Biol. Cell (2013) **105**, 109–117 DOI: 10.1111/boc.201200063

Review

Transcellular tunnel dynamics: Control of cellular dewetting by actomyosin contractility and I-BAR proteins

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Dewetting is the spontaneous withdrawal of a liquid film from a non-wettable surface by nucleation and growth of dry patches. Two recent reports now propose that the principles of dewetting explain the physical phenomena underpinning the opening of transendothelial cell macroaperture (TEM) tunnels, referred to as cellular dewetting. This was discovered by studying a group of bacterial toxins endowed with the property of corrupting actomyosin cytoskeleton contractility. For both liquid and cellular dewetting, the growth of holes is governed by a competition between surface forces and line tension. We also discuss how the dynamics of TEM opening and closure represent remarkable systems to investigate actin cytoskeleton regulation by sensors of plasma membrane curvature and investigate the impact on membrane tension and the role of TEM in vascular dysfunctions.

Introduction

Cell membrane homeostasis results from a precise balance between elongation and contraction forces at the cortex level, coupled to vesicle traffic contributing to the buffering of membrane tension (see recent reviews: Gauthier et al., 2012; Salbreux et al., 2012). Excessive contraction or disruption of the actin cytoskeleton cortex leads to the local detachment of the membrane associated with induction of membrane blebs (Sheetz et al., 2006; Ridley, 2011; Spangler et al., 2011; Salbreux et al., 2012). In contrast, a reduction of actomyosin cable contractility produces large tunnels across thin endothelial cells, referred to as transendothelial cell macroaperture tunnels (TEMs) (Boyer et al., 2006; Maddugoda et al., 2011; Gonzalez-Rodriguez et al., 2012). Internal cellular regulation usually inhibits the spontaneous formation of such TEMs, but some bacterial toxins or leucocytes have the capability to induce TEMs to cross the endothelial barrier (Lemichez et al., 2010). While cell blebs have been extensively studied from biological and physical perspectives, much remains to be learned on TEM dynamic. Recently, studies conducted on a group of bacterial toxins, now referred to as tunnel-forming toxins (TFTs), have shed light on the molecular and biophysical aspects of the dynamic of TEM formation (Maddugoda et al., 2011; Gonzalez-Rodriguez et al., 2012). These toxins reduce actomyosin contractility and allow for the spontaneous opening of large apertures (macroapertures) across endothelial cells (Boyer et al., 2006; Maddugoda et al., 2011). Tunnels open up to a maximum diameter of about 10–20 μm within 1–2 min, depending on the type of cell intoxication (Figure 1). Next, membrane waves rich in actin filaments (F-actin) extend around the edge of the TEM driving aperture closure in about 3–5 min (Figure 1) (Boyer et al., 2006; Maddugoda et al.,

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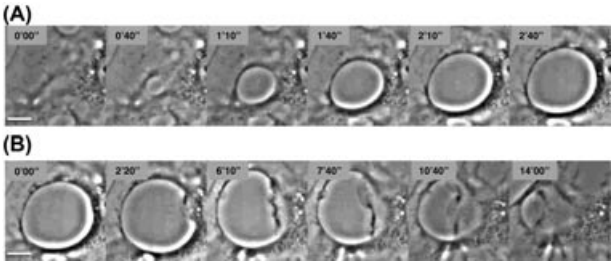
Key words: Actin, Bacteria, cAMP signalling, Computational methods/theoretical biology, Cytoskeleton.

Abbreviations: AFM, atomic force microscope; EF/ET, oedema factor/toxin; LF/LT, lethal factor/toxin; MIM, missing in metastasis; MLC, myosin light chain; NMII, non-muscle myosin II; ROCK, Rho kinase; TED, transendothelial cell diapedesis of leucocytes; TEM, transendothelial cell macroaperture; TFTs, tunnel-forming toxins.

Figure 1 | Series of video images of TEM dynamics
TEM opening (**A**) and closure (**B**) in a HUVEC intoxicated with the EDIN homologue C3-exoenzyme of *Clostridium botulinum* for 24 h. Bar = 10 μm.



2011; Gonzalez-Rodriguez et al., 2012). As discussed in the following section *from liquid dewetting to cellular dewetting*, recent physical modelling of TEM dynamics suggests a new framework to help decipher the relationship between actomyosin contractility and membrane tension, and how this interplay affects cell shape organisation (Gonzalez-Rodriguez et al., 2012).

From liquid dewetting to cellular dewetting

General aspects of liquid and cellular forms of dewetting

Two recent studies have revealed that TEM tunnel opening can be described as a cellular form of dewetting, that is, the physical processes governing TEM are analogous to those governing the opening of dry patches in liquid films (Box 1 and see Maddugoda et al., 2011; Gonzalez-Rodriguez et al., 2012 for biological and physical details). Liquid dewetting is a very common phenomenon that occurs for instance when water is forced to spread on a hydrophobic substrate or when oil is heated on a non-adhering pan. The liquid tends to reduce its contact with the substrate, producing dry patches that grow. Different modes of liquid dewetting have been described (de Gennes et al., 2004). It has been observed that the cellular mode of dewetting leading to TEM opening has similarities and differences with the dewetting of a metastable film of viscous liquid on a rigid substrate. Similar to dewetting of metastable liquid films, cellular dewetting exhibits the nucleation and growth of irregularly distributed dry patches, rather than the appearance of periodic undulations on the surface that grow in size, which would be characteristic of the spinodal mode of dewetting (de Gennes et al., 2004). Cellular dewetting is a transient phenomenon leading to the opening of tunnels with limited radius. The driving surface force is thus not constant as for simple liquids. The dynamics of cellular dewetting, with a typical time scale on the order of minutes, are dominated by viscous dissipation

Box 1. Key physical concepts of cell dewetting

Driving force: Dewetting requires the existence of a driving force pulling on the tunnel (of radius R). Pulling is due to the tension in the cell membrane (σ), which is partially counteracted by a line tension around the tunnel (T). Consequently, the driving force consists of two contributions:

$$F_D = 2\sigma - \frac{T}{R} \quad (1)$$

If this driving force is positive ($F_D > 0$), dewetting proceeds.

Nucleation radius: Dewetting starts with the formation of a small initial transcellular hole, which can be induced by membrane fluctuations. For this initial tunnel to grow by dewetting, its radius (nucleation radius) must be large enough to yield a positive initial driving force (Eq. 1). This condition requires a nucleation radius of the order of 100 nm.

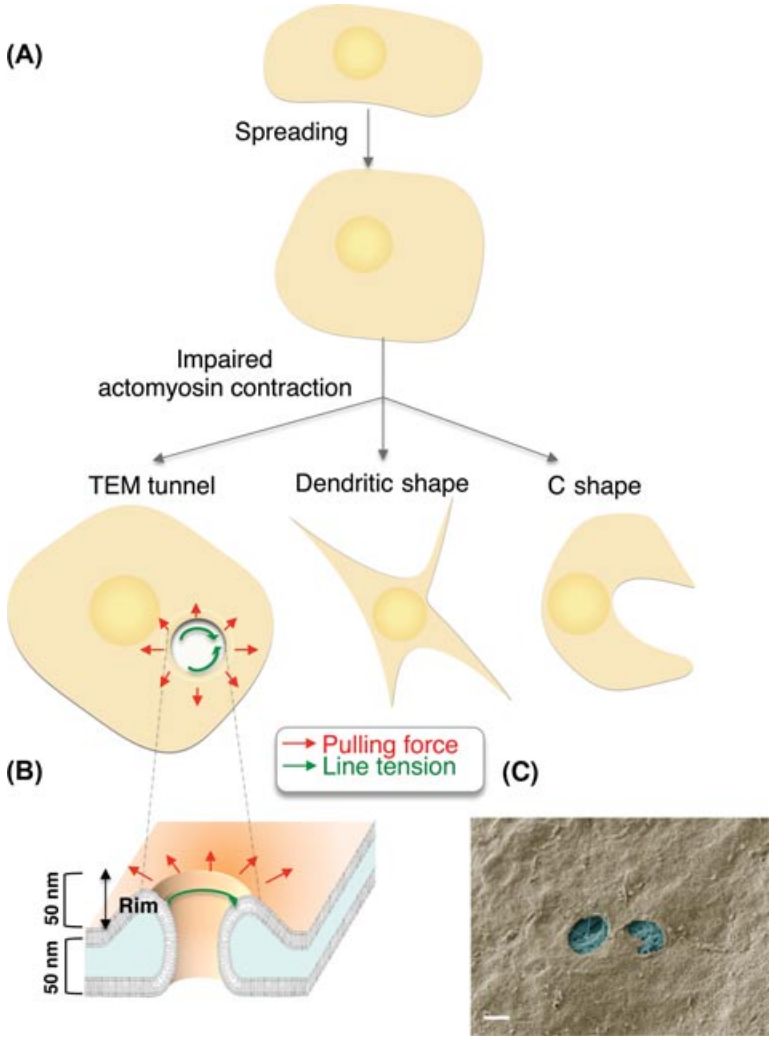
Membrane tension: The membrane tension (σ) itself depends on the tunnel radius R. As the tunnel radius increases, the membrane is relaxed and its tension rapidly decreases, as described by Helfrich's law:

$$\sigma = \sigma_0 \exp\left(-\frac{R^2}{R_c^2}\right) \quad (2)$$

where σ_0 is the initial membrane tension in the absence of TEM, and R_c is a characteristic radius proportional to the cell size. According to Eq. 2, as the tunnel opens, the membrane tension decreases. Eventually, the membrane tension exactly equilibrates with the line tension in Eq. 1, yielding $F_D = 0$ and halting the tunnel growth process. Such an equilibrium configuration is reached for a typical tunnel radius of several micrometers.

Line tension: The line tension T is a resisting force along the edge of the tunnel that opposes the membrane tension and limits dewetting. A cell can increase the line tension by recruiting some proteins (such as MIM) to the membrane surrounding the tunnel. As a consequence of Eq. 1, increasing line tension reduces the maximum tunnel radius.

Figure 2 | Schematic representations
(**A**) of membrane retractions resulting from a reduction of actomyosin contractility of cells undergoing spreading, (**B**) of the forces applied to the membrane at the edge of transcellular tunnels together with the distance between membranes determined by AFM. (**C**) Colourised scanning electron micrograph of TEM induced by *B. anthracis* ET.



effects, whereas inertial effects, that is, the resistance to acceleration due to the mass and velocity of an object, are by comparison negligible. Thus, a physical analogy to the dewetting of polymer films (Redon et al., 1994; Gonzalez-Rodriguez et al., 2012) is appropriate to model the dynamics of cellular dewetting as a liquid film slipping on a weakly adhesive, rigid substrate.

Specificity of the mode of cellular dewetting

The newly described cellular form of dewetting is driven by surface forces that result from the tension of an overspread membrane when actomyosin contraction is partially relaxed (Figure 2 and Box 1 for physical considerations). Cellular dewetting processes spontaneously progress once the tunnel has formed (nucleated), probably due to the increased probability of fusion of apical and basal membranes when cells are spread. The details of this fusion mechanism are still unknown. As the TEM opens, atomic force microscope (AFM) analysis has revealed the presence of a rim, formed by cytoplasmic matter removed from the tunnel at the edge of TEMs, which is a major characteristic of liquid dewetting (see Figure 2B and

Maddugoda et al., 2011). The pulling force is the membrane tension, which tends to relax as the hole grows. In addition to the pulling forces, another force called line tension acts at the edge of the rim and tends to close the hole (Gonzalez-Rodriguez et al., 2012) (Figure 2B and Box 1). Dewetting proceeds when the driving pulling forces are larger than the resisting line tension forces. This implies that, for dewetting to proceed, the radius of the hole initially formed must be larger than a threshold, known as the nucleation radius [see for details (Gonzalez-Rodriguez et al., 2012)]. Otherwise, the hole will spontaneously shrink and disappear. For dewetting of endothelial cells in the presence of toxins, physical considerations suggest a nucleation radius on the order of 100 nm (Box 1 and Gonzalez-Rodriguez et al., 2012 for determination of nucleation radius). The process underpinning the formation of such a nucleation hole is the subject of current research. In cellular dewetting, the membrane tension tends to decrease as the hole opens, in analogy with transient pores in vesicles (Sandre et al., 1999). TEM grows up to a maximum radius and stops when surface and line tension forces are balanced (Figure 2 and Gonzalez-Rodriguez et al., 2012).

Analogy with tunnels formed during leucocyte transcellular diapedesis

TEM tunnels also contribute to endothelium permeability and form during transendothelial cell diapedesis (TED) of leucocytes (Aird, 2007a,b; Carman and Springer, 2008). Notably, the actin cytoskeleton within endothelial cells reorganise at sites of leucocyte transcytosis to form transmigratory cups with lamellipodia-like extensions (Carman et al., 2003; Carman and Springer, 2008). Tunnel formation during TED involves a complex array of interactions between adhesion molecules of both cell types and the downstream modulation of signalling pathways currently under study (Carman and Springer, 2008). How transcellular diapedesis is initiated is still a major question to be addressed. Existing reports indicate that it can be thought of as an active process driven by leucocytes extending podosome-like structures that indent the endothelium surface, in search of a low-resistance migration path, and/or actively softening cellular regions to dig transcellular tunnels (Carman et al., 2007; Isac et al., 2011). This likely favours a close apposition between endothelial cell luminal and abluminal plasma membranes for fusion (Carman and Springer, 2008). TIRF analysis of actin filaments (F-actin) and the phosphorylated form of non-muscle myosin II (NMII)-light chain (phospho-MLC) at the site of leucocyte transmigration show some examples of local reduction of actomyosin filament content, which may favour endothelial cell membrane apposition for fusion (Isac et al., 2011). Vesicle fusion is also thought to favour membrane deformation during TED induction by leucocytes (Mamdouh et al., 2009). Further studies are required to evaluate whether dynamics of TEM and TED tunnels involve common molecular determinants.

Actomyosin-based regulation of TEM dynamics

TEM opening: Actomyosin relaxation and cell spreading

The actomyosin cytoskeleton is a critical player in cell-shape organisation and dynamics as it provides forces to restrain or trigger membrane deformations (Levayer and Lecuit, 2012). Actin filaments and NMII associate into different structures, and along with an assortment of actin-binding proteins produce actomyosin cables and rings as well as a contractile network across the cytosol and at the cell cortex underneath plasma membrane (Levayer and Lecuit, 2012; Salbreux et al., 2012; Gauthier et al., 2012). Actin and upstream regulatory factors are direct targets of numerous bacterial virulence factors (Aktories and Barbieri, 2005). TFTs corrupt several signalling pathways, which converge upon a reduction of NMII activity through dephosphorylation of MLC (Maddugoda et al., 2011). TEM opening is thought to result from the increase in membrane tension during the spreading of cells on the substrate. This likely involves a reduction of the actomyosin-based contraction/organisation of the cell cortex and/or a reduction of the stabilising effect of the transcellular actomyosin network (Salbreux et al., 2012; Gauthier et al., 2012). Indeed, an elegant study recently quantitatively revealed the existence of a transcellular network of contractile actomyosin fibres crucial to balancing the spreading forces exerted at opposite epithelial cell edges during spreading (Cai et al., 2010). Consequently, inhibition of NMII during the spreading of mouse embryonic fibroblasts, as well as other cell lines, produces retractions initiated either at the periphery of cells, yielding C-shapes and

dendritic shapes, or within cells inducing TEM tunnels (Figure 2A; Cai et al., 2010). Such retractions initiated at the edge of cells were also observed in Vero cells intoxicated with RhoA inhibitory toxins (Boyer et al., 2006). The molecular basis of these differences of retraction between cell types is yet to be determined.

TEM opening by toxins and modulation of MLC phosphorylation

TFTs have the common property of reducing actomyosin contraction and triggering cell spreading. A first group of TFTs virulence factors corresponds to ADP-ribosylating toxins notably expressed *by Staphylococcus aureus* and catalysing the posttranslational modification of the asparagine-41 of the small GTPase RhoA for inactivation (Chardin et al., 1989; Aktories and Barbieri, 2005; Boyer et al., 2006). Hence, the central role played by RhoA activity in preventing opening of TEM was confirmed by direct RNAi-mediated depletion (Boyer et al., 2006). The small GTPase RhoA primarily controls actomyosin contractility through activation of Rho kinase (ROCK) and downstream induction of phosphorylation of MLC (Jaffe and Hall, 2005). Indirect induction of MLC phosphorylation driven by ROCK involves primarily the inhibition of MLC phosphatase (Kimura et al., 1996). Inhibition of ROCK with the molecule Y27632 also promotes the opening of dynamic TEM tunnels (Boyer et al., 2006). The second group of TFTs corresponds to adenylate cyclase toxins of *Bordetella pertussis* (cyaA) and *Bacillus anthracis* oedema toxin (ET) (Tang and Guo, 2009). The extreme virulence determinant of *B. anthracis* resides primarily in both pXO1 and pXO2 plasmids-encoded factors, notably the three-component protein toxin (Moayeri and Leppla, 2009). Protective antigen (PA) is the toxin component, which primarily binds to capillary morphogenesis protein-2 (CMG2) cell receptor to drive endocytosis and injection of enzymatic factors [oedema factor (EF) and lethal factors (LF)] into the cytosol (Deuquet et al., 2012). The LF is a zinc-metalloprotease that cleaves the amino-terminal part of MAPK kinases thereby interrupting these signalling cascades. The EF is a calmodulin-dependent adenylate cyclase that catalyses the production of high amounts of cyclic-AMP, a broad signalling molecule, at the perinuclear region (Dal Molin et al., 2006; Tang and Guo, 2009). This leads to the downstream activation of PKA and EPAC/Rap pathways. Production of cAMP by ET (PA + EF), or synergistic stimulation of EPAC and PKA pathways, triggers the opening of TEM tunnels in primary endothelial cells of different origins (Maddugoda et al., 2011). ET also triggers TEM opening in the endothelium of rat arteries (Figure 2C). Moreover, inhibition of the cAMP phosphodiesterase PDE4 also sensitises cells to form TEMs (Dodge-Kafka et al., 2005). Conversely, inhibition of PKA, EPAC or Rap has a major blocking effect on the formation of TEM by ET (Maddugoda et al., 2011). Thus, induction of TEM by ET requires a cAMP-driven activation of both PKA and EPAC pathways. The increase in cAMP level triggers the downstream modulation of actomyosin cytoskeleton regulatory proteins (Lorenowicz et al., 2007). Notably, PKA phosphorylates MLCK and decreases its binding affinity to calmodulin, an essential co-activator, thereby reducing the phosphorylation of MLC (Horman et al., 2008). In parallel, the engagement of the EPAC/Rap pathway favours cell spreading (Lorenowicz et al., 2007). This raises the question of whether the production of cAMP during leucocyte diapedesis favours a transcellular mode of migration (Mehta and Malik, 2006; Lorenowicz et al., 2008).

TEM maximum diameter: Specificity of the cellular form of dewetting

TEMs triggered by bacterial toxins reach a maximum diameter. This is a distinguishing feature between cellular and liquid dewetting, as the latter proceeds indefinitely until the liquid film has completely retracted into droplets (de Gennes et al., 2004; Gonzalez-Rodriguez et al., 2012). These contrasting behaviours result from the different driving forces in both types of dewetting. In liquid dewetting, the driving force arises from the surface energetic gain in draining the non-wettable surface; the magnitude of this force remains constant throughout the dewetting process. In cellular dewetting, the driving force is the membrane tension (Gonzalez-Rodriguez et al., 2012). As the TEM tunnel opens, the surface covered by the membrane decreases. Consequently, Gonzalez-Rodriguez et al. (2012) have described the resulting decrease in membrane tension by applying Helfrich's law (see Box 1 and detailed description of the model in Gonzalez-Rodriguez et al., 2012). This quantitatively linked the decrease of the driving force of cellular dewetting to the increase of the tunnel radius.

Eventually, the tunnel reaches a maximum radius and the opening stops. This equilibrium is attained when the decrease in membrane tension is exactly balanced by the line tension, and thus the driving force becomes null. This physical phenomenon is analogous to the opening of pores in lipid vesicles, which is also driven by membrane tension; pores also reach a maximum size limited by the line tension at its edge (Sandre et al., 1999). Although in vesicles, line tension results from the exposure of lipid chains to the aqueous solvent, in TEMs line tension partly results from the accumulation of the Missing in Metastasis (MIM) proteins at their edges (Gonzalez-Rodriguez et al., 2012). Moreover, in contrast to pores in vesicles that close after leaking of the internal volume and membrane tension reduction, cells do not leak but TEM nevertheless also close (Boyer et al., 2006). This implies that cells have the intrinsic capacity to perceive transcellular holes to limit their opening and close them.

TEM closure and the sensing of membrane curvature

Although the existence of a maximum TEM size can be explained by physical principles analogous to those governing inert systems, the closing mechanism is a biological phenomenon specific to living cells. Recent advances show that spatial determinants, such as the sensing of newly formed plasma-membrane curvature generated by TEM might account for cellular TEM perception and closure (Maddugoda et al., 2011). Closure operates by extension of actin-rich lamellipodia-like membrane waves that originate from the edge of TEM. This involves a local recruitment of the inverse-BAR (I-BAR) domain containing proteins MIM and ABBA (Saarikangas et al., 2009). These proteins are bifunctional with an amino-terminal I-BAR domain for membrane curvature sensing and/or deformation and the remaining part driving actin polymerisation. The I-BAR domain of MIM and ABBA contains positively charged amino acid-residues distributed along a curve-shaped interface and an amphipathic alpha-helix (Saarikangas et al., 2009). Both of these molecular determinants play key roles in the accumulation of MIM and ABBA and their I-BAR domain at TEM edges (Maddugoda et al., 2011). This occurs rapidly after the opening event providing further evidence in cells that MIM (similarly ABBA) I-BAR domain senses newly formed membrane curvature (Saarikangas et al., 2009; Maddugoda et al., 2011). Next, other domains of MIM, as yet identified, drive the recruitment/activation of Arp2/3 for local actin polymerisation and extension of membrane waves. This phenomenon likely generates the forces required to close TEMs, as measured during extension of the membrane wave of migrating cells (Mogilner and Oster, 1996). Depletion of MIM has a blocking effect on the closure of TEMs and increases the diameter of the holes (Gonzalez-Rodriguez et al., 2012). Physical arguments deduced from the cellular dewetting model suggest that an increase in the maximum hole size is likely associated with a decrease in line tension. Indeed, it can be deduced from the cellular dewetting model that if the line tension is smaller this leads to a larger equilibrium hole size (Gonzalez-Rodriguez et al., 2012). On the basis of this reasoning, a recent study has proposed that the physical mechanism by which MIM limits maximum TEM size involves a control of the line tension around the hole (Gonzalez-Rodriguez et al., 2012). Nevertheless, cellular dewetting does not proceed up to the formation of droplets in MIM depleted cells, implying a contributing role of other systems in the regulation of line tension at the edge of TEMs.

Cellular dewetting in pathophysiology

The endothelial cell monolayer forms a semi-permeable film lining the blood circulatory system, regulating exchange between the blood and tissues both by para- and transcellular pathways (Aird, 2007a,b). Transcellular tunnels in the endothelium of specific vessels foster exchanges and form a path for leucocyte transcellular extravasation from the blood stream (Braet and Wisse, 2002; Aird, 2007a,b). As discussed in the following paragraph, a strong correlation has been established between the phenomenon of TEM opening and major vascular dysfunctions.

Vascular dysfunctions and sepsis are hallmarks of Anthrax. This infection caused by the agent *Bacillus anthracis* is primarily a zoonosis. An epidemic that occurred in 1979 in Ekaterinburg, Russia documented in humans the pathologic lesions resulting from inhalational anthrax (Abramova et al., 1993). This documentation, reviewed by Abramova and colleagues, determined that the route of how the infection spreads and identified the presence of oedema (including gelatinous oedema), pleural effusion and

hemorrhages as the most characteristic symptoms. Studies conducted in mice have revealed that toxemia largely recapitulates major vascular dysfunctions and death resulting from Anthrax disease (Moayeri and Leppla, 2009; Firoved et al., 2005). Pathophysiological symptoms triggered by intravascular injections of ET/LT toxins comprise a combination of vascular dysfunctions–notably massive oedema at the gastrointestinal tract (GIT; ET = PA + EF) – and pulmonary vascular leakages (LT = PA + LF) associated with massive hypotension. Several reports aimed at studying the cytotoxic effects of Anthrax toxin on endothelial cells have started to reveal major reorganisation of the actin cytoskeleton (Trescos and Tournier, 2012). After long periods of intoxication, the lethal toxin triggers the formation of thick actin cables parallel to each other due to interruption of MAP kinase signalling (Warfel et al., 2005; Rolando et al., 2010). This phenomenon is associated with an elongation of endothelial cells and the opening of intercellular gaps. ET triggers the opposite effect in the actin cytoskeleton leading to a reduction of contractile actomyosin cables via induction of MLC dephosphorylation (Maddugoda et al., 2011). Infection experiments have revealed similar effects to those produced by purified toxin and demonstrated that ET has a dominant effect over LT on actin reorganisation during early time periods of infection (Maddugoda et al., 2011). Interestingly, ET during infection or intoxication leads to a rupture of the skin and GIT endothelium barrier, monitored by Evans blue dye (Guichard et al., 2010; Maddugoda et al., 2011). This dye has a high affinity for serum albumin allowing albumin extravasation monitoring after intravascular injection. Gelatinous oedema triggered by ET at the level of the GIT of mice is associated with massive decrease of the endothelium barrier function (Maddugoda et al., 2011). A strong correlation has also been reported between induction of TEM by the *S. aureus* secreted toxin EDIN and the rupture of the endothelium barrier. Consistently, replacement of the ET catalytic domain by EDIN is sufficient to rupture the GIT endothelium barrier (Rolando et al., 2009; Maddugoda et al., 2011). Induction of TEM correlates with *S. aureus* dissemination (Lemichez et al., 2010; Edwards and Massey, 2011). Indeed, EDIN-expressing *S. aureus* have a higher capacity to disseminate into mice tissues following bacteremia (Munro et al., 2010) and a higher prevalence in human pathogenic strains isolated from deep-seated infections (Munro et al., 2011). Thus, induction of TEMs triggered by bacterial TFTs strongly correlates with the induction of major endothelium barrier dysfunctions, such as oedema and hemorrhage, thereby also favouring bacterial sepsis.

Concluding remarks

The discovery of the dynamics of TEM tunnels and of the physical principles underpinning this phenomenon have opened new avenues for characterising important cell biology aspects of curved membrane-regulated actomyosin cytoskeleton organisation and the control of membrane tension. Thus, these studies point to the role of membrane shape in controlling cell morphology and movement of the edges. Moreover, they show the intrinsic capability of the cell to recruit actin-based machinery to close the TEM in non-pathological situations such as transcellular diapedesis of leucocytes. Eventually, these studies should also improve our understanding of the molecular basis of vascular diseases associated with inflammation and infection.

Funding

E.L. laboratory is supported by an institutional funding from INSERM, a grant from the Agence Nationale de la Recherche (ANR-07-MIME-007) and ARC Foundation (SFI20111203659).

Acknowledgements

We thank S. Bourdoulous, D. Fletcher, K. Webster and C. Stefani for critical reading of the manuscript and figure artwork.

Conflict of interest statement

The authors have declared no conflict of interest.

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Received: 17 September 2012; Accepted: 21 November 2012; Accepted article online: 28 November 2012

