Actin and PIP3 waves in giant cells reveal the inherent length scale of an excited state

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Running title: Actin and PIP3 waves in giant cells

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Keywords: actin waves, PIP3 signals, excitable systems, cell polarity, cell fusion.

Author contributions
Carsten Beta and Günther Gerisch designed the research project. Mary Ecke, Matthias Gerhardt, Andreas Stengl and Michael Walz performed experiments and analyzed data. Carsten Beta, Matthias Gerhardt and Günther Gerisch evaluated the results and wrote the paper.
Abstract

The membrane and actin cortex of a motile cell can autonomously differentiate into two states, one typical of the front, the other of the tail. On the substrate-attached surface of Dictyostelium cells, dynamic patterns of front-like and tail-like states are generated that are best suited to monitor transitions between these states. To image large-scale pattern dynamics independent of boundary effects, we produced giant cells by electric-pulse induced cell fusion. In these cells actin waves are coupled to the front and back of PIP3-rich bands that have a finite width. These composite waves propagate across the plasma membrane of the giant cells with undiminished velocity. After any disturbance, the bands of PIP3 return to their intrinsic width. Upon collision, the waves locally annihilate each other and change direction; at the cell border they are either extinguished or reflected. Accordingly, expanding areas of progressing PIP3 synthesis become unstable beyond a critical radius, their center switching from a front-like to a tail-like state. Our data suggest that PIP3 patterns in normal-sized cells are segments of the self-organizing patterns that evolve in giant cells.
Introduction

Actin-based force generation is the principal mechanism of motility in eukaryotic cells. A signature of directional locomotion in many cell types is the polarization in well-defined front and tail regions that differ in composition and dynamics of the actin cytoskeleton and in the phosphoinositide signaling system of the membrane. In the front region, actin is polymerizing to drive the formation of membrane protrusions, whereas in the tail region, the cell body retracts due to the contractile action of myosin II. The leading edge corresponds to a PIP3 enriched membrane at the front. The front is distinguished from the back of the cell, which is dominated by the PIP3 degrading phosphatase PTEN (Funamoto et al., 2002; Iijima and Derrestos, 2002). Front and tail type regions can also self-organize into dynamic patterns on the planar bottom surface of a cell, where transition zones between the two states travel along the substrate-attached membrane (Arai et al., 2010; Schroth-Diez et al., 2009; Tanigushi et al., 2013). These zones are decorated with an actin-rich band, called an actin wave.

An overview on various types of actin-based waves has been provided by Allard and Mogilner (2013). The actin waves studied by us in Dictyostelium cells differ in principle from waves that are produced in an in-vitro motility assay by instabilities in the myosin-driven translocation of pre-established actin filaments (Schaller et al., 2010). As revealed by FRAP, the actin waves in Dictyostelium travel by the net polymerization of actin at their front and net depolymerization at their back (Bretschneider et al., 2009). Similarly, waves of Hem-1, a subunit of the WAVE complex that promotes Arp2/3-mediated actin polymerization, propagate by the recruitment of Hem-1 at their front and its release at their back (Weiner et al., 2007). In human osteosarcoma cells, ventral actin waves can generate trans-membrane signals by integrin activation (Case and Waterman, 2011). These adhesive waves are associated with the Arp2/3 complex, as the actin waves in Dictyostelium.

No membrane folds have been detected beneath the actin waves on the substrate-attached surface of Dictyostelium cells (Gerisch et al., 2004). The planar membrane on which these
waves are traveling distinguishes them from circular dorsal ruffles, which are shaped by curved proteins that bind to convex or concave portions of the membrane and activate the polymerization of actin (Peleg et al., 2011).

Actin waves on a planar substrate-attached cell surface are not necessarily associated with cell movement or changes in cell shape, and thus differ from waves that reflect spatio-temporal patterns in the protrusive activity of a motile cell. The latter class of waves comprises periodic actin-based protrusions in various mouse and Drosophila cells (Döbereiner et al., 2006) and curvature waves in Dictyostelium that travel from the leading edge to the tail of a cell (Driscoll et al., 2012).

The actin waves propagating on the substrate-attached surface of Dictyostelium cells are supposed to be instrumental in the search for bacteria to be taken up by phagocytosis (Gerisch et al., 2009). These waves typically display a closed, ring-shaped structure and circumscribe a PIP3-rich inner territory that differs from an external area decorated with PTEN, a PIP3 degrading PI3-phosphatase. Several criteria relate the inner territory to the front and the external area to the tail region of a polarized cell. The membrane of the inner territory is distinguished not only by the enrichment in PIP3 and the lack of PTEN, but also by the activation of Ras (Gerisch et al., 2011). Correlated with differences in the membrane are differences in the structure and composition of the underlying actin network: the inner territory of densely packed actin filaments is enriched in the Arp2/3 complex, the external area consists of wide-meshed actin bundles that are associated with myosin-II and the actin-bundling protein cortexillin (Schroth-Diez et al., 2009). Thus the wave patterns on the substrate-attached cell surface provide an opportunity to image, within one plane of focus, transitions between two states of the cell membrane in line with changes in the underlying actin cortex (Gerisch et al., 2012).

Excitability of the membrane and cortical actin layer has been implicated in the periodicity of actin polymerization at the leading edge (Ryan et al., 2012) and in the chemotaxis of
eukaryotic cells (Shi and Iglesias, 2013; Nishikawa et al., 2014). The wave dynamics in
Dictyostelium provides a system to study the propagation of an excited state within a single
cell (Gerisch et al., 2012). The wave patterns previously analyzed were formed within the
boundaries of a single cell where the lateral borders confined the space for wave expansion to
10 – 20 μm, allowing only sections of the pattern to develop. To eliminate these restrictions of
pattern development, we used cells in which the total membrane area is greatly enlarged such
that the cell borders are far off.

Giant cells were either produced by cultivating myosin-II deficient cells in suspension
where the mutant cells are unable to divide (DeLozanne and Spudich, 1987; Knecht and
Loomis, 1987), or they were generated in a wild-type background by electric-pulse induced
cell fusion (Gerisch et al., 2013). The giant cells enabled us to investigate the unpinned and
undamped propagation of actin waves over distances that by an order of magnitude exceeded
the radius of a normal cell. We will demonstrate that PIP3 patterns self-organize into dynamic
zones with an intrinsic length scale. Independent of cell size, this length scale determines the
distance between leading and trailing actin waves that are coupled to the borders of the PIP3-
rich territories.
Results

*Dictyostelium* cells fused by electric pulses form an intact actin cortex that supports long-range wave propagation

To monitor actin waves on the substrate-attached surface of giant cells, we fused cells that expressed markers for both the front and tail region of a normal motile cell: mRFP-LimEΔ, a label for filamentous actin that is enriched at the front (Fischer et al., 2004), and GFP-myosin-II heavy chain, a marker for the tail region (Moores et al., 1996). The electro-fused cell shown in Figure 1 exemplifies three features that are typical of the wave patterns in giant cells: (1) the waves can propagate with undiminished velocity over long distances, largely undisturbed by the cell boundary, (2) when waves collide, they tend to extinguish each other, and (3) there is no persistent pacemaker that serves as the origin of a periodic wave pattern. In this large cell, a zone enriched in myosin-II was formed at an average distance of 6 µm behind the peak of the actin wave, independently of the cell border.

Features of actin patterns in giant cells

A variety of wave patterns in giant cells is illustrated in Figure 2, showing a large myosin-II-null cell that expressed LimEΔ-GFP as a label for filamentous actin. Dominant features of the patterns are actin enriched bands with peaks of actin accumulation at their border (Fig. 2A). These bands are polarized and mobile. They propagate across the substrate-attached surface of the cell with one of their broadsides ahead with an average velocity of 0.12 µm x s⁻¹ ± 0.06 (s.d.). The actin rich border of the band corresponds to the actin waves previously studied in normal-sized cells (Gerisch et al., 2011). In the giant cells, these waves consist of a leading and a trailing segment (Fig. 2B and Movie 1). In accord with the terms employed for normal-sized cells, we designate the band-shaped regions surrounded by the actin waves as “inner territory” and the regions outside the wave as “external area” (Gerisch et al., 2011).
normal-sized cells, the inner territory has been shown to be enriched in the Arp2/3 complex, the external area to be associated with myosin-II (Schroth-Diez et al., 2009).

Typically, pattern formation begins with the local clustering of actin, followed by the circular spreading of a wave from the initiation site (Fig. 2C, D). The concentric pattern eventually breaks and gives rise to waves that continue to propagate as actin-rich bands in radial direction (Fig. 2E 553 and 561 s frames). When the bands collide, they extinguish each other locally followed by their fusion, as shown in Figure 2E (582 to 618 s frames) and in Movie 1.

**Expanding waves maintain a constant width**

The overview of shape dynamics shown in Figure 2 and Movie 1 reveals a preference for band-shaped waves with a preserved width. To substantiate this observation, we have developed an image processing tool to represent the wave width and its changes over time. If a pattern is dominated by a specific width this will produce a peak in the distribution plot. The evolution of a dominant width is reflected in changing positions of the most prominent peak as a function of time. This analysis is illustrated in Figure 3A to D for two scenarios, the expansion of a circular wave and the propagation of a band-shaped wave.

The actin wave shown in Figure 3A undergoes expansion until, at a critical size, a circular trailing wave is inserted that leads to a “doughnut” pattern. In the evolution of the width distribution, the circular expansion is reflected in a gradual shift of the dominant peak to larger values, until at 48 s the preferred width of around 12 µm is restored by the emerging doughnut shape (Fig. 3E).

The width of the wave displayed in Figure 3B and Movie 3 stays constant at about 15 µm for more than five minutes until, after 400 s, a segment of the wave widens, resulting in a second peak in the distribution that gradually shifts to larger values (Fig. 3F). After 470 s, the widened area is converted into an arc-shaped structure and the distribution returns to a single
peak between 10 and 15 µm. The overview of Movie 3 adds further examples for relaxation of
the system into that preferred width between the outer borders of the actin wave. In
conclusion, key principle of the wave pattern is an inherent length scale that does not vary
with cell size.

**PIP3 dynamics in the wave patterns of giant cells**

The territory enclosed by the leading and trailing segments of an actin wave propagates as a
coherent PIP3 enriched band (Fig. 4A, B and Movie 4). To explore the PIP3 dynamics during
wave propagation, we measured fluorescence intensities of the marker GFP-PHcrac (Parent
and Devreotes, 1999) at multiple points on the substrate-attached surface of giant cells. For an
unbiased sampling of temporal changes in a field of PIP3 waves, points of measurement were
distributed on a rectangular grid at a distance of 10 µm (Fig. 4C). This is about the diameter of
a non-fused, normal-sized cell. At each site, the passage of a wave produced a transient
increase in PIP3. As an example, the time series obtained at the point encircled in Fig. 4C is
displayed in Figure 4D. The distribution of peak-to-peak intervals extends over a wide range,
suggesting that the system did not oscillate at a specific frequency (Fig. 4E).

Among the transient PIP3 increases, we selected those corresponding to waves that were
not influenced by the cell border or by the interference with another wave. Averaging the
scans shown in Figure 4F resulted in a curve with a width at half-maximum of 44 s. A rise
time of 17 s from half-maximum to maximum and a slower decay time of 27 s from maximum
down to half-maximum results in an asymmetric shape of the temporal PIP3 profile. No
extended plateau of PIP3 accumulation was observed. The spatial profile of PIP3 normal to
the direction of wave propagation is shown in Figure 4G. This profile is characterized by a
distance from half-maximum to maximum of 2.1 µm, and a distance from maximum back to
half-maximum of 3.0 µm. The mean velocity of propagation was 0.13 µm x s⁻¹ ± 0.03 (s.d.).
Inner territory converts to external area when a trailing wave is inserted

As shown in Figure 4F and G, the PIP3 increase during passage of a band-shaped wave across a point of the cell surface has a limited lifetime of about 44 seconds and a width in space of 5 µm at half maximum. This pattern evolves from circular waves that radially expand from their site of origin, as shown for an early stage of pattern formation in Figure 1. If the lifetime of the PIP3-rich state of the membrane is limited, there should be a critical radius beyond which the territory within a circular wave becomes unstable such that its center will turn from the PIP3-rich into a PIP3 depleted state. Figure 5 shows three examples of how the conversion of a circular PIP3 pattern into a propagating band pattern takes place. In the case of Figure 5A, also shown in Movie 5, the circular area is almost symmetrically divided into two bands by the lateral ingression of external area from two opposite sides. The measurement of PIP3 dynamics at different points in the field indicate a change from longer to shorter persistence times during evolution of the circular area into propagating bands (Fig. 5 B). It appears, therefore, that during expansion of a circular wave, PIP3 is in a metastable state, before it relaxes into the steady state of synthesis and degradation that dominates the band pattern of PIP3 in giant cells. Often the external area ingresses only from one side, converting the circular PIP3-rich area into a horseshoe-shaped band with two open ends.

The two cases shown in Figure 5 C and D are distinguished by a prolonged preservation of symmetry. Here the coherent PIP3-rich area is transformed into a “doughnut” pattern consisting of a PIP3-rich annulus and a PIP3 depleted area in the center of the doughnut (Fig. 5C and Movie 6). During the decline of PIP3 in this area, a circular actin wave is inserted. To provide evidence that the PIP3 depleted area changes its specification, we have colabeled cells with GFP-myosin-II heavy chains. This label indicates that filamentous myosin-II strongly
assembles in the central area of a doughnut pattern, thus underscoring the transition from a front-like into a tail-like state (Fig. 5D and Movie 7).

The preference for band patterns with a defined width is also maintained during splitting, curling and budding of PIP3-rich territories. In particular, lateral expansion contributes to the elongated shape of the bands (Fig. S1).

Variability of refractory phases

In an excitable system, a period of stimulation is typically followed by a refractory phase during which a stimulus cannot evoke a second response. The unidirectional propagation of PIP3 waves suggests that after a period of activation the PI3 kinases pass a state of refractoriness. Moreover, when two waves collide, they most often extinguish each other, as shown in Figure 2E and Movies 4 and 8. This behavior is typical of waves in an excitable system that are followed by a refractory phase. Nevertheless, a detailed analysis of pattern dynamics in giant cells indicated that the refractory phase can be short, and an absolute refractoriness is sometimes lacking. In the giant cells, we investigated the variability of refractory phases using waves that collide with the cell border.

Frequently, waves disappeared from the substrate-attached cell surface after collision with the cell border, as expected if excitation is followed by a refractory phase (Fig. 6A, B). In other cases, the waves were not completely extinguished at the border of the cell; remnants persisted and propagated backward along the substrate-attached cell membrane. In these remnants of a wave, PIP3 remained at a high level for a longer period of time than during unperturbed wave propagation (Fig. 6C, D as compared to Fig. 4F).
Discussion

Self-organization of wave patterns in giant cells

Subject of the present paper is the self-organization of PIP3 and actin waves on the inner face of the substrate-attached membrane in giant cells of *D. discoideum*. We produced these cells by electric-pulse induced cell fusion or by myosin-II knockout in order to investigate pattern dynamics not limited by the narrow borders of normal-sized cells, which have been studied previously (Gerisch et al., 2012). As the wave patterns in normal cells, those in giant cells are generated spontaneously. The patterns develop independently of external chemoattractant gradients or any other structured impact from the environment, indicating that they evolve by self-organization.

The principal pattern elements in giant cells are PIP3-rich bands of finite length that propagate along the membrane. These bands are flanked at their front by the leading segment of an actin wave and at their back by a weaker (sometimes missing) trailing segment. This means, each segment of the actin wave separates two states of the membrane from each other, a PIP3-rich and a PIP3 depleted state. The pattern-generating system can be considered as an excitable medium the excited state of which consists of a composite wave embracing the PIP3-rich territory together with the flanking segments of an actin wave (Fig. 7). This implies that the actin pattern is coupled to the on and off of PIP3 synthesis, and that segments of an actin wave are formed at two transition states: one from low to high, the other from high to low PIP3.

In previous experiments we have enhanced the wave formation by pre-treating cells with latrunculin A to block actin polymerization (Gerisch et al., 2004). During recovery from the drug, the cells pass consistently a stage of profuse wave formation. However, actin waves are also formed on the substrate-attached surface of untreated cells, in particular during the first few hours of starvation (Bretschneider et al., 2004; Taniguchi et al., 2013), and the giant cells maintain this behavior.
Wave dynamics in giant cells as compared to normal ones

In normal-sized cells, actin waves have been shown to alternately expand and retract (Gerisch et al., 2011), resulting in the presence of actin waves at the front of an expanding and at the back of a shrinking PIP3-rich area (Asano et al., 2008). The pattern in giant cells shows that actin waves at the front and at the back of a PIP3-rich area are in fact sectors of a wave that are coupled to each other through the finite width of the PIP3-rich zone. The expanding waves correspond to the leading segments of an actin wave in giant cells, and the retracting waves to its trailing segments. This means that the wave patterns previously observed in normal-sized cells are confined sectors of the multitude of configurations that, due to the larger space available, can freely evolve in giant cells (Fig. 8). Notably, the leading segment of an actin wave in giant cells propagates from a PIP3-rich territory toward the PIP3 depleted external area, while the trailing segment moves the opposite way toward increasing concentrations of PIP3.

Waves in giant cells can propagate with undiminished velocity and amplitude over distances almost one order of magnitude larger than the radius of a normal cell (Fig. 1). The average velocity of 0.11 µm x s⁻¹ for the propagation of PIP3 bands in electro-fused-cells is similar to that in normal-sized cells where an average of 0.14 µm x s⁻¹ was found (Gerisch et al., 2012). In the cell shown in Figure 1, a velocity of 0.043 µm x s⁻¹ was found for an actin wave (Fig. 1 B). This exceptionally low value is probably due to the fact that in this case a circular wave was addressed at an early stage of pattern development.

The actin waves are independent of myosin-II (Bretschneider et al., 2009). Cells lacking myosin-II heavy chains profusely form waves (Fig. 2 and Movie 1), which propagate at a velocity of 0.12 µm x s⁻¹ similar to those in wild type cells, indicating that the filamentous myosin has no substantial influence on wave propagation. As in wild-type cells, the actin waves in myosin-II-null cells enclose a dense fabric of actin filaments and separate this inner
territory from an area with a loose filamentous network (Fig. 2 B). These data indicate that myosin-II, although being enriched in the external area, is not required for the switch from inner to external area nor for differentiation of the cortical actin structure.

**PIP3-rich territories relax to a defined width**

The evolution of spatio-temporal patterns in giant cells starts with expanding circular waves. When their radius exceeds a critical length, the PIP3-rich membrane area becomes unstable. Depletion of PIP3 and assembly of filamentous myosin-II indicates a state transition that corresponds to a switch from the “front” to the “tail” state of membrane and cortex organization (Fig. 5D). As a consequence of the limited width, curved bands are generated that are rich in PIP3 and surrounded by an actin wave. These PIP3-rich bands have an average width at half-maximum of 5.1 μm (Fig. 4G). This width is determined by the temporal sequence of PIP3 synthesis and degradation, which limits the persistence of the high-PIP3 state to 44.5 s at half-maximum (Fig. 4F). This characteristic lifetime of the PIP3-rich state in giant cells is similar to 46 s ± 7 (s.d.), the lifetime in normal, non-fused cells (Gerisch et al., 2012).

The giant cells show that the width of the PIP3-rich bands is not determined by the cell border or any other compartmentalizing membrane. The defined width appears to be unique to PIP3-rich regions of the membrane; we did not find a fixed length scale for PIP3 depleted areas. As a consequence, the PIP3 bands are not generated by a stable pacemaker that sets a specific frequency. Instead, the peak-to-peak intervals of PIP3 waves measured at various points on the cell surface show a broad distribution (Fig. 4E).

The mechanism that determines the intrinsic width of a PIP3 band may be relevant to the polar organization of a normal-sized cell with a front-to-tail distance of 10 – 20 μm. This mechanism seems to limit not only the front region in a motile cell but also the length of the PIP3-rich zone in a phagocytic cup. In phagocytic cups that enclose a long cylindrical particle,
the PIP3-rich section of the membrane tube that encloses the particle has a limited length of about 8 µm, even when the entire phagocytic cup is longer (Gerisch et al., 2009).

Characteristics of the excitable system as disclosed in giant cells

The wave-generating cell membrane together with the associated cortical region behaves as an excitable system consisting of multiple components that are coupled to each other. Two features of this system unveiled in giant cells illustrate specific properties of the underlying excitable system. First, sites of wave initiation are generated stochastically. The wave-generating system in Dictyostelium cells thus behaves as an excitable medium with random fluctuations that occasionally cross the excitation threshold so that a wave is initiated at a random location. This is reminiscent of an “extreme event” that may occur in an excitable system modeled by diffusively coupled FitzHugh-Nagumo units (Ansmann et al., 2013).

A second characteristic feature is that refractory phases are variable and sometimes indistinct. Refractory phases are critical when waves interact with each other or with the cell border. If a refractory phase does exist, waves annihilate each other at the site of collision (Fig. 2E and Movie 4). First, the leading segments of the actin waves fuse and disappear while the trailing segments continue to propagate until both types of segments are extinguished at the site of collision (Fig. 2E, 582 and 612 s frames). Left and right to this site, the waves will fuse such that two new waves are formed that propagate in opposite directions, each of them normal to the previous axis of wave propagation (Fig. 2E, 618 s frame and Movie 8).

For waves interacting with the cell border we observed two different scenarios attributable to variability in refactororiness (Fig. 6). Consistent with full refactororiness, a wave may be annihilated upon collision with the cell border (Fig. 6A, B). However persist at the border until the PIP3-rich territory expands in reverse direction, so that the wave appears to be reflected, implying the absence of a refractory phase (Fig. 6C, D). Lack of a refractory phase is also evident when a wave changes direction, as shown in Figure 3B and diagrammed in Figure 8A. In this case the trailing segment of an actin wave is converted into a leading one.
and the PIP3-rich territory turns from shrinkage to expansion. From this flexibility in behavior a broad range of dynamical regimes of the underlying pattern generating system may be deduced.

Relevance of the data to models of wave dynamics

Wave reflection at non-flux boundaries has been reported for various excitable systems (Petrov et al., 1994; Argentina et al., 1997; Hayase and Otha, 1998). Among them are different versions of the Oregonator model of the Belousov-Zhabotinsky reaction (Kosek and Marek, 1995; Bordyugov and Engel, 2008) and of the Hodgkin-Huxley model (Aslanidi and Mornev, 1997). In a system with three coexisting steady states discussed by Petrov et al. (1994), the waves revert direction without annihilation of their maxima, similar to the waves reflected at the border of a Dictyostelium cell (Fig. 6C, D). The reversal of direction in the model system has been attributed to the effect of a boundary on the diffusive fluxes of reactants into and out of the wave.

Recent models of actin waves focus on the interactions of small GTPases with the machinery of actin polymerization, consistent with the finding that actin waves in Dictyostelium are coupled to patterns of PIP3 (Asano et al., 2008; Gerisch et al., 2009) and of Ras signaling (Gerisch et al., 2011) on the underlying membrane. In the model analyzed by Mata et al. (2013), the GTPase enhances its own activation by positive feedback, and is inactivated by negative feedback from F-actin. It is essential for this model that the concentration of a GTPase, i.e. the sum of its concentrations in the active and inactive state, is constant. This is a reasonable assumption for the closed volume of a living cell that continues to generate waves for more than an hour. The model allows wave reflection on a diffusion barrier, a behavior that we have obtained. In a certain parameter space, the model proposes pinning of the actin waves, which we did not observe for waves that travel along the extended membrane area of the giant cells.
The model of Khamviwath et al. (2013) proposes a positive feedback circuit between GTPase, PIP3 and F-actin, based on experimental data by Sasaki et al. (2007), and a second positive feedback loop due to Arp2/3-mediated branching of actin filaments, as suggested by Carlsson (2010). The decay on the back of the waves is thought to be caused by local exhaustion of one of the constituents of the actin network. This model is consistent with the observed changes of direction in the propagation of actin waves and with their annihilation at the sites of collision.

The patterns obtained in giant cells provide a basis for explicit models of traveling actin waves as they are generated spontaneously in Dictyostelium cells. A characteristic of these waves is their division into two segments that are separated by a finite width of a PIP3 enriched membrane space.
Materials and methods

Cell strains and culture conditions

Cells of *D. discoideum* strain AX2–214 were transfected with integrating vectors to express GFP and/or mRFP fusion proteins as compiled in Supplemental Table I. Cells were cultivated in HL5 or modified maltose-containing medium with selection markers blasticidin, hygromycin, and/or G418. The PIP3 marker GFP-PHcrac was used in two versions: for Figure 4A, B it was expressed on an extrachromosomal vector in AX3 cells (Parent and Devreotes, 1999); otherwise as a superfolding GFP construct in AX2 cells (Müller-Taubenberger and Ishikawa-Ankerhold, 2013). Cells of the AX2-derived myosin-II heavy-chain null mutant HS2205 (Manstein et al., 1989) were cultivated for 2 days in shaken suspension to produce large cells by the prevention of cytokinesis. Temperature was within 20 to 23 °C throughout the experiments.

Cell fusion

Transformed cells were fused by electric pulses using one of two different protocols. 

Protocol 1. Cells were harvested from non-confluent Petri dishes, washed twice in 17 mM K/Na-phosphate buffer pH 6.0, adjusted in the buffer to 1.5 x 10⁷ cells/ml, and gently shaken for 3 h in roller tubes, allowing the cells to agglutinate. Using a pipet with the tip cut-off to prevent dissociation, aliquots of the suspension were transferred to electroporation cuvettes with an electrode distance of 4 mm and fused in a BioRad Gene Pulser Model 1652077 (Bio-Rad Laboratories, Hercules, CA94547) by applying 3 pulses of 1kV and 1 or 3 µF at 1 s intervals. A 20 µl aliquot of the fused cell suspension was transferred into an open chamber on a glass coverslip. After 5 minutes 1 ml of the phosphate buffer supplemented with 2 mM CaCl₂ and 2 mM MgCl₂ was added, and after settling the cells were subjected to imaging.
Protocol 2. Cells were cultivated in suspension up to the end of exponential growth. Then the cells were washed in an aqueous solution of 340 mM glucose and resuspended at 1×10^7 cells/ml in this dielectric medium. After shaking for 12 h the cells were washed twice and adjusted to 5×10^6 cells/ml in the same solution. Cells were fused within glass-bottom culture dishes equipped with aluminum electrodes spaced by 4 mm. An ECM 2001 Electro Cell Manipulator (Harvard Apparatus, Holliston, MA 01746-1388) was set to loop nine times over a dielectrophoresis step of 70 V for 8 s, followed by a 1 kV pulse for 50 µs and another dielectrophoresis step for 1 s. The entire procedure was repeated and the cells were gently transferred to 35 mm glass bottom culture dishes where they were stepwise equilibrated with 17 mM K/Na-phosphate buffer pH 6.0, supplemented with 2 mM CaCl_2 and 2 mM MgCl_2.

**Image acquisition and analysis**

Confocal images were acquired at a Zeiss LSM 780 equipped with a Plan-Apo 63x/NA 1.46 or with a Plan-Apo 40x/NA 1.4 oil immersion objective (Carl Zeiss Microscopy, 07745 Jena, Germany). An algorithm was designed to analyze the shape of waves as follows. At a pixel size of 0.181 µm for Fig. 3 A and 0.191 µm for Fig. 3 D, the actin wave was defined manually by pointing on its perimeter and rendered automatically by a polygon with a point-to-point distance of 10 pixels. A subroutine calculated the length of all secants directed from each point of the polygon perpendicularly across the wave (for examples, see Figure 3). The probability of secant lengths was color-coded and the temporal evolution of the length distribution presented as a kymograph (Fig. 3C and F).

Fluorescence intensities were processed using the image processing package Fiji (http://Fiji.sc/Fiji) developed by Schindelin et al. (2012) on the basis of ImageJ (http://imagej.nih.gov/ij). Plugins of Fiji were used for point scans with an oval area of 4 x 4
pixels and for line scans with a width of 30 pixels. Data were copied in a Microsoft Excel spreadsheet for calculation and chart plotting.

Acknowledgements

We thank Kirsten Krüger (Institute of Physics and Astronomy, University of Potsdam), Jana Prassler (MPI for Biochemistry) for providing cell cultures, and Annette Müller-Taubenberger, LMU München, for superfolding GFP-PHcrac. G. G. thanks the Max Planck Society for support.

The authors declare no competing financial interest.
References


Ansmann, G., Karnatak, R., Lehnertz, K. and Feudel, U. (2013). Extreme events in 

organization of the phosphatidylinositol lipids signaling system for random cell 

Argentina, M., Coullet, P. and Mahadevan, L. (1997). Colliding waves in a model excitable 


*Chaos* **18**, 026104.

Bretschneider, T., Diez, S., Anderson, K., Heuser, J., Clarke, M., Müller-Taubenberger, 

Bretschneider, T., Anderson, K., Ecke, M., Müller-Taubenberger, A., Schroth-Diez, B., 
dynamics of actin waves, a model of cytoskeletal self-organization. *Biophys. J.* **96**, 
2888-900.

Carlsson, A. E. (2010). Dendritic actin filament nucleation causes traveling waves and 


Figure Legends

Fig. 1. Propagation of actin waves followed by myosin-II enriched zones. (A) Development of a wave pattern in an electro-fused cell expressing mRFP-LimEΔ as a label for filamentous actin (red), and GFP-myosin-II heavy chains (green). The confocal images show the substrate-attached cell surface. The left panel begins with indistinct actin clustering throughout the entire surface area, and ends with the fusion and extinction of three actin waves. Myosin-II assembles into filaments preferentially in a zone traveling behind the actin waves. The right panel begins with the re-start of wave formation, continues with extinction of the waves, and ends with a second re-start at new sites of origin. Numbers indicate seconds after the first frame. Bar, 10 μm. (B) Plot of wave propagation obtained by recording movement of the actin peak along the scan indicated in the 95 s frame of (A).

Fig. 2. Patterns of actin waves in a large myosin-II-null cell that expressed LimEΔ-GFP as a label for filamentous actin. Fluorescence images are focused on the substrate-attached cell surface where the wave patterns are formed. (A) Overview of an actin pattern. The left image is overexposed to visualize the shape of the cell with actin-rich filopodia on its border and the typical arrangement of filamentous actin in branched bands. The right image, at lower exposure, highlights at the border of the bands the actin waves by their prominent accumulation of filamentous actin. These waves envelop an “inner territory” covered with a dense texture of actin filaments, and are surrounded by an “external area” that contains a loose actin network. Arrows point to two normal-sized cells. (B) Fluorescence intensities (I₀) in arbitrary units, scanned across an actin band as indicated in the right panel of (A). Movement of the actin band in the direction of the arrowheads in (A) and (B) distinguishes a leading segment from a trailing one. Because of the dense texture of the actin network in the “inner territory” between these segments, the fluorescence intensity of the actin label is higher in this territory than in the external area. (C) Images of a time series from the cell shown in (A). The
images illustrate the initiation and propagation of an actin wave, followed by insertion of a trailing wave. (D) Scans of fluorescence intensities corresponding to the images in (C). Position of the scans are indicated in the first panel of (C). The scans show initiation and propagation of leading and trailing waves. The actin label drops when inner territory turns into external area. (E) Time series showing fusion of two wave systems followed by splitting. Both events are indicated by arrowheads. This sequence is a continuation of the one shown in (C). The entire recording is documented in Movie 1. Numbers in the frames of (A), (C) and (E) designate seconds in accord with frame numbers of the movie. Bars, 10 µm.

**Fig. 3. Quantitative analysis of the shape of actin waves.** (A) Sequence of images depicting the insertion of a trailing wave during the expansion of a circular actin wave in a large myosin-II-null cell. (B) Images displaying the dynamics of a band-shaped actin wave (red) in a large cell obtained by electro-fusion. The cell also expressed GFP-myosin-II heavy chains (green). The arrowhead in the 560 seconds frame points to an arc-shaped indentation where internal area, surrounded by the wave, is converted to external area. Time in (A) and (B) is indicated in seconds in accord with (E) and (F). Image 1 in (A) corresponds to the 460-second image of Figure 2C. Bars, 10 µm. The full image series for (B) is shown in Movie 2. (C) and (D) Measuring the width of actin waves. For an expanding circular wave (C) or a band-shaped traveling wave (D), the dominant width is computed by approximating the wave by a polygon. At each point of the contour, the width is calculated by taking the distance to the opposite side in a direction normal to the contour at the point of interest (arrows). Then the probability distribution of the wave width is computed for each time point. (E) and (F) Time series from top to bottom of probability distributions for the waves shown in (A) and (B), respectively. The insertion of a trailing wave seen in the 48-seconds frame is reflected in a switch of the distribution to smaller values (arrowhead in (E)). The local expansion of the wave seen in the 470-seconds frame of (B) is reflected in a branching of the distribution (arrowhead in (F)).
Fig. 4. **PIP3 dynamics associated with actin waves.** (A) An electro-fused cell labeled for PIP3 with GFP-PHcrac (green) and for filamentous actin with mRFP-LimEΔ (red). The arrowhead on the bar points into the direction of wave propagation. (B) Fluorescence intensities scanned along the bar shown in (A) at 0 s (top) and 26.5 s later (bottom), the time at which the image of (A) was taken. The arrowhead in the 0-second frame indicates the direction of propagation of the combined PIP3 and actin wave. (C) PIP3 pattern in an electro-fused cell used for the analysis of PIP3 dynamics. The cell changed shape during the recording as shown in Movie 4. The actual perimeter at the 2464 s frame of the movie is indicated by a white line. The array of white dots demarcates points of measurement. (D) Temporal pattern of fluorescence intensities (In) at the dot encircled in (C). The time at which the image of (C) was acquired is indicated by an arrowhead. (E) Probability distribution (P) of the time intervals between PIP3 peaks, compiled from 138 measurements in time series like the one shown in (D). (F) Temporal profile of PIP3 during the passage of a wave over single points at the array shown in (C), averaged from 23 measurements. (G) Spatial profile of PIP3 in the direction of wave propagation, averaged from 22 scans at different positions of the cell membrane. The scans are adjusted and normalized to the points of highest fluorescence intensity. Bars in (A) and (C), 10 µm.

Fig. 5. **Conversion of circular waves into the band patterns of giant cells.** The labels are mRFP-LimEΔ for filamentous actin, GFP-myosin-II heavy-chains for myosin-II, and GFP-PHcrac for PIP3 in giant cells produced by electro-fusion. In the left panels of (A), (C) and (D), three different image series are depicted. In the corresponding right panels, scans of fluorescence intensities are displayed. Positions of the line scans are indicated in the first
image of each series. (A) An expanding wave that is converted into two bands of PIP3 by
ingression of external area from two opposite sides. The cell was labeled for filamentous actin
(red) and PIP3 (green). In this example the trailing waves are rudimentary. The full sequence
for (A) is presented in Movie 5. (B) Temporal dynamics of PIP3 at different positions of the
wave propagating to the upper left in the images of (A). Temporal patterns of fluorescence
intensities were scanned at positions 1 to 6 demarcated in the image on top. These scans
indicate that the persistence time of PIP3 shortens during conversion from the circular wave to
the band pattern. Fluorescence intensities of GFP-PHcrac (I\textsubscript{fl}) were measured at 1-\textmu m
distances, beginning at the initiation site of the wave and progressing in the direction of its
propagation. Positions are numbered 1 to 6 in the top panel. This panel shows the fluorescence
image of the PIP3 label at 0 s, the time of the peak intensity at position 1. (C) Circular wave
converted into a doughnut pattern. The giant cell was labeled as in (A) for filamentous actin
(red) and PIP3 (green). The insertion of a trailing actin wave (arrows in the 74 s scan) is
linked to the decay of PIP3 in the central area. The entire time series is presented in Movie 6.
(D) Similar wave in a cell double-labeled for filamentous actin (red) and myosin-II (green).
The scans show the accumulation of myosin-II in the central area simultaneously with the
insertion of a trailing actin wave (arrows in the 120 s scan). The entire time series is shown in
Movie 7. Bars in the images, 10 \textmu m.

Fig. 6. \textbf{Annihilation or return of PIP3 waves at the cell border}. Giant cells expressing
GFP-PHcrac were produced by electro-fusion. The upper panels show image series of PIP3
waves that collide with the cell border, which is demarcated by a solid line in the first frame
of each series. The bottom panels display fluorescence intensities as a function of time,
scanned at single points close to the cell border. Positions of these scans are indicated by
graticules I to IV in the upper panels. (A and B) Two examples of waves that fade away at the
cell border. (C and D) Waves that persist at the border as a PIP3 enriched area and return from
there as a laterally expanding wave. The scans I to IV show PIP3 peaks of typical duration when the waves are annihilated (I and II), and prolonged periods of PIP3 enrichment for returning waves (III and IV). In scan III, PIP3 increases again before the baseline is reached. In scan IV, PIP3 remains high for a prolonged period of time. The entire time series from which the images are taken is covered in Movie 4. Time is indicated in seconds aligned with the time scales of the corresponding scans. Bars, 10 µm.

**Fig. 7. A composite wave viewed as an excited state.** Based on fluorescence intensities (I<sub>n</sub>) measured at single points of the substrate-attached cell surface, the diagram envisages a propagating PIP3 wave (green) in the cell membrane coupled to two segments of an actin wave (red) in the cell cortex as a composite wave. The external area in front of the leading segment and behind the trailing segment is viewed as the resting state of an excitable medium.

**Fig. 8 Diverse transition scenarios of actin waves (red) that enclose PIP3-rich territories on the membrane (green).** (A) Conversion of an expanding circular wave into propagating bands. A wave is typically initiated by actin clusters embedded in a PIP3 patch. This patch expands while the actin becomes concentrated at its border (1), forming an expanding circular wave (2). When the radius of this wave exceeds a critical size, two modes of transition into arc-shaped waves are observed. Either asymmetry arises early by the local ingestion of external area into the circular wave (3 and 4), or a trailing wave is inserted that encircles a new external area (5). The resulting doughnut pattern is unstable and breaks up into an arc-shaped wave (6). In either way the PIP3-rich territory assumes an elongated shape and travels perpendicular to its long axis (7). Each of the propagating PIP3 bands is confined by the leading segment of an actin wave at its front (solid red lines) and the trailing segment at its back (dotted red lines). The PIP3 bands can elongate (8) or split into two (9). Alternatively, two bands can unite by end-to-end fusion (10). Two examples illustrate the generation of
more complex patterns. First, the PIP3 band can broaden beyond the critical width (11), causing correction of the width by the ingression of external area (12). Second, the open ends of the band may curl (13) and either fuse into a closed expanding circle or split off (14). The diagram illustrates two cases of reversal of direction (blue arrows). During conversion of a circular wave into an arc-shaped structure, part of an expanding wave is turned into a retracting segment (3-4). Conversely, the trailing segment of a wave can be turned into a leading one (11-12). (B) Collision of waves. Two bands of PIP3 enveloped by the leading and trailing segments of an actin wave extinguish each other at the point of collision (1-4). Simultaneously, the lateral portions of the two PIP3 bands fuse, thus generating two bands that propagate in opposite directions at angles of about ±90° to the directions of the previous bands (4-5).
Figure 2
Figure 6

Wave Extinction at Border

Wave Return at the Cell Border

[A] 0, 71, 95
[B] 7, 62, 76
[C] 63, 108, 217
[D] 50, 121, 158

Legend:

- [I] Wave Extinction at Border
- [II] Wave Return at the Cell Border

Graphs:

- Graph [I]: Time vs. Intensity
- Graph [II]: Time vs. Intensity
- Graph [III]: Time vs. Intensity
- Graph [IV]: Time vs. Intensity
Figure 7

[Diagram showing a graph with time on the x-axis and an unknown y-axis labeled as $ln$. The graph shows two segments labeled as ‘leading segment’ and ‘trailing segment’. The graph also shows transitions between ‘resting state’, ‘excited state’, and ‘external area’ labeled on the graph.]