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Membrane Resealing in Cultured Rat Septal Neurons after Neurite Transection: Evidence for Enhancement by Ca^{2+}-triggered Protease Activity and Cytoskeletal Disassembly

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Neurites of cultured septal neurons were transected with a laser under sterile conditions, and the subsequent membrane resealing was assayed using a dye exclusion method. In agreement with findings in other preparations, Ca^{2+} enhanced resealing: in normal culture medium the percentage of lesioned neurons that resealed within 20–30 min after transection increased with increasing bath $[Ca^{2+}]$ over the range 10^{-7} to 2×10^{-3} M; about 75% of cells resealed in 2 mM Ca^{2+}. Mn^{2+} and Sr^{2+} also enhanced resealing, but Mg^{2+} inhibited it.

The percentage of resealing neurons was sensitive to agents known to alter the stability of cytoskeletal components. Agents that tend to disassemble microtubules and/or neurofilaments (e.g., colchicine, low-ionic-strength media) strongly promoted resealing, whereas treatments that tend to stabilize microtubules (taxol, Mg^{2+}) inhibited resealing. Addition of exogenous proteases (papain, trypsin, or dispase) enhanced resealing, whereas inhibitors of cysteine proteases (including a specific inhibitor of calpain, a Ca-activated neutral protease) strongly inhibited resealing. Calmodulin inhibitors inhibited resealing, consistent with reports that calmodulin facilitates calpain-mediated proteolysis of fodrin, a component of the cortical cytoskeleton.

Based on these results, we hypothesize that one of the major mechanisms involved in resealing is activation of endogenous proteases by Ca^{2+} entry into the injured neurite. The resulting changes in the cellular cytoskeleton might promote fusion and resealing of the cut ends of the plasma membrane by enhancing membrane mobility and/or by removing structures that normally prevent membrane–membrane contact.

Membrane resealing is critical for neuronal recovery following axonal transection, but little is known about the mechanisms underlying this resealing. The electrophysiological measurements of Yawo and Kuno (1983, 1985) indicate that Ca^{2+} is required for resealing of lesioned cockroach giant axons. Structural studies have shown that axons constrict following axotomy and that this constriction is accompanied by "softening" of the axoplasm and loss of axoplasmic proteins (Lubinska, 1956; Gallant, 1988). Cytoskeletal disruption is often observed following nerve injury (Meiri et al., 1983; Lucas et al., 1985; Emery et al., 1987; Gross and Higgins, 1987), and this disruption has been attributed to Ca^{2+} influx (Schlaepfer and Bunge, 1973; Schlaepfer and Zimmerman, 1985).

We describe here a series of experiments designed to explore the mechanisms by which Ca^{2+} entry into the injured neurite promotes membrane resealing. We used a nitrogen laser to transect the neurites of cultured rat septal neurons and developed a dye exclusion technique that facilitates measurement of membrane resealing in a population of neurons. We then tested how the percentage of neurites that resealed varied in the presence of agents that influence cellular processes known to be enhanced by Ca^{2+} entry (including cytoskeletal disruption, protease and phospholipase activity, and calmodulin activation). Our results suggest that resealing of injured neurites is strongly facilitated by disruption of certain elements of the cytoskeleton and by activation of endogenous cysteine proteases in the calpain family.

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Materials and Methods

Neuronal culture. Monolayer cultures of dissociated septal neurons were prepared from fetal Sprague–Dawley rats at 14–15 d gestation. The embryonic septal tissue was mechanically dissociated by gentle trituration. The cell suspension was plated into 35-mm-diameter tissue culture dishes (Nunc, Kamstrup, Denmark) coated with collagen followed by poly-L-lysine (Kaufman et al., 1985). To increase plating efficiency, the dishes were also pretreated with lung fibroblast–conditioned medium (Dribin and Barrett, 1982a,b) at 35°C for 1 hr immediately before plating. Cells were plated at a density of 1000–5000 cells/cm² and grown in N5 culture medium supplemented with an acid-stable 55 kDa serum fraction (Kawamoto and Barrett, 1986). To enhance survival of these low-density cultures (needed to minimize overlap of neuronal processes), four to six pieces of fetal septal brain explant, 0.2–0.5 mm in diameter, were placed among the dissociated cells. Under these conditions, single neurons (including those not touching the explants) survived, sent out processes, and could maintain those processes for 2 months or more. Neurite transections (see below) were performed at room temperature on neurons that had been in culture 3–7 d.

Transection of neurites. In most experiments neurites were transected by one or more shots from a pulsed nitrogen laser (Laser Service Corp., Rochester, NY) operating at 337 nm wavelength and 12 nsec pulse duration (Higgins et al., 1980). Pulses were delivered at 7 Hz through a 32× quartz objective on an inverted microscope. Focusing the laser beam through the bottom of the culture dish (thickness, 0.75 mm) allowed experiments to be performed under sterile conditions. Lesions were produced with energy densities ranging from 1 to 12 μJ/μm². This surgical process involves shock waves produced by substrate vaporiza-

tion (Higgins et al., 1980). When the laser beam was instead focused within 2 μm of cell processes without directly hitting them, the cell processes showed no signs of damage and the neurons continued to survive. Since the beam is small (about 2 μm in diameter at the plane of focus) and easy to control, the laser provides a precise and rapid way to transect neuronal processes. In each experiment the neurites of about 30–40 neurons in a culture dish were transected under microscopic control over a time course of 10–15 min. The cultures were incubated in 5% CO_2 to maintain normal pH.

To check the generality of our results with laser-transected neurites, in a few experiments neurites were instead transected using the broken tip (5–10 μm diameter) of a glass micropipette (see also Shaw and Bray, 1977; Wessels et al., 1978).

Measurement of membrane resealing. Membrane integrity was measured by exclusion of dye, either Lucifer yellow CH (445 Da; Molecular Probes, Eugene, OR) or dextrans labeled with fluorescein or rhodamine isothiocyanate (FITC, RITC; 42 and 71 kDa; Sigma, St. Louis, MO). This technique is justified in Figures 2–4, below. Labeled dextrans were separated from free FITC or RITC using Sephadex G25 gel chromatography and then concentrated with a speed vacuum concentrator. Dyes (2 mg/ml) were dissolved in test medium of the same composition as that used during neurite transection; dye concentrations were more than 100 times that needed for detection by fluorescence microscopy in a glass capillary 1 μm in diameter. Dyes were added 20–30 min after the final transection, unless otherwise specified. Neurons were incubated with dye for 10–15 min at 37°C, then washed several times with dye-free culture medium. Nikon epifluorescence filters (set B for Lucifer yellow and FITC–dextran and set G for RITC–dextran) were used for dye imaging. Neurons that had resealed before dye addition excluded the dye, whereas neurons that had not yet resealed took up the dye. Dye uptake was so rapid that there was a marked distinction between labeled and unlabeled neurons. The percentage of neurons showing membrane resealing was calculated as $(1 - N/T) \times 100\%$, where N = dye-positive neurons and T = total transected neurons. Unless otherwise stated, the averages presented in figures, tables, and text are means (±SEM) of results from at least three experiments, each including transections of at least 30–40 neurons.

Ca^{2+} buffering. 1,2-Bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) was used as a calcium chelator because it is highly selective for Ca^{2+} over Mg^{2+} and is little affected by pH changes (Tsien, 1980), and was not toxic to neurons. For $[Ca^{2+}]$ in the range 0.01–10 μM, the total BAPTA concentration was fixed at 100 μM, and free $[Ca^{2+}]$ was varied by changing total [Ca], using the BAPTA dissociation constants for Ca^{2+} (0.1 μM) and for Mg^{2+} (17 mM; Tsien, 1980) to calculate the total [Ca] needed to achieve the desired free $[Ca^{2+}]$ in the presence or absence of Mg. Solutions containing 100 μM Ca^{2+} were buffered with 1 mM BAPTA. Higher concentrations of Ca^{2+} were unbuffered. In solutions containing no added Ca, the total [Ca], measured with an absorption spectrophotometer (Perkin-Elmer 3030), was less than 10 μM.

Low-ionic-strength media. Low-ionic-strength media consisted of NaCl or KCl (1–75 mM), 5 mM $KHCO_3$, and 5.5 mM glucose, with sorbitol (or sucrose or metrizamide) added to adjust the total osmolarity to 300 mOsm. In experiments requiring low $[Ca^{2+}]$, 100 μM BAPTA was added to buffer free $[Ca^{2+}]$ to less than 0.01 μM.

Reagents. Calpain inhibitor II and dispase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and BAPTA, from Molecular Probes (Eugene, OR). Taxol was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Other reagents were obtained from Sigma.

Results

In initial experiments we verified that neurons whose neurites were lesioned using the laser could indeed survive and regrow a new process from the proximal neurite stump. Neuronal survival was measured in normal medium (2 mM Ca^{2+} and 2 mM Mg^{2+}) 24 hr after neurite transection. Neurons rarely survived when their neurite was transected at distances <25 μm from the cell body. The survival rate was about 20% with lesions approximately 50 μm from the soma, and increased to 60–80% for lesion distances ≥100 μm (two experiments, with transections of 35 neurons in each experiment at each distance). Similar distance-dependent survival following neurite transection was also reported by Lucas et al. (1985). In all subsequent experiments neurites were transected at distances >100 μm from the soma.

Most neurons that survived 24 hr after transection grew a new process from the proximal cut end of the neurite. Figure 1 shows one example in which serial micrographs were made of one neurite over times ranging from 5 min to 40 hr after neurite transection. The lesioned neurite showed changes similar to those described by Shaw and Bray (1977) following glass needle lesions: both proximal and distal neurite segments developed rounded, beadlike swellings, which persisted for 5–10 min (5 min micrograph). The proximal neurite retracted (2.5 hr) and subsequently regrew (21 and 40 hr). The distal stump initially recovered (1.5 hr) but later degenerated (21 hr).

Dye exclusion as a measure of membrane resealing

Figure 2 presents evidence that injured neurons exclude dye once their membrane reseals. The upper photographs (labeled *1A–3A*) show three different transected neurons. When Lucifer yellow was added to the culture medium 3 min posttransection, all three lesioned neurons became labeled with dye (Fig. 2, *1B–3B*). Then a second dye, RITC–dextran, was added at 5, 7, or 10 min posttransection. This second dye was taken up following the 5 and 7 min delays (*1C, 2C*) but was excluded when added after the 10 min delay (*3C*). A likely explanation for this dye exclusion is that the injured membrane had resealed by the time the second dye was added (i.e., within 10 min).

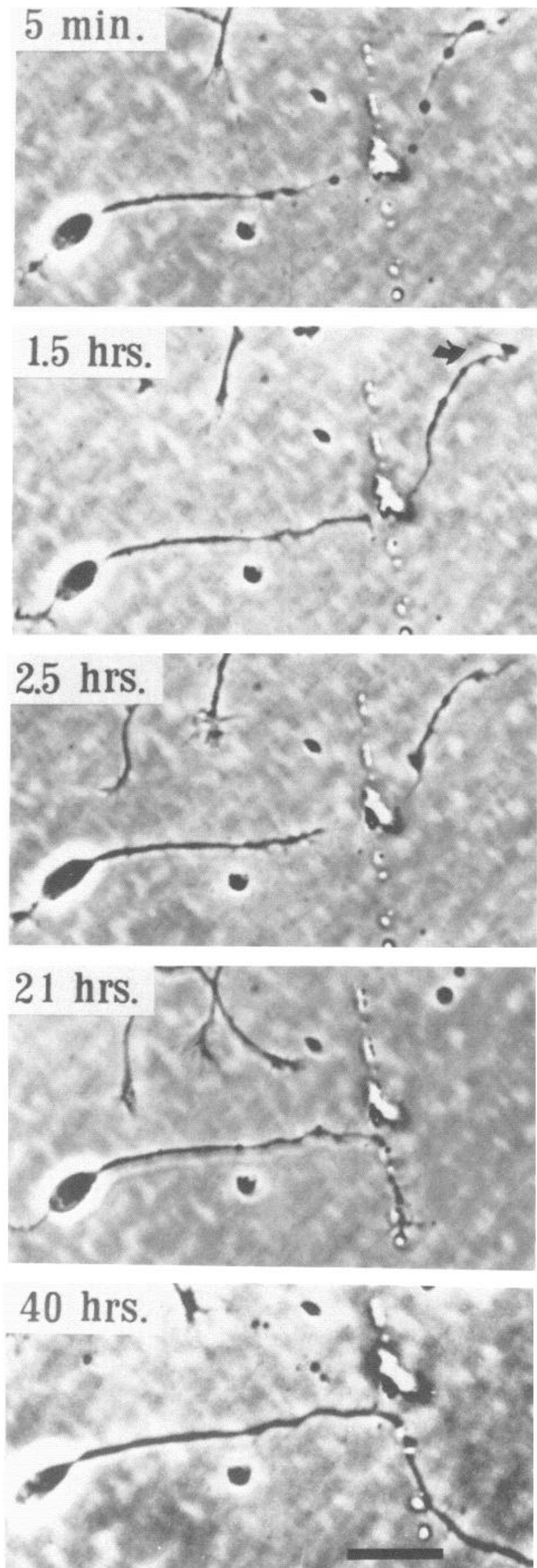
Dye entry did not appear to influence the resealing process. Dye-filled neurons were able to reseal and exclude a second dye (Fig. 2, *3C*), and in another series of experiments the percentage of lesioned neurons excluding one dye was similar whether or not another dye had been present during lesioning.

Figure 3 shows the posttransection time course of dye exclusion measured with three sizes of dye (445 Da Lucifer yellow, 42 kDa RITC–dextran, and 71 kDa FITC–dextran) under identical conditions. The percentage of lesioned neurons that excluded dye increased as the interval between transection and dye addition increased, reaching a plateau by about 20 min. Each dye yielded approximately the same percentage and time course of dye exclusion. Since FITC- (or RITC-) labeled dextran is too large to pass through any known membrane channel, we conclude that exclusion of any of these three dyes gives an accurate measure of membrane resealing. This conclusion is also supported by the similarity between the time courses of dye exclusion and of the electrophysiological measurements of membrane resealing used in earlier studies (see Discussion).

In most of the following experiments, resealing was assayed 20–30 min after neurite transection, during which 60–80% of lesioned neurites resealed in the normal culture medium containing 2 mM Ca^{2+} and 2 mM Mg^{2+}.

Route of dye entry following neurite transection

Because dye reached the soma so quickly, we had to develop a special technique for rapid immobilization of dye in order to determine the route of dye entry. This technique, described in the Figure 4 caption, permitted visualization at a stage when dye had entered both the proximal and distal segments of the transected neurite, but had not yet reached the soma (Fig. 4). This experiment demonstrates that dye entry occurs through the cut ends of the neurite (or through membrane regions very close to the site of transection).



We considered whether the membrane depolarization produced by the lesion might increase the permeability of intact membranes enough to allow some small dyes to pass directly through otherwise uninjured regions of membrane. To test this possibility, we bathed neurons in isotonic KCl and found that Lucifer yellow (the smallest dye used in this study) did not enter unlesioned cells. This result indicates that depolarization by itself is not sufficient to permit dye entry.

Ca^{2+} enhances membrane resealing and neuronal survival after neurite transection

Figure 5 shows that membrane resealing (measured by dye exclusion 30 min after transection) and neuronal survival at 24 hr are both dependent on extracellular [Ca^{2+}]. For bath [Ca^{2+}] less than 1 μM, membrane resealing and neuronal survival were both very low (less than 10%). Both increased with increasing [Ca^{2+}], with the maximal increase occurring between 10 and 100 μM. Maximal neuronal survival and membrane resealing occurred in 2 mM Ca^{2+}. Resealing was slightly less at 5 mM Ca^{2+} (data not shown).

Membrane resealing correlated well with neuronal survival, consistent with the idea that resealing is an important determinant of survival following neurite transection. However, a higher percentage of neurons survived for 24 hr after transection than resealed within 30 min after transection, suggesting that some neurons (a minority) resealed with delays exceeding 30 min. This result is consistent with other experiments (not shown) in which neurons were held in a nonsealed state in low-[Ca^{2+}] medium for 30 min and then exposed to normal [Ca^{2+}]. About 65% of these neurons then resealed, indicating that (at least in low [Ca^{2+}]) lesioned neurons can survive in a dye-permeable state for at least 30 min and later reseal.

Effects of other divalent cations on resealing

Figure 6*A* shows that 2 mM Mg^{2+} inhibited membrane resealing at all [Ca^{2+}] tested. The inhibition of resealing by Mg^{2+} was greater at low [Ca^{2+}] (Fig. 6*B*), with 100% inhibition at (or below) 1 μM Ca^{2+}, but only 20% inhibition at 2 mM Ca^{2+}.

Sr^{2+} and Mn^{2+} enhanced resealing. At low concentrations these cations were more effective than Ca^{2+}. For example, in one experiment performed on sister cultures in 2 mM Mg^{2+}, 1 ± 4% of lesioned neurons resealed in Mg^{2+} alone, 15 ± 4% resealed after addition of 100 μM Ca^{2+}, 31 ± 5% after addition of 100 μM Mn^{2+}, and 50 ± 6% after addition of 100 μM Sr^{2+}. However, resealing decreased as [Mn^{2+}] was increased to 2 mM and did not change significantly as [Sr^{2+}] was increased to 2 mM. Thus, the 75% resealing observed in 2 mM Ca^{2+} was greater than that seen in any tested concentration of Sr^{2+} or Mn^{2+}.

←

Figure 1. Phase-contrast micrographs of a septal neuron (4 d in culture) at the indicated times following a laser-induced neurite transection 149 μm from the soma. Note the "bead" formation along both cut ends *5 min* after transection. By *1.5 hr,* this beading had disappeared from both proximal and distal segments, and a microspike (marked by an *arrow*) was present at the tip of the distal segment. By *2.5 hr,* the proximal and distal segments had withdrawn slightly from the site of transection, and the proximal segment had developed microspikes. At *21 hr,* the distal segment had disappeared, and the proximal segment was elongating. The rate of regrowth during the interval between 2.5 and 21 hr was 49 μm/d. After *40 hr,* the rate of process growth was 73 μm/d. The near-vertical *row of dots* in each micrograph are dents in the plastic dish made by additional laser pulses. Scale bar, 50 μm.

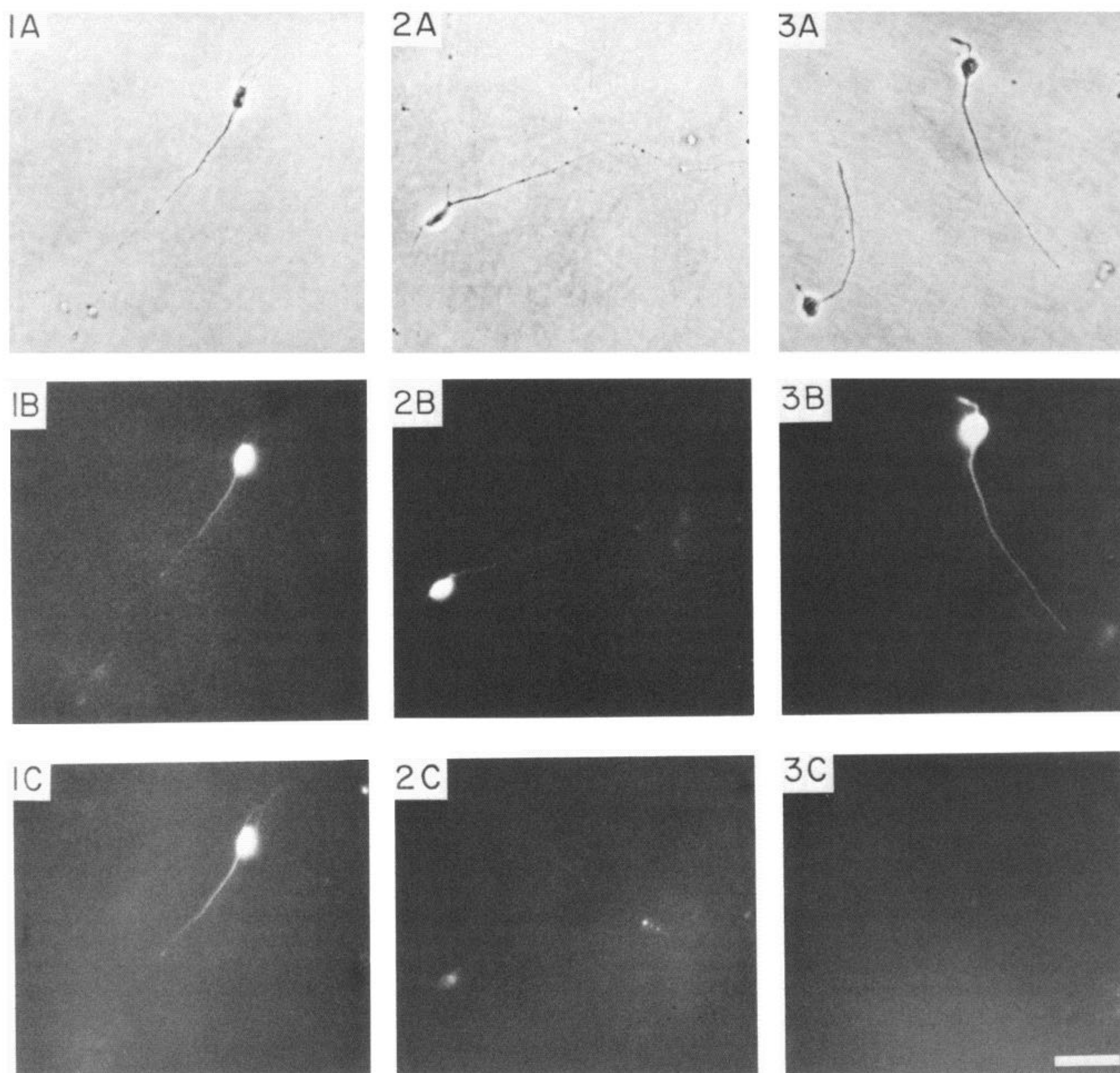


Figure 2. Micrographs of dye uptake before, and dye exclusion after, membrane resealing following neurite transection. Septal neurons from embryonic day 15 (E15) fetal rat were grown in culture 3 d before transection. *1A–3A* are phase-contrast micrographs of three different neurons immediately following neurite transection. An unlesioned neuron is also present at left in *3A*. Lucifer yellow (CH; 445 Da) was added 3 min after each transection (*1B–3B*). RITC–dextran (42 kDa) was added to the same dishes 5 min (*1C*), 7 min (*2C*), and 10 min (*3C*) after the transections. Each lesioned neuron took up Lucifer yellow, indicating that none had resealed by 3 min posttransection. The neurons in *columns 1* and *2* also took up RITC–dextran, indicating that they remained unsealed 5 and 7 min posttransection, respectively. The lesioned neuron to the *right* in *column 3* did not take up RITC–dextran, indicating that it had fully resealed by 10 min posttransection (*3C*). The unlesioned neuron in *column 3* excluded both dyes. Scale bar, 25 μm.

These results suggest that Sr^{2+} and Mn^{2+} can mimic some, but not all, of the enhancing effects of Ca^{2+}, and/or that millimolar concentrations of Sr^{2+} and Mn^{2+} may have additional toxic effects that interfere with resealing.

Resealing is enhanced by colchicine and reduced by taxol

Colchicine (Andreu and Timasheff, 1986; Wilson and Farrell, 1986), vinblastine and vincristine (Luduena et al., 1986) all disrupt microtubules. When these drugs were added individually to normal culture medium, they appeared to reduce the thickness of neurites, and some intact neurons showed the "beaded" morphology usually seen only after neurite transection. However, as indicated in Table 1, these microtubule-disrupting drugs all enhanced membrane resealing following neurite transection. Lumicolchicine, a modified form of colchicine that does not affect microtubules (Borisy et al., 1972), did not significantly affect resealing.

Because the microtubule-disrupting drugs produced beading and thinning of intact (nonlesioned) neurites, it seemed possible that the increased dye exclusion seen with these drugs might

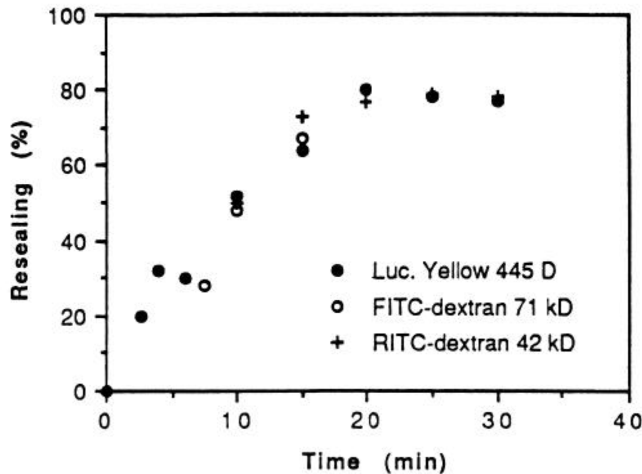


Figure 3. The time course of membrane resealing after neurite transection was measured by exclusion of three sizes of dye, as indicated in the *legend.* These results were obtained from a single plating of E15 septal neurons that had been grown in culture 3–5 d before laser-induced neurite transection. All media contained 2 mM Ca^{2+} and 2 mM Mg^{2+}. Each *point* represents the average percentage of dye-excluding neurons measured from a population of 35 or more lesioned neurons. The expected SD was estimated to be less than 5% of each data point by modeling the experiment with a binomial distribution (the probability of resealing was set to give the observed resealing rate, and the number of trials was set equal to the number of neurites transected).

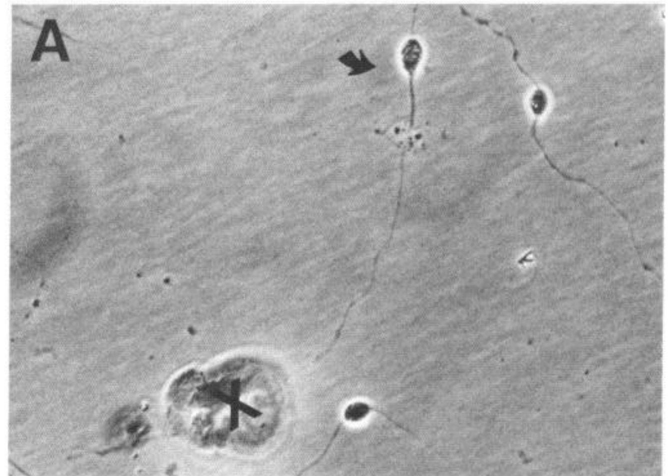
have been due to restricted diffusion of dye along the neurite rather than to enhanced membrane resealing. This alternative explanation is unlikely, however, because when a group of neurons was pretreated for 30–60 min with colchicine and then transected in the presence of colchicine and Lucifer yellow dye (instead of the usual procedure of adding dye 20–30 min after transections), the soma of every lesioned neurite became labeled with dye ($n = 25$).

Taxol, which stabilizes microtubules (Schiff and Horwitz, 1980; Horwitz et al., 1986), reduced resealing by 77% (Table 1).

Resealing is enhanced in low-ionic-strength media

Low-ionic-strength solutions have been reported to dissociate microtubules and neurofilaments (Schlaepfer, 1971; Olmsted and Borisy, 1975; Liem et al., 1978). We found that low-ionic-strength solutions (6 mEq, with sorbitol added to maintain normal osmolarity) produced no visible disruption of neuronal geometry in intact neurons. When neurites were lesioned close to the soma in divalent cation–free media of low ionic strength, there was a marked increase in Brownian motion within the soma cytoplasm, often accompanied by loss of particles through the lesion site. These observations suggest that low-ionic-strength solutions did indeed disrupt the cytoskeleton near a lesion site.

We then tested how low-ionic-strength solutions (6–150 mEq) affected resealing of neurites lesioned at the usual ≥ 100 μm from the soma and found that divalent cation–free, 6 mEq solutions yielded 100% resealing (Fig. 7). Increasing ionic strength above 6 mEq with either NaCl (solid circles) or KCl (open circles) decreased resealing. Addition of 2 mM $MgCl_2$ to these low-ionic-strength solutions abolished resealing (×s in Fig. 7), an effect consistent with inhibition of resealing by Mg^{2+} in solutions of normal ionic strength (Fig. 6) and with stabilization of microtubules and neurofilaments by Mg^{2+} (Weisenberg, 1972; Inagaki et al., 1987).



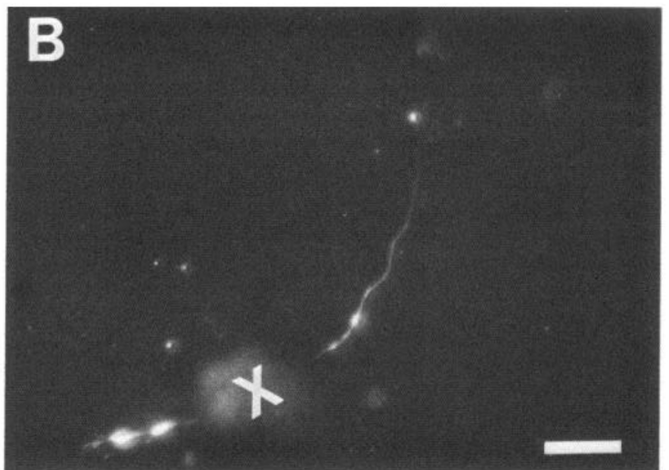


Figure 4. Initial dye entry through the cut ends of a laser-transected neurite. The *arrow* in the phase-contrast micrograph in *A* indicates the soma of the neuron, and the *X* indicates the site of neurite transection. The corresponding fluorescence micrograph in *B* shows the distribution of RITC–dextran in the proximal and distal ends of the transected neurite. In the proximal segment, fluorescence decreases with distance from the site of transection. Dye is not yet detectable in the soma (the fluorescent *dot* near the soma is extracellular debris). Note that the two unlesioned neurons visible in *A* did not take up dye. Most laser lesions produced much less substrate disruption than that illustrated here (see Figs. 1, 2). Scale bar, 25 μm. *Method:* Because dye entry and diffusion were normally so rapid, the following special procedure was devised for fast fixation of the initial stages of dye entry. Septal neurons were transected in Ca-free culture medium containing 2 mM Mg to retard resealing (see Fig. 6*A*). Immediately following completion of all transections, RITC–dextran was added for 30 sec, and then cells were exposed for 5 sec to a low-ionic-strength medium (6 mEq) to speed resealing (see Fig. 7). The cultures were then washed with normal medium for 20 sec, with cold ethanol (−20°C) for 30 sec, and finally with room temperature ethanol. Ethanol precipitates the dye-labeled dextrans, and thus quickly fixes the dye in place.

Addition of 1 mM Ca^{2+} to low-ionic-strength media had relatively little effect on resealing (squares in Fig. 7). Resealing at low ionic strength differed from resealing at normal ionic strength by being only minimally (6%) inhibited by taxol.

Membrane resealing in low-ionic-strength solutions was not dependent on the specific sugar used to maintain osmolarity: replacement of sorbitol by metrizamide or sucrose also yielded 100% resealing in 6 mEq solutions. Adding extra sorbitol to

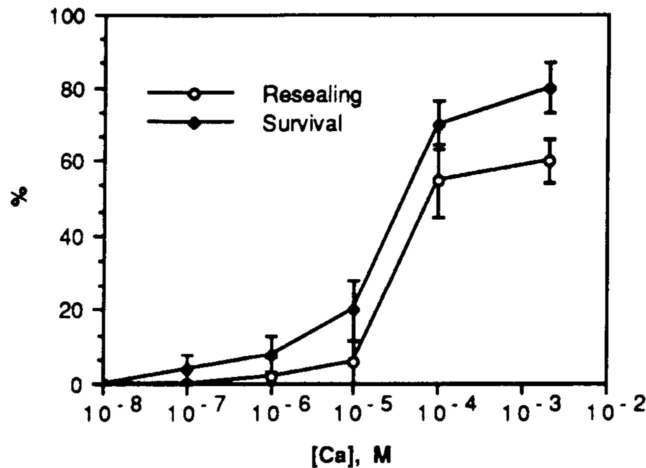


Figure 5. Membrane resealing and neuronal survival in various [Ca^{2+}]. Septal neurons were grown in medium containing 2 mM Ca^{2+} and 2 mM Mg^{2+} for 3–6 d, and then were exposed to the indicated [Ca^{2+}] during neurite transection and thereafter. [Ca^{2+}] in the range of 10^{-8} to 10^{-4} M was buffered with 100 μM to 1 mM BAPTA, as described in Materials and Methods. Lucifer yellow dye was added to cultures 30 min after the final transection, and exclusion of dye from the cell bodies of transected neurites was used to indicate membrane resealing. Neuronal survival was scored 24 hr after transection. *Error bars* represent SEM. *Lines* were drawn by eye.

make the solution hypertonic (400 mOsm) reduced resealing from 100% to 30%.

In low-ionic-strength solutions, high concentrations of vincristine (1 mM) stabilize neurofilaments of rat sciatic nerve (Schlaepfer, 1971). If the high rate of resealing in Ca^{2+}-free, low-ionic-strength solutions was due in part to dissociation of neurofilaments, one would predict that addition of vincristine would inhibit membrane resealing. As shown in Figure 8, high concentrations of vincristine did indeed reduce resealing at low ionic strength. These high concentrations also inhibited resealing in normal medium, though less dramatically. In contrast, colchicine (50 μM to 1 mM) always enhanced resealing in normal medium (Fig. 8, Table 1).

Membrane resealing is inhibited by inhibitors of cysteine proteases and enhanced by exogenous proteases

Microtubules (as well as other cytoskeletal components) can also be disassembled by proteolytic cleavage. To determine whether activation of endogenous proteases might facilitate resealing, we tested the effects of various protease inhibitors, with results summarized in Table 2. Leupeptin and antipain, which inhibit many cysteine proteases [including papain and calpain (Ca^{2+}-activated protease); Rich, 1986a] dramatically inhibited resealing. Likewise, calpain inhibitor II, a specific inhibitor of calpain I and calpain II (Boehringer Mannheim Biochemicals, 1989), significantly reduced resealing. In contrast, protease inhibitors that do not inhibit cysteine proteases were less effective in reducing resealing. For example, aprotinin, an inhibitor of serine proteases (Gebhard et al., 1986), did not significantly inhibit resealing, and pepstatin, an inhibitor of aspartic proteases (Rich, 1986b), reduced resealing by only 33%.

Effects of exogenous proteases on membrane resealing were tested in Ca^{2+}-depleted culture medium, in which resealing is otherwise minimal (Fig. 5). Papain (a cysteine protease), trypsin (a serine protease), and dispase (a metalloprotease) all dramatically promoted resealing (Table 3). The enhancement of resealing by papain and trypsin was inhibited by leupeptin. However, the enhancement by dispase was only slightly reduced by leupeptin, consistent with the fact that leupeptin does not inhibit the proteolytic activity of dispase.

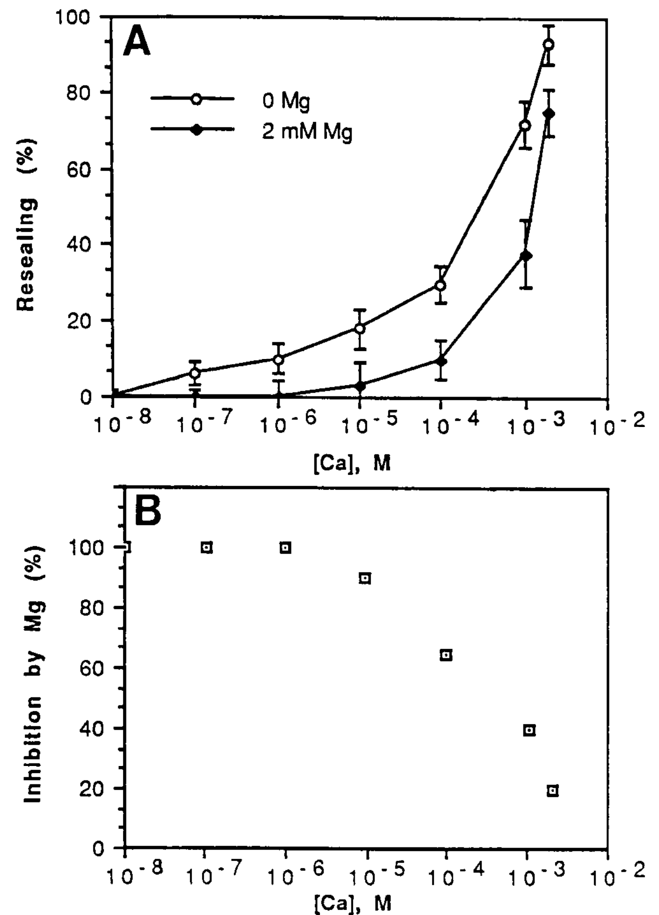


Figure 6. *A,* Measurements of resealing in the presence and absence of Mg^{2+} at various [Ca^{2+}] (buffered as described in Materials and Methods). Immediately prior to neurite transection, all dishes were incubated in 100 μM EGTA for 1 min, and then washed several times with the test medium. *Error bars* represent SEM. *Lines* were drawn by eye. *B,* Percent inhibition of resealing by Mg^{2+}, as a function of bath [Ca^{2+}], calculated as $(C - CM)/C$, in which C = percentage resealing in medium containing Ca^{2+} alone and CM = percentage resealing in medium containing both Ca^{2+} and Mg^{2+}. Inhibition of resealing by Mg^{2+} was greater at low [Ca^{2+}].

Table 1. Agents that alter microtubule stability affect resealing

Condition	Resealing (% ± SEM)	% enhancement	% inhibition
Control	66 ± 4.5		
Colchicine (50 μM)	91 ± 1.2	38	
Vincristine (50 μM)	75 ± 5.8	14	
Vinblastine (50 μM)	74 ± 2.5	12	
Lumicolchicine (50 μM)	70 ± 3	6	
Taxol (20 μM)	15 ± 3.2		77

Controls were transected in normal culture medium containing 2 mM Ca^{2+} and 2 mM Mg^{2+}. Colchicine, vincristine, vinblastine, and lumicolchicine were added to cultures 1 hr before transections. Taxol was added 5–10 min before transection. Percentages of enhancement and inhibition were normalized to control.

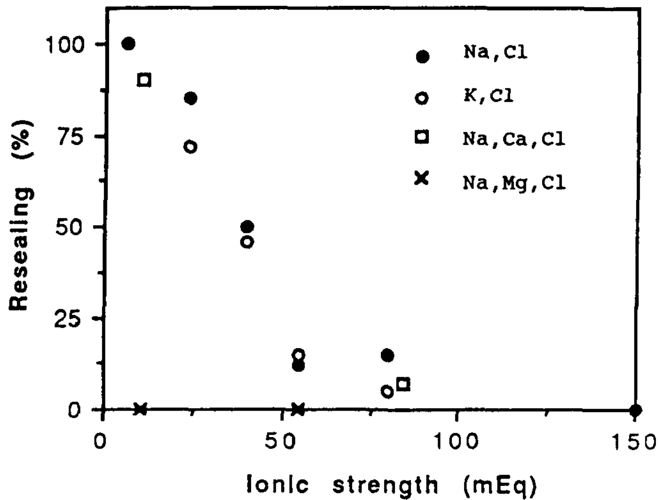


Figure 7. Effects of ionic composition and ionic strength (6–150 mEq) on resealing. All solutions contained 5 mM $KHCO_3$; *symbols* indicate additions of NaCl (*solid circles*), KCl (*open circles*), NaCl plus 1 mM $CaCl_2$ (*open squares*), or NaCl plus 2 mM $MgCl_2$ (*crosses*). Sorbitol was added to maintain a total osmolarity of 300 mOsm. Except as indicated, no divalent cations were added, and 100 μM BAPTA was added to ensure low free [Ca^{2+}] (10^{-8} M) in Ca-free solutions. Lucifer yellow dye was added 20 min after the final neurite transection in each experiment. Each *point* represents the average from transections of 30–40 neurons. The expected SD for each point is less than 5%, calculated using the binomial distribution. Note that 100% of neurons resealed in low-ionic-strength (6 mEq), Ca-free medium.

These results suggest that endogenous protease activity is important for membrane resealing and that endogenous cysteine proteases play a more important role than endogenous serine or aspartic proteases. The ability of exogenous proteases to enhance resealing in Ca^{2+}-depleted medium suggests that the lack of resealing observed in low-[Ca^{2+}] solutions may be due to lack of activation of Ca^{2+}-dependent endogenous proteases.

Resealing is inhibited by calmodulin inhibitors

Calmodulin greatly accelerates the calpain-mediated degradation of fodrin (also called brain spectrin), a cytoskeletal protein that in its tetrameric form helps link filamentous (F)-actin to the plasma membane [Goodman and Zagon, 1986 (review); Seubert et al., 1987; Harris et al., 1989; Harris and Morrow, 1990]. Consistent with the hypothesis that cytoskeletal disassembly facilitates resealing, we found that resealing in normal medium was inhibited by any of three different calmodulin inhibitors: trifluoperazine (5 μM) inhibited by 67%; W7 (50 μM), by 71%; and troponin I (40 μM), by 88%. Trifluoperazine and W7 can also bind directly to calmodulin-like domains on calpain I (Brumley and Wallace, 1989), but this alternative mode of action requires drug concentrations considerably higher than those used here. Trifluoperazine had a much smaller effect on resealing (only 7% inhibition) in divalent cation–free, low-ionic-strength media.

Resealing is inhibited by destabilization of F-actin

Contrary to our expectation, cytochalasin E, which destabilizes F-actin (Lin et al., 1978; Cooper, 1987), strongly inhibited resealing: in experiments performed in normal medium, addition of 2 and 4 μg/ml cytochalasin E inhibited resealing by 31% and 76%, respectively (Table 4). However, this inhibitory effect of cytochalasin E was overcome by the facilitatory effect of colchicine and was greatly reduced in low-ionic-strength solutions (Table 4). These results suggest that resealing is improved when F-actin remains intact, but that the integrity of F-actin is less important for membrane resealing than is the disassembly of microtubules and neurofilaments.

Table 2. Protease inhibitors inhibit resealing

Condition	Resealing (% ± SEM)	% inhibition (Normalized to control)
Control	75 ± 5	
Leupeptin (1 mM)	9 ± 1.3	88
Antipain (1 mM)	6 ± 1.5	92
Calpain inhibitor II		
25 μM	27 ± 1.5	64
50 μM	20 ± 1.4	73
100 μM	19 ± 2.0	75
500 μM	9 ± 1.8	88
Aprotinin (1 mM)	71 ± 4	5
Pepstatin (200 μM)	50 ± 7	33

Controls were transected in normal medium containing 2 mM Ca^{2+} and 2 mM Mg^{2+}. The indicated inhibitors were added to this medium 5 min prior to transection. The 1 mM concentration of leupeptin is in the range required to inhibit calpain in other systems (Banik et al., 1983) and to inhibit fusion of rat erythrocytes, which has been shown to involve calpain activation (Kosower et al., 1983; Glaser and Kosower, 1986). The 1 mM concentration of aprotinin is much greater than the dissociation constant of the inhibitor (6×10^{-14} M; Gebhard et al., 1986). The 200 μmM concentration of pepstatin was saturating and exceeds the concentrations that produce 50% inhibition of most aspartic proteases (1 nM to 1 μM; Umezawa and Aoyagi, 1977).

Energy depletion has little effect on membrane resealing

To test whether resealing might involve an ATP-dependent contraction involving F-actin, we added two metabolic inhibitors (50 μM dinitrophenol to uncouple oxidative phosphorylation and 50 μg/ml sodium azide to inhibit glycolysis) to culture medium 1 hr before neurite transection. The rate of resealing in the presence of these metabolic inhibitors was 80%, not significantly different from the 75% measured in control medium. In other experiments neurons were preincubated in glucose-depleted culture medium containing 50 μM dinitrophenol and 40 μM apyrase for 1 hr prior to transection. This medium should drastically reduce cellular [ATP], since dinitrophenol and lack of glucose prevent formation of new ATP, while apyrase entering at the lesion site should deplete preformed ATP by converting

Table 3. Exogenous proteases enhance resealing

Condition	Resealing (% ± SEM)
Control	8 ± 3
Papain (0.13 mg/ml)	100 ± 0
Papain (0.13 mg/ml) plus leupeptin (0.48 mg/ml)	1 ± 3
Trypsin (1 μg/ml)	90 ± 3.4
Trypsin (1 μg/ml) plus leupeptin (0.48 mg/ml)	3 ± 3
Dispase (0.17 mg/ml)	100 ± 0
Dispase (0.17 mg/ml) plus leupeptin (0.48 mg/ml)	90 ± 1.7

Controls were transected in Ca-depleted medium of normal ionic strength, in the absence of serum. Proteases were added to this medium immediately prior to transections. In experiments listed as protease alone, the protease inhibitor leupeptin (1 mM = 0.48 mg/ml) was added 5 min after the final transection to limit protease activity and thus present cell detachment from the substrate. In protease plus protease inhibitor experiments, both the protease and the inhibitor were added at the same time prior to transections.

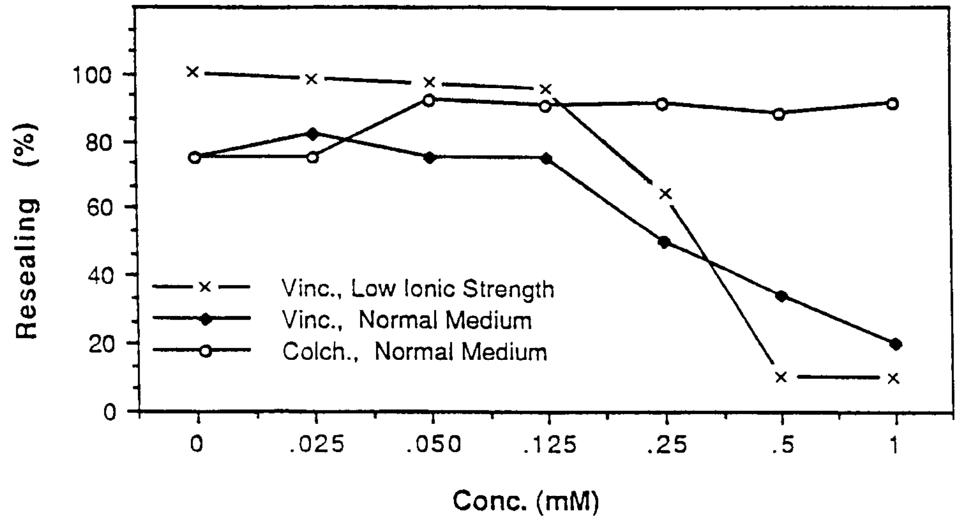


Figure 8. Concentration-dependent effects on resealing of vincristine (*vinc.*) and colchicine (*colch.*) in normal culture medium and of vincristine in low-ionic-strength (6 mEq) medium. Cells were incubated with drug for 1 hr before, as well as during and after, transection. Each *point* represents the average from transections of 30–40 neurons. The expected SD was less than 5% as calculated from the binomial distribution.

it to AMP. In this ATP-depleted condition, resealing was slightly reduced (from 75% to 65%), but this reduction was not statistically significant. Thus, membrane resealing does not appear to require a major expenditure of metabolic energy.

Effects of phospholipase A2 inhibitors

Yawo and Kuno (1983) presented evidence that the Ca^{2+}-dependent membrane resealing they measured in lesioned cockroach axons was mediated by activation of phospholipase A2. We attempted to test this hypothesis, but found that the phospholipase A2 inhibitors they used, *p*-bromophenacryl bromide (10 μM) and mepacrine (2 mM), produced morphological signs of toxicity in unlesioned neurons. For example, in 2 mM mepacrine, 51% of 145 *unlesioned* neurons took up dye. These nonspecific effects made it difficult to test the phospholipase A2 hypothesis rigorously in our cultured neurons. We did find that reducing the concentration of *p*-bromophenacryl bromide to 5 μM reduced the toxicity (unlesioned neurons were no longer dye permeable) and permitted 52% resealing of lesioned neurons, compared to 75% resealing of neurons lesioned in the absence of the drug (mean of two experiments, each involving transections of 35 neurons).

Similarity of resealing mechanisms following micropipette-produced neurite transections

To test whether the results reported here might be unique to laser-lesioned neurites, we repeated certain key experiments on neurites cut with the broken tips of glass micropipettes. For a sample of 90 transected axons, the resealing rate in control solution was 91 ± 4.3%. Similar to our results with laser-transected neurites, resealing of micropipette-transected neurites was strongly inhibited in nominally Ca^{2+}-free solutions containing 1 mM Mg^{2+} (15 ± 8% resealing), and addition of colchicine to this Ca^{2+}-free medium 20 min prior to transection increased the resealing rate to 74 ± 4%. In normal medium (2 mM Ca^{2+}), resealing following micropipette-produced transections was similarly reduced by inhibitors of cysteine proteases (only 15 ± 2% resealing in 1 mM leupeptin, and 32 ± 11% resealing in 1 mM antipain). These results suggest that the mechanisms governing resealing following micropipette-induced and laser-induced neurite transections are qualitatively similar.

Discussion

Methods for measuring membrane resealing

In previous studies, membrane resealing after neurite or axon transection was monitored by measuring changes in cellular resting potential and input resistance with an intracellular microelectrode (Yawo and Kuno, 1983; Lucas et al., 1985). Transection of neuronal processes produced dramatic depolarization of the resting potential and reduction of input resistance, both of which recovered to near pretransection levels after a delay of 5–30 min in cockroach giant axons (Yawo and Kuno, 1983, 1985) and 2–5 min in laser-transected spinal cord neurons (Gross et al., 1983). This recovery of electrical properties is probably associated with, or occurs after, membrane resealing, since even a 0.1 μm hole in the cell membrane would produce a large electrical leak and so depolarize the cell. Constriction of the cut ends of severed squid axons, which might be an initial step in resealing, also occurs with a time course of 5–30 minutes (Gallant, 1988). For most laser-lesioned neurons, our dye exclusion assay yielded a postlesion resealing time course compatible with these previous studies: 60–80% of neurons lesioned in normal culture medium excluded dye within 20–30 min (Fig. 3).

A minority (5–20%) of the lesioned neurites appear to have resealed after this initial 30 min period, since the percentage of lesioned neurons that survived for 24 hr was greater than the percentage of neurons that resealed by 30 min (Fig. 5). Perhaps the more severely damaged neurites required longer times for complete resealing. A slowly resealing population of neurites might not have been detected in previous electrophysiological studies, because of the difficulty of maintaining intracellular impalements over prolonged times.

Possible mechanisms involved in membrane resealing

In agreement with previous studies on cockroach giant axons (Yawo and Kuno, 1985), we found that resealing of lesioned septal neurites in normal culture medium is dependent on extracellular Ca^{2+} (Fig. 5). Thus, it seems likely that membrane resealing involves processes triggered by Ca^{2+} entry into the injured neurite.

Disassembly of microtubules and neurofilaments

Several lines of evidence suggest that one mechanism by which Ca^{2+} entry facilitates resealing involves disassembly of micro-

Table 4. Agents that alter F-actin stability affect resealing

Condition	Resealing (% ± SEM)	% enhancement	% inhibition
I. Control	80 ± 3		
Cytochalasin E			
2 μg/ml	55 ± 8		31
4 μg/ml	19 ± 2		76
II. Control	70 ± 5		
Cytochalasin E (4 μg/ml)			
plus colchicine (50 μM)	94 ± 5	34	
III. Low ionic strength,			
no divalent cations	100 ± 0		
plus cytochalasin E (4 μg/ml)	91 ± 1		9

Controls in conditions I and II were transected in normal culture medium containing 2 mM Ca^{2+} and 2 mM Mg^{2+}. All experiments in condition III were performed in 6 mEq solutions containing 100 μM BAPTA. Cytochalasin E was added 10–20 min prior to transections. Percentages of enhancement and inhibition were normalized to control.

tubules and neurofilaments. First, treatments that favor disassembly of these structures (e.g., colchicine, exogenous proteases, low-ionic-strength solutions) promoted resealing, whereas treatments that stabilize these cytoskeletal structures (Mg^{2+}, taxol) inhibited resealing (Figs. 5–7, Tables 1–3). Second, the concentration-dependent effects of vincristine are consistent with this hypothesis: micromolar concentrations of vincristine disrupt microtubules and slightly enhance resealing in medium of normal ionic strength (Table 1), whereas millimolar concentrations of vincristine stabilize neurofilaments and inhibit resealing in medium of low ionic strength (Fig. 8). Third, treatments that disassemble microtubules and neurofilments were able to overcome the inhibitory effects of other treatments. For example, low-ionic-strength solutions enhanced resealing in the presence of taxol, trifluoperazine, and cytochalasin E. Also, low-ionic-strength solutions and exogenous proteases promoted resealing in Ca^{2+}-depleted solutions (Table 3, Fig. 7).

These findings suggest that a mechanism involving disruption of microtubules and neurofilaments promotes the resealing of injured neurites. Disassembly of these cytoskeletal components may act by reducing or modifying the interaction between the cytoskeleton and the plasma membrane (Klausner et al., 1981; Fach et al., 1986; Regula et al., 1986), thus enhancing the mobility of membrane components needed for resealing. In addition, disassembly of microtubules and neurofilaments may help remove cytoskeletal components that would otherwise protrude from the injury site and thus sterically hinder resealing.

In contrast, membrane resealing was inhibited by cytochalasin E, which destabilizes F-actin (Table 4). Perhaps tension exerted by contraction of intact F-actin promotes resealing by helping to collapse the cut end of the neurite. Consistent with this idea, cytochalasin D, an F-actin inhibitor, blocks the neurite retraction often observed after transection (Heidemann et al., 1986). Our results suggest that the role of F-actin in resealing does not require high levels of ATP, and that F-actin integrity is not as important for resealing as is disassembly of microtubules and neurofilaments, since the inhibitory effects of cytochalasin E could be overcome by colchicine and low-ionic-strength solutions (Table 4).

Activation of Ca^{2+}-dependent endogenous proteases

Ca^{2+} influx into the injured neurite might promote disassembly of microtubules and/or neurofilaments in several different ways: via direct actions (Weisenberg, 1972; Olmsted, 1976; Margolis, 1983), via calmodulin-mediated mechanisms (Yamamoto et al., 1983; Yamauchi and Fujisawa, 1983; Harris and Morrow, 1990), and via activation of neutral proteases in the calpain family (Schlaepfer and Bunge, 1973; Schlaepfer, 1974; Schlaepfer and Zimmerman, 1985). Consistent with these possible mechanisms of action, resealing was inhibited by calmodulin inhibitors (trifluoperazine, W7, and troponin I) and by inhibitors of Ca^{2+}-activated cysteine proteases (leupeptin, antipain, and calpain inhibitor II; Table 3).

Evidence for involvement of calpain in resealing seems especially strong. This family of Ca^{2+}-dependent neutral cysteine proteases appears to be activated at the cut ends of axons (Schlaepfer and Hasler, 1979; Banik et al., 1982) and can degrade cytoskeletal components such as neurofilaments (Vitto and Nixon, 1986) and microtubule-associated protein (Sandoval and Weber, 1978), as well as proteins that anchor cytoskeletal components to the plasmalemma (e.g., fodrin; Siman et al., 1984; reviewed by Mellgren, 1987). Fusion of lipid membranes is thought to require removal of certain membrane proteins, and, consistent with this idea, degradation of spectrin by calpain facilitates membrane fusion in red blood cells (Lucy, 1984; Glaser and Kosower, 1986). Degradation of fodrin (= brain spectrin) alters cell shape and increases the lateral mobility of membrane proteins (Burridge et al., 1982; Levine and Willard, 1983). Degradation of fodrin by calpain is greatly facilitated by calmodulin (Harris and Morrow, 1990), suggesting that calpain and calmodulin may act cooperatively in promoting membrane resealing.

Calpain I has an affinity for Ca^{2+} in the micromolar range, whereas full activation of calpain II requires 2 mM Ca^{2+} (Murachi, 1983; Vitto and Nixon, 1986), the optimal Ca^{2+} concentration for resealing at normal ionic strength. These different Ca^{2+} affinities of calpain I and II might help account for the broad range of Ca^{2+} concentrations (micromolar to millimolar) that affect resealing (Fig. 5). Mg^{2+} does not activate calpain II (David and Shearer, 1986), consistent with failure of Mg^{2+} to promote membrane resealing. Micromolar Mn^{2+} can activate erythrocyte calpain (Pontremoli et al., 1985), and Sr^{2+} increases proteolysis in skeletal muscle (Baracos et al., 1986), consistent with these cations' ability to support some resealing.

In sum, based on our results with cultured neurons, we suggest that resealing of injured neurites is facilitated by (and may re-

quire) degradation of certain cytoskeletal components (microtubules, neurofilaments, fodrin) and/or their attachment to the plasma membrane, and that this degradation is mediated by endogenous neutral cysteine proteases (calpains) activated by Ca^{2+} entry into the injured neurite. Other actions of Ca^{2+} that may also contribute to resealing include interaction with membrane surface charges, direct fusion-promoting effects on membrane lipids (Papahadjopoulos et al., 1979; Duzgunes et al., 1985), and activation of membrane phospholipase A2 (Yawo and Kuno, 1983). We hope that the results presented here help to stimulate further work on the involvement of the cytoskeleton and proteases in membrane resealing; improved understanding of the mechanisms involved may lead to improved treatments for speeding recovery from nerve injury.

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