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Actomyosin pulsing in tissue integrity maintenance during morphogenesis

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Summary

The actomyosin cytoskeleton is responsible for many changes in cell and tissue shape. For a long time, the actomyosin cytoskeleton has been known to exhibit dynamic contractile behavior. Recently, discrete actomyosin assembly/disassembly cycles have also been observed in cells. These so-called actomyosin pulses have been observed in a variety of contexts, including cell polarization and division, and in epithelia, where they occur during tissue contraction, folding, and extension. In epithelia, evidence suggests that actomyosin pulsing, and more generally, actomyosin turnover, is required to maintain tissue integrity during contractile processes. This review explores possible functions for pulsing in the many instances during which pulsing has been observed, and also highlights proposed molecular mechanisms that drive pulsing.

The pulsatile behavior of nonmuslce myosin 2 and filamentous actin

Nonmuscle myosin 2 (Myo2) and filamentous actin (F-actin) comprise a molecular machine capable of generating contractile force and changing cell shape (Kasza and Zallen, 2011; Lecuit et al., 2011; Vicente-Manzanares et al., 2009). Localization of F-actin and Myo2 (actomyosin) to specific subcellular structures, like the lamella, stress fibers, apical networks or adherens junctions, is critical to cellular actomyosin function (Murrell et al., 2015). Dynamics in both the spatial and temporal regulation of actomyosin have been observed in a growing number of biological contexts, especially in developmental systems, raising the question of how spatiotemporal actomyosin dynamics affect actomyosin function. One class of temporal actomyosin dynamics is a process broadly described as actomyosin pulsing, in which F-actin and Myo2 first assemble in a subcellular structure and then disassemble, on the time scale of minutes or less (Figure 1). Neither the mechanism nor the function of actomyosin pulsing is fully understood. In this review, we discuss observations of actomyosin pulsing in cellular and developmental contexts, we explore possible biological functions for pulsing, and we evaluate proposed molecular mechanisms for actomyosin pulsing.

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Pulsed actomyosin accumulation is thought to generate contractile (i.e. tensile) stress on the actomyosin cytoskeleton, which transmits force to cellular neighbors and substrates when attached to a cell's adhesion receptors (Gardel et al., 2008; Roh-Johnson et al., 2012; Thievessen et al., 2013). This transmitted force can either persist as tension in the epithelium, or it can resolve with the displacement of cell junctions and actomyosin toward the center of the contraction. Actomyosin pulses also often correspond with an accumulation and dissipation of F-actin (Blanchard et al., 2010; He et al., 2010; Mason et al., 2013; Rauzi et al., 2010; Valencia-Expósito et al., 2016). In some biological contexts, repetitive actomyosin pulses exhibit *bona fide* oscillatory behavior that can be identified as a dominant frequency using Fourier analysis (Gorfinkiel, 2016; Koride et al., 2014; Sokolow et al., 2012; Solon et al., 2009). In other contexts, however, actomyosin pulses may not occur at a well-defined frequency and are possibly stochastic (Xie and Martin, 2015). Another important distinction in discussing actomyosin spatiotemporal dynamics is that between pulses and traveling waves, recently reviewed by (Allard and Mogilner, 2013). These two phenomena are probably related, and sometimes occur together (Munro et al., 2004; Rauzi et al., 2010; Saravanan et al., 2013). We limit this review to a discussion of pulsing.

Actomyosin pulsing has been observed in cultured cells, and in developmental contexts like single-celled embryos or epithelia undergoing morphogenesis. Work in cultured cells has demonstrated that cortical actomyosin pulses can emerge after microtubule depolymerization (Bornens et al., 1989; Paluch et al., 2005; Pletjushkina et al., 2001). Cortical pulses have also been observed during mitosis, where they push cytosol back and forth across the cytokinetic cleavage furrow to maintain symmetry in the volume of the two presumptive daughter cells (Sedzinski et al., 2011). In endothelial cells, retraction cycles were shown to be triggered by bursts of Ca^{2+} that activate myosin (Tsai and Meyer, 2012). In developmental contexts, actomyosin pulsing was first reported in the *C. elegans* zygote, where pulsing is involved in establishing anterior-posterior polarity preceding the first cell division (Munro et al., 2004). Pulsing was later observed in the *Drosophila* embryo in several contexts: in apical constriction of epithelial cells of the ventral furrow (Martin et al., 2009), salivary gland (Booth et al., 2014), and renal tubules (Saxena et al., 2014); in constrictions of the amnioserosa cells during dorsal closure (Blanchard et al., 2010; David et al., 2010; Solon et al., 2009); during extension of the germband (Fernandez-Gonzalez and Zallen, 2011; Rauzi et al., 2010; Sawyer et al., 2011); and in the follicular epithelium surrounding and shaping the developing oocyte (He et al., 2010; Valencia-Expósito et al., 2016). They appear to shrink pre-existing cell contacts (Rauzi et al., 2010), and drive elongation of junctions, leading to tissue extension (Collinet et al., 2015; Yu and Fernandez-Gonzalez, 2016).

With improvements in live microscopy and the adaptation of F-actin and Myo2 fluorescent probes for more animal models, pulsing has also been observed in vertebrates. In the 8-cell mammalian blastocyst, actomyosin pulsing corresponds to an increase in cortical tension leading to embryo compaction (Maître et al., 2015). During *Xenopus* neural tube formation, Ca^{2+} bursts trigger accumulation of F-actin across the apical surface of neuroepithelial cells, driving apical constriction (Christodoulou and Skourides, 2015). In addition, actomyosin pulsing occurs during *Xenopus* gastrulation and mesoderm convergent extension (Kim and Davidson, 2011; Skoglund et al., 2008), and corresponds to dynamics in cell-cell adhesive

adherens junction complexes and F-actin (Pfister et al., 2016). Thus, actomyosin pulsing is a widely conserved mechanism for generating contractile force both in individual cells and tissues.

Biological functions of actomyosin pulsing

It is clear that pulsing can be correlated with cell shape change, suggesting that increased actomyosin concentration in contracting domains promotes temporary increases in mechanical force. However, pulsing may do more than change cell shape. As mentioned above, in individual cells, actomyosin pulsing may maintain cell volume symmetry across the cleavage furrow during cytokinesis (Sedzinski et al., 2011). In tissues, it is tempting to speculate that pulsing coordinates tissue-wide cell shape changes and tissue deformation. While there have been some observations of spatial patterns in pulsing behavior (Saravanan et al., 2013; Solon et al., 2009; Xie and Martin, 2015), it has been difficult to determine the importance of these patterns because perturbations of pulsing behavior intrinsically affect Myo2 motor activity. Nevertheless, several intriguing models have emerged.

In epithelia, there is substantial evidence suggesting that pulsing and actomyosin turnover are critical for tissue mechanical integrity during morphogenesis. A challenge in addressing the function of pulsing is to find perturbations that disrupt pulsing without obliterating the actomyosin machine. In a system involving a folding epithelium, the *Drosophila* ventral furrow, numerous perturbations have been found that abrogate actomyosin pulsing, while leaving the actomyosin machinery more-or-less intact (Jodoin et al., 2015; Mason et al., 2016; Vasquez et al., 2014). These perturbations include disrupting either Myo2 or F-actin turnover directly, or disrupting this dynamic turnover by interfering with upstream RhoA signaling dynamics. Interestingly, both types of perturbation lead to a phenotype where cytoskeletal networks of neighboring cells separate from each other, at least in part through the actomyosin network separating from intercellular junctions. This has led to a model for actomyosin pulsing in maintaining tissue integrity during intercellular actomyosin contractility (Figure 2). In the *Drosophila* ventral furrow cells, the connection between the actomyosin network and the adherens junction is dynamic in the unperturbed, and contracting, epithelia (Figure 2i); this connection is sometimes lost (Figure 2ii), and subsequently repaired through turnover of actin and Myo2 (Figure 2iii) (Jodoin et al., 2015). In the absence of turnover, intercellular connections cannot be repaired, leading to additional breaks and the tearing of the epithelium (Figure 2iv).

The importance of turnover during contraction reveals a paradox regarding contractility in a tissue. Cortical Myo2 and F-actin can generate a strong force, yet contraction also clusters Myo2 and F-actin, leading to disconnection of the intercellular actomyosin networks. To continuously transmit force to the cell junctions and to neighboring cells, the actomyosin network in the middle of a cell or apical domain has to maintain connections to distal adhesive structures at the cell periphery (Jodoin et al., 2015; Martin et al., 2010; Roh-Johnson et al., 2012). Modeling studies have demonstrated that F-actin turnover is an effective mechanism for dispersing contracted networks and sustaining force generation across a surface (Mak et al., 2016). Clustering can lead to the loss of global connectivity of the cytoskeletal network (Figure 2ii). Actin turnover allows actomyosin clusters to dissipate,

redistributing the network to enable further large contractions (Figure 2iii) (Mak et al., 2016). Therefore, pulsing may allow an intercellular actomyosin network to sustain contractility by counter-acting the clustering tendency of contracting actomyosin networks.

Pulsing may also (or alternatively) represent a transition state in actomyosin contractility. In several systems that exhibit pulsing, actomyosin pulses precede a more sustained contraction. For example, during *Drosophila* ventral furrow formation, early actomyosin pulses initially produce reversible, or “unratcheted”, contractions, where cell area expands after the pulse induces contraction (Xie and Martin, 2015). Relaxation was recently found to depend on the presence of a negative regulator of RhoA activity (see below), suggesting that pulse relaxation, or the unratcheted state, represents transient RhoA inactivation (Mason et al., 2016). This state then evolves to one in which more persistent actomyosin correlates with sustained, or “ratcheted”, cell constriction (Xie and Martin, 2015). A similar pattern appears in *Drosophila* dorsal closure, where early actomyosin pulses correspond with constricting and relaxing cell area, followed by sustained contraction and persistent Myo2 (Blanchard et al., 2010; Sokolow et al., 2012; Solon et al., 2009). Actomyosin pulsing might therefore reflect an intermediate state in the activation of Myo2 contractility where there is limited cell shape change. One can think of the “unratcheted” pulses as an idling state of the actomyosin motor, which actomyosin passes through before achieving a state that allows sustained contraction (Teo and Yap, 2016).

Mechanisms for actomyosin pulsing

Actomyosin contractility is regulated by the small GTPase RhoA. RhoA in turn is regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which respectively promote the inactive GDP-bound or active GTP-bound states of RhoA (Ridley, 2015). Two RhoA effectors that are particularly important for contraction are the formin Diaphanous (Dia), which nucleates/elongates unbranched F-actin, and Rho-Kinase (ROCK), which activates Myo2 through direct phosphorylation of the myosin regulatory light chain (Amano et al., 1996), and indirectly through inhibition of myosin phosphatase (Kimura et al., 1996). Importantly, actomyosin contractility and pulsing has only been observed with Myo2, and not other non-contractile myosins. Phosphorylation of the regulatory light chain assembles Myo2 into bipolar filaments, which are capable of binding and contracting F-actin (Sellers, 1991). Starting with this well-established parts list, the past five years have seen a growing effort to determine a molecular mechanism for actomyosin pulsing.

ROCK, “active” RhoA, and even a RhoGEF (*Drosophila* RhoGEF2) have all been observed to pulse in *Drosophila* epithelia (Mason et al., 2016; Munjal et al., 2015; Vasquez et al., 2014). This suggested the possibility that there is dynamic regulation of the RhoA GTPase. Such dynamics would require a negative regulator of RhoA. In the *Drosophila* ventral furrow, negative regulation was recently found to be mediated by a RhoGAP, C-GAP (Mason et al., 2016). In this system, the peak in RhoGEF2 signal precedes the peak Myo2 signal by about 10 seconds. C-GAP depletion prevents the Myo2 disappearance after increases in Myo2. C-GAP overexpression causes myosin to disappear completely after a Myo2 pulse. Thus, in the ventral furrow, RhoGEF2 appears to initiate pulses, presumably by

increasing the actomyosin assembly rate, and C-GAP terminates the pulse, either through constant disassembly (Figure 3, curve 1) or by increasing the disassembly rate after assembly (Figure 3, curve 2). Interestingly, removal of C-GAP from the ventral furrow disrupts not only pulsing, but also the spatial organization of RhoA pathway components (Mason et al., 2016). Future insight into the timing of C-GAP and its mechanism of recruitment will be important for elucidating the mechanism of pulsing. Additionally, it will be important to determine whether negative regulation of RhoA is a conserved mechanism to regulate pulsing dynamics in other cellular and developmental contexts.

A non-mutually exclusive hypothesis, is that actomyosin pulsing is a self-organized processes (Munjal et al., 2015). Pulses could be initiated through a low level of actomyosin activity resulting in “advection” or flow of F-actin, Myo2, and membrane associated proteins, such as ROCK, into a cluster (Munjal et al., 2015). Flow of proteins that regulate actomyosin contractility, such as ROCK, would result in a more sustained contraction. Disassembly of the pulse would occur through delayed negative feedback, possibly through the recruitment of myosin phosphatase to advection-assembled actomyosin structures (Munjal et al., 2015; Vasquez et al., 2014). In this model, pulsing results from the balance between a protein’s advective flow into the protein cluster and the dissociation of that protein from the membrane. A pulse cycle would represent the net flow of a protein into a cluster, slowing of this flow, and then dissociation outcompeting flow, which would represent pulse disassembly (Figure 3). Although such a mechanism can explain pulsing without the need for active regulation by RhoA, it is possible that advection could also mediate the behavior of proteins that regulate RhoA (i.e. GEFs and GAPs).

Evidence from other systems suggests that RhoA activity is dynamic and that RhoA dynamics mediates actomyosin dynamics. In mitosis, RhoA flux through the GTP hydrolysis cycle regulates the formation of the actomyosin cytokinetic furrow (Miller and Bement, 2009; Zanin et al., 2013). In addition, bursts or “flares” of Rho-GTP correlate with bursts of F-actin accumulation at apical adherens junctions in interphase cells depleted of the RhoA-binding protein anillin (Reyes et al., 2014), and cortical waves of RhoA activity have been observed after anaphase of frog or echinoderm embryonic cells (Bement et al., 2015). The protrusion-retraction cycle at the leading edge of migrating fibroblasts requires negative regulation of RhoA through Protein Kinase A (PKA) induced recruitment of Rho GDP-dissociation inhibitor (RhoGDI) (Tkachenko et al., 2011). Overall, these studies suggest that actomyosin contractile systems with behaviors such as pulsing, cycling, or waves are associated with dynamic regulation of RhoA, which could serve as a “pacemaker”. Processes such as advection could serve as a source of delayed negative feedback to regulate RhoA, for instance by recruiting an inhibitor of RhoA. Identifying further regulators of RhoA activity and how they are recruited is thus likely to be important for understanding what regulates the timing of different pulsing behaviors.

Finally, we note that actomyosin networks have been studied intensely *in vitro*, where variables such as myosin concentration, cross linker concentration, and actin filament orientation affect the contractile behavior of the network (Ennomani et al., 2016; Murrell et al., 2015; Reymann et al., 2012). However, to our knowledge, pulsing has not yet been reconstituted in an *in vitro* actomyosin network. This raises two interesting points. First,

what is the effect of varying network properties such as myosin and crosslinker concentration and actin filament orientation on *in vivo* actomyosin networks, and second, what allows *in vivo* actomyosin networks to pulse? We speculate that *in vitro* pulsing requires actin and myosin filament turnover rates above those that have been achieved *in vitro* and may require RhoA signaling to coordinate actin and myosin turnover.

Concluding remarks

One of the areas for which actomyosin pulsing presents an exciting explanatory potential is in regard to neural tube defects, which represent a relatively common human birth defect (Wallingford et al., 2013). Pulsatile actin dynamics were recently observed in *Xenopus* neural tube closure (Christodoulou and Skourides, 2015), and disrupting pulses by elevating cytosolic Ca^{2+} levels promoted neural tube defects. It was separately observed that the actin severing protein, cofilin, is important for neural tube closure (Escuin et al., 2015; Gurniak et al., 2005; Mahaffey et al., 2013). This suggests that actin turnover, and possibly actomyosin pulsing, is critical for neural tube morphogenesis. Because of the evidence from *Drosophila* tissue folding, it would be productive to investigate epithelial integrity during folding of the neural tube in these cases where actomyosin turnover is defective.

Pulsing has been observed and characterized in a growing number of biological contexts, and we suspect that the list will grow as fluorescent actin and Myo2 probes are visualized in more tissues. Though the mechanism of pulsing is not fully understood, actomyosin pulses have already been implicated in cell shape change by correlating the appearance of pulses with cell shape change. Current challenges are to determine the biological functions and the molecular mechanism(s) governing this widespread phenomenon (see Outstanding Questions).

Outstanding Questions Box

- In addition to being important for epithelial integrity during morphogenesis, does actomyosin pulsing serve other functions, such as modulating force production in a tissue?
- What is the complete parts-list for regulating the assembly, disassembly, and location of an actomyosin pulse?
- What sets the pace of actomyosin pulsing and what are the feedbacks involved?

In addition to exploring the models we review here, we see several opportunities for productive inquiry. First, it will be important to determine the function of actomyosin pulsing in additional systems. This requires careful analysis of mutants in genes that promote actomyosin assembly and disassembly, which could include: 1) actin regulators, 2) myosin phosphatase or genes that regulate Myo2 filament assembly, such as Protein Kinase C and Casein Kinase II, which phosphorylate the Myo2 heavy chain (Murakami et al., 1998; 1995), and 3) RhoA inhibitors, such as GAPs, RhoGDI, ubiquitin ligases, and kinases (Hodge and Ridley, 2016). Second, we propose adding additional complexity to

reconstituted actomyosin assemblies to determine the molecular mechanism by which dynamic cellular actomyosin networks generate and sustain force. Specifically, it is important to determine how actin filament turnover, Myo2 minifilament turnover, and adherens-junction-like boundaries affect the assembly and force-generating capacity of *in vitro* networks assembled with purified components. Third, because actomyosin dynamics have been correlated in several contexts with cell shape change, we look forward to experimental approaches that build functional evidence for this connection. For these experiments, we advocate the use of point-mutants with precise effects on biochemical activities, rather than global inhibition of myosin or actin with drugs, null mutants, or knockdown experiments. Fourth, we still do not understand what directs the location of an actomyosin pulse. Fifth, we advocate searches for proteins outside of the core actomyosin machinery, for example aPKC (David et al., 2013) and Crumbs (Flores-Benitez et al., 2015), that impinge on actomyosin dynamics. Finally, we are excited about using the newest generation of *in vivo* biomechanical sensors, like lipid droplets embedded in epithelia (Campàs et al., 2014) or single-walled carbon nanotubules (Tan et al., 2016) that can provide direct measurements of the material properties of cells and tissues experiencing dynamic actomyosin contractility. These tools will allow researchers to determine the relationship between actomyosin dynamics and cell and tissue forces and determine how tissue form emerges from dynamic and heterogeneous cell behaviors.

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Trends Box

- Actomyosin pulsing has been observed in many cellular contexts, in systems ranging from cell culture to developing epithelia in both invertebrates and vertebrates.
- The functions of actomyosin pulsing *in vivo* are not fully elucidated. However, evidence suggests they play a role in maintaining intercellular mechanical connections in tissues that undergo morphogenesis.
- Actomyosin pulsing involves actin and Myo2 turnover, and may be regulated by a combination of Rho signaling dynamics and self-organized advective flows.

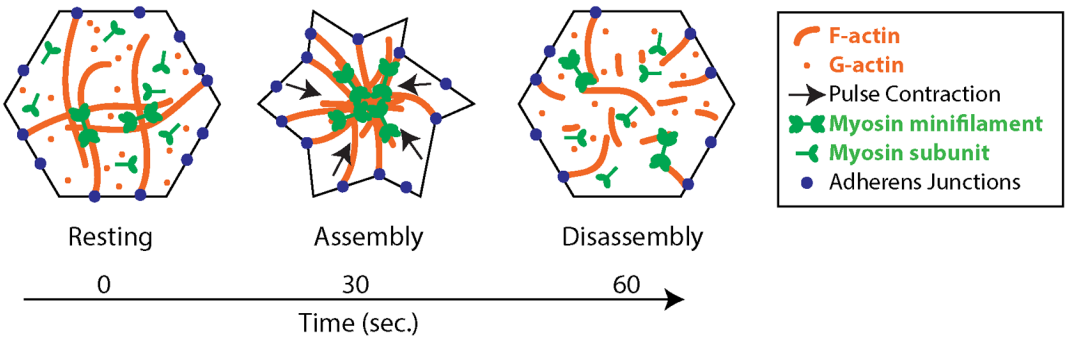

Figure 1.

A generalized schematic of actomyosin pulsing. Over the course of approximately one minute, Myo2 minifilaments assemble and contract an F-actin cytoskeletal network. This contraction can pull on cell adhesion receptors like adherens junctions or focal adhesions. Pulsed contraction is often followed by relaxation involving disassembly of Myo2 and the F-actin cytoskeleton.

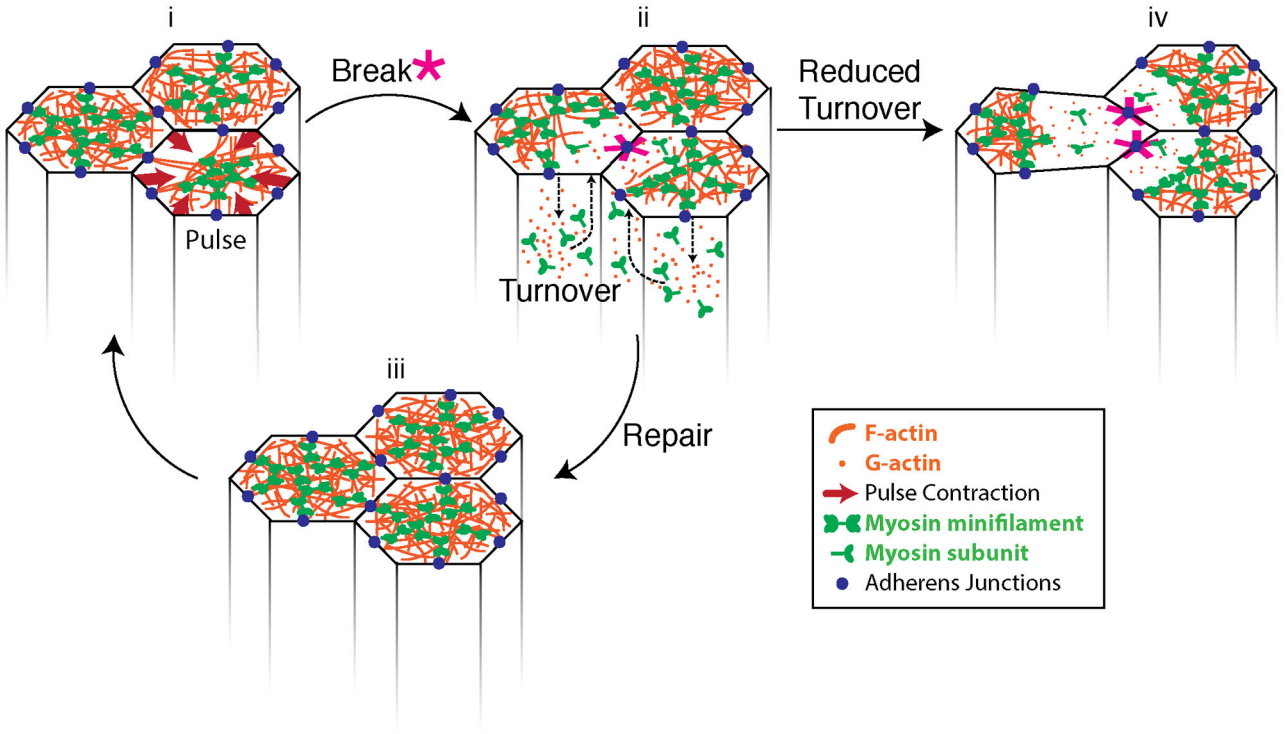

Figure 2.

Actomyosin turnover sustains intercellular actomyosin connectivity. **(i)** During epithelial morphogenesis, actomyosin pulses (dark red arrows) lead to contractions, **(ii)** contractility can disrupt intercellular actomyosin connections to adherens junctions (magenta asterisk). **(iii)** actomyosin turnover repairs these connections in wild-type cells, **(iv)** but with reduced turnover, tears appear in the epithelium between cells, interfering with morphogenesis.

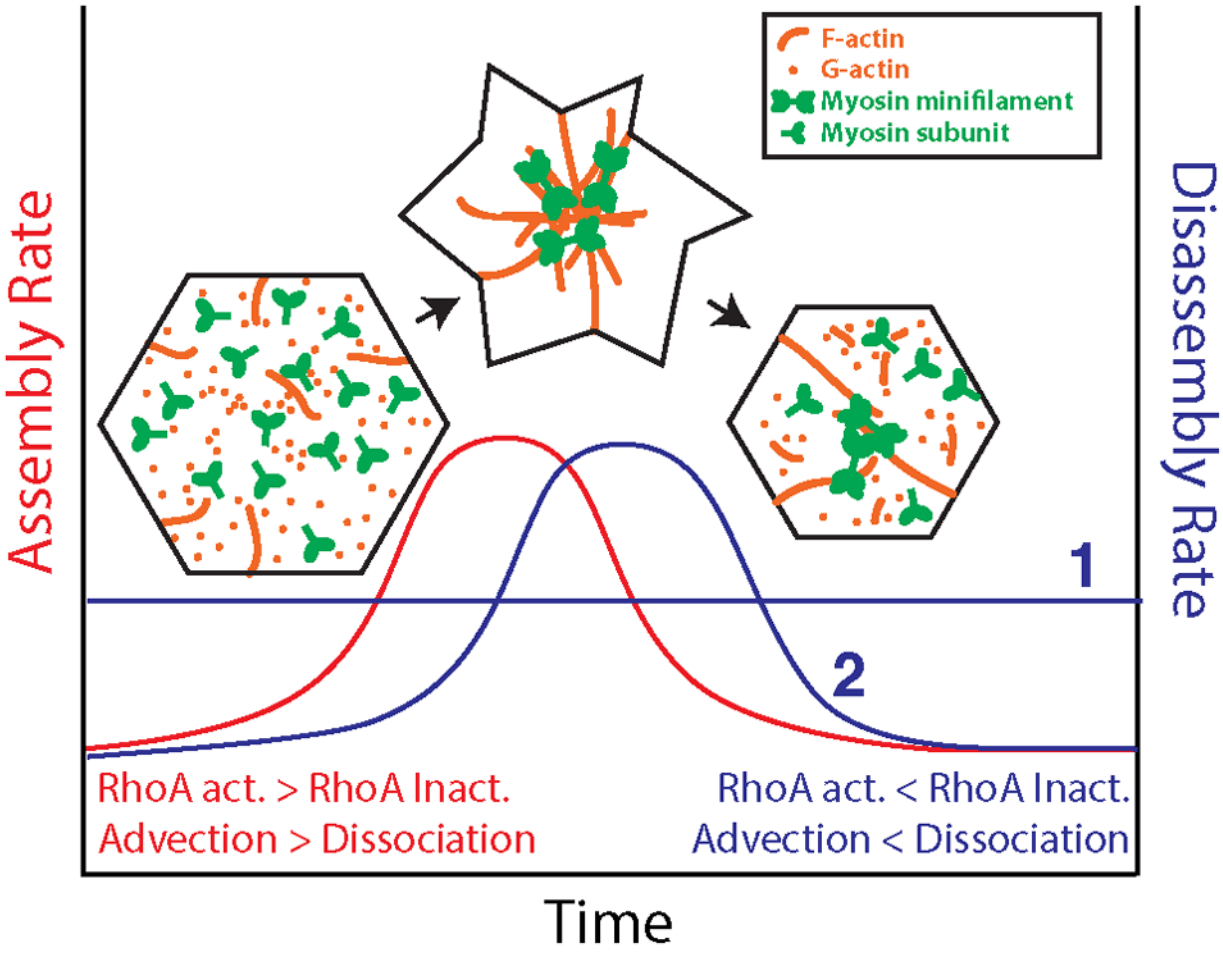

Figure 3.

Model for Myo2 and actin turnover in actomyosin pulses. Actomyosin pulse assembly occurs through a combination of RhoA activation via a RhoGEF, and actomyosin advection. Disassembly occurs through a combination of RhoA inactivation through a RhoGAP, and through dissociation of actomyosin from the apical actin cortex. The balance between assembly and disassembly is determined by the relative amount of RhoA activation/deactivation, or advection/dissociation. It is unclear whether disassembly is occurring at a constant rate (blue curve 1) or spikes after pulse assembly (blue curve 2).