins that are required to seal the cut ends of transected invertebrate GAs (1, 2, 9) and neurites of cultured mammalian cells (3, 20). Yawo and Kuno (19) suggested that Ca\textsuperscript{2+}-activated phospholipase A\textsubscript{2} facilitates fusion of membranes by hydrolysis of membrane phospholipids to induce sealing of cockroach GAs (19). Barrett and coworkers (20) have reported that transected neurites of mammalian cells in vitro and spinal cord axons in vivo (10) do not seal in the absence of calpain activity. Godell et al. (9) have reported that inhibition of calpain prevents sealing of transected crayfish GAs and that addition of calpain facilitates sealing of transected squid GAs.

Ca\textsuperscript{2+} also plays a constructive role in plasmalemmal sealing by activating vesicle proteins that promote membrane fusion such as synaptotagmin, synaptobrevin, and syntaxin. [Exocytosis of docked presynaptic vesicles are triggered by Ca\textsuperscript{2+} binding to synaptic vesicle proteins, such as synaptotagmin, which presumably regulate interaction of other membrane proteins (synaptobrevin and syntaxin) at presynaptic terminals (see references in Ref. 2).] These highly conserved proteins promoted plasmalemmal sealing after injury. For example, microinjection of tetanus toxin, which disrupts exocytosis of synapses by specifically cleaving synaptobrevin, prevented sealing of the disrupted plasmalemma of sea urchin eggs (16). Severe invertebrate GAs did not seal when antibodies to synaptotagmin or syntaxin were injected before transection (2); severed neurites of cultured rat pheochromocytoma (PC12) cells in culture did not exclude dye when antibody to synaptotagmin was internally loaded before neurite transection (3). These data clearly indicate that proteins with well-established roles in intracellular fusion processes are also involved in plasmalemmal repair.

Mechanisms of plasmalemmal repair are conserved in mammalian cells

As described above, data from crayfish, earthworm, and squid GAs and sea urchin eggs strongly support a vesicle-mediated sealing mechanism following various types of plasmalemmal damage. Recent data from a mammalian neuronal model (a PC12 cell line) suggest that vesicles also mediate the sealing of a damaged plasmalemma (3). Additional evidence...
that vesicles from various sources mediate sealing in the plasmalemma of mammals is the recent report that lysosomal membrane proteins (synaptotagmin VII and Lamp-1) promote the plasmalemmal sealing of injured fibroblasts (15). These data from invertebrate GAs, sea urchin eggs, and various mammalian neuronal and nonneuronal cells suggest that plasmalemmal sealing in most eukaryotic cells is an evolutionarily conserved process involving vesicles, Ca\textsuperscript{2+}, calpain, and proteins that facilitate vesicle fusion with other membranous cellular structures such as the Golgi apparatus and synaptic terminals. A description of the molecular processes that enable vesicles to restore a plasmalemmal seal is now essential to our understanding of plasmalemmal repair.

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References


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