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Topical Review

Cell Surface Area Regulation and Membrane Tension

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Abstract. The beautifully orchestrated regulation of cell shape and volume are central themes in cell biology and physiology. Though it is less well recognized, cell surface area regulation also constitutes a distinct task for cells. Maintaining an appropriate surface area is no automatic side effect of volume regulation or shape change. The issue of surface area regulation (SAR) would be moot if all cells resembled mammalian erythrocytes in being constrained to change shape and volume using existing surface membrane. But these enucleate cells are anomalies, possessing no endomembrane. Most cells use endomembrane to continually rework their plasma membrane, even while maintaining a given size or shape. This membrane traffic is intensively studied, generally with the emphasis on targeting and turnover of proteins and delivery of vesicle contents. But surface area (SA) homeostasis, including the controlled increase or decrease of SA, is another of the outcomes of trafficking.

Our principal aims, then, are to highlight SAR as a discrete cellular task and to survey evidence for the idea that membrane tension is central to the task. Cells cannot directly "measure" their volume or SA, yet must regulate both. We posit that a homeostatic relationship exists between plasma membrane tension and plasma membrane area, which implies that cells detect and respond to deviations around a membrane tension set point. Maintenance of membrane strength during membrane turnover, a seldom-addressed aspect of SA dynamics, we examine in the context of SAR.

SAR occurs in both animal and plant cells. The review shows the latter to be a continuing source of groundbreaking work on tension-sensitive SAR, but is principally slanted to animal cells.

Key words: Mechanosensitive — Cytomechanics — Membrane traffic — Membrane skeleton — Vacuole — Exocytosis — Endocytosis — Protoplast — Neuron — Stretch — Osmotic

Tension Hypothesis for Surface Area Regulation (SAR)

Neurons and guard cells have been especially informative about mechanosensitive SAR. Their biology makes strong demands on SAR and their large shape/volume changes facilitate the studies. During neuronal arborization, the tractile tension exerted by any neurite determines whether that neurite will extend (high tension) or retract (low tension). In a leaf, changing ambient conditions repeatedly drive guard cells to swell then shrink, and in doing so to open and then close the stomata for gas exchange. These and many other situations require these radically different cytomechanical objects to perform mechanosensitive SAR. Is there a common thread?

Stated minimally, a tension hypothesis of SAR is: *When membrane tension goes high locally, SA is added locally from widespread, mechanically accessible endomembrane reserves. When tension goes low locally, excess SA is retrieved locally.*

For idealized cells experiencing isotropic forces (i.e., nonadherent, spherical cells), plasma membrane tension changes would be uniform, so "locally" could be dropped from the hypothesis. But for most cells *in situ*

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(i.e., adherent irregular-shaped cells), localized forces and hence localized tension perturbations would be the norm.

For freely behaving cells, monitoring membrane tension is difficult. Cells can be inflated or patches of membrane aspirated and Laplace's law (*see* Table) relating transmembrane pressure and membrane tension applied, but these are invasive and/or mechanically disruptive approaches. Membrane tension changes can now be monitored via laser tweezer tether force, an approach developed by Sheetz and colleagues (Dai et al., 1995*b*). Dai, Ting-Beall & Sheetz, (1997) examined membrane traffic in rat basophilic leukemia (RBL) cells, looking for dependence between rates of exocytosis and endocytosis and levels of membrane tension. As this introduces many issues, it is a useful starting place.

In RBL cells, hypotonia (50%) causes membrane tension to increase. After a sometimes unmeasurable overshoot (the tweezers lose their hold) tension subsides, over 20 min, to a lower subcontrol level. Simultaneously, endocytosis decreases (monitored over 20 min in a cell sorter, using a fluid phase uptake indicator). With stimulated secretion, <10 sec after using a serotonin secretogogue (release measured radioisotopically), membrane tension falls dramatically. Subsequently (by 5 min) a compensatory increase in endocytosis is detectable and is sustained during 20 min stimulation. Bearing in mind the complex factors (in-plane bilayer tension, adhesion of cytoskeleton, bending stiffness) coming between tether force values and membrane tension estimates, Dai et al. (1997) conclude that their findings support the hypothesis that membrane tension regulates plasma membrane SA via tension-altered rates of endocytosis and exocytosis. They caution, however, that along with direct SA/tension feedback effects, nontrivial second order effects arise. For instance, membrane skeleton changes accompanying secretion (e.g., Zhang et al., 1996; Valentijn, Gumkowski & Jamieson, 1999) inevitably alter the intensity of bilayer/membrane skeleton interactions that profoundly affect membrane tension.

Tension was monitored, too, (Raucher & Sheetz, 1999*b*) during mitosis. As Hela cells enter mitosis, membrane tension rises and endocytosis decreases and vice versa when they exit mitosis. This timing, plus evidence that amphyphilic compounds decrease tension and increase endocytosis, suggests that tension directly regulates endocytosis rates, possibly via the assembly of endocytic machinery.

Membrane tension is "continuous" over the plasma membrane, a point crucial to membrane tether work. In other words, pulling a tether perturbs the tension of all contiguous plasma membrane. For small cells like RBLs and fibroblasts, continuity is experimentally observable when any point of the accessible (nonadherent) surface is tested (Raucher & Sheetz, 1999*a*). Does this mean that

Table 1. Membrane tension and mechanical quantities

Force, tension, pressure units, seen in the context of membranes (and apples):

1 Newton is an enormous ***force,*** ~10^{11-12}-fold greater than needed to pull a membrane tether

1 N ~ = 100 g (. . . ~1 apple in Earth's gravity)

1 Newton/meter is a large ***tension*** that would easily rupture a membrane

1 N/m = 10^3 dyne/cm (. . . ~1 apple dangling from a meterwide banner)

1 Newton/square meter is an unmeasureably small ***pressure*** if applied via a micropipette

1 N/m^2 = 1 Pa = ~0.0001 mmHg (. . . ~1 apple per coffee table)

Membrane tensions*

0.12 mN/m—resting tension, plant protoplast membrane (*see* Kell & Glaser, 1993)

0.003 mN/m—resting tension growth cones of chick neurons (Hochmuth et al., 1996)

0.04, 0.12, 0.02 mN/m—tensions in normal, swollen and reshrunken molluscan neurons (Dai et al., 1998)

~1 mN/m—activation of mechanosensitive channels (*see* Sachs & Morris, 1998)

3–4 1 mN/m—lytic tension of large lecithin bilayer vesicles (Kwok & Evans, 1981)

4 mN/m—lytic tension for plant protoplast (*see* Kell & Glaser, 1993)

5–8 mN/m—lytic tension for mast cells inflated under whole cell clamp (Solsona et al., 1998)

12 mN/m—lytic tension skeletal muscle membrane (Nichol & Hutter, 1996)

*NB—"in-plane" tension (not interfacial surface tension, though the units are the same).

The spring constant (elasticity or stiffness) of Hooke's Law, like tension, has the units of N/m.

Forces* (for references, *see* Dai et al., 1998; Sachs & Morris, 1998)

3 pN . . . force generated by myosin molecule

7 pN . . . force to pull membrane tether from a neuron

10–20 pN . . . calculated force for activation of a "typical" MS channel

20 pN . . . actin-gelsolin bond

50 pN . . . force to pull erythrocyte membrane tethers

30,000 pN . . . carbon-carbon bond

**Sometimes, forces are loosely referred to as "tensions" (the magnitude of a force exerted, say, via a string) but a "tension" that counteracts a force is a force.

Pressure Conversions (*see also*, Table 1 in Sachs & Morris, 1998)

1 N/m^2 = 1 Pa, 1 kPa = 7.5 mmHg, 100 mmHg = 13.3 kPa = 133 mbar, 1 bar = 100 kPa

1 mmHg = 1.36 cm H_2O, 760 mmHg = 1 atmosphere = 101.3 kPa

1 mosmol = 18 mmHg

Laplace's Law: governs the tension of a ideal thin-walled sphere:

Tension = Pressure × radius of curvature × ½

Thus, the more curved a membrane, the less tension experienced for a given transmembrane pressure. (e.g., in an osmotically swelling cell, microvillar bilayer feels less tension than adjacent flat membrane)

"local tension" (*see* the tension hypothesis) is a nonadmissible term? Does continuity imply, say, that deforming a neuron terminal in a human toe alters bilayer tension a meter away near the spine? In practice, no (though it is a thought to give comfort to reflexologists!). The plasma membrane is not an idealized (Hookean) spring and a local step tension perturbation is not felt instantaneously over the entire plasma membrane. A tension perturbation propagates at a finite speed, creating a spreading front. With distance, cumulative effects of thermal noise would swamp out evidence of a transient perturbation. In effect, tension continuity exhibits a length constant. Moreover, if plasma membrane operates a tension-SA feedback system, compensatory SA changes will cancel the initial perturbation, even for a step change rather than a tension transient. *Provided that machinery for tension-SA feedback is homogeneously available, a local tension perturbation will yield local compensatory SA adjustments.* This is akin to injecting inward current into a tetrodotoxin-poisoned axon: current flows to ground across the entire axonal membrane, but current density is greatest at the injection point and will elicit there the biggest negative feedback (outward current through homogeneously available depolarization-activated channels) to counter the original perturbation. Likewise, continuity of membrane tension is consistent with the idea of *locally* acting tension. Indeed, locally acting SA/tension feedback requires it.

SAR and Bilayer Properties

SAR cannot just piggyback on volume regulation because a cell surface is not the outer limit of a volume but a topologically and biophysically distinct entity. By virtue of the plasma membrane's lipid bilayer the surface is governed by discrete rules. This bilayer is the cell's defining osmotic barrier, across which swelling and shrinking forces are exerted and the selective permeability barrier across which electrochemical gradients develop. Donnan effects associated with these gradients yield hydrostatic pressure. This, along with local bilayer deformation and curvature effects, is a principal determinant of bilayer tension.

Its thinness makes the bilayer an effective capacitor. Determinations from inflated spherical cells give 0.5 $\mu F/cm^2$ as the specific capacitance for plasma membrane (Solsona, Innocenti & Fernandez, 1998), half the "textbook" value of 1 $\mu F/cm^2$ and close to 0.7 $\mu F/cm^2$ obtained for membrane patches imaged at high resolution (Sokabe, Sachs & Jing, 1991). A cell's capacitance scales linearly with its plasma membrane area, and so SA determines both the rate at which transmembrane voltage can change and the absolute magnitude of ionic flux needed for any given voltage change. Consequently: (i) cell SA affects the speed of electrical signaling and (ii) cell SA affects metabolic load, since the quantity of "pump" energy needed to recharge cellular batteries after capacitative discharge depends on the absolute number of ions pumped. Thus, a large SA/volume ratio aids facilitated transport (e.g., gut epithelia) but imperils high frequency electrical signaling in small caliber fibers (hence dendrites are short and axons smooth, with capacitance further reduced by myelination).

The high lateral diffusivity of bilayer molecules and the ease of bulk lipid flow in the plane of the membrane define the lipid bilayer as a two-dimensional fluid; this fluidity figures in SAR as the means by which in-plane tension re-equilibrates following a local perturbation (e.g., Monck et al., 1991; Dai & Sheetz, 1995*a,b;* Hochmuth et al., 1996). Equilibrating flows occur as tethers are pulled from the plasma membrane (hence the observation that membrane tension is continuous) or when bilayer flows out over a cytoskeletal protrusion (e.g., an F-actin bundle). Freely diffusible bilayer components are aptly regarded as "two-dimensional solvent" molecules in which any fixed or adherent components are "solutes" (e.g., tethered integral membrane proteins, anchored lipids, adherent membrane skeleton, adherent extracellular matrix). The solutes generate a so-called membrane osmotic pressure (Dai & Sheetz, 1995*b*). In cytoplasm (3-D), aqueous osmolytes determine water distribution. The 2-D bilayer's solutes, likewise, determine the equilibrium distribution of solvent—i.e., diffusible bilayer molecules (Discher, Mohandas & Evans, 1994). Accordingly, to pull a bilayer tether from the plasma membrane, one component of the pulling force is that used in overcoming 2-D osmotic pressure. When membrane skeleton disassembly eliminates "osmotically active" bilayer molecules from the 2-D system, the pulling force should decrease. Indeed, cytochalasin, which promotes F-actin depolymerzation, has this effect (Dai & Sheetz, 1995*b*). Unnatural everted tethers like those pulled by tweezers, lack membrane skeleton, as do membrane blebs (Dai & Sheetz, 1999). But living cells regularly make "inverted tethers": surface invaginations like pinosomes or retrieval vacuoles (Dailey & Bridgman, 1993). For these stable invaginations (i.e., stable compared to the ephemeral spontaneous invaginations of large liposomes (Lipowsky, 1991)), bilayer/membrane skeleton interactions (Fujimoto & Ogawa, 1989; Herring et al., 2000) are almost certainly mandatory. Inverted tethers would form whenever membrane skeleton was drawn towards the cell interior (either actively by motor proteins or passively by retracting cytoplasmic structures), taking with it osmotically obliged bilayer constituents.

Plasma membrane bilayers have "rafts" of sphingolipid and cholesterol-rich domains that exist as phase-separated liquid-ordered regions (Brown & London, 1998). Since the mechanical properties of such deter-

gent-insoluble rafts must differ from those of the more purely liquid bilayer regions, they may prove important for tension-sensitive SAR. Tension continuity effects, for instance, would be quantitatively different for lipids in rafts *vs.* more fluid regions of bilayer. Levitan et al. (2000) report variations in the activation of volume-sensitive anion channels with membrane cholesterol suggesting that cholesterol increases the deformation energy for the channel (*c.f.* Okada, 2000).

Fluids, by definition, have neither elasticity nor rigidity. Since a lipid bilayer has a degree of both, it is in some respects a solid (*see* Lipowsky, 1991, 1995; Dai & Sheetz, 1995*b;* Hochmuth et al., 1996). Artificial bilayers and biological membranes stretch elastically until their area increases by ~2–3% then, at ~1–12 mN/m tension (their elastic limit) they rupture (*see* Table, which provides some context for mechanical units). Additionally, a bilayer is a solid in that, in a manner dependent on its molecular constituents, it resists bending and takes preferred shapes (its spontaneous curvature). Finally, a bilayer's two leaflets can shear past each other; shearing of domains is a property of solids. All the solid properties of the bilayer come into play during SAR, though the elastic limit (or tensile strength) is perhaps the most dramatic.

For fibroblasts, some maximum length of bilayer tether can be pulled from the cell surface using a fixed force (Raucher & Sheetz, 1999*a*); this, it is argued, identifies the magnitude of a small bilayer reservoir, amounting to 0.3–1% of the total surface area. This reservoir could buffer minor increases in membrane tension and until depleted, obviate a need for tension-sensitive SAR. The nature of the reservoir is ill-defined, but interactions between membrane-associated proteins and bilayer could foster micro-undulations that flattened as tension increased. How this might operate for cells whose membrane (unlike the "dorsal" surface of cultured fibroblasts) is predominantly adherent, is open to question. The buffer effect relies on continuity of tension, and how continuity operates across, say, a tight junctional zone or into cell-cell contacts such as neuromuscular junctions, is unknown.

Size Matters, Curvature Matters

The bilayer's limited tensile strength dictates a need for SAR and, where bilayer is not highly curved, a need for mechanoprotection. To illustrate, consider two spherical liposomes, 2 and 20 μm diameter, in a salty pond. Rain dilutes the pond by ~1 mosmol. If they are perfect osmometers, the liposomes experience 2.4 kPa of hydrostatic pressure (24×10^2 N/m^2 = 1 mosmol = 18 mmHg; *see* Table for conversions). For liposomes that leak solutes, less pressure is felt: arbitrarily, say 0.13 kPa (= 1 mmHg). Laplace's Law ($T = Pr/2$; *see* Table) predicts the leaky 2 and 20 μm liposomes would have in-plane membrane tensions of ~0.1 mN/m (= 0.1 dyn/cm) and ~1 mN/m respectively. 1 mN/m is perilously close to lytic for biological bilayers (Table); these liposomes might "survive" this seemingly inconsequential (1 mosmol) osmotic perturbation but only because leak dissipated most of the potential energy.

Protection of the bilayer would have been provident from the earliest stages of cellular evolution. Procaryote (and plant cell) plasma membranes rely on heavily crosslinked load-bearing walls. Large hydrostatic pressures (turgor) transfer perpendicularly through the essentially noncompressible bilayer, generating tension not in the bilayer itself, but in the closely apposed wall. In animal cells, the primary strategy for keeping plasma membrane tension sublytic (by ~2 orders of magnitude e.g., Hochmuth et al., 1996; Dai et al., 1998) is to pump and leak osmolytes as appropriate. Also, the ubiquitous spectrin-actin-myosin membrane skeleton (= cortical cytoskeleton), typically ~0.05–0.1 μm thick (Zhelev et al., 1994) modulates bilayer stress, but "how" needs work.

Membrane Skeleton Strength

Though membrane skeleton can be extracted essentially intact from some cells (e.g., erythrocytes, Ursitti & Wade, 1993; sea urchin zygotes, Walker, Kane & Burgess, 1994) this has not produced data telling us how responsibility for plasma membrane strength is apportioned between bilayer and membrane skeleton. Further *in situ* studies on the mechanics of skeleton proteins using mutant and recombinant forms (e.g., Mencke & Jockusch, 1991; Glogauer et al., 1998; Dai et al., 1999; Weber, Niewohner & Faix, 1999) should illuminate the role of the extensible, dynamic, contractile mesh of membrane skeleton proteins in membrane strength (Hitt & Luna, 1994; Discher et al., 1994).

Lytic tensions reported for artificial bilayers and for plasma membranes overlap (*see* Table). Unfortunately, conditions used in determining plasma membrane tensile strength damage the membrane skeleton. For skeletal muscle membrane, for example, Nichol & Hutter (1996) used large blebs shed into a high KCl bath. They concluded that any membrane skeleton remaining did not augment tensile strength (lytic tension was 12 mN/m) beyond what could be imputed to the bilayer. This is less than compelling grounds for discarding the idea that membrane skeleton is mechanoprotective for the bilayer. Mechanoprotection is evident when, in renal cells, progressive membrane skeleton disassembly makes aspiration of blebs progressively easier (Doctor, Zhelev & Mandel, 1997). Terakawa & Nakayama (1985) showed that, exposed directly to KCl (but not KF), squid giant axon membrane skeleton "dissolves", as seen by scan-

ning EM. Perfused with KCl (but not KF), axons show reversible stretch-induced impairment of ion channel function. Evidently, intact membrane skeleton protects bilayer components (including integral membrane proteins like channels) from tension. Patch-clamp recordings also point to membrane skeleton integrity as the major determinant of whether, during membrane stretch, mechanosusceptible channels will feel tension via the bilayer (Hamill & McBride, 1997; Wan, Juranka & Morris, 1999; Tabarean, Juranka & Morris, 1999; *see also* Glogauer et al., 1998).

How much passive load-bearing the membrane skeleton does remains unanswered. The issue is complex since active "off-loading" of tension from the bilayer by active membrane skeleton components (cortical actomyosin (Dai et al., 1999)) may contribute. If this creates a bilayer reservoir (Raucher & Sheetz, 1999*a*) this "active" buffer could also enhance *passive* strength via "corrugation" (decreasing the bilayer's average radius of curvature). Microvilli are the epitome of passive bilayer strengthening. Consider, for example, the microvilli-rich exteriors of molluscs, where provision of transport SA is surely of minor importance. Instead, in this mechanically challenging environment, the maximized tensile strength of the highly curved bilayer would seem critical.

"Membrane strength" *in situ* depends, thus, on passive and active factors and there is no standard approach for quantifying this strength. Global (bioassay) measures such as osmotic fragility continue to be used. Dystrophic muscle cells, with their compromised membrane skeletons, are more osmotically fragile than controls (Menke & Jockusch, 1991). With extreme hyposmia in neurons, rupture occurs ~4-fold sooner with cortical contractility blocked (Wan, Harris & Morris, 1995). With hyperosmia in *Dictyostelium* (Kuwayama et al., 1996; Zischka et al., 1999), surface contractility in shrinking cells generates a spheroid shell rigid enough to ensure retention of cytoplasmic water. An exciting but apparently unexplored possibility is that membrane strength changes could be altered by hydration-sensitive protein conformation changes (Zimmerberg, Bezanilla & Parsegian, 1990; Colombo, Rau & Parsegian, 1992; Grazi et al., 1995) that cause membrane skeleton cross-linkages (and hence passive and active membrane strength) to vary adaptively when subsurface water activity fluctuates.

Walled eukaryotes—fungi and plants—also possess membrane skeletons with spectrinlike proteins (Bisikirska & Sikorski, 1997), spectrin's anchor protein, ankyrin (Davis & Bennett, 1990), and F-actin (Mulholland et al., 1994; Braun et al., 1999) which contributes to membrane trafficking, but their involvement in membrane strength here is even more speculative than in animal cells.

If "membrane strength" is taken as "tensile strength", then membrane skeleton contributions to bilayer mechanoprotection *in situ* are unresolved. If concerted passive + active contributions are included, then membrane skeleton is unquestionably mechanoprotective, though it might be more appropriate to refer to "membrane resilience" (the generalized ability to withstand mechano/osmotic insults). High precision cytomechanical investigations such as are possible for erythrocytes (e.g., Discher et al., 1995) elude more complex cells (e.g., Mencke & Jockusch, 1991; Hutter, 1992; Glogauer et al., 1998) but it is abundantly clear that bilayer/membrane skeleton interactions (Raucher et al., 2000) yield a resilience impossible for a bilayer alone. Returning to SAR, we take as a given that membrane skeleton is mechanoprotective in living cells.

The Membrane Skeleton During SAR: SA Expansion

If membrane skeleton fortifies the plasma membrane and tension is a sensor/effector for SA expansion, then the dynamics of membrane skeleton are critical to SAR. With membrane skeleton intervening physically between the surface bilayer and endomembrane, how can fusion occur? Another conundrum: when plasma membrane is under unremitting tension (e.g., during osmotic inflation, growth, imposed stretch) how is the danger of rupture minimized while new membrane is incorporated? If the submembranous actin web disaggregates (e.g., Czekay, Kinne-Saffran & Kinne, 1993), this seems particularly important. Fig. 1 depicts various options for SA expansion. A naked mechanically vulnerable zone of bilayer could be formed as in *A*1 or *B*2. It could be formed then fortified with new membrane skeleton as in *A*3, *B*3 or, prefortified endomembrane could be added in a single step as in *C* or *D*. Substantial evidence exists for *A*1 and *B*1, albeit for contexts where the plasma membrane is not considered to be at elevated tension. For some of the other steps and options, evidence is only circumstantial.

*A*1 is like presynaptic zone exocytosis (e.g., Bernstein, DeWit & Bamburg, 1998) and like cortical granule fusion in sea urchin eggs (Chestkov et al., 1998) (though not embryos). Such fusion of naked vesicles to naked planar bilayer may be safe where surrounding structures predictably offer mechanical support. In one class of fusion events, fortifying new SA is not an issue because vesicle bilayer is retained around a solid lumenal matrix and retrieved after the diffusible contents are discharged (Angelson et al., 1999). If, however, the plasma membrane acquires new bilayer while under tension (steps *A*2, *B*2), new membrane skeleton will be required (step 3). The steps for remodeling a continuous membrane skeleton mesh are not known but probably involve adding new filaments and exploiting the extensibility (Ursitti & Wade, 1993) of the existing mesh. The mesh could

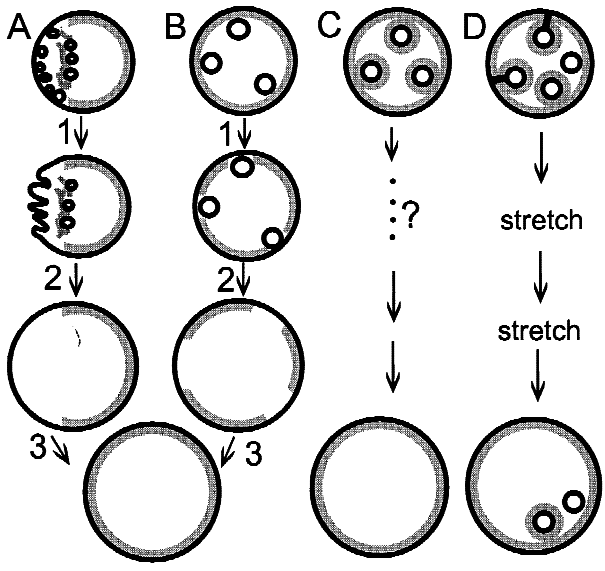

Fig. 1. Adding endomembrane to the surface in the face of a swelling or stretching force. (A) Membrane skeleton-free endomembrane fuses to a surface zone that lacks membrane skeleton. (B) Naked endomembrane adds at membrane skeleton-lined surface; the intervening membrane skeleton must disassemble to allow fusion. Naked surface bilayer is then refurbished with membrane skeleton. (C) Membrane skeleton-linked endomembrane fuses with membrane skeleton-lined plasma membrane—this seems topologically untenable. (D) Membrane skeleton-lined bilayer invaginations are in contact (via a high series resistance) with membrane skeleton-lined plasma membrane. The invaginations open out under tension and surface area increases without disrupting membrane skeleton integrity. Endomembrane which does not contact the surface bilayer (two examples depicted) not participate. In contrast, during steps *A*1 or *B*1, stretch might hasten fusion, but the surface membrane would be unprotected till after steps *A*3 or *B*3.

relax passively over "bald spots" or be actively drawn and knitted together by a surface motor. Cibert et al. (1999) postulate that myosin II may knit rips in the erythrocyte membrane skeleton. Because SA expansion is an obligate component of neurite outgrowth and because neurites are under tension, growth cones may be ideal for examining these issues. Calpain, a protease for spectrin and other membrane cytoskeleton elements, must be active for successful growth cone extension (Gitler & Spira, 1998), consistent with the view that membrane skeleton remodeling precedes incorporation of new membrane, as in step *A*3 (or step *B*2).

Option *B* steps 1,2 is like chromaffin cell exocytosis. There, cortical F-actin represents a negative control on secretion and is locally disassembled under the enzymatic control of a gelsolin enzyme, thereby allowing exocytosis to proceed (Zhang et al., 1996). Mast cell degranulation (Norman et al., 1996) too requires disassembly of the F-actin barrier, though it is not the rate-limiting step. In *Dictyostelium* (Rauchenberger et al., 1997) excretion occurs by exocytosis of postlysosomal endomembrane vacuoles. These are F-actin coated along the entire postlysosomal pathway except just prior to exocytosis when both the vacuolar coat and the cortical cytoskeleton locally dissolve, allowing for fusion.

Option *C* is a straw man, to contrast against option *D*. While no experimental evidence establishes "*D* and not *C*", it seems impossible that prefortified endomembrane could fuse to the membrane skeleton-lined plasma membrane without a bilayer–bilayer contact structure.

Option *D*, then, predicates a contact structure. Mechanical continuity between reserve and plasma membranes could be established via bilayer tethers supported by fusion pore type structures (Lindau & Almers, 1995), a bilayer groove, or merely an area(s) where cell stretch could induce direct bilayer–bilayer apposition at gaps in the membrane skeleton. The contact would electrically isolate the reserve membrane from the surface.

Wherever cell stretch routinely presages a need for SA expansion, option *D* would be particularly appropriate. Preparations worth revisiting include bladder epithelium, where stretch reversibly recruits membrane (Lewis & de Moura, 1982, 1984), alveolar cells, where a single stretch markedly stimulates exocytosis (Wirtz & Dobbs, 1990), and neurons, where a neurite's outgrowth rate depends on the magnitude of traction forces exerted by the growth cone (Lamoureux, Buxbaum & Heidmann, 1998). Since growth cone advance requires no local influx of calcium (Campenot & Draker, 1989), tension produced by the growth cone's traction might trigger SA expansion, not calcium-driven exocytosis. Active growth cones *in situ* (chick optic tectum, fast-frozen to capture endomembrane structure for electron microscopic reconstructions (Cheng & Reese, 1987)) show stacks of flattened vacuoles separated by a filamentous network (plausibly, spectrin). The outermost of these lined (prefortified?) vacuoles is juxtaposed to the plasma membrane and, on fusing, leaves the next filament-lined element poised for incorporation. Other observations too, suggest that SA expands directly under tension. In molluscan neurons, capacitance increases during swelling are ascribed to mechanically accessible endomembrane (Wan et al., 1995), possibly the subsurface cisternae evident in High Voltage EM and putatively described as hot spots of mechanically accessible membrane (Fejtl et al., 1995). At mammalian growth cones (Dailey & Bridgman, 1993), HVEM images capture fleeting retrieval and fusion events involving vacuolar membrane; the vacuoles are a major component of growth cone traffic, as shown by analysis of high resolution DIC movies taken prior to fast-fixing for HVEM. In sensory cells (Baumann, 1998), immuno-EM reveals submembranous cisternae to be spectrin-lined. Direct evidence that spectrin-fortified vacuolar membrane moves to and from the plasma membrane comes from hyperstimulated secretory cells (Fujimoto & Ogawa, 1989), from vacuolar myopathies (De bleeker, Engel &

Winkelmann, 1993) and from the invagination of surface membrane in shrinking muscle (Herring et al., 2000).

Surface-coating proteins, clathrin and caveolin, mediate traffic of submicron endocytic vesicles. Though not covered in Fig. 1, these proteins are like a dynamic, short-range, short-term membrane skeleton. Their assembly and disassembly rates and other aspects of their interactions with trafficking molecules, one would anticipate to be sensitive to bilayer tension. This could mediate mechanosensitive endocytosis and exocytosis, as in stretched epithelia (Lewis & de Moura, 1982; Fink & Cooper, 1996), axons (Dai & Sheetz, 1995*a*) and circulatory cells (Dai et al., 1997).

SAR: Evidence from Plant Cells

Turgor pressure in the range of 1 MPa (~10 atmospheres) prevents plant cell plasma membrane from maintaining infoldings that could provide excess SA. Since plant cell protoplasts are fully distended in their "resting" state, their need for SAR is obvious. Indeed, they provided the first evidence that SA and membrane tension are components in a regulatory feedback loop. We therefore cover the state of the art for plants, then resume with animal cells.

Adjustment of Plant Protoplast SA in Response to Osmotic Imbalances

Early interest in plant cell volume and SA changes developed from attempts to understand freezing injury. Protoplasts cooled at 0 to −10°C shrink in response to osmotic potentials created by extracellular ice formation. When subsequent warming reverses the process, swelling can rupture the plasma membrane. Protoplasts of cold-acclimated plants swell without rupture to about twice the volume of those from nonacclimated plants (Wolfe, Dowgert & Steponkus, 1985). Plants also generate osmotic potentials, using the attendant cell volume changes to do work like moving leaves and regulating gas exchange. The latter involves control of the stomatal pore aperture by specialized epidermal guard cells. Accumulation of potassium salts and subsequent water influx swells the guard cells, opening the stomatal pore. The reverse process closes the pore. Going from open to closed, guard cell volume can differ by a factor of two (Raschke, 1979).

To address mechanisms of plasma membrane SA change, Wolfe & Steponkus (1983) studied mechanical properties of rye leaf protoplast plasma membrane via micropipette aspiration. They reported a resting tension of ~0.1 mN/m and an area elastic modulus of 230 mN/m. Given that the membrane lyses at 4–6 mN/m, they estimated intrinsic elastic expansion to be limited to 2–3%. This led to the following tension/SA hypothesis (Wolfe et al., 1985): Hyposmia causes water influx and elevated membrane tension, resulting in incorporation of membrane material into the plasma membrane. Under hyperosmia, water efflux causes decreased tension and removal of membrane material from the plasma membrane.

Insights into tension/SA changes have recently been gained by high resolution whole cell patch-clamp capacitance measurements of guard cell protoplasts (Homann, 1998). Osmotically driven swelling and shrinking is indeed associated with incorporation and removal, respectively, of membrane material, with dSA/dt dependent on osmotic potential. This supports the hypothesis that membrane trafficking is modulated by membrane tension. Hydrostatic pressure also led to changes in SA in these protoplasts (*personal observation,* U. Homann) as well as in barley aleurone protoplasts (Zorec & Tester, 1993); in both cases tension-modulated exo- end endocytosis was calcium-independent.

The large tension-driven changes in SA raise the question of the origin of new membrane during swelling and its fate during shrinkage and net loss. Fluid phase uptake experiments on onion epidermal cell (Oparka, Prior & Harris, 1990), protoplasts from (non-cold-acclimated) rye leaves (Gordon-Kamm & Steponkus, 1984*a*), *Chenopodium album* suspension culture cells (Wartenberg et al., 1992) and *Vicia fabia* guard cells (Diekmann et al., 1993) reveal that shrinkage results in formation of large endocytotic vesicles (or "vacuoles") of ~0.3–1.5 μm. Except for the *Chenopodium* protoplasts, however, this vacuolar membrane is not readily reincorporated into expanding plasma membrane upon reswelling, but remains in the cytoplasm. Thus, retrieval of membrane material during large hyperosmotic stimuli is not demonstrably reversible in most preparations.

After osmotic shrinkage, electronmicrographs show tethered spherical extrusions or surface polyps on cold-acclimated but not control protoplasts (Gordon-Kamm & Steponkus, 1984*b*). These are reversible on return to isotonic conditions. Thus, in cold-acclimated protoplasts, no irreversible loss of membrane material occurs. The differential behavior of acclimated *vs.* nonacclimated protoplasts during shrinkage may be consequent on plasma membrane lipid alterations that protect against freeze-thaw rupture by providing an SA reserve to extrude during shrinkage thereby preventing lysis on reswelling (Lynch & Steponkus, 1987; Steponkus et al., 1988). Tethered extrusions are also observed in shrinking guard cell protoplasts (Lambrechts, Schroeder & Verbelen, 1992). The relationship, if any, between shrinkage-induced extrusions and invaginations (Wartenberg et al., 1992) is unknown; neither has been examined in the presence of the cell wall.

In guard cell protoplasts, the importance of exo- and

endocytosis of small vesicles during osmotically driven SA changes is confirmed by recent confocal imaging of the fluorescent membrane marker FM1-43 and by patch-clamp capacitance measurements. During shrinkage, FM1-43 labeled membrane (Kubitscheck, Homann & Thiel, 2000) was retrieved from the plasma membrane and internalized. Protoplast shrinkage was occasionally associated with formation of large (~3 μm) FM1-43 labeled endocytotic vacuoles. But since SA decrease was always correlated with diffuse fluorescent labeling of the cytosol it was concluded that endocytosis of small vesicles is an obligatory process by which guard cells accommodate an osmotically driven decrease in SA. This is supported by patch-clamp measurements (Homann & Thiel, 1999) where the fusion and fission of single vesicles with the plasma membrane could be resolved. Under hypo- and hyperosmotic conditions exo- and endocytosis, respectively, of vesicles with a median diameter of ~300 nm could be detected.

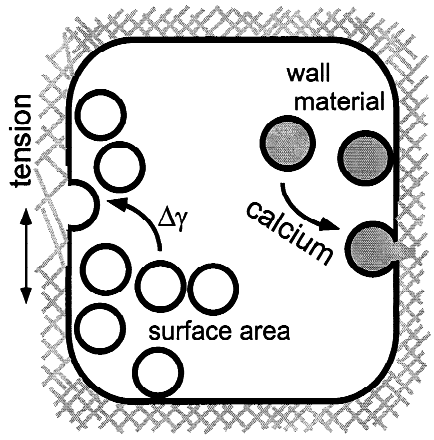

Fig. 2. Plant cell exocytosis: schematic diagram of the two independent pathways. One exocytotic pathway is calcium-dependent and recruits membrane from a small pool. The other calcium-independent pathway is stimulated by increased membrane tension ($\Delta\gamma$) and draws membrane from a larger pool. In growing plant cells calcium-stimulated exocytosis may be involved in secretion of cell wall material whereas tension driven exocytosis may account for incorporation of new membrane material.

SA Changes During Plant Cell Growth

As they mature, plant cells may enlarge 10–1,000-fold in volume. To do so, they loosen their wall (wall stress relaxation), take up water and expand (for review *see* Cosgrove, 1997). The large volume increase requires a corresponding SA increase and thus delivery of membrane material to the plasma membrane. In addition, new cell wall material has to be synthesized and secreted. How plant cells coordinate cell growth, wall synthesis and secretion and incorporation of new membrane material into the plasma membrane is poorly understood. Several studies implicate calcium in controlling polarized growth, where elongation is restricted to specific zones. Tip growth in pollen tubes (for review *see* Hepler, 1997), root hairs (Felle & Helper, 1997; Bibikova, Zhigilei & Gilroy, 1997) and rhizoids from *Fucus* (Brownlee & Wood, 1986) is coupled to calcium influx and a steep tip-focused calcium gradient that may direct secretory vesicles (Brownlee et al., 1999) to the site of cell elongation, where calcium then promotes fusion. An apical acid domain (Feijo et al., 1999) may also facilitate vesicle movement and exocytosis.

Calcium-controlled secretion is not, however, the whole story. Comparisons of vesicle production *vs.* plasma membrane turnover in growing pollen tubes (Picton & Steer, 1983) and elongating coleoptile cells (Phillips, Preshaw & Steer, 1988) reveal a mismatch between the vesicle membrane delivered to the cell surface and that required for cell elongation. Most (≥65%) of the delivered membrane must recycle to the cytoplasm, since fusion and retention of all the secretory vesicles would exceed the membrane material requirements for SA increase. Work on maize coleoptile cells provides evidence for two independent exocytotic pathways (Thiel, Sutter & Homann, 2000). One is stimulated by calcium and recruits membrane from a small pool. The other is calcium independent and osmotically driven and draws membrane from a much larger reservoir. Calcium stimulated exocytosis is proposed to be a key element in hormone stimulated cell growth (Thiel, Rupnik & Zorec, 1994), but the osmotically evoked increase in SA (Thiel et al., 2000) suggests that tension-driven exocytosis also plays a role in cell elongation. During cell expansion, wall stress relaxation and subsequent water influx may create plasma membrane tensile stress sufficient to stimulate incorporation of new membrane material. We speculate (Fig. 2) that delivery of compounds for cell wall synthesis occurs via a calcium-dependent pathway, whereas insertion of new membrane material involves tension-driven exocytosis. Two exocytotic pathways may be necessary because vesicles delivering cell wall material may be inappropriate as plasma membrane material. In coleoptile cells, transient fusion of vesicles with the plasma membrane has been reported (Weise et al., 2000); during transient fusion the vesicular content may be released while the vesicular matrix is retained for re-use in a subsequent cycle (Albillos et al., 1997), providing an economic mechanism for secretion of wall compounds, while tension-driven exocytosis accommodated SA increase.

SAR: Evidence from Cultured Neurons

We now return to animal cell SAR, using findings of one of the authors (CM), mostly on *Lymnaea* neurons, as a

focus for discussions. Molluscan neurons are large, readily cultured. Some re-arborize extensively, others remain spherical (Sigurdson & Morris, 1989) and each configuration is needed for certain experiments. Our original interest was in their stretch channels (Morris & Sigurdson, 1989) but two startling observations drew our attention to SAR in these neurons. Trying to maximally stimulate the stretch channels, we used distilled water (~2 mosmol) and astonishingly, most neurons were unruptured at 1 hr (Wan et al., 1995). That was observation 1. Observation 2 was that on return to normal medium the hugely bloated neurons immediately vacuolated (Reuzeau et al., 1995). "Everybody knows" cells rupture instantly in distilled water and everybody knows shrinking cells should not have rapidly expanding vacuoles. These pieces of conventional wisdom, however, are "erythrocytocentric" and need revamping. Epithelial and skin cells routinely handle extreme swell/shrink challenges: drinking a tall glass of water, for instance, is not dangerous, nor is eating a spoonful of sugar. Additionally, neurons' reputation as osmotically and mechanically fragile is unwarranted. Vertebrate neurons, for example, arborize vigorously in culture medium diluted to 50% normal (Bray et al., 1991), and, far from avoiding tension, both peripheral and CNS neurons absolutely require tension for outgrowth (Lamoureux et al., 1989; Chada et al., 1997).

What makes neuronal membrane so robust and what explains the dramatic vacuolation of shrinking neurons? Work summarized below shows that neurons are rupture-resistant not because their plasma membrane is inordinately tough but because it responds to changing mechanical stress with changes of area. Shrinkage-induced vacuolation is one consequence of this tension sensitivity. During neural development, an ability to adjust plasma membrane area according to prevailing tension is probably indispensible (Fig. 3) and in adults it would allow for SA adjustments during physiological and pathological volume and shape changes. Tension-sensitive SAR would be deployed when "membrane tone"—dependent on subsurface actomyosin (e.g., Wan et al., 1995; Dai et al., 1999; Raucher & Sheetz, 1999*a*)—failed to keep bilayer stress at a safe level. The emerging picture of mechanosensitive SAR in neurons makes no reference to the abundant mechanosusceptible potassium channels of neurons (molluscan and mammalian; Vandorpe et al., 1994; Patel et al., 1998) which seem not to be physiological mechanotransducers (Morris & Horn, 1991; Wan et al., 1995, 1999).

Extreme Swelling

Live cell confocal microscopy of isolated cultured neurons using bath and bilayer dyes reveals no evidence of deep infoldings amplifying the SA (Mills & Morris, 1998). Unarborized neurons can swell ~5-fold without rupture (Wan et al., 1995) but this may exceed the *in situ* capacity, since during isolation, endomembrane reserves may have been augmented by retraction of neurites. Assuming a smooth plasma membrane surface, 5-fold swelling requires SA to increase to 300% its original value. Elastic bilayer expansion could account for only 2–3% and a "tension buffer reservoir" (Raucher & Sheetz, 1999*a*) for at most 1%. The much-inflated neurons are fully viable since, returned to normal saline, they shrink, retrieve SA, and over the next day, can re-arborize.

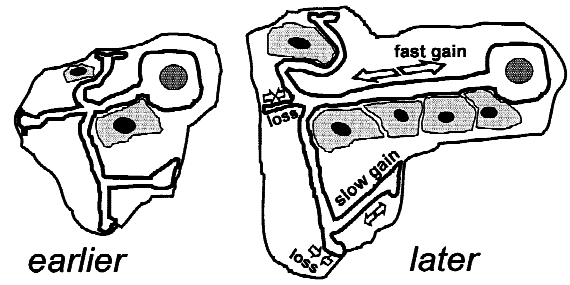

Fig. 3. Membrane tension and morphogenesis in the CNS. Any widely ramifying neuron, whether in an ancient sponge or a modern vertebrate (likewise a branching phloem cell in a plant), would benefit from tension-sensitive SAR. During morphogenesis of the CNS, neurites embedded in an enlarging tissue mass will stretch and need to gain SA rapidly, while simultaneously, those in diminishing masses will go slack and need to retrieve SA (or they may retract after failing to adhere to any target). No global directive to the whole cell could organize SAR, but tension-sensitive SAR would automatically organize plasma membrane addition and retrieval at rates commensurate with the local tension. The simplicity of tension-sensitive SAR may be crucial in allowing morphogenetic forces to optimize CNS wiring (Van Essen, 1997).

SA Changes During Volume Changes

Voltage-clamped and made to swell then shrink over tens of minutes as their diameter and capacitance are monitored, unarborized neurons do not maintain a fixed SA. Rather, they add and subsequently retrieve plasma membrane, exhibiting apparent (based on spherical geometry) SA increases then decreases of ~0.7 $\mu F/cm^2$ (Wan et al., 1995) (*see* Fig. 4*a*). In these experiments extracellular ions were held constant (extracellular sucrose was varied) and cytoplasmic integrity was preserved via perforated patch clamp. The SA changes are consistent with the tension theory of SAR postulated at the outset, though the nature of SA sources and sinks is not revealed.

Swell/Shrink-Induced SA Changes Are Not Calcium Dependent

These swell/shrink-induced SA changes occur whether 0.5 μM or 3.5 mM extracellular calcium is used (Wan et

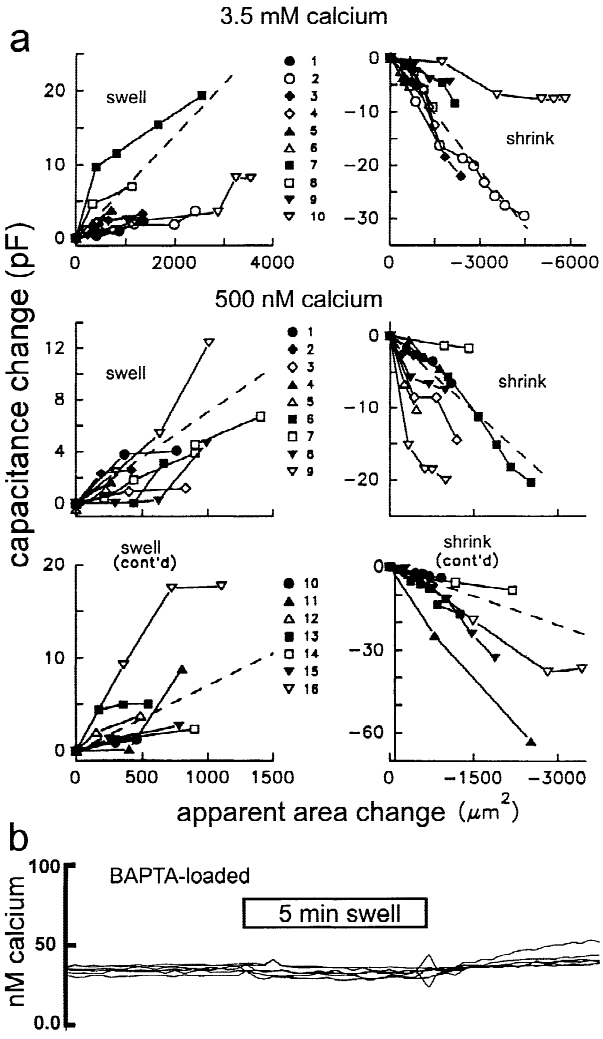

Fig. 4. Tension-sensitive SAR is calcium independent. (*a*) Capacitance changes as *Lymnaea* neurons first swell (~50% normal osmolarity) then shrink (180% normal osmolarity) in control and low extracellular calcium solutions. The dashed line in each graph corresponds to 0.7 μF/cm^2. Symbols identify the same neuron during swelling and shrinking (from Wan et al., 1995). (*b*) cytoplasmic calcium (fura-2 data) in BAPTA-loaded neurons is demonstrably invariant during and after swelling in 50% saline. Nevertheless, VLDs form in these neurons just after the swell stimulus (i.e., as the neurons reshrink in normal saline) (from Herring et al., 1998).

al., 1995) (Fig. 4*a*). Conventional exocytosis might underlie the SA increases if swelling released calcium from stores but Fura2 signals show that swell/shrink episodes negligibly alter intracellular calcium. Moreover, clamping cytoplasmic calcium below 50 nM with BAPTA (Fig. 4*b*) does not abolish swell/shrink-induced membrane reorganization (Herring et al., 1998). If calcium does not drive swelling-induced SA increases, physical effects involving high plasma membrane tension seem plausible. From the viewpoint of cellular energy economy, using osmomechanical potentials to mediate SAR seems more elegant than dissipating ion gradients (e.g., calcium) in an already-compromised cell. High tension could be both signal and potential energy source for incorporation of new membrane. Low plasma membrane tension should facilitate most known or conceivable SA retrieval mechanisms. Compared, say, to relying on stretch-activated calcium-permeant channels to trigger exocytosis, direct tension-induced addition of endomembrane should be fail-safe. Thus, both evidence and *a priori* reasoning converge on membrane tension as the sensor/effector for osmomechanically driven SA changes in neurons.

Vacuole-like Dilations in Shrinking Cells

Shrinking neurons retrieve their excess plasma membrane as invaginations of the substratum-adherent surface (Fig. 5). The initially tubular (~1 μm diameter) invaginations dilate, yielding, within minutes, a collection of ballooned-out structures (some branched) up to 10 μm across and penetrating ~5 μm into the cytoplasm (Reuzeau et al., 1995). Shrinkage in hyperosmotic medium elicits this response. However, with swelling-followed-by-reshrinkage (in which case shrinkage occurs in normal medium), substantially more bilayer is recruited to the invaginations. Evidently, swelling augments SA and, accordingly, more excess SA is invaginated upon reshrinking. The invaginations—vacuole-like dilations (VLDs)—have a lumen initially contiguous with the bath solution and VLD bilayer is contiguous with plasma membrane bilayer (Mills & Morris, 1998). Neurons show an upper limit to the number of VLDs that can form, and the longer a cell swells the closer it gets to its limit, as tested by the subsequent shrink-step (Reuzeau et al., 1995). Also, using more abrupt osmotic perturbations increases the effectiveness of milder (i.e., less hyposmotic, less prolonged) swelling stimuli (*cf.* Reuzeau et al., 1995 *vs.* Dai et al., 1998).

Not all cells types form VLDs. Mammalian neurons (*see* Fig. 6*a*), glia (C.E. Morris and L. Mills, *unpublished observation*) and myotubes do so, but undifferentiated myoblasts in the same cultures as the myoblasts do not (Herring et al., 2000). Likewise, undifferentiated neuroblastoma swell and reshrink without forming VLDs (C.E. Morris, *unpublished observation*).

Cell-Mediated Shrinkage also Elicits VLDs

Spontaneous vacuole-like structures occur (infrequently compared to shrinkage-induced VLDs) in molluscan neurons (Herring et al., 1999) but their trafficking dynamics are unknown. In sympathetic neurons, however, the dynamics of small spontaneous vacuoles (~0.15–1.5 μm) interacting directly with the surface have been analyzed and shown to be important in SA retrieval (Dailey

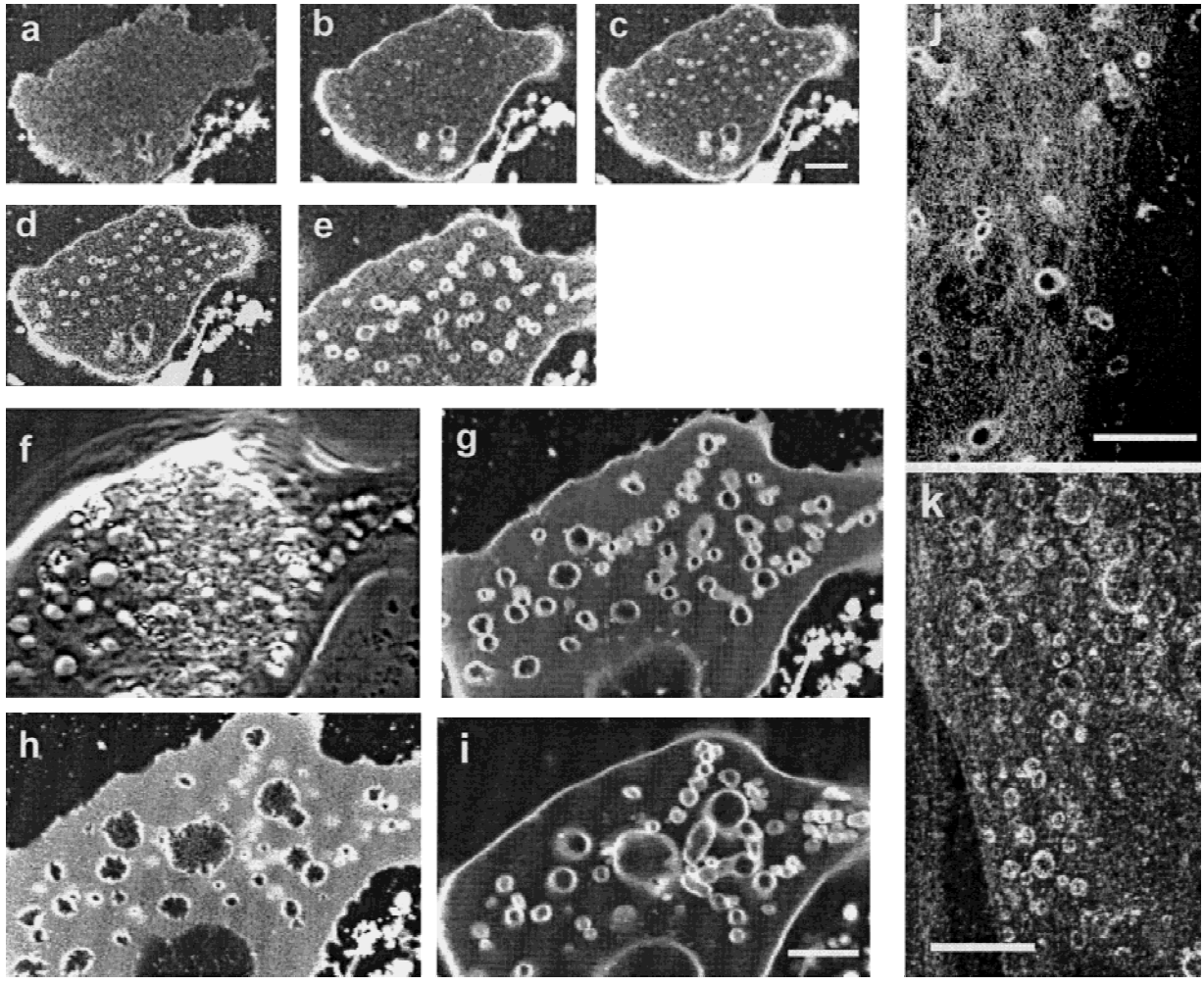

Fig. 5. VLD formation and early stages of recovery. (*a–d*) Confocal fluorescence images of a live DiI-stained *Lymnaea* neuron forming VLDs (*f*, simultaneous phase contrast image for *g*). In panel *a* the neuron is swollen in hyposmotic medium, in all others it is in normal saline. *h* and *i* are DiI images taken within moments of each other, but *h* is at the substratum (where it is evident that some VLD mouths are already closing or constricting) whereas *i* is 2.5 μm up in the dilated part of the VLDs. (from Mills & Morris, 1998) Scale bar, *a–d*, 1 μm (with e zoomed 1.5×) and bar for *f–i*, 10 μm. (*j*) A neuron, fixed within 1–2 min of initiating VLDs, stained for F-actin. Scale bar, 10 μm (from Herring et al., 1999). (*k*) A rat myotube, fixed with 3 min of initiating VLDs, immunostained for spectrin. Scale bar, 10 μm (from Herring et al., 2000).

& Bridgman, 1993). If shrinkage-induced VLDs are exaggerated versions of such retrieval vacuoles, then VLDs would be expected during cell-mediated shrinkage. Regulatory volume decrease (RVD) is a form of cell-mediated shrinkage whereby cells suddenly exposed to hyposmotic medium regain normal volume. Mammalian kidney cells exhibit a strong and rapid RVD response and indeed, exposed to hyposmotic medium, they form multiple extracellular-fluid-filled "vessicles", i.e., VLDs (Czekay et al., 1993) (Fig. 7). Over ~5 min, their well characterized RVD (the result of regulated sorbitol loss) is accompanied by the invagination of surface membrane, revealing what appears to be coordinated adjustments of volume and SA. Though *Lymnaea* neurons exhibit no RVD (as determined by cell body diameter) they swell more sluggishly than other *Lymnaea* cells, as if under some braking influence (Morris et al., 1989). During prolonged (15–60 min) hyposmia, they develop small dynamic VLDs (Reuzeau et al., 1995) when cell-mediated shrinking/retraction of lamellipodia begins (Herring et al., 1999). Arthropod neurons, too, make VLDs (Grau & Cooke, 1992).

VLDs Are Reversible and Repeatable Invaginations of Surface Membrane Initiated at Discrete Sites

VLDs repeatedly form/reverse at their previous sites when shrink/swell cycles are repeated (say, every 2–3

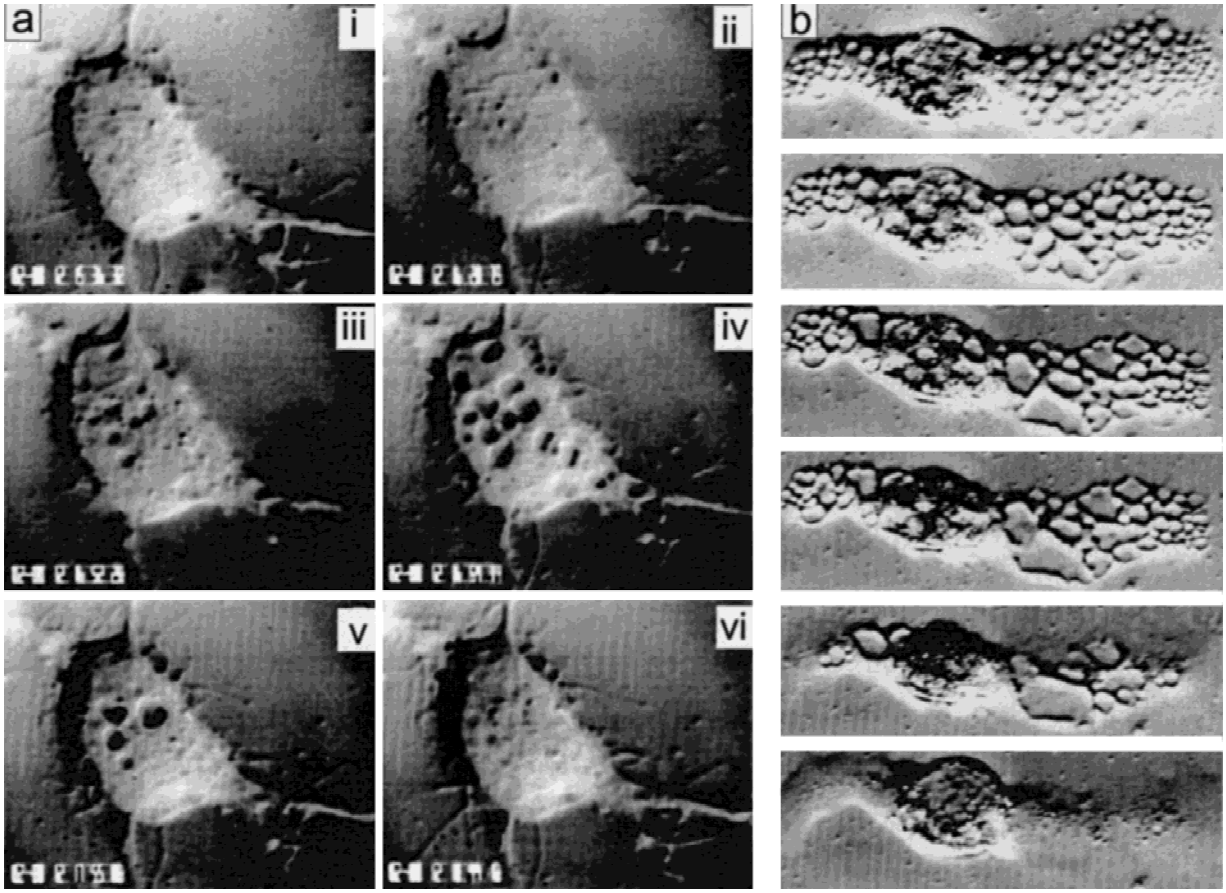

Fig. 6. Hippocampal neurons and snail neurons: cell-mediated VLD recovery. In *a* (i–vi), a hippocampal neuron at 37°C swells in 50% saline (ii), forms VLDs (iii–iv) and recovers (v–vi), all within 3 min (normal saline except in ii). In a second round, just after vi, VLDs formed at the same sites as in iv (from Dai et al., 1998). In *b* (top to bottom) a *Lymnaea* neuron with newly elicited VLDs (just previously, there had been a brief swell in distilled water) undergoes recovery in normal saline over a period of 135 min. Some VLDs shrink and disappear, others first fuse then disappear (from Reuzeau et al., 1995). This cell-mediated *recovery* differs from mechanically driven *reversal:* had the neuron been made to reswell in hypsomotic medium just after the top panel, VLDs would have disappeared by reversal in tens of seconds. On return to normal saline, the neuron would again have formed VLDs, using the same sites, as illustrated in Reuzeau et al. (1995). Whatever it is that defines VLD sites survives both reversal and, as mentioned for *a,* fast recovery. Scale bar, 6 and 20 μm for *a* and *b,* respectively.

min). Some substratum-adherent membrane structure evidently survives the turmoil, providing a morphological "memory". Similar reversibility and repeatability is reported for VLDs in frog muscle t-tubule (Krolenko et al., 1995, 1998). The basis for the memory is unknown but in neurons VLDs form in reduced adhesivity zones which are, presumably, *not* where endoplasmic reticulum links to substratum-adherent plasma membrane, since both DiO fluorescence and enhanced DIC show the reticulum surviving perturbations that elicit VLDs (C.E. Morris, *unpublished observation* with C. Cohan and L. Mills). Live-cell confocal microscopy of DiI-stained bilayer (Fig. 5(*a–i*); Mills & Morris, 1998) shows VLDs originating as invaginating plasma membrane and, during swelling-induced reversal, returning to the substratum plane. Live cell observations (Hoffman modulation (Reuzeau et al., 1995), phase contrast, fluorescent aqueous bath dyes or membrane dyes (Mills & Morris, 1998)) all reveal VLDs forming repeatedly at discrete loci. In neurons fixed just as VLDs are initiated, VLD membrane skeleton (F-actin, spectrin) is seen at the ~1 μm invaginations (Fig. 5*j* and *k*) (Herring et al., 1999, 2000). Perhaps spectrin skeleton specializations define the "sites". In any case, when VLDs are generated repeatedly, the ~1 μm invagination starts at precisely the same location (Mills & Morris, 1998). As a VLD invaginates and dilates, it takes in bath dye, and when VLDs are made to reverse by reswelling the neuron, dye is expelled to the bath. Under no condition does dye disperse through the cytoplasm. In renal cells, VLDs that form during RVD are like neuronal VLDs in that they, too, reverse (indicated by dye loss) with reswelling of the cell. In plant

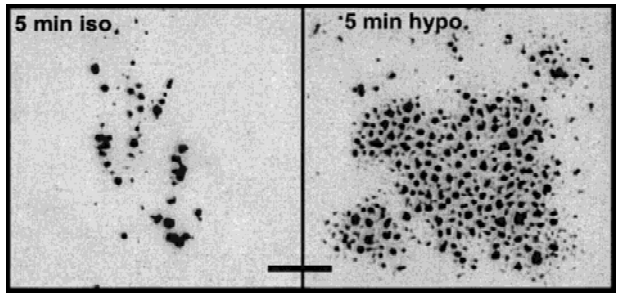

Fig. 7. Cell-mediated shrinkage and SAR. During regulatory volume decrease (hypotonic medium, right) in rat inner medullary collecting duct cells, bath dye (black) becomes incorporated into vesicles (<1 μm) invaginated from the surface. The process occurs in control cells (isotonic medium, left) but is less pronounced than in shrinking cells. Scale bar, 8 μm (excerpted from Czekay et al., 1993).

cells, large shrinkage-induced bath fluid filled endocytic vesicles also reverse on reswelling (Wartenberg et al., 1992).

SAR in Relation to Adhesive Contacts

Except at VLD sites, the adherent footprint of cultured neurons is remarkably stable over multiple VLD cycles (Mills & Morris, 1998). Insofar as VLDs represent SAR, SAR forgoes involving the entire cell surface, occurring instead at special low-adhesion loci. *In situ* in the CNS, this could be nontrivial (*see below*, "SAR in the CNS").

Mechanical Forces Drive VLD Formation and Reversal

VLDs form as cells shrink, whether shrinkage is via a normal-to-hyperosmotic step or a hyposmotic-to-normal step (Reuzeau et al., 1995). Since absolute osmolarity is irrelevant, chemical aspects of an osmomechanical perturbation (other than net water movement) are not what elicit VLDs. Mechanical forces associated with shrinking initiate the invagination of a tubule of surface membrane and subsequently dilate it to yield a VLD. Actomyosin motor forces are not involved since VLD formation is not blocked by cytochalasin or NEM (N-ethylmaleimide) Reuzeau et al., 1995; Herring et al., 1999). The same applies for VLD reversal: swelling mechanics (not chemistry) is responsible (Reuzeau et al., 1995) and like VLD formation, reversal is unaffected by cytochalasin and NEM.

VLDs Have a Spectrin and F-actin Membrane Skeleton

Failed bilayer-membrane skeleton interactions promote "blebbing" (Doctor et al., 1997; Dai & Sheetz, 1999). Since VLDs can form without F-actin (Reuzeau et al., 1995; Herring et al., 1999) are VLDs "inverted blebs"? Presumably not, since normally they have a healthy F-actin skeleton. They seem more akin to spectrin ("fodrin")-positive macrovacuoles that enlarge in fibroblasts when cytochalasin impairs actin-dependent membrane reprocessing (Brett & Godman, 1984; *see* Reuzeau et al., 1995). In cells fixed < 2 min into VLD formation, VLDs are lined with both spectrin and F-actin. We examined F-actin in neurons by phallotoxin staining and (for live neurons) by injecting fluorescent G-actin (Herring et al., 1999). Spectrin we examined in myotubes, using antibodies for muscle spectrin/fodrin (Herring et al., 2000)). The spectrin lining of VLDs is essentially uniform (though very large VLDs may have naked patches) and indistinguishable from the plasma membrane spectrin skeleton. All VLDs also have an F-actin skeleton which, when VLDs are first evident as ~1 μm invaginations (Fig. 5*j*), is relatively uniform. But within minutes F-actin dynamics yield extreme VLD-to-VLD differences (Fig. 8*b*). Whether existing F-actin filaments rearrange or *de novo* polymerization occurs is unclear. Nevertheless, before 5 min (Fig. 8*b*), some VLDs are heavily invested with F-actin while others (some immediately adjacent) have but a light coating. VLD-associated F-actin motility is discussed below.

Mechanisms for VLD Initiation and VLD Enlargement

VLDs appear inflated, as if water exiting the shrinking cell via nascent VLDs creates local hydrostatic pressure. This may also explain the formation of frog t-tubule VLDs ("reversible vacuoles" in the terminology of Krolenko et al., (1995, 1998)). Transient hydrostatic pressure may drive VLD enlargement (dilation), but envisaging how it could *initiate* invagination in neurons is trickier, and transient hydrostatic pressure would not stabilize VLDs once formed. Confocal microscopy with a bath dye shows that, prior to a shrink step, dye has full access (except at actual contacts) to the narrow space between neuron and substratum. At this stage, DiI stained bilayer gives no hint of where VLDs will be initiated (Reuzeau et al., 1995; Mills & Morris, 1998). Just after shrinkage (i.e., ~1–2 min), aqueous bath dye in VLDs is not detectably dilute: there is no apparent diffusion barrier (Mills & Morris, 1998). Moreover, on bath washout, most VLDs lose dye, so the "mouths" of new-made VLDs are open to the bath. Local inflationary pressures must dissipate almost as quickly as they develop. Continuous monitoring of nascent VLDs might reveal as-yet-undetected rapidly changing bath-to-VLD-lumen dye gradients. Given the complexity of osmotic flux in restricted cellular spaces (Pohl, Saparov & Antonenko,

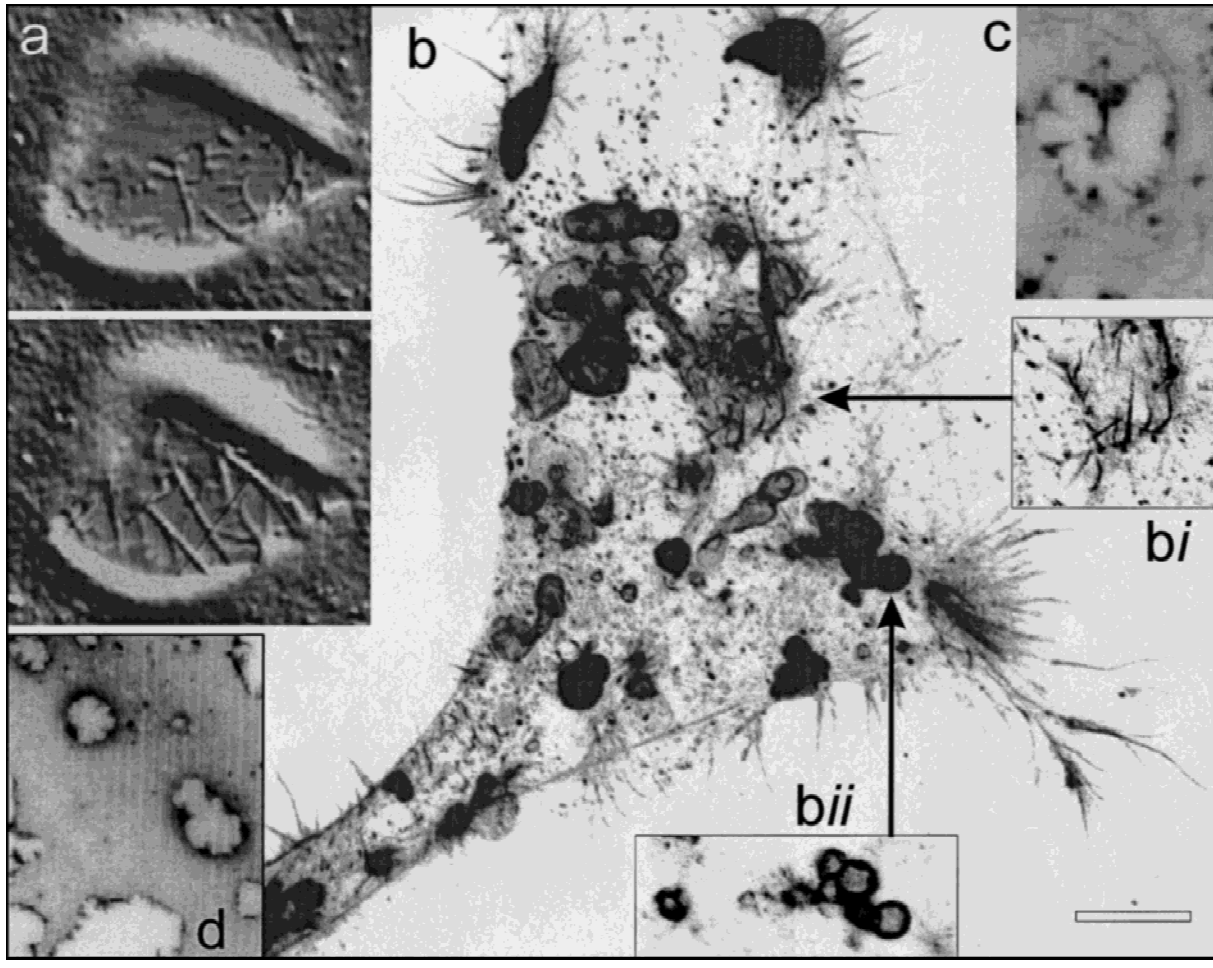

Fig. 8. F-actin reorganizes where plasma membrane invaginates in response to membrane stretch. (*a*) A pair of enhanced DIC images taken 3 min apart showing motile leading edge (lamellipodia and filopodia) at the mouth of a VLD. (*b–d*) Fluorescence images (black = actin stain). (*b*) Composite confocal image of phalloidin staining, 5 min after initiation of VLDs (*bi* and *bii* are sections from the bottom and middle, respectively, of the composite). (*c*) High resolution epifluorescence image at the substratum, similar treatment as in *b.* (*d*) High resolution epifluorescence image showing VLD leading edge at the substratum in a live neuron previously injected with fluorescent G-actin. Scale bar 12, 10, 4 and 10 μm for *a–d*, respectively (modified from Herring et al., 1999).

1997), it seems tenable that hydrostatic pressure transients do the work of dilating VLDs. As outlined below (*see* Fig. 10), in the CNS, restricted extracellular spaces might support transient dilation-producing pressures at sites where water exits shrinking neurons.

In neurons treated with cytochalasin or NEM, VLDs persist indefinitely (Reuzeau et al., 1995) so dilation represents a plastic not an elastic change of cell structure. Their "ballooned" look notwithstanding, VLDs do not "deflate" when osmotic equilibrium is reattained. Rather, VLD disappearance demands cell-mediated work (*see* next two sections).

A working hypothesis for VLD formation, therefore, is as follows: At discrete low adhesion sites on otherwise adherent plasma membrane, ~1 μm tubules invaginate where shrinking cytoplasm pulls inward on the membrane skeleton (Fig. 9). F-actin is not the puller (Herring et al., 1999) but other spectrin-linked structures may do the job (Herring et al., 2000). Spectrin-lined retrieval vacuoles occur in diverse contexts (Brett & Godman, 1984; Fujimoto & Ogawa, 1989; De bleeker et al., 1993; Herring et al., 2000) and ankyrin, which connects spectrin to membrane proteins, is implicated in surface membrane invagination (Michaely et al., 2000). Whatever the molecular specifics, pulling creates high tension in the invaginating bilayer, causing a net flow of more bilayer material to the invagination point. The flowing bilayer of nascent VLDs may be inordinately water permeable (water permeability increases with membrane fluidity; Lande, Donovan & Zeidel, 1995). If so, the following positive feedback can be expected as a cell abruptly shrinks: at the inflowing bilayer of the invagination point, water exits the cell at an abnormally high rate, creating hydrostatic pressure that further increases VLD

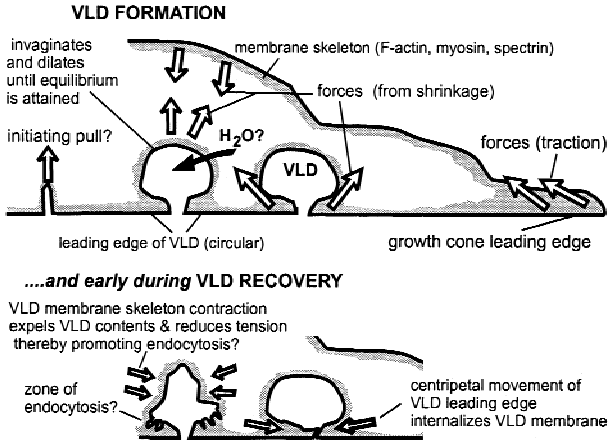

Fig. 9. Working model for how VLDs form and how they undergo recovery. The diagram highlights similarities in the characteristics of leading edge at VLDs and at growth cones.

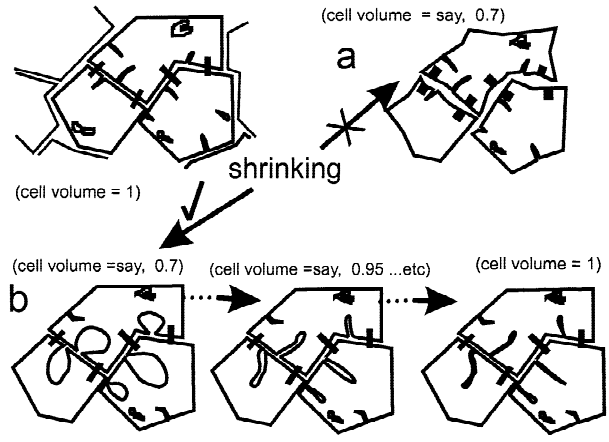

Fig. 10. A VSAR model for neurons *in situ.* If shrinking neurons mutually pulled away from each other (*a*), synapses would be under mechanical stress and, at the extreme, synaptic contacts would fail. Alternately (*b*), if shrinking forces generate transient VLDs at discrete extrasynaptic sites, cytoplasmic volume could decrease while cell volume was maintained. The dilated pseudo-intracellular compartment could later collapse as cytoplasmic volume was restored.

bilayer tension, causing additional flow of bilayer to the invagination. Water exits to the VLD lumen across an ever-enlarging area of flowing, high-water-permeability, high-tension bilayer. Hydrostatic pressure (which dissipates, but not instantaneously) sustains VLD inflation until osmotic equilibrium is attained. The extensible spectrin skeleton uniformly lines the dilating VLD; this reconfiguration of bilayer and spectrin is plastic and does not passively "recoil" at equilibrium.

VLD Recovery Retrieves SA

VLDs disappear not passively but by a cell-mediated process termed VLD recovery, which is actomyosin-dependent and Brefeldin A sensitive (Reuzeau et al., 1995). In normal saline, recovery requires <3 min or as much as 3 hr depending on cell type, temperature and severity of osmotic insults (*see* Fig. 6*a*, and *b*). Submicromolar external calcium impairs recovery. Some recovering VLDs pinch off as vacuoles made of retrieved SA (Mills & Morris, 1998). Where pinching fails, the membrane is eventually flattened by actomyosin-generated forces. Inhibition by Brefeldin A implicates endocytosis and regions of intensely DiI-stained VLD membrane seen during recovery may be undergoing endocytosis (Mills & Morris, 1998).

Constriction of VLDs mouths is evident a few minutes into recovery. One manifestation is that during washout, some VLDs retain bath dye, even while their immediate neighbors may not (Mills & Morris, 1998). Time-course imaging with DiI during recovery (Mills & Morris, 1998) (*see* Fig. 5) and fixed cell images of VLD membrane skeleton (Herring et al., 1999) also show VLDs internalized as vacuoles. Membrane retrieval by this means, plus endocytosis, may underlie the capacitance decrease of shrinking neurons (Fig. 4*a*) (Wan et al., 1995; Dai et al., 1998). In secretogogue-stimulated pancreatic acinar cells (Valentijn et al., 1999), ultrastructural evidence points to actin-dependent SA compensatory endocytosis that, presumably, coincides with endocytic mechanisms identified in secretory cells by capacitance measurements. Specifically, high resolution capacitance in chromaffin cells shows that post-exocytotic endocytosis occurs mostly via a compensatory recycling form of endocytosis (quantum size, 6 fF) but there is another form (quantum size ~40× greater; associated with clathrin-based endocytosis) seemingly for "excess" membrane (Smith & Neher, 1997). Such "excess mop-up" might well contribute to (cytochalasin- and Brefeldin-sensitive) VLD recovery.

Leading Edge: F-actin Dynamics at VLDs

The process that constricts VLD mouths corresponds with the building of a rim of F-actin-rich leading edge—a mix of lamellipodia and filopodia—at the VLD mouth (Herring et al., 1999). Except that VLD leading edge is circular and advances centripetally, it is remarkably like growth cone leading edge, even advancing at the same average speed as growth cone leading edge. During recovery, motile VLD leading edge can be seen by DIC time lapse (Herring et al., 1999) (Fig. 8*a*), DiI fluorescence (Mills & Morris, 1998) (Fig. 5*h*), fluorescent actin in live neurons (Fig. 8*a*). Cytochalasin prevents both VLD recovery (Reuzeau et al., 1995) and VLD leading edge formation like that in Fig. 8 (Herring et al., 1999). VLD recovery is also blocked by the strong

myosin ATPase inhibitor NEM and it requires a source of calcium (Reuzeau et al., 1995). Convoluted VLD bilayer distal to the VLD leading edge may reflect endocytosis (Mills & Morris, 1998). Macropinocytosis and pagocytosis are actin-dependent and resemble VLD constriction expect the latter requires adhesion to a substratum.

During VLD recovery, NEM/cytochalasin-sensitive writhing "kneads" VLDs as they progressively diminish in size (Reuzeau et al., 1995). VLD actomyosin is likely responsible, and VLDs with particularly intense F-actin staining (Herring et al., 1999) may be particularly contractile. As VLDs contract, VLD lumen contents are probably expelled to the bath (Mills & Morris, 1998) past the constriction, since with rare exceptions, even VLDs retaining dye after bath washout eventually shrink (over tens of minutes) and lose dye to the bath (C.E. Morris, *unpublished observation*). VLD contraction would lower VLD bilayer tension, thereby facilitating endocytosis (*see* Dai & Sheetz, 1997). This could explain the zone of convoluted VLD bilayer distal to the leading edge during recovery (*see* Fig. 9 bottom). These events, seen as mediating SAR, resemble the picture emerging for acinar cell amylase secretion, where F-actin dependent expulsion of vesicle contents and actin-dependent endocytosis are both implicated (Valentijn et al., 1999).

Plasma Membrane Tension Estimated by the Laser Tweezers Tether Method

Freely diffusing bilayer molecules adhere to appropriately coated beads which can then be captured by laser tweezers and pulled. From the force needed to hold a tether of bilayer thus drawn from a cell surface, plasma membrane tension can be estimated (Hochmuth et al., 1996). For molluscan neurons, a steady-state tension of 0.04 mN/m prevails and after swelling in 50% medium, tension is 0.12 mN/m (Dai et al., 1998). In spite of the major osmotic shock, this tension—0.12 mN/m—is comfortably below lytic levels. On return to normal saline (which elicits VLDs), tension (0.02 mN/m) undershoots the control level as it does in reshrinking RBL cells (Dai et al., 1997).

Plasma membrane is not a simple Hooke's Law spring so what do these estimates mean? Tether force depends on in-plane bilayer tension but also on "membrane osmotic pressure" (principally, adhesion of bilayer to membrane skeleton (e.g., Dai & Sheetz, 1999). This labile contributor obviates simple interpretations of tether force during swelling and shrinking. Consider the post-swelling "undershoot". One explanation is bilayer flaccidity resulting from recent osmolyte loss. But diverse cellular structures compete for limited actin-binding proteins (Gerisch, Maniak & Neujahr, 1999; Chubb et al., 2000), so the undershoot probably also reflects reconfiguration of F-actin (from the subsurface network to VLD leading edges).

The tension of swollen neurons is intriguingly low. For cells in 50% medium to maintain a low tension is consistent with SA recruitment whenever tension exceeds some threshold, as suggested by the capacitance data. Additionally, any counter-contraction by cortical actomyosin would help keep bilayer tension sublytic, an idea backed by the observation that a myosin inhibitor (NEM) renders neurons osmotically fragile (Wan et al., 1995). Interestingly, in *Dictyostelium*, myosin II shows osmosensitive phosphorylation and is osmoprotective (Zischka et al., 1999) and myosin I (used in pseudopod formation and macropinocytosis) generates measurable cortical tension (micropipette aspiration; Dai et al., 1999).

Monitoring tether force dynamically during osmotic perturbations is difficult. The observations available indicate that tension is not monotonic as swelling gets under way. Irregular force oscillations in the first minutes of neuronal swelling (Dai et al., 1998) may reflect plasma membrane tension-SA homeostasis: the cell swells, membrane tension increases, mechanically accessible membrane is recruited, tension decreases. More work is needed and in particular, capacitance of swelling neurons (Dai et al., 1998) needs to be correlated temporally with membrane tension. A critical test of the tension-SA homeostasis will be to determine if, during swelling, tension increases precede capacitance increases, with a latency inversely related to the swelling rate.

Although neurons with intracellular calcium clamped below 60 nM by BAPTA are osmotically fragile, their resting tension is indistinguishable from normal (Dai et al., 1998). Either the contribution of cortical contractility to resting tension can be maintained in this calcium range, or else it makes a negligible contribution. Likewise, calcium-driven membrane traffic is not important for resting tension. With swelling, however, BAPTA neurons show tether force excursions exceeding the range of the laser tweezers ("escaped" tethers draw back into the plasma membrane). Such excursions may result because with BAPTA, swelling-induced tension increases go uncountered by cortical contractility. With tension measurements truncated on the upswing, it remains unknown if tension peaked then descended i.e., whether tension was oscillating as predicted if the high membrane tension excursion recruited more membrane.

Mechanoprotective Contractile "Membrane Tone" May Reduce the Need for SAR

Membrane skeleton is passively and actively a variable-strength mechanoprotector for the in-parallel bilayer (*see*

Sachs & Morris, 1998). This is evident in molluscan neurons, the susceptibility of whose TREK-type channels to stretch provides a useful "report" on the mechanoprotective status of the membrane skeleton. Sundry membrane skeleton disruptors (cytochalasin, NEM, osmotic swelling, calcium ionophores, mechanical trauma) increase the ease by which stretching the plasma membrane stresses the bilayer enough to activate channels (Wan et al., 1999). In shrunken neurons, by contrast, the channels show below-normal susceptibility stretch.

During SAR, mechanoprotection of the bilayer necessarily has to be updated wherever surface changes occur. F-actin is recruited to rapidly invaginating membrane (Fig. 8*b;* Herring et al., 1999). Likewise, growth cone membrane subjected to pulling forces recruits F-actin (Fig. 9). In both cases, this may strengthen vulnerable expanses of plasma membrane. F-actin dynamics at VLDs may echo those in stretched fibroblast cell margins; there, F-actin realigns parallel to the tension vector, whilst deeper in the cytoplasm, intermediate filaments remain in place (Kolega, 1986). In stromal fibroblasts, force-mediated (calcium and tyrosine phosphorylation dependent) accumulation of F-actin at the plasma membrane (Glogauer et al., 1997) correlates with increased membrane rigidity. This is construed as a mechanoprotective response, counteracting membrane extension at force transfer points.

It will be critical to learn if mechanoprotection of stretched membrane and reactive actomyosin contractility are correlated; increased bilayer tension may stimulate not only F-actin accumulation at stress points but contraction of cortical actomyosin as a counterforce. If so, then, within limits, stretch-induced "membrane tone" could reduce the need for SAR, deferring recruitment of mechanically accessible reserves until higher tension was felt.

The curious phenomenon of "axonal beading" may reflect an axon simultaneously mechanoprotecting its surface bilayer while defending a fixed volume and SAR. Axons in mammals, molluscs and other organisms respond to a sudden mild stretch by immediately producing undulations. As stretch increases, undulations become beads. A model for beading (Markin et al., 1999) invokes a balance between membrane tension and hydrostatic pressure operating at fixed axonal SA and volume. The model alludes to undefined "special properties" of the membrane skeleton, of which contractility (V. Markin, *personal communication*) is a prime candidate. An axon would prefer not to increase its capacitance, so during transient mechanical stress, actomyosin-dependent axonal undulations might allow axons to avoid SAR. For neurons, the first need is to conserve structure unless structural change serves information processing. If axonal membrane tone can actively fight cell volume and bilayer tension fluctuations, it may relegate SAR to a second line of defense against swelling or stretch transients.

Cytomechanical Stimuli

For both plant and animal cells, anisosmotic volume changes are experimentally useful but, we emphasize, not interchangeable with mechanical stimuli. Hypertonia e.g., inhibits clathrin-mediated though not fluid-phase endocytosis (Novak et al., 1988; Synnes et al., 1999) whereas decreased membrane tension should favor both. Extreme hypertonicity stimulates exocytotic synaptic transmitter release of (apparently) docked secretory vesicles (Rosenmund & Stevens, 1996) and exocytotic fusion in turn stimulates endocytosis (e.g., Smith & Neher, 1997). Both shrinking and swelling may create locales of high tension in adherent neurons. We argue e.g., that VLDs form where shrinkage yields discrete high tension points. Inflation under whole-cell conditions (e.g., Solsona et al., 1998), too, carries penalties because of cytoplasm washout. Cytomechanics is unavoidably plagued with the untidiness of mechanically stimulating soft reactive material, but determining if diverse stimuli produce converging answers helps get past the caveats.

Tension-sensitive SAR in the CNS

Whenever SA increases, the cost of electrical signals increases and their speed decreases (e.g., Pinsker et al., 1976) so neurons must avoid excess SA. Neurites *in situ* maintain a nonzero tension and, it is assumed (Van Essen, 1997; Markin et al., 1999), a nonzero hydrostatic pressure. A steady-state, nonzero hydrostatic pressure would seem to create an *a priori* need for SAR to be tension-sensitive and to use prefortified membrane stores for adding area.

Neuronal architecture constitutes information. In the CNS, therefore, volume and surface area regulation (VSAR) should operate to preserve information-rich contact surfaces, i.e., synapses. If shrinking neurons mutually pulled away, as in Fig. 10*a*, synapses would at best be stressed and at worst disconnected. Synapse preservation during cell-mediated shrinkage requires that both neuronal shape and volume be preserved and the use of discrete, low adhesion sites for VSAR as depicted in Fig. 10*b* might achieve this goal.

The VSAR model (elaborated elsewhere (Morris, 2000)) assumes that extrasynaptic plasma membrane performs SAR. An important question for the future is whether membrane tension is fully continuous between extrasynaptic and synaptic membrane. Compensatory balancing of synaptic exocytosis by endocytosis occurs in close proximity (e.g., Boudier et al., 1999). Perhaps the

machinery subserving the highly ordered synaptic zone "feels" an attenuated version of tension fluctuations experienced in adjacent (extrasynaptic) membrane and so is spared during VSAR. Because it invokes no calcium signaling, the model avoids crosstalk problems between VSAR and snaptic transmission; this is justified in the model because neither neuronal RVD (Morán et al., 1997), nor VLD formation and reversal, nor swell/shrink-induced SA changes (Wan et al., 1995; Herring et al., 1998) depend on calcium signaling.

Swell/shrink-induced VLDs can be visualized in hippocampal brain slices by confocal microscopy (L. Mills, *personal communication*) but are dauntingly hard to study. Where CNS tissue is everywhere unique, striated muscle tissue is quintessentially repetitive. In muscles, swell/shrink-induced VLDs are easily located along the t-tubules near Z-lines (Franzini-Armstrong et al., 1978; Krolenko et al., 1995). Like VLDs in cultured neurons, muscle VLDs use discrete sites during repeat VLD formation episodes. It is reassuring to observe VLD phenomena *in situ* in muscle tissue, but adequate testing of the VSAR model will require brain slice preparations.

Stretch and Membrane Dynamics in Fibroblasts

Fibroblasts, which routinely experience whole-cell stretch, display a panoply of mechanosensitive responses (e.g., Glogauer et al., 1997). Stretch-induced release of endosomal contents from fibroblasts (Hagmann, Dagan & Burger, 1992) may represent tension-sensitive SAR. When a pipette is used to pull an adherent fibroblast, stretching plasma membrane at a physiological rate, endosomes release their prelabeled fluorescent contents. This presumed stretch-induced fusion of endosomal membrane with the surface is abolished by cytochalasin (but not a microtubule reagent) and by low free calcium in the medium. An important unanswered question is the fate of endosomal membrane during and after release of endosomal contents.

Electrorotation is a noninvasive way to estimate membrane capacitance. In fibroblastic cells studied by electrorotation, changes in capacitance correlate with swelling (Sukhorukov, Arnold & Zimmermann, 1993). Over a wide range of osmolarities, swelling cells evidently obtain membrane first by smoothing out microvilli, then below ~200 mOsm (280 mOsm is iso-osmotic) a progressive incorporation of new membrane into the cell surface is indicated. Plausibly, membrane tension is the signal for recruitment.

Fibroblast spreading and motility require actin assembly dependent plasma membrane extension. Raucher & Sheetz (2000) report that compounds that expand the fibroblast plasma membrane decrease its apparent tension and stimulate lamellipodial extension. High extension rates are associated with lower apparent membrane tension and with decreased membrane-cytoskeleton adhesion, through hydrolysis of phosphatidylinositol diphosphate (*see also* Raucher et al., 2000). Conversely, osmotic swelling increases membrane tension and decreases lamellipodial extension rates. It will be informative to test this with mammalian neurons, where both osmotic swelling (Bray et al., 1991; Lin et al., 1995) and neurite stretch (Lamoureux et al., 1989) stimulate net outgrowth of processes and where both osmotic shrinkage (Bray et al., 1991) and local pulling (Zheng et al., 1991) stimulate the formation of leading edge.

Stretch and Membrane Turnover in Epithelial Cells

Upon stretch or swelling, bladder epithelium reversibly recruits membrane to the surface (Lewis & de Moura, 1982, 1984). Transepithelial apical cell capacitance measurements indicate that the apical face SA changes in response to tissue tension, changes that are complete within 5 min of applying and releasing stretch, respectively. It is argued that as bladder cells stretch from goblet to disk shaped, they maintain a constant cell volume by incorporating apical membrane vesicles from a cytoplasmic pool 3-fold greater than the resting microvillar surface. Increases are 18–75% of initial apical capacitance in different experimental paradigms and effects of cytoskeletal reagents point to cytoskeletal involvement.

The enveloping layer epithelium of early killifish embryos is another dynamic epithelium showing evidence of tension-sensitive SAR (Fink & Cooper, 1996). Cellular remodeling by exocytosis and endocytosis occurs as the cells break contacts and reform tight junctions. The attendant membrane dynamics are followed by labeling the epithelium with fluorescent lectins and lipids. In epiboly cells are actively rearranging, and membrane turnover accelerates at cell-cell contacts in a peripheral band of apical membrane close to tight junctions. This membrane turnover increase, it is argued, depends on local mechanical forces responsible for the rearrangements. The conclusion is based on the fact that post-epiboly, when cells are no longer restructuring, sustained mechanical deformation of the embryos (using compression between coverslips) accelerates apical membrane turnover at precisely the cell-cell contact regions that had been dynamic during epiboly.

Avoiding SAR

It would be surprising if cells always took the same approach to SAR, and indeed, there seem to be "SARophile" and "SARophobe" strategies. Unfinished (embryonic or re-arborizing) neurons and guard cells are saro-

philes. Their lifestyles create a demand for mechanosensitive SAR, with mechanically accessible endomembrane reserves ever at the ready. In sharp contrast are erythrocytes, which, lacking endomembrane, are necessarily complete sarophobes. Nucleated blood and lymph cells, which can be free-floating or adherent (crawling), are sarophobic in at least some contexts, relying on excess SA rather than tension-sensitive membrane recruitment. Whole cell recording capacitance measurements indicate that these cells simply smooth out with osmotic or hydrostatic swelling. Mast cells hydrostatically double their volume at fixed capacitance (Solsona et al., 1998) and lymphocytes exhibit similar behavior while swelling osmotically (Ross, Garber & Cahalan, 1994). Surface irregularity flattening can probably handle most physiologically conceivable osmotic challenges. However, this may not be the whole story, since whole cell clamp is physically and biochemically invasive and may uncouple response systems. In osmotically inflated intact white blood cells (intact RBL cells; Dai et al., 1997), inflation decreases the rate of endocytosis and all else being equal, this should produce an increased capacitance in the swelling cell. If exocytosis increased, as in plant cells, the effect would be even more marked. Corey & Neher (1997) compared endocytosis using perforated patch and whole cell configurations and found that whole cell diminishes endocytosis rates. Using perforated patch in molluscan neurons yielded larger osmotically induced SA changes than with whole cell (Wan et al., 1995). Nevertheless, what of mast cells? Hydrostatically inflated under whole cell clamp, their major notable capacitance change is a dramatic (~70-fold) reversible decrease at ~4 mN/m in the frequency of exocytotic fusion events. The biophysical implication is that the assembly and activity of exocytotic fusion pores is sensitive to plasma membrane tension (another reason to mechanoprotect surface bilayer!). The mast cell response is in stark contrast to that of plant cells, where tension promotes exocytosis; presumably, physiologic stretch-induced exocytosis in plants occurs at membrane tensions well below those which disable the exocytotic machinery.

Another cell whose excess SA allows for swelling at fixed capacitance is the *Xenopus* oocyte. This "megacell", being adapted for transfer from oviduct to freshwater, has a tough fibrous vitelline layer. It is argued (Zhang &Hamill, 2000) that a robust sarophobe strategy—reliance on unfolding of surface irregularities—ensures that *in situ* the oocyte's mechanosusceptible channels never feel mechanostimuli. In molluscan neurons, by contrast, a sarophile strategy (stretch-induced SA increase (Wan et al., 1995)) protects mechanosusceptible channels from tension (Morris & Horn, 1991).

Skeletal muscle cells use precisely timed depolarizations so teams of cells will exert contractile force appropriately. A need for invariant depolarization rates probably favors the sarophobe strategy (maintaining an excess, fixed SA) over adjusting SA to the prevailing tension. Caveolae may ensure that muscle fibers can endure abnormally large stretches without SA changes. Almost half the SA of a skeletal muscle fiber is caveolae (of which there are ~40 μm^{-2}). By reversibly flattening under excessive tension (after simple surface irregularities have been used up) caveolae appear to act as mechanical safety devices (Dulhunty & Franzini-Armstrong, 1975). Ultrastructure/electrophysiology comparisons in cardiac muscle fibers suggest that caveolae contribute appreciably to surface capacitance (mitchell, Powell & Sturridge, 1986). Stretch-induced flattening of cardiac caveolae (as in skeletal muscle) would probably occur, therefore, at a fixed "electrical" SA. In cardiac atrial cells, ultrastructural evidence for stretch-induced release of natriuretic peptide is thought to show that at caveolae, (as in skeletal muscle) would probably occur, therefore, at a fixed "electrical" SA. In cardiac atrial cells, ultrastructural evidence for stretch-induced release of natriuretic peptide is thought to show that at caveolae, stretch transiently opens a pathway for release of peptide from contiguous vesicles (Page, Upshaw-Earley & Goings, 1994). If so, here is a case of stretch-induced peptide secretion at fixed SA. Caveolae and coated pits are reported in tubular invaginations of the surface in spleen cells, but whether these are mechanically responsive is unknown (Uehara & Miyoshi, 1999).

In astrocytes and hepatocytes excess SA allows swelling to trigger volume regulatory mechanisms without recruiting additional capacitance (Graf et al., 1995; Olson & Li, 1997). This is also true for lymphocytes, though an upper limit to which lymphocytes swell at fixed capacitance was noted: beyond ~2-fold, swelling recruits new SA, coincident with the onset of ionic leakiness (Ross et al., 1994).

Tissues that regularly experience both passive stretch and osmotic swelling like bladder (Lewis & de Moura, 1982, 1984), are likely sarophiles and this may explain the case of shark rectal gland epithelial cells. Under whole-cell clamp, these cells (in contrast to hepatocytes, which do not derive from a stretch-prone epithelium) respond to hyposmotic conditions with a capacitance increase and to hyperosmotic conditions with a capacitance decrease (Thiele et al., 1998). These SA changes are likely to be physiologically relevant as they are accompanied by conductance changes associated with cell volume regulation.

When SAR Fails

When SAR fails, the membrane ruptures at a point of high tension and the cytoplasm is flooded locally with

calcium. To a surprising extent, healthy cells, including neurons (Eddleman et al., 1997; Togo et al., 1999) can seal off a region of ruptured membrane by recruiting endomembrane of mixed and uncertain identity. Unspecified calcium-dependent mechanisms create a new plasma membrane across the wound (Terasaki, Miyake & McNeil, 1997). Whether these emergency measures overlap with SAR in terms of cellular machinery (endomembrane used, membrane skeleton dynamics) remains to be seen. For example calpain, a key membrane skeleton remodeling enzyme is required both for neuronal wound repair (Godell et al., 1997) and for growth cone extension (Gitler & Spira, 1998) which likely involves mechanosensitive recruitment of "prefortified" membrane.

Conclusion

In an essay, "From molecular to modular cell biology", Hartwell et al. (1999) present an evolutionary engineering-oriented perspective in which cell biology is seen as a collective of modules. A module uses a small fraction of the cell's components to accomplish a relatively automonous function. Cells generate differentiated states by a cut-and-paste use of modules such as signal transduction, protein synthesis, DNA replication, action potential generation. We suggest that mechanosensitive SAR may eventually be recognized as a module. Cells may employ mechanosensitive SAR in conjunction with imposed osmotic changes (e.g., in the swelling/shrinking bladder), with cell-mediated osmotic changes that produce desired shape changes (e.g., in guard cells), with externally imposed shape changes (e.g., for stretched neurites in embryos) and with cell-mediated shape change (e.g., during embryonic epithelial cell remodeling). The molecular and ultrastructural correlates of SAR are mostly not known, but, generically, tension-sensitive exocytosis and endocytosis plus tension-sensitive evagination and invagination of larger quantities of SA are implicated. Certain cellular features (e.g., caveolae, contractile subsurface actomyosin) may temper the need for SAR. In different cell types, SAR undoubtedly uses different collections of components, but that would not disqualify it as a module; the module "action potential generation", for instance, uses an assortment of different channels and pumps in different cells.

Though membrane tension has been central to our discussions, this review evinces a distinct shortage of mN/m". Membrane tension, while easy to allude to, is far from easy to quantify in live irregular-shaped cells. The paucity of measured tensions is confounded, moreover, by the fact that bilayer tension and plasma membrane tension (that of the bilayer plus ill-defined and dynamic membrane skeleton) are not the same thing (*see* Raucher & Sheetz, 1999*a*). Generally, a poorly characterized hybrid of the two is what is monitored. Tension can be estimated by application of Laplace's Law, but frequently, conditions that allow for the necessary radius of curvature determinations seriously comprise cell function (digestion of plant cell walls to form protoplasts, inflation under whole cell clamp, aspiration of a patch of membrane into a micropipette). For SAR, however, the critical question that needs a precise answer is not "how many mN/m" but this: What parameter(s) do cells measure in order to determine whether SAR is called for? As studies of tension-sensitive SAR continue, it will be of fundamental importance to establish what is used as a set point for SAR. Is it the tension in the bilayer? the tension in some component of the membrane skeleton? some combination of the two? And is the set point mechanism universal, so that cells as different as plant coleoptile cells and embryonic neuronal cells monitor the same parameter? Another glaring unknown is the identity(ies) of mechanically accessible endomembrane. Are there distinct pools used only for SAR or does mechanically accessible endomembrane overlap in part or in full with other endomembrane pools?

Currently, SAR is a "putative module". Major questions of a fundamental nature remain to be answered: 1. What is the identity of the surface structure whose tension controls the rate and direction of SAR? 2. Once tension has been probed, to what other structures is the information conveyed? 3. How are these structures used to alter the quantity of surface membrane? 4. As membrane is added or subtracted at the surface, how is membrane strength maintained? 5. How does a cell's SAR module interact with its other modules, such as volume regulation, protein secretion, motility? Thinking of SAR as a cell biological module whose invariant components and variable features need to be defined may prove useful as work continues.

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References

Albillos, A., Dernick, G., Horstmann, H., Almers, W., Alvarez de Toledo, G., Lindau, M. 1997. The exocytotic event in chromaffin cells revealed by patch amperometry. *Nature* **389:**509–512

Angelson, J.K., Cochilla, A.J., Kilic, G., Nussinovitch, I., Betz, W.J. 1999. Regulation of dense core release from neuroendocrine cells revealed by imaging single exocytic events. *Nat. Neurosci.* **2:**440–446

Baumann, O. 1998. Association of spectrin with a subcompartment of the endoplasmic reticulum in honeybee photoreceptor cells. *Cell Motil. Cytoskeleton* **41:**74–86

Bernstein, B.W., DeWit, M., Bamburg, J.R. 1998. Actin disassembles reversibly during electrically induced recycling of synaptic vesicles in cultured neurons. *Brain Res. Mol. Brain Res.* **53:**236–251

Bibikova, T.N., Zhigilei, A., Gilroy, S. 1997. Root hair growth in

Araibidopsis thaliana is directed by calcium and an endogenous polarity. *Planta* **203:**495–505

Bisikirska, B., Sikorski, A.F. 1997. Some properties of spectrin-like proteins from *Pisum sativum. Z Naturforsch* [*C*] **52:**180–186

Boudier, J.A., Martin-Moutot, N., Boudier, J.L., Iborra, C., Takahashi, M., Seagar, M.J. 1999. Redistribution of presynaptic proteins during alpha-latrotoxin-induced release of neurotransmitter and membrane retrieval at the frog neuromuscular junction. *Eur. J. Neurosci.* **11:**3449–3456

Braun, M., Baluska, F., von Witsch, M., Menzel, D. 1999. Redistribution of actin, profilin and phosphatidylinositol-4,5-biphosphate in growing and maturing root hairs. *Planta* **209:**435–443

Bray, D., Money, N.P., Harold, F.M., Bamburg, J.R. 1991. Responses of growth cones to changes in osmolality of the surrounding medium. *J. Cell. Sci.* **98:**507–515

Brett, J.G., Godman, G.C. 1984. Macrovacuolation induced by cytochalasin: its relation to the cytoskeleton; morphological and cytochemical observations. *Tissue Cell* **16:**311–324

Brown, D.A., London, E. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14:**111–136

Brownlee, C., Goddard, H., Hetherington, A.M., Peake, L.-A. 1999. Specificity and integration of responses: Ca^{2+} as a signal in polarity and osmoregulation. *J. Exp. Bot.* **50:**1001–1011

Brownlee, C., Wood, J.W. 1986. A gradient of free cytosolic calcium in rhizoid cells of *Fucus seratus. Nature* **320:**624–626

Campenot, R.B., Draker, D.D. 1989. Growth of sympathetic nerve fibers in culture does not require extracellular calcium. *Neuron* **3:**733–743

Chada, S., Lamoureux, P., Buxbaum, R.E., Heidemann, S.R. 1997. Cytomechanics of neurite outgrowth from chick brain neurons. *J. Cell Sci.* **110:**1179–1186

Cheng, T.P.O., Reese, T.S. 1987. Recycling of plasmalemma in chick tectal growth cones. *J. Neurosci.* **7:**1752–1759

Chestkov, V.V., Radko, S.P., Cho, M.S., Chrambach, A., Vogel, S.S. 1998. Reconstitution of calcium-triggered membrane fusion using "reserve" granules. *J. Biol. Chem.* **273:**2445–2451

Chubb, J.R., Wilkins, A., Thomas, G.M., Insall, R.H. 2000. The *Dictyostelium* RasS protein is required for macropinocytosis, phagocytosis and the control of cell movement. *J. Cell Sci.* **113:**709–719

Cibert, C., Pruliere, G., Lacombe, C., Deprette, C., Cassoly, R. 1999. Calculation of a gap restoration in the membrane skeleton of the red blood cell: possible role for myosin II in local repair. *Biophys. J.* **76:**1153–1165

Colombo, M.F., Rau, D.C., Parsegian, V.A. 1992. Protein solvation in allosteric regulation: a water effect on hemoglobin. *Science* **256:**655–659

Cosgrove, D.J. 1997. Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. *Plant Cell* **9:**1031–1041

Czekay, R., Kinne-Saffran, E., Kinne, R.K.H. 1993. Membrane traffic and sorbitol release during osmo- and volume regulation in isolated rat renal inner medullary collecting duct cells. *Eur. J. Cell. Biol.* **63:**20–31

Dai, J., Sheetz, M.P. 1995*a.* Axon membrane flows from the growth cone to the cell body. *Cell* **83:**693–701

Dai, J., Sheetz, M.P. 1995*b.* Mechanical properties of neuronal growth cone membranes studied by tether formation with laser optical tweezers. *Biophys. J.* **68:**988–996

Dai, J., Sheetz, M.P. 1999. Membrane tether formation from blebbing cells. *Biophys. J.* **77:**3363–3370

Dai, J., Sheetz, M.P., Wan, X., Morris, C.E. 1998. Membrane tension in swelling and shrinking molluscan neurons. *J. Neurosci.* **18:**6681–6692

Dai, J., Ting-Beall, H.P., Hochmuth, R.M., Sheetz, M.P., Titus, M.A. 1999. Myosin I contributes to the generation of resting cortical tension. *Biophys. J.* **77:**1168–1176

Dai, J., Ting-Beall, H.P., Sheetz, M.P. 1997. The secretion-coupled endocytosis correlates with membrane tension changes in RBL 2H3 cells. *J. Gen. Physiol.* **110:**1–10

Dailey, ME., Bridgman, P.C. 1993. Vacuole dynamics in growth cones: correlated EM and video observations. *J. Neurosci.* **13:**3375–3393

Davis, L.H., Bennett, V. 1990. Mapping the binding sites of human erythrocyte ankyrin for the anion exchanger and spectrin. *J. Biol. Chem.* **265:**10589–10596

De bleecker, J.I., Engel, A.G., Winkelmann, J.C. 1993. Localization of dystrophin and β-spectrin in vacuolar myopathies. *Am. J. Pathology* **143:**1200–1208

Diekmann, W., Hedrich, R., Raschke, K., Robinson, D.G. 1993. Osmocytosis and vacuolar fragmentation in guard cell protoplasts: their relevance to osmotically-induced volume changes in guard cells. *J. Exp. Bot.* **44:**1569–1577

Discher, D.E., Mohandas, N., Evans, E.A. 1994. Molecular maps of red cell deformation: hidden elasticity and in situ connectivity. *Science* **266:**1032–1035

Discher, D.E., Winardi, R., Schischmanoff, P.O., Parra, M., Conboy, J.G., Mohandas, N. 1995. Mechanochemistry of protein 4.1's spectrin-actin-binding domain: ternary complex interactions, membrane binding, network integration, structural strengthening. *J. Cell. Biol.* **130:**897–907

Doctor, R.B., Zhelev, D.V., Mandel, L.J. 1997. Loss of plasma membrane structural support in ATP-depleted renal epithelia. *Am. J. Physiol.* **272:**C439–C449

Dulhunty, A.F., Franzini-Armstrong, C. 1975. The relative contributions of the folds and caveolae to the surface membrane of frog skeletal muscle fibres at different sarcomere lengths. *J. Physiol.* **250:**513–539

Eddleman, C.S., Ballinger, M.L., Smyers, M.E., Godell, C.M., Fishman, H.M., Bittner, G.D. 1997. Repair of plasmalemmal lesions by vesicles. *Proc. Natl. Acad. Sci. USA* **94:**4745–4750

Feijo, J.A., Sainhas, J., Hackett, G.R., Kunkel, J.G., Hepler, P.K. 1999. Growing pollen tubes possess a constitutive alkaline band in the clear zone and a growth-dependent acidic tip. *J. Cell. Biol.* **144:**483–496

Fejtl, M., Szarowski, D.H., Decker, D., Buttle, K., Carpenter, D.O., Turner, J.N. 1995. Three-dimensional imaging and electrophysiology of live *Aplysia* neurons during volume perturbation: confocal light and high-voltage electron microscopy. *J. Microscop. Soc. Am.* **1:**75–85

Felle, H.H., Hepler, P.K. 1997. The cytosolic Ca^{2+}-concentration gradient of *Sinapis alba* root hairs as revealed by Ca^{2+}-selective microelectrode tests and fura-dextran imaging. *Plant Physiol.* **114:**39–45

Fink, R.D., Cooper, M.S. 1996. Apical membrane turnover is accelerated near cell-cell contacts in an embryonic epithelium. *Dev. Biol.* **174:**180–189

Franzini-Armstrong, C., Heuser, J.E., Reese, T.S., Somlyo, A.P., Somlyo, A.V. 1978. T-tubule swelling in hypertonic solutions: a freeze substitution study. *J. Physiol.* **283:**133–40

Fujimoto, T., Ogawa, K. 1989. Retrieving vesicles in secretion-induced rat chromaffin cells contain fodrin. *J. Histochem. Cytochem.* **37:**1589–1599

Gerisch, G., Maniak, M., Neujahr, R. 1999. Patterns of cellular activities based on protein sorting in cell motility, endocytosis and cytokinesis. *Biochem. Soc. Sympt.* **65:**1–14

Gitler, D., Spira, M.E. 1998. Real time imaging of calcium-induced localized proteolytic activity after axotomy and its relation to growth cone formation. *Neuron* **20:**1123–1135

Glogauer, M., Arora, P., Chou, D., Janmey, P.A., Downey, G.P., Mc-

Culloch, C.A.G. 1998. The role of actin-binding protein 280 in integrin-dependent mechanoprotection. *J. Biol. Chem.* **273:**1689–1698

Glogauer, M., Arora, P., Yao, G., Sokholov, I., Ferrier, J., McCulloch, C.A. 1997. Calcium ions and tyrosine phosphorylation interact coordinately with actin to regulate cytoprotective responses to stretching. *J. Cell Sci.* **110:**11–21

Godell, C.M., Smyers, M.E., Eddleman, C.S., Ballinger, M.L., Fishman, H.M., Bittner, G.D. 1997. Calpain activity promotes the sealing of severed giant axons. *Proc. Natl. Acad. Sci. USA* **94:**4751–4756

Gordon-Kamm, W.J., Steponkus, P.L. 1984*a*. The behavior of the plasma membrane following osmotic contraction of isolated protoplasts: implications in freezing injury. *Protoplasma* **123:**83–94

Gordon-Kamm, W.J., Steponkus, P.L. 1984*b*. The influence of cold acclimation on the behavior of the plasma membrane following osmotic contraction of isolated protoplasts. *Protoplasma* **123:**161–173

Graf, J., Rupnik, M., Zupancic, G., Zorec, R. 1995. Osmotic swelling of hepatocytes increases membrane conductance but not membrane capacitance. *Biophys. J.* **68:**1359–1363

Grau, S.M., Cooke, I.M. 1992. Peptidergic neurons of the crab. *Cardisoma carnifex*, in defined culture maintain characteristic morphologies under a variety of conditions. *Cell. Tissue Res.* **270:**303–317

Grazi, E., Magri, E., Schwienbacher, C., Trombetta, G. 1995. Osmotic properties of myosin subfragment 1: implications of the mechanism of muscle contraction. *Arch. Biochem. Biophys.* **322:**97–102

Hagmann, J., Dagan D., Burger, M.M. 1992. Release of endosomal content induced by plasma membrane tension: video image intensification time lapse analysis. *Exp. Cell Res.* **198:**298–304

Hamill, O.P., McBride, D.W. Jr. 1997. Induced membrane hypo/hypermechanosensitivity: a limitation of patch-clamp recording. *Annu. Rev. Physiol.* **59:**621–631

Hartwell, L.H., Hopfield, J.J., Leibler, S., Murray, A.W. 1999. From molecular to modular cell biology. *Nature* **402:**C47–C52

Hepler, P.K. 1997. Tip growth in pollen tubes: calcium leads the way. *Trends Plant Sci.* **2:**79–80

Herring, T.L., Cohan, C.S., Welnhofer, E.A., Mills, L.R., Morris, C.E. 1999. F-actin at newly invaginated membrane in neurons: implications for surface area regulation. *J. Membrane Biol.* **171:**151–169

Herring, T.L., Juranka, P.F., McNally, J., Lesiuk, H., Morris, C.E. 2000. The spectrin skeleton of newly invaginated plasma membrane. *J. Mus. Res. Cell Mot.* **21:**67–77

Herring, T.L., Slotin, I.M., Baltz, J.M., Morris, C.E. 1998. Neuronal swelling and surface area regulation: elevated intracellular calcium is not a requirement. *Am. J. Physiol.* **274:**C272–C281

Hitt, A.L., Luna, E.J. 1994. Membrane interactions with the actin cytoskeleton. *Curr. Opin. Cell Biol.* **6:**120–130

Hochmuth, F.M., Shao, J.Y., Dai, J., Sheetz, M.P. 1996. Deformation and flow of membrane into tethers extracted from neuronal growth cones. *Biophys. J.* **70:**358–369

Homann, U. 1998. Fusion and fission of plasma-membrane material accommodates for osmotically induced changes in the surface area of guard-cell protoplasts. *Planta* **206:**329–333

Homann, U., Thiel, G. 1999. Unitary exocytotic and endocytotic events in guard-cell protoplasts during osmotic-driven volume changes. *FEBS Lett.* **460:**495–499

Hutter, O.F. 1992. The membrane hypothesis of Duchenne muscular dystrophy: quest for functional evidence. *J. Inherit. Metab. Dis.* **15:**565–577

Kolega, J. 1986. Effects of mechanical tension on protrusive activity and microfilament and intermediate filament organization in an epidermal epithelium moving in culture. *J. Cell Biol.* **102:**1400–1411

Krolenko, S.A., Amos, W.B., Brown, S.C., Tarunina, M.V., Lucy, J.A. 1998. Accessibility of T-tubule vacuoles to extracellular dextran and DNA: mechanism and potential application of vacuolation. *J. Muscle Res. Cell Motil.* **19:**603–611

Krolenko, S.A., Amos, W.B., Lucy, J.A. 1995. Reversible vacuolation of the transverse tubules of frog skeletal muscle: a confocal fluorescence microscopy study. *J. Muscle Res. Cell. Motil.* **16:**401–411

Kwok, R., Evans, E. 1981. Thermoelasticity of large lecithin bilayer vesicles. *Biophys. J.* **35:**637–652

Kubitscheck, U., Homann, U., Thiel, G. 2000. Osmotic evoked shrinking of guard cell protoplasts causes retrieval of plasma membrane into the cytoplasm. *Planta* (*in press*)

Kuwayama, H., Ecke, M., Gerisch, G., Van Haastert, P.J. 1996. Protection against osmotic stress by cGMP-mediated myosin phosphorylation. *Science* **271:**207–209

Lambrechts, D., Schroeder, J.I., Verbelen, J.P. 1992. The influence of osmolarity on the surface properties of the plasma membrane of isolated guard cell protoplasts from *Vicia faba* L. *Plant Physiol.* **11:**25–32

Lamoureux, P., Buxbaum, R.E., Heidemann, S.R. 1989. Direct evidence that growth cones pull. *Nature* **340:**149–162

Lamoureux, P., Buxbaum, R.E., Heidemann, S.R. 1998. Axonal outgrowth of cultured neurons is not limited by growth cone competition. *J. Cell Sci.* **111:**3245–3252

Lande, M.B., Donovan, J.M., Zeidel, M.L. 1995. The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *J. Gen. Physiol.* **106:**67–84

Levitan, I., Christian, A.E., Tulenko, T.N., Rothblat, G.H. 2000. Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. *J. Gen. Physiol.* **115:**405–416

Lewis, S.A., de Moura, J.L. 1982. Incorporation of cytoplasmic vesicles into apical membrane of mammalian urinary bladder epithelium. *Nature* **297:**685–688

Lewis, S.A., de Moura, J.L. 1984. Apical membrane area of rabbit urinary bladder increases by fusion of intracellular vesicles: an electrophysiological study. *J. Membrane Biol.* **82:**123–136

Lin, C., Lamoureux, P., Buxbaum, R.E., Heidemann, S.R. 1995. Osmotic dilution stimulates axonal outgrowth by making axons more sensitive to tension. *J. Biomechanics* **28:**1429–1438

Lindau, M., Almers, W. 1995. Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. *Curr. Opin. Cell Biol.* **7:**509–517

Lipowsky, R. 1991. The conformation of membranes. *Nature* **349:**475–481

Lipowsky, R. 1995. The morphology of lipid membranes. *Curr. Opin. Struct. Biol.* **5:**531–540

Lynch, D.V., Steponkus, P.L. 1987. Plasma membrane lipid alterations associated with cold acclimation of wintry rye seedlings (*Secale cereale* L. cv Puma). *Plant Physiology* **83:**761–767

Markin, V.S., Tanelian, D.L., Jersild, R.A. Jr., Ochs, S. 1999. Biomechanics of stretch-induced beading. *Biophys. J.* **76:**2852–2860

Menke, A., Jockusch, H. 1991. Decreased osmotic stability of dystrophin-less muscle cells from the *mdx* mouse. *Nature* **349:**69–71

Michaely, P., Kamal, A., Anderson, R.G., Bennett, V. 1999. A requirement for ankyrin binding to clathrin during coated pit budding. *J. Biol. Chem.* **274:**35908–35913

Mills, L.R., Morris, C.E. 1998. Neuronal plasma membrane dynamics evoked by osmomechanical perturbations. *J. Membrane Biol.* **166:**223–238

Mitchell, M.R., Powell, T., Sturridge, M.F., Terrar, D.A., Twist, V.W. 1986. Electrical properties and response to noradrenaline of individual heart cells isolated from human ventricular tissue. *Cardiovasc. Res.* **20:**869–876

Monck, J.R., Oberhauser, A.F., Alvarez de Toledo, G., Fernandez, J.M. 1991. Is swelling of the secretory granule matrix the force that dilates the exocytotic fusion pore? *Biophys. J.* **59:**39–47

Morán, J., Morales-Mulia, S., Hernandez-Cruz, A., Pasantes-Morales, H. 1997. Regulatory volume decrease and associated osmolyte fluxes in cerebellar granule neurons are calcium independent. *J. Neurosci. Res.* **47:**144–154

Morris, C.E. 2001. Tension-sensitive membrane traffic and an optimal strategy for volume and surface area regulation in CNS neurons. *Am. Zoologist* (*in press*)

Morris, C.E., Horn, R. 1991. Failure to elicit neuronal macroscopic mechanosensitive currents anticipated by single-channel studies. *Science* **251:**1246–1249

Morris, C.E., Sigurdson, W.J. 1989. Stretch-inactivated ion channels coexist with stretch-activated ion channels in neuronal membranes. *Science* **243:**807–809

Morris, C.E., Williams, B., Sigurdson, W.J. 1989. Osmotically-induced volume changes in isolated cells of a pond snail. *Comp. Biochem. Physiol.* **92A:**479–483

Mulholland, J., Preuss, D., Moon, A., Wong, A., Drubin, D., Botstein, D. 1994. Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. *J. Cell Biol.* **125:**381–391

Needham, D., Nunn, R.S. 1990. Elastic deformation and failure of lipid bilayer membranes containing cholesterol. *Biophys. J.* **58:**997–1009

Nichol, J.A., Hutter, O.F. 1996. Tensile strength and dilatational elasticity of giant sarcolemmal vesicles shed from rabbit muscle. *J. Physiol.* **493:**187–198

Norman, J.C., Price, L.S., Ridley, A.J., Koffer, A. 1996. The small GTP-binding proteins, Rac and Rho, regulate cytoskeletal organization and exocytosis in mast cells by parallel pathways. *Mol. Biol. Cell* **7:**1429–1442

Novak, J.M., Ward, D.M., Buys, S.S., Kaplan, J. 1988. Effect of hypo-osmotic incubation on membrane recycling. *J. Cell Physiol.* **137:**235–242

Okada, Y. 2000. A scaffolding for regulation of volume-sensitive Cl-channels. *J. Physiol.* **520:**2

Olson, J.E., Li, G.Z. 1997. Increased potassium, chloride, and taurine conductances in astrocytes during hypo-osmotic swelling. *Glia* **20:**254–261

Oparka, K., Prior, D., Harris, N. 1990. Osmotic induction of fluid-phase endocytosis in onion epidermal cells. *Planta* **180:**555–561

Page, E., Upshaw-Earley, J., Goings, G.E. 1994. Localization of atrial natriuretic peptide in caveolae of in situ atrial myocytes. *Circ. Res.* **75:**949–954

Patel, A.J., Honore, E., Maingret, F., Lesage, F., Fink, M., Duprat, F., Lazdunski, M. 1998. A mammalian two pore domain mechano-gated S-like K^+ channel. *EMBO J.* **17:**4283–4290

Phillips, G.D., Preshaw, C., Steer, M.W. 1988. Dictyosome vesicle production and plasma membrane turnover in auxin-stimulated outer epidermal cells of coleoptile segments from *Avena sativa* (L.). *Protoplasma* **145:**59–65

Picton, J.M., Steer, M.W. 1983. Membrane recycling and the control of secretory activity in pollen tubes. *J. Cell Sci.* **63:**303–310

Pinsker, H., Feinstein, R., Sawada, M., Coggeshall, R. 1976. Anatomical basis for an apparent paradox concerning conduction velocities of two identified axons in Aplysia. *J. Neurobiol.* **7:**241–253

Pohl, P., Saparov, S.M., Antonenko, Y.N. 1997. The effect of a transmembrane osmotic flux on the ion concentration distribution in the immediate membrane vicinity measured by microelectrodes. *Biophys. J.* **72:**1711–1718

Raschke, K. 1979. Movements of stomata. *In:* Encyclopedia of Plant Physiology, NS, vol 7. W. Haupt and E. Feinlieb, editors. pp. 383–441. Springer, Berlin Heidelberg New York

Rauchenberger, R., Hacker, U., Murphy, J., Niewohner, J., Maniak, M. 1997. Coronin and vacuolin identify consecutive stages of a late, actin-coated endocytic compartment in *Dictyostelium. Curr. Biol.* **7:**215–218

Raucher, D., Sheetz, M.P. 1999*a.* Characteristics of a membrane reservoir buffering membrane tension. *Biophys. J.* **77:**1992–2002

Raucher, D., Sheetz, M.P. 1999*b.* Membrane expansion increases endocytosis rate during mitosis. *J. Cell. Biol.* **144:**497–506

Raucher, D., Sheetz, M.P. 2000. Cell spreading and lamellipodial extension rate is regulated by membrane tension. *J. Cell. Biol.* **148:**127–136

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J.D., Sheetz, M.P., Meyer, T. 2000. Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell* **100:**221–228

Reuzeau, C., Mills, L.R., Harris, J.A., Morris, C.E. 1995. Discrete and reversible vacuole-like dilations induced by osmomechanical perturbation of neurons. *J. Membrane Biol.* **145:**33–47

Rosenmund, C., Stevens, C.F. 1996. Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron* **16:**1197–1207

Ross, P.E., Garber, S.S., Cahalan, M.D. 1994. Membrane chloride conductance and capacitance in Jurkat T lymphocytes during osmotic swelling. *Biophys. J.* **66:**169–178

Sachs, F., Morris, C.E. 1998. Mechanosensitive ion channels in nonspecialized cells. *Reviews of Physiol. Biochem. Pharmacol.* **132:**1–78

Sigurdson, W.J., Morris, C.E. 1989. Stretch-sensitive ion channels in growth cones of snail neurons. *J. Neurosci.* **9:**2801–2808

Smith, C., Neher, E. 1997. Multiple forms of endocytosis in bovine adrenal chromaffin cells. *J. Cell. Biol.* **139:**885–894

Sokabe, M., Sachs, F., Jing, Z.Q. 1991. Quantitative video microscopy of patch clamped membranes stress, strain, capacitance, and stretch channel activation. *Biophys. J.* **59:**722–728

Solsona, C., Innocenti, B., Fernandez, J.M. 1998. Regulation of exocytotic fusion by cell inflation. *Biophys. J.* **74:**1061–1073

Steponkus, P.L., Uemura, M., Balsamo, R.A., Arvinte, T., Lynch, D.V. 1988. Transformation of the cryobehaviour of rye protoplasts by modification of the plasma membrane lipid composition. *Proc. Natl. Acad. Sci. USA* **85:**9026–9030

Sukhorukov, V.L., Arnold, W.M., Zimmermann, U. 1993. Hypotonically induced changes in the plasma membrane of cultured mammalian cells. *J. Membrane Biol.* **132:**27–40

Synnes, M., Prydz, K., Lovdal, T., Brech, A., Berg, T. 1999. Fluid phase endocytosis and galactosyl receptor-mediated endocytosis employ different early endosomes. *Biochim. Biophys. Acta* **1421:**317–328

Tabarean, I.V., Juranka, P., Morris, C.E. 1999. Membrane stretch affects gating modes of a skeletal muscle sodium channel. *Biophys. J.* **77:**758–774

Terakawa, S., Nakayama, T. 1985. Are axoplasmic microtubules necessary for membrane excitation? *J. Membrane Biol.* **85:**65–77

Terasaki, M., Miyake, K., McNeil, P.L. 1997. Large plasma membrane disruptions are rapidly resealed by Ca^{2+}-dependent vesicle-vesicle fusion events. *J. Cell. Biol.* **139:**63–74

Thiel, G., Rupnik, M., Zorec, R. 1994. Raising the cytosolic Ca^{2+} concentration increases the membrane capacitance of maize coleoptile protoplasts: evidence for Ca^{2+}-stimulated exocytosis. *Planta* **195:**305–308

Thiel, G., Sutter, J.-U., Homann, U. 2000. Ca^{2+}-sensitive and Ca^{2+}-insensitive exocytosis in maize coleoptile protoplasts. *Pfluegers Arch.* **439:**R152–R153

Thiele, I., Warth, R., Bleich, M., Waldegger, S., Lang, F., Greger, R. 1998. Osmotically induced conductance and capacitance changes in

in vitro perfused rectal gland tubules of Squalus acanthias. *Kidney Blood Press Res.* **21:**317–324

Togo, T., Alderton, J.M., Bi, G.Q., Steinhardt, R.A. 1999. The mechanism of facilitated cell membrane resealing. *J. Cell. Sci.* **112:**719–731

Uehara, K., Miyoshi, M. 1999. Tubular invaginations with caveolae and coated pits in the sinus endothelial cells of the rat spleen. *Histochem. Cell. Biol.* **112:**351–358

Ursitti, J.A., Wade, J.B. 1993. Ultrastructure and immunocytochemistry of the isolated humane erythrocyte membrane skeleton. *Cell. Motil. Cytoskel.* **25:**30–42

Valentijn, K.M., Gumkowski, F.D., Jamieson, J.D. 1999. The subapical actin cytoskeleton regulates secretion and membrane retrieval in pancreatic acinar cells. *J. Cell. Sci.* **112:**81–96

Vandorpe, D.H., Small, D.L., Dabrowski, A.R., Morris, C.E. 1994. FMRFamide and membrane stretch as activators of the *Aplysia* S-channel. *Biophys. J.* **66:**46–58

Van Essen, D.C. 1997. A tension-based theory of morphogenesis and compact wiring in the central nervous system. *Nature* **385:**313–318

Walker, G.R., Kane, R., Burgess, D.R. 1994. Isolation and characterization of a sea urchin zygote cortex that supports in vitro contraction and reactivation of furrowing. *J. Cell. Sci.* **107:**2239–2248

Wan, X., Harris, J.A., Morris, C.E. 1995. Responses of neurons to extreme osmomechanical stress. *J. Membrane Biol.* **145:**21–31

Wan, X., Juranka, P., Morris, C.E. 1999. Activation of mechanosensitive currents in traumatized membrane. *Am. J. Physiol.* **276:**C318–C327

Wartenberg, M., Hamann, J., Pratsch, I., Donath, E. 1992. Osmotically induced fluid-phase uptake of fluorescent markers by protoplasts of *Chenopodium-album*. *Protoplasma* **166:**61–66

Weber, I., Niewohner, J., Faix, J. 1999. Cytoskeletal protein mutations and cell motility in *Dictyostelium. Biochem. Soc. Symp.* **65:**245–265

Weise, R., Kreft, M., Zorec, R., Homann, U., Thiel, G. 2000. Transient and permanent fusion of vesicles in *Zea mays* coleoptile protoplasts measured in the cell-attached configuration. *J. Membrane Biol.* **174:**15–20

Wirtz, H.R., Dobbs, L.G 1990. Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science* **250:**1266

Wolfe, J., Dowgert, M.F., Steponkus, P.L. 1985. Dynamics of membrane exchange of the plasma membrane and the lysis of isolated protoplasts during rapid expansion in area. *J. Membrane Biol.* **86:**127–138

Wolfe, J., Steponkus, P.L. 1983. Mechanical properties of the plasma membrane of isolated plant protoplasts. *Plant Physiol.* **71:**276–285

Zhang, Y., Hammill, O.P. 2000. On the discrepancy between whole-cell and membrane patch mechanosensitivity in *Xenopus* oocytes. *J. Physiol.* **523:**101–115

Zhang, L., Marcu, M.G., Nau-Staudt K., Trifaro, J.M. 1996. Recombinant scinderin enhances exocytosis, an effect blocked by two scinderin-derived actin-binding peptides and PIP2. *Neuron* **17:**287–296

Zhelev, D.V., Needham, D., Hochmuth, R.M. 1994. Role of the membrane cortex in neutrophil deformation in small pipettes. *Biophys. J.* **67:**696–705

Zheng, J., Lamoureux, P., Santiago, V., Dennerll, T., Buxbaum, R.E., Heidemann, S.R. 1991. Tensile regulation of axonal elongation and initiation. *J. Neurosci.* **11:**1117–1125

Zischka, H., Oehme, F., Pintsch, T., Ott, A., Keller, H., Kellermann, J., Schuster, S.C. 1999. Rearrangement of cortex proteins constitutes an osmorprotective mechanism in *Dictyostelium. EMBO J.* **18:**424–429

Zimmerberg, J., Bezanilla, F., Parsegian, V.A. 1990. Solute inaccessible aqueous volume changes during opening of the potassium channel of the squid giant axon. *Biophys. J.* **57:**1049–1064

Zorec, R., Tester, M. 1993. Rapid pressure driven exocytosis-endocytosis cycle in a single plant cell. *FEBS Lett.* **333:**283–286