Coordinated oscillations in cortical actin and Ca$^{2+}$ correlate with cycles of vesicle secretion

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The actin cortex both facilitates and hinders the exocytosis of secretory granules. How cells consolidate these two opposing roles was not well understood. Here we show that antigen activation of mast cells induces oscillations in Ca$^{2+}$ and PtdIns(4,5)P$_2$ lipid levels that in turn drive cyclic recruitment of N-WASP and cortical actin level oscillations. Experimental and computational analysis argues that vesicle fusion correlates with the observed actin and Ca$^{2+}$ level oscillations. A vesicle secretion cycle starts with the capture of vesicles by actin when cortical F-actin levels are high, followed by vesicle passage through the cortex when F-actin levels are low, and vesicle fusion with the plasma membrane when Ca$^{2+}$ levels subsequently increase. Thus, cells employ oscillating levels of Ca$^{2+}$, PtdIns(4,5)P$_2$ and cortical F-actin to increase secretion efficiency, explaining how the actin cortex can function as a carrier as well as barrier for vesicle secretion.

The regulated exocytosis of secretory granules is a fundamental process in all eukaryotic cells$^1$. Successful secretion requires the delivery of vesicles from inside the cell to the plasma membrane before fusion can occur. Forty years ago, it was realized that cells have a peripheral microfilament web, later identified as the actin cortex, that acts as a mechanical barrier preventing dense-core secretory vesicles from docking to the plasma membrane in unstimulated cells$^2$–$^8$. This observation contrasted with the previously studied synaptic vesicles that were pre-docked at the plasma membrane and ready to rapidly fuse. These dense-core vesicles are ubiquitously present in cells and are characterized by slower regulated secretion kinetics when compared with synaptic vesicles. However, in addition to having a barrier function for vesicles, the actin cortex also acts as a carrier that has to bind myosin V actin motors to capture and transport vesicles to the plasma membrane to mediate vesicle fusion$^9$–$^{15}$. This simultaneous function as a barrier for vesicles and as a matrix required for vesicle transport to the plasma membrane raises the question of how cells consolidate these opposing barrier and carrier roles of the actin cortex.

RESULTS
Cortical actin acts to facilitate and hinder secretion

We investigated the role of the actin cortex in secretion by focusing on mast-cell-receptor (FcεRI)-triggered granule exocytosis in rat basophilic leukaemia (RBL) cells, a model for studying antigen-triggered mast-cell activation and allergic responses$^{16}$. Consistent with a previously proposed barrier role for cortical actin$^{1,8}$, depolymerization of cortical actin by the addition of latrunculin caused a small increase in total secretion (Fig. 1a). However, in agreement with a carrier role that increases rather than blocks secretion rates$^{10}$–$^{15}$, the initial rate of exocytosis was reduced after the depletion of cortical actin (Fig. 1b–d and Supplementary Movie S1). Single-cell secretion measurements using fluorescence de-quenching of previously endocytosed dextran–FITC showed that activation of the FcεRI receptor results in cell-wide Ca$^{2+}$ oscillations and exocytosis events that occur at peak Ca$^{2+}$ in each cycle$^{17}$ (Fig. 1e). On the basis of this pulsatile secretion dynamics, we reasoned that corresponding oscillating changes in the actin cortex may explain why it has antagonistic barrier and carrier roles in cells.

Activation of the FcεRI causes oscillations in actin polymerization at the cortex

To investigate the dynamics of the actin cortex during secretion we used live-cell total internal reflection fluorescence (TIRF) microscopy of mCherry-tagged F-tractin, a biosensor that measures the local concentration of polymerized actin (F-actin; Fig. 2a and Supplementary Fig. S1a–c)$^{18}$–$^{22}$. In this imaging mode, F-tractin molecules are selectively excited within $\sim$100 nm of the membrane adhesion surface. Strikingly, antigen stimulation of the FcεRI pathway resulted in regular oscillations (Fig. 2a, right panel, bottom, and Supplementary Movie S2) as well as more irregular pulsatile changes in the local F-actin concentration (top right panel). Quantitative cluster analysis (see

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Figure 1 Depolymerization of cortical F-actin increases the total amount of secreted enzyme but with slower initial kinetics. (a) Population measurement of total secreted β-hexoamidase 30-min post pharmacological activation with ionomycin (1 μM) and PdbU (100 ng/ml) addition to cells pre-treated with 4 μM latrunculin (green) or dimethylsulphoxide (DMSO) control (blue). (P < 0.001, two-sample t-test, error bars s.e.m., n = 64). (b) Time course of initial loss of secretory granules (SG) monitored with LysoTracker. Loss of secretory granules is defined as the relative drop in fluorescence intensity from the intensity before antigen addition. Cells were stimulated with ionomycin (1 μM) and PdbU (100 ng/ml); control- (DMSO) and latrunculin-A (4 μM) pre-treated (5 min before drug addition) cells are shown in blue and green, respectively. The error bars show the 95% confidence intervals (n = 1,258 and 2,623). (c,d) The disappearance of the secretory granule marker LysoTracker corresponds to an increase in the exocytosis marker VAMP7–pHluorin. (c) Time series of the secretory granule marker and pH-sensitive VAMP7–pHluorin. The black vertical line marks the addition of 1 μM ionomycin and 100 ng/ml PdbU. The dashed lines show the time points of the snapshots shown in d. Scale bar, 5 μm. Note that both a and c are based on LysoTracker marking of secretory granules, with a showing their loss and c showing the remaining secretory granule intensity corresponding to the images in d. (e) Time series of Ca²⁺ (orange) signals and dextran–FITC (red) release following activation of the mast-cell receptor FccRI. The increase in pH during vesicle fusion results in de-quenching of FITC, causing a transient fluorescent secretion signal.

Methods) identified two distinct power spectrum patterns (Fig. 2b, left, and Supplementary Fig. S2). Regular oscillations mapped to the centre region of the adhesion surface whereas more irregular fluctuations mapped to the peripheral region (Fig. 2b, right). Furthermore, the peripheral F-actin fluctuations operated locally with a characteristic length scale of ~1 μm similar to the spatial range of previously observed lamellipodium dynamics²³,²⁴, whereas the oscillatory region in the centre operated globally with a length scale of >5 μm (Fig. 2c; see Methods). The relative change in cortical F-actin concentration during the oscillations was ~15%, as estimated from calibration measurements using phalloidin staining (Supplementary Fig. S1).

Integration of the spatial location of individual vesicle fusion events (Fig. 3a, left and middle panels) showed that exocytosis occurs exclusively in the centre and not in the peripheral region (Fig. 3a, right, Supplementary Movie S3). We therefore focused our mechanistic studies on the centre region.
Figure 2 Receptor-stimulated cell-wide and local F-actin oscillations. (a) Live measurements of local cortical F-actin oscillations using an F-tractin biosensor and TIRF optical sectioning (left). Distinct local cortical F-actin concentration changes are observed at different sites; two representative regions of interest are shown in the periphery (blue) and in the cell centre (green; right). (b) Two types of F-actin oscillation with distinct power spectra. The amplitudes of the power spectra (decibels, dB) were converted to a colour code (left panel, x axis; period time labels) for each pixel in a cell (y axis), and clustered into two groups. The two subtypes of oscillation map to centre and peripheral cell adhesion regions, respectively. The map (right) shows the projection of the major period of each local F-tractin power spectrum onto the cell image. (c) Oscillations are synchronized between pixels across the entire centre region but not in the peripheral region. The left panel shows single-site examples with exponential fits to the spatial correlation function for pixels in the centre and peripheral regions, respectively. The image on the right shows a map of the fitted spatial correlation length for each pixel. Scale bars, 5 μm.

Figure 3 Oscillations in the PtdIns(4,5)P₂ lipid precede F-actin oscillations. (a) Secretion occurs exclusively in the centre and not in the peripheral region. The two micrographs show a cell loaded with the secretory marker dextran–FITC before (left) and 1 s after (right) an exocytosis event. Scale bar, 5 μm. The yellow square marks an exocytosis event. The kymograph in the middle is taken through the centre of the yellow square. The right panel shows a map projecting 155 exocytosis events from 12 cells according to their distance from the periphery (monitored in the first 20 min after antigen addition). The red line marks the normalized area where central F-actin oscillations are observed. (b) Synchronized phase-shifted oscillations in PtdIns(4,5)P₂ (monitored with PH-PLCδ) and cortical F-actin. (c) Example of a time series of signals measured with epi-fluorescence of Fura-2 (orange; Ca²⁺ signals) and a second PtdIns(4,5)P₂ biosensor, the Tubby domain from the Tubby protein tagged with YFP and imaged in TIRF (cyan). Raw data for b,c are presented in Supplementary Table S1. (d) Cross-correlation between the time series shown in c was used to estimate the lag and significance of the correlation between the two time series. (e) Comparison of the lag between Ca²⁺ and PH-PLCδ with that between Ca²⁺ and the Tubby domain. The bars show the median lag and the error bars represent the median absolute deviation. P = 0.57, Wilcoxon rank sum test, n = 15.
Coupled Ca²⁺, PtdIns(4,5)P₂ and N-WASP oscillations drive F-actin dynamics

The observed F-tractin oscillation time period was reminiscent of the period of antigen-triggered Ca²⁺ oscillations in RBL and other cells (Fig. 1c and Supplementary Fig. S3 and Movie S6). Such Ca²⁺ oscillations are driven by the generation of inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃) through phospholipase C (PLC)-mediated phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) hydrolysis, Ins(1,4,5)P₃-mediated opening of Ins(1,4,5)P₃ receptor Ca²⁺ channels in the endoplasmic reticulum, and positive feedback from Ca²⁺ back to the Ins(1,4,5)P₃ receptor and to phospholipase C. This raised the possibility that Ca²⁺ oscillations result in oscillations of the levels of PtdIns(4,5)P₂ in the plasma membrane. Furthermore, because several regulatory pathways connect PtdIns(4,5)P₂ to actin dynamics this raised the possibility that Ca²⁺ oscillations result in oscillations of the plasma membrane concentration of PtdIns(4,5)P₂ that then result in oscillatory changes in cortical F-actin. To directly investigate whether the levels of PtdIns(4,5)P₂ oscillate, we monitored cortical actin using F-tractin and PtdIns(4,5)P₂ using the biosensor YFP–PH-PLCδ. We found a marked correlation between the two (Fig. 3b and Supplementary Movie S5), with PtdIns(4,5)P₂ increasing first followed by F-tractin. The average lag between PtdIns(4,5)P₂ and F-tractin was 11.1 ± 1.5 s and was statistically significant larger than zero (P < 0.001, single-sample t-test). As a control, we used a second biosensor based on a Tubby domain that binds PtdIns(4,5)P₂ with a higher selectivity and observed the same oscillations, arguing that the biosensors measure oscillations in PtdIns(4,5)P₂ levels (Fig. 3c–e).

Several pathways connect PtdIns(4,5)P₂ to actin dynamics. As the positively charged poly-lysine motif in N-WASP (neural Wiskott–Aldrich syndrome protein) cooperatively binds to plasma membrane PtdIns(4,5)P₂ (ref. 27) in vitro, we examined whether the observed oscillation in PtdIns(4,5)P₂ might be responsible for oscillations in recruitment of N-WASP that would then create oscillations in polymerized actin (Fig. 4a).

The observed oscillations in plasma membrane polymerized actin were synchronized with PtdIns(4,5)P₂ oscillations (Fig. 4a and Supplementary Movie S5). PtdIns(4,5)P₂ signals were followed by N-WASP recruitment and then F-actin polymerization. Lags between N-WASP and PH-PLCδ to F-tractin were significantly bigger than zero (P < 0.05 n = 8 and P < 0.01 n = 7, respectively) with the average lag between PH-PLCδ and F-tractin significantly bigger than the lag between N-WASP and F-tractin (P < 0.05 n = 15, error bars show s.e.m.). A reduction in PtdIns(4,5)P₂ by forced translocation of a 5' PtdIns(4,5)P₂phosphatase rapidly decreases the level of N-WASP plasma membrane localization. Partial recruitment of YFP–FKBP–Inp54p to the plasma membrane (upper panel) by the addition of 0.5 µM rapamycin (to trigger binding to plasma-membrane-localized FRB; t = 620 s) triggers a small reduction in the level of PtdIns(4,5)P₂ as reported by the PH-PLCδ–mTurquoise biosensor (middle panel). A small relative reduction in PtdIns(4,5)P₂ was sufficient to cause a rapid reduction in the localization of mCherry–N-WASP to the plasma membrane (lower panel). Raw data for a,c are presented in Supplementary Table S1.

FIGURE 4

Oscillations in the plasma membrane levels of N-WASP connect PtdIns(4,5)P₂ and F-actin oscillations. (a) Synchronized phase-shifted oscillations in the N-WASP (purple) and cortical F-actin (F-tractin, green) concentrations in the centre region. (b) PtdIns(4,5)P₂ signals are followed by N-WASP recruitment and then F-actin polymerization. Lags between N-WASP and PH-PLCδ to F-tractin were significantly bigger than zero (P < 0.05 n = 8 and P < 0.01 n = 7, respectively) with the average lag between PH-PLCδ and F-tractin significantly bigger than the lag between N-WASP and F-tractin (P < 0.05 n = 15, error bars show s.e.m.). (c) A reduction in PtdIns(4,5)P₂ by forced translocation of a 5' PtdIns(4,5)P₂phosphatase rapidly decreases the level of N-WASP plasma membrane localization. Partial recruitment of YFP–FKBP–Inp54p to the plasma membrane (upper panel) by the addition of 0.5 µM rapamycin (to trigger binding to plasma-membrane-localized FRB; t = 620 s) triggers a small reduction in the level of PtdIns(4,5)P₂ as reported by the PH-PLCδ–mTurquoise biosensor (middle panel). A small relative reduction in PtdIns(4,5)P₂ was sufficient to cause a rapid reduction in the localization of mCherry–N-WASP to the plasma membrane (lower panel). Raw data for a,c are presented in Supplementary Table S1.

FIGURE 5

Inhibition of N-WASP dynamics inhibits F-actin oscillation without affecting Ca²⁺ and PtdIns(4,5)P₂ oscillations. (a) Wiskostatin-mediated inhibition of N-WASP and actin dynamics. Co-oscillation of pathway components after stimulation with antigen. The dashed line indicates the addition of N-WASP (final concentration, 8.33 µM). (b) Inhibition of N-WASP dynamics through chemically induced FRB/FKBp dimerization of an inhibitory N-WASP construct. Co-oscillation of pathway components after stimulation with antigen. In this experiment, N-WASP was tagged with an FKBP and a non-fluorescent plasma-membrane-targeted FRB domain was co-expressed. The dashed line indicates the addition of 5 µM rapamycin, which induced dimerization of the FKBP and FRB domains. This, in turn, caused anchoring of N-WASP to the plasma membrane that resulted in inhibition of actin oscillations without any significant change in PtdIns(4,5)P₂ oscillations. Raw data for a,b are presented in Supplementary Table S1.
Figure 6 Synchronized phase-shifted oscillations of Ca\(^{2+}\), PtdIns(4,5)P\(_2\), N-WASP and F-actin. Ca\(^{2+}\) oscillations drive cortical F-actin oscillations. (a) Example of a live-cell 5-channel analysis of Ca\(^{2+}\) (Fura2), PtdIns(4,5)P\(_2\), N-WASP and F-actin signals showing synchronized oscillations. Stopping Ca\(^{2+}\) oscillations using the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) pump inhibitor thapsigargin blocks all oscillations. (b) Changes in the intensity of the four biosensors in a representative cell. The time at which actin oscillations were inhibited using 4 \(\mu\)M latrunculin is indicated. (c) Suppressing actin polymerization using cytocholasin D blocked F-actin oscillations but not the other oscillations. Raw data for a–c are presented in Supplementary Table S1. (d) Schematic representation of the Ca\(^{2+}\) pathway that generates coordinated cortical F-actin oscillations. P indicates the probability that the lag between sequential biosensors is bigger than zero on the basis of cross-correlation analysis of 83 cycles in 4 cells. (e) Average normalized cycle intensities of all cycles. The colour codes are the same across all panels. The dashed lines in a–c indicate the time of drug addition.

Supplementary Movie S4). The average lag between N-WASP and F-actin was 6.7 ± 1.1 s (P < 0.01 for lag bigger than zero). Figure 4b shows a quantitative analysis of a longer delay from the PtdIns(4,5)P\(_2\) increase to the F-tractin peak when compared with the delay from the N-WASP to the F-tractin peak, consistent with the interpretation that the changes in PtdIns(4,5)P\(_2\) drive N-WASP recruitment (P < 0.05, two-sample t-test). Furthermore, partial lowering of the levels of PtdIns(4,5)P\(_2\) by recruiting the PtdIns(4,5)P\(_2\) phosphatase Inp54p to the plasma membrane (using a FRB/FKBP dimerization method\(^{23}\)) reduced the plasma membrane concentration of N-WASP (Fig. 4c). Thus, although other PtdIns(4,5)P\(_2\)-sensitive F-actin regulators are probably involved\(^{28}\), these results suggest that N-WASP is a link between PtdIns(4,5)P\(_2\) and F-actin oscillations in the centre region of cells.

Together, these data suggest a mechanism for the observed actin oscillations. The positive-feedback-based Ca\(^{2+}\) oscillations cause oscillations in the plasma membrane concentration of PtdIns(4,5)P\(_2\) that then result in oscillatory recruitment of N-WASP to the plasma membrane. N-WASP oscillatory recruitment to the membrane then causes oscillations in cortical actin polymerization. To further
test this mechanism we developed an assay that allowed us to visualize this entire pathway live in a single cell by performing simultaneous 5-channel live-cell monitoring of cytoplasmic Ca\(^{2+}\) levels using Fura-2 by epi-fluorescence imaging and N-WASP, F-actin and PH-PLC\(\beta\) by TIRF imaging. As predicted by this model, all four signals oscillated with the same frequency but shifted phases (Figs 5 and 6). Pharmacological agents were then used to further examine this model by inhibiting the oscillations at different levels in the pathway. Inhibition of N-WASP dynamics using the small-molecule inhibitor wiskostatin (Fig. 5a) dissociated N-WASP from the plasma membrane, blocked F-actin oscillations and reduced total cortical F-actin without affecting Ca\(^{2+}\) or PtdIns(4,5)P\(_2\). Similarly, rapamycin-mediated plasma membrane recruitment of an inhibitory N-WASP–FKBP12 fusion construct\(^{28}\) suppressed actin oscillations without affecting Ca\(^{2+}\) or PtdIns(4,5)P\(_2\) oscillations (Fig. 5b). The addition of the endoplasmic reticulum Ca\(^{2+}\) pump inhibitor thapsigargin rapidly stopped all four oscillations (Fig. 6a). This argued that the observed PtdIns(4,5)P\(_2\) oscillations are probably a result of Ca\(^{2+}\) oscillations periodically activating Ca\(^{2+}\)-sensitive PLC. Finally, blocking the most downstream oscillation of F-actin by the addition of latrunculin B (Fig. 6b) or cytochalasin D (Fig. 6c) showed that oscillations in Ca\(^{2+}\), PtdIns(4,5)P\(_2\) and N-WASP continued after the inhibition of actin oscillations as predicted by the linear pathway mechanism. Thus, linked Ca\(^{2+}\) and PtdIns(4,5)P\(_2\) oscillations and F-actin are shown: average, where the F-actin and Ca\(^{2+}\) levels are the mean of the oscillatory inputs, and optimal, where the levels were chosen to maximize the total secretion. (d) Monitoring antigen-triggered recruitment of myosin Va, a motor protein that binds vesicles to F-actin and transports them. The left micrographs show that recruitment of myosin Va occurs in the centre where fusion occurs (maximum projection before and 2 min after antigen addition; scale bars, 5 \(\mu\)m). The right bar graph shows that, during F-actin oscillation cycles, the recruitment preferentially occurs when the F-actin levels are high. As a control, the average intensity of a GFP membrane marker (Lyn) is compared with GFP–myosin Va (MyoVa) during low-actin (light green) and high-actin (dark green) phases during oscillations. Error bars are s.e.m., \(n = 83\), ***\(P < 0.001\), Student’s \(t\)-test; NS, not significant.

To map the sequence of events during each of the oscillation cycles, we averaged the signals from different cycles using the peak Ca\(^{2+}\) level as an anchor (Fig. 6e). This analysis showed that a Ca\(^{2+}\) increase (orange) and concomitant PtdIns(4,5)P\(_2\) reduction (cyan) precede the drop in plasma-membrane-localized N-WASP (purple), which in turn precedes a drop in F-actin polymerization (green). As the Ca\(^{2+}\) levels decrease and the PtdIns(4,5)P\(_2\) levels increase, N-WASP is again recruited to the plasma membrane, followed by an increase in F-actin polymerization.

**Computational model linking oscillating cortical actin to enhanced secretion efficiency**

We next considered the possibility that coupled Ca\(^{2+}\) and actin oscillations may explain the antagonistic barrier and carrier function of the actin cortex. We reasoned that oscillatory actin dynamics would allow cytoplasmic vesicles to be captured when actin is elevated, pass to the plasma membrane when cortical actin is lowered and fuse with the plasma membrane when Ca\(^{2+}\) is subsequently increased. To evaluate how such a system may work, we constructed a simplified mathematical model (Fig. 7a–b and Supplementary Note). The scheme in Fig. 7a shows how the model defines the F-actin and Ca\(^{2+}\) dependencies of the vesicle capture, passage and fusion processes. The right panel shows the output, the secretion rate. The lower panel shows the output, the secretion rate.

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**Figure 7 Mathematical model of how phase-shifted oscillations in cortical F-actin and Ca\(^{2+}\) correlate with a repetitive cycle of vesicle capture, passage and fusion to enhance secretion rates.**

(a) Scheme of the mathematical model. Inset: The F-actin and Ca\(^{2+}\) dependencies of the three vesicle-state transitions: capture, passage and fusion. \(V_{cortex}\) and \(V_{pm}\) are the respective concentrations of vesicles bound to the actin cortex and plasma membrane. SG, secretory granule. (b) Simulation output of the model. The upper panel shows the model input: Ca\(^{2+}\) oscillations and F-actin oscillations. The middle panel shows the changes in the concentration of actin-bound (blue) and plasma-membrane-bound (green) vesicles. The lower panel shows the output, the secretion rate. (c) Comparison of model predictions of secretion rates for oscillatory and constant F-actin and Ca\(^{2+}\) input levels. Two levels of constant Ca\(^{2+}\) model inputs: Oscillatory

| Model input                         | Oscillatory | Constant average | Constant optimal |
|-------------------------------------|-------------|------------------|------------------|
| Actin                               | 0.1         | 0.1              | 0.1              |
| Capture                             | 0.001       | 0.001            | 0.001            |
| Fusion                              | 0.001       | 0.001            | 0.001            |
| Secretion rate                     | 0.001       | 0.001            | 0.001            |

(b) Model output: Secretion rate (a.u.)

| Model output                          | Secretion rate |
|---------------------------------------|----------------|
| Actin                                 | 0.1            |
| Capture                               | 0.001          |
| Fusion                                | 0.001          |
| Secretion rate                       | 0.001          |

The right panel shows the output, the secretion rate. The lower panel shows the output, the secretion rate.
Figure 8 Direct measurements of enhancement in secretion due to Ca$^{2+}$ and F-actin oscillations. (a) Bar diagram showing that secretion rates are more efficient for cells that oscillate (blue) when compared with cells with elevated but non-oscillating Ca$^{2+}$ levels (red). The addition of thapsigargin or cytocholasin D to oscillating cells abolishes the enhanced secretion without significantly affecting the secretion when added to non-oscillating cells. The addition of jasplakinolide abolishes the oscillating cell’s advantage and causes a further overall reduction in secretion. The addition of DMSO is included as a control. (n oscillating = 2,891, n non-oscillating = 4,642 cells; error bars are s.e.m). The results of t-tests comparing the exocytosis level of oscillating cell to non-oscillating cell secretion before the addition of drug or secretion after the addition of DMSO are presented above the bar plot; NS, not significant, * P < 0.05 and *** P < 0.001. (b) Example of an oscillating cell treated with jasplakinolide, showing that secretion slows even though the average Ca$^{2+}$ level is higher. (c) Example of an oscillating cell treated with thapsigargin, showing that stabilizing the actin cortex inhibits secretion. (d) Schematic representation of the proposed secretion cycle based on phase-shifted F-actin and Ca$^{2+}$ oscillation cycles. In each cycle, capture occurs at high F-actin, followed by passage at low F-actin, followed by fusion when the Ca$^{2+}$ levels subsequently increase. SG, secretory granule; PM, plasma membrane. Note that changes in the diagram are not to scale and are for illustration purpose alone.
most of the vesicle fusion occurs (Fig. 3a). Finally, a statistical analysis showed that myosin Va recruitment occurs preferentially during peak compared with trough F-actin levels during oscillations (Fig. 8d, right), supporting the model assumption of a cyclic capture of vesicles at peak levels of F-actin.

We then tested the prediction that oscillations make secretion more effective by comparing secretion rates in cells exhibiting oscillating Ca\(^{2+}\) levels with cells exhibiting persistently elevated Ca\(^{2+}\) levels. Both types of cell can be found in response to the same antigen stimulus. Indeed, oscillating cells (blue, Fig. 8a and Supplementary Fig. S5) show ~30% higher secretory rates when compared with cells with persistently elevated Ca\(^{2+}\) levels (red). To directly compare secretion during oscillatory Ca\(^{2+}\)/F-actin changes versus persistent Ca\(^{2+}\) elevation, we added the endoplasmic reticulum Ca\(^{2+}\) pump blocker thapsigargin that increases Ca\(^{2+}\) levels and found that cells with oscillations before the addition of thapsigargin subsequently reduced exocytosis rates to levels comparable to those of non-oscillating cells (Supplementary Movie S7). An example trace is shown in Fig. 8b, where the addition of thapsigargin keeps Ca\(^{2+}\) at a persistently high level while reducing the rate of secretion (Fig. 8c). We further tested whether the advantage of oscillating cells is eliminated when we block actin oscillations with either an actin polymerization inhibitor cytochalasin D, or an actin filament stabilizer jasplakinolide (Fig. 8b). Both inhibitors reduced the secretion rates of oscillating cells by approximately ~30%, to the same rate as those of non-oscillating cells (Fig. 4a). In agreement with a barrier role for the actin cortex, jasplakinolide, which stabilizes cortical actin, reduced the level of secretion in both cases even further (Fig. 8a,c).

**DISCUSSION**

The evolution of biological control systems such as the one of regulated secretion involves optimization between conflicting requirements. Our study suggests that an oscillating system design can be employed by cells to optimize the reliability and efficiency of dense-core vesicle secretion. The challenge for regulated secretion is that the actin cortex is critically required for cells to maintain cell shape and prevent the formation of membrane blebs\(^{30}\). Yet, as appreciated 40 years ago\(^{3}\), the same cortical actin that is critical for a cell’s integrity prevents the access of secretory granules to the plasma membrane. This barrier effect of the actin cortex is probably important in many cell types to minimize inadvertent secretion in response to short stimuli that are too short or weak to lower the barrier. At the same time, secretory granules need to be transported from the cell interior to the vicinity of the plasma membrane in a process requiring microtubules to get the vesicles closer to the periphery and actin-motor-mediated transport to get them docked to the plasma membrane\(^{1}\). This apparent barrier–carrier paradox therefore provides an excellent example of the evolution of dynamic regulatory strategies to optimize cell function given multiple constraints on the system.

Our study introduces a dynamic secretory system design that uses oscillations in cortical actin to temporally partition the opposing functions of the actin cortex. We show that cells employ phase-shifted oscillations of Ca\(^{2+}\), PtdIns(4,5)P\(_2\), N-WASP and F-actin to enhance secretion. The coupled Ca\(^{2+}\) and F-actin oscillations correlate with a vesicle secretion recruitment and fusion engine that repeatedly transitions between three strokes: capture at high F-actin levels, passage through the actin cortex at low F-actin levels, followed by Ca\(^{2+}\)-triggered fusion (Fig. 8d). Thus, cells can consolidate opposing roles of a control element by oscillating its concentration and temporally partition the associated regulatory processes. Given the ubiquitous presence of oscillating systems in biology, it is possible that many of them evolved to resolve similar opposing constraints. As in the case of dense-core vesicle secretion, such optimized oscillatory systems probably have a general role in enhancing the effectiveness and robustness of different cellular outputs.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary Information is available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

T.M. and R.W. designed experiments and wrote the manuscript. R.W. performed all experiments and data analysis.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Methods

Nucleic acid reagents. mTurquoise–NWASP, mCitrine–NWASP, mCitrine–PH-PLCδ and mTurquoise–PH-PLCδ were cloned into Clontech-C1-like mTurquoise and mCitrine vectors. F-trac- GFP was a gift from M. Schell (Department of Pharmaceutical Sciences, University, USA) and was sub-cloned to replace the fluorescent protein with mCerulean, mCherry and tdTomato. GFP–myosin Va was a gift from J. Spudich (Department of Biochemistry, Stanford University, USA). siRNA reagents for N-WASP were purchased from Sigma Aldrich (cat. no. SASI_Rn02_00231193, 5′-GGACAAAGGCCATATAAG-3′ and SASI_Rn02_00231194, 5′-GGGAAAGAACGCTATAA-3′), siRNA against myosin Va was purchased from Dharmacon (cat. no. MQ-094195-00-0005, 5′-GAUAUAGGUGGCUCAUCU-3′, 5′-GAUACUGGCGAGCAGGU-3′. 5′-CCAAACUAUUAUUAAGCUA-3′). Control non-targeting siRNA was purchased from Dharmacon (cat. no. D-001206-14-05, 5′-GGUUGUCAUUGUCCGAAU-3′).

Chemicals. The Fura-2 and Flou-4 Ca^{2+} dyes were used at 1–4 μM and thapsigargin was used at 1 μM. (Invitrogen). The N-WASP inhibitor wiskostatin (Sigma) was used at 8.33 μM. Latrunculin B (used at 4 μM), cytochalasin D (used at 2.5 μM) and jasplakinolide (used at 2 μM) were purchased from EMD Chemicals.

Cell culture and electroporation. RBL-2H3 cells (ATCC) were cultured in DMEM supplemented with 10% FBS (Invitrogen). In all experiments with the exception of that in Fig.1, cells were sensitized overnight with anti-TNP IgE (BD Biosciences) and stimulated by the addition of 10 μM ionomycin and 100 ng ml^{-1} PdBu (Sigma). For all fluorescent reporter experiments and siRNA knockdowns, cells were electroporated using Amaxa nucleofactor following the manufacturer’s instructions (kit T, program X001).

Microscopy. Cells were imaged using a custom-built prism TIRF microscope with 5 laser lines (442, 473, 514, 532 and 650). The microscope was equipped with a Lambda Sutter LS arclamp with an internal filter wheel and another shutters were controlled using the through-the-lens signal generated using an Arduino control board. To combine epi-fluorescence we added a 10/90 broad band-pass filter. The exposure time was 100–500 ms using gain values of 250–600. To correct for differences in the loading of dextran–FITC between experiments, the number of peaks in each cell was divided by the average number of peaks per cell in a movie. Oscillatory cells were identified on the basis of the Fura-2 ratio time series using spectrally analysis with a frequency window of 1/15 to 1/60 s.

Time-series processing. Time-series data were estimated either per pixel or for a user-defined region of interest. The pixel intensity data per frame and the time stamp for that frame were used to interpolate the data to a uniform grid (T_{pix}). An equal spacing time grid was chosen (T_{pix}) such that the time difference between two interpolation points in the grid is the average time difference between channels. Then a linear interpolation procedure was employed for each channel time series separately to estimate the channel intensities on the time grid T_{pix}. This procedure resulted in a matrix where all intensities are from the same time point. This interpolated matrix was then used for further analysis. This analysis procedure made the analysis independent of the order of channel acquisition. An example of a time series of oscillation times and the grid matrix is shown in Supplementary Fig. S14d. For the cross-correlation analysis, a high-pass filter was applied to the time series and the output was normalized to a maximal autocorrelation value of 1. To aid visualization of the oscillatory dynamics all time-series data were filtered using a high-pass filter with a width >300 s. An example of the analysis routine with intermediate results is provided in Supplementary Fig. S8.

Clustering analysis of fluctuating actin regions. Time-lapse images were first corrected for uneven illumination and photobleaching and registered in x–y drift as described above. The aligned stack was used to create ∼12,000 time series for intensity changes for the biosensor F-tracin. Frequency power spectrum analysis was used to identify the regularity of the oscillations and their major period. In addition, the range (1–99% percentile interval) was calculated for all of the time series. These three dimensions (regularity, major period and range) were used as inputs to GMM clustering analysis for two populations. The boundary between the two populations was the two-dimensional manifold where the posterior distribution for each of the two populations was 0.5. Supplementary Fig. S3 shows an example of a scatter plot used for clustering.

Spatial correlation analysis. The correlation coefficient between every two time series in the cells as well as their Euclidean distance was measured (∼80,000,000 pairs per cell). The data were binned (single-pixel width; 0.26 μm) and fitted with an exponentially decaying function (Fig. 2c). The length scale was defined as the distance over which the correlation decays.

Measurement of F-actin levels after thapsigargin and EGTA treatment. Cells were transfected with EGFP–PH-PLCδ channel that was interrupted every 3–5 s to acquire the Fura-340 and Fura-380 epi-fluorescence channels. Exocytosis was quantified by first identifying peaks in each cells’ dextran–FITC intensity time series. Peaks were defined as local maxima above a threshold to eliminate noise. For correction in using dextran–FITC between experiments, the number of peaks in each cell was divided by the average number of peaks per cell in a movie. Oscillatory cells were identified on the basis of the Fura-2 time series using spectrally analysis with a frequency window of 1/15 to 1/60 s.
to the manufacturer’s protocol. Several hundred cells (743 for the control, 895 for thapsigargin+EGTA and 1,559 for thapsigargin) were imaged per treatment. Binary masks were generated using Otsu thresholding on the DAPI intensity and the phalloidin intensity within each mask per cell was quantified.

**Statistical analysis.** The following statistical tests were used throughout the paper.

To test for the existence of a lag, cross-correlation analysis on two time series was performed (as explained above) for multiple cells. In each cell, the cross-correlation analysis provided a single value of lag where cross-correlation is maximal. Using this population of lags, a single-sample *t*-test was used to estimate whether the lag value was significantly different from zero. The *P* values in Fig. 4b are the values for which the lags between N-WASP and F-actin and between PtdIns(4,5)P₂ and N-WASP were significantly different from zero.

To test the difference between lags of different biosensors, first a population of lags for each marker was estimated as described above and a statistical test to compare the two populations was performed. In Fig. 4d we show average lag values and therefore use a parametric two-sample *t*-test. In Supplementary Fig. S5c, owing to the existence of a few outliers, the median value is presented and a non-parametric Wilcoxon rank-sum test was performed.

To test the significance of different treatments on β-hexoamidase secretion (Supplementary Figs S1 and S10), multiple wells were measured and a two-sample *t*-test was performed comparing each of the treatment against controls.

To test the significance of the change in cortical F-actin after treatment with thapsigargin (Supplementary Fig. S4a) and thapsigargin + EGTA, cells were treated as described above and two sample *t*-tests were performed comparing them to control cells.
**Figure S1** Changes in the intensity of the biosensor F-Tractin reflect changes in total actin concentration. 

**a** Co-oscillation of YFP-actin and F-Tractin-mCerulean measured in a 12mm x 12mm ROI in the center of the cell. F-Tractin and YFP actin has the identical phase with F-Tractin oscillations. F-Tractin has higher relative amplitude of oscillations due to an improved signal to noise compared to yfp-actin that reports filamentous actin compared to soluble monomers plus filamentous actin. These oscillations are not seen when monitoring a plasma membrane marker WGA-647 (Wheat Germ Agglutinin conjugated to alexa 647). This shows that actin oscillations are not a result of changes in the Z-position of the cell. 

**b** Quantification of the improved signal to noise of F-Tractin compared to actin. Time series of F-Tractin-mCerulean and YFP-Actin where normalized to show % change from average and plotted in a scatter plot against each other. The slope of the linear regression line (1.46) shows that F-Tractin has a ~46% improved signal to noise over directly measuring Actin in our TIRF imaging set-up.

**c** Time series of F-Tractin, showing the base line actin fluctuation levels (T=0 to T=200) compared to the oscillations post oscillations (T>200).

**d-g** Estimation of the change in relative F-actin concentration from peak to trough in an oscillatory cycle. 

- **d** To capture cells at either peak or trough we used the addition of either Thapsigargin alone or Thapsigargin with EGTA to antigen stimulated cells. The levels of actin cortex after these drug manipulations was estimated using TIRF imaging of phallodin-alexa 594 stained cells. Cells with low calcium (Thaps + EGTA) had a 14% increase in their actin cortex compared to control whereas cell with high calcium (Thaps) had 50% reduction in their actin cortex (P-value <0.005 for both treatments, N=743, 895 and 1559 for Control, Thaps and Thaps+EGTA. Errorbars show s.e.m.). 

- **e, f, g** Measurements of calcium (e) and F-Tractin (f) during oscillations and after the addition of Thapsigargin. 

  - **g** The magnitude of F-Tractin change during oscillation (Osc) was 23% of the level of F-tracin change seen after the addition of Thapsigargin. At the same time, addition of Thapsigargin (as shown in panel a), the actin levels were reduced by 64% from their peak levels without calcium. Therefore it can be estimated that the approximate change of cortical F-actin during oscillation is ~15% (=23% * 64%).
Figure S2 Cluster analysis of the F-Tractin timeseries. a Maximum projection of F-Tractin-mCherry timelapse data shows higher levels of polymerized actin in the periphery of cells. b An intensity based Gaussian mixture model clusters the pixels in the cells into two regions. c Map of the range of actin oscillations defined as the 1% to 99% of the intensities in the F-Tractin timeseries. d For each pixel, a major period was identified in the power-spectral analysis and converted to a color coded amplitude. The center pixels all have identical major periods. e Scatter plot representing the three dimensional data used for the cluster analysis; the range of intensity during oscillations the magnitude of the maximal regular oscillation and the period (color coded). The dashed line shows the 2D projection of the identified clusters boundary. f Color matrix shows the obtained secretion rates from simulation with various levels of constant Calcium and Actin. The bottom panel shows the product of the Capture and Passage transitions as a function of Actin. The right panel shows the fusion transition as a function of Calcium. Scale-bar 5 μm.
**Figure S3** Control analysis of the spatial and temporal scale of calcium oscillations and results of the mathematical model when Calcium and Actin are kept constant. 

**a** Example of a time series of Ca2+ at the bottom of the cell as imaged by TIRF microscopy of a flou4 calcium sensitive dye showing uniform Ca2+ oscillations. 

**b** and **c**. A period map analysis of Ca2+ oscillation on a pixel basis identical to the actin analysis shown in Fig 1 and S3. Analysis routine used is in panels **b, c** is identical to the one used figure 1c. This analysis showed that Ca2+ oscillations have the same frequency everywhere in the cell. 

**d** Analysis of the spatial scale of calcium oscillations shows that oscillations are synchronized across the entire cell (after occasionally observed wave propagations in the first few oscillations). Analysis routine used is identical to the one used in figure 1d. 

**e** Maximal projection of the Flou4 intensity, cell was segmented manually to separate it from neighboring cells. Scale-bar 5 μm.
**Figure S4** Control experiments on the role of GFP-Myosin Va and N-WASP in secretion.  

**a-b** Total secretion using b-hexoamidase assay after siRNA knockdown of non targeting control and knockdown of Myosin Va (a) and N-WASP (b). Western blots confirm knockdown levels and boxplot show total secretion. (** P-value < 0.01, *** P-value < 0.001, two sample t-test comparing with control wells, N=8 per treatment). Panel b also shows that pharmacological inhibition of N-WASP with Wiskostatin reduces secretion to similar levels as the siRNA treatment (two sample t-test, p-value>0.15, N=8 per treatment).  

**c** Quantification of levels of GFP-Myosin Va in cells after antigen stimulation. Red line shows the average distance of the boundary between the pulsatile and oscillatory regions from cluster analysis that was performed on F-Tractin time series from 45 cells. A total of 9 cells where used to estimate Myosin Va recruitment, errorbars are s.e.m.  

**d** Control experiments, showing that addition of 2 mM Jasplakinolide completely stops actin oscillations.
Figure S5 Additional examples of calcium and dextran-FITC time series. 

**a** Additional examples are shown of time series of calcium (orange) and dextran FITC (red) in cells activated with antigen. 

**b** Histogram of timing of exocytosis peaks in relation to the average calcium cycle.
Figure S6 Description of microscopy set-up and time series analysis. 

(a) Schematic of the light path on the prism-based TIRF used in this study. 5 laser lines were combined on an optical table using single edge dichroics where each laser has its own shutter. The combined laser line was targeted to a prism such that it will pass through the immersion oil and reflect from the water-coverslip interface at a shallow angle. The reflection at shallow angle results from a evanescent wave that penetrates ~100 nm into the sample. Epi illumination was performed through a 10/90 beamsplitter and a Sutter arclamp.

(b) Spectral map of the main fluorescent proteins and dyes used in this work overlaid on the excitation (for Fura-2) laser lines (dotted-dashed lines) and emission (rectangles) used.

(c) Example of the distribution of time between frames of same channel. The small population with dt > 10 sec is the frames where autofocus routine was employed. Inset shows a zoom of the region between 4-5 seconds where majority of time intervals were.

(d) Demonstration of the interpolation routine used to "gridify" timeseries to the same grid. Raw measurements for all channels are shown with blue x marker and interpolated values are shown in red points. Note that all red points have the same time values, allowing for comparisons of delays between channels independent of the order of acquisition and duration of exposures.
Figure S7  Quantification of level of bleed-through and auto-fluorescent signals in the TIRF setup used in this study. Bar plot show the % signal seen in the “bleed-through” channel for all pairs of channels that are close to each other within the spectra. Arrows are used to point to cells transfected with only one color of fluorescent protein (eg CFP and not YFP). Note that cellular auto-fluorescent will also contribute to apparent bleed-through and was not corrected in the images. All images show pairs of channels with potential bleed-through between channels. In all cases the intensity of the correct marker could be seen but not any signal in the cells not expressing that marker. This demonstrates that channel bleed-through makes only a minimal contribution to the measured signals. Scale-bar 5 μm.
**Figure S8** Step-by-step tracking of the time series analysis.  

- **a** PH-plcd (top left) and F-Tractin (top right) images compared to the result of the actin dynamics cluster analysis (bottom left) and an overlay of PH-plcd and F-Tractin (bottom right). Scale-bar 5 mm.
- **b** The raw intensity time series averages in the central oscillatory region (shown as white pixels in panel a bottom left).
- **c** Time series after correction for photobleaching, background subtraction and rescaling.
- **d** Removal of trends in the time series. Frequencies at least an order of magnitude lower than the measured main oscillation frequency are removed, allowing for a reliable cross-correlation analysis.
- **e** Rescaling of the result.
- **f** Alternative normalization by rescaling to the average signal.
- **g** Zoomed view of panel f that is also shown in main Fig. 2c.

In panels b-g: x-axis shows the time of antigen additions in seconds and the y-axis shows corrected fluorescence intensity.
Supplementary Movies

**Movie S1** Secretion after pharmacological activation. Low resolution (10x) TIRF timelapse of SG marker after the addition of Ionomycin and PdbU. Movie is sped up x25 times (7fps imaging dt = ~3.5 sec), scale bar 20 mm.

**Movie S2** F-Actin Oscillations. High resolution (60x) TIRF timelapse of F-Actin (left) and the temporal derivative (dF-Actin/dt) on the right. Movie is sped up x60 times (15 fps imaging dt = ~4 sec), scale bar 5 mm.

**Movie S3** High resolution imaging of exocytosis after physiological activation. Exocytosis of dextran-FITC after stimulation with antigen. Movie is sped up x7 times (7 fps imaging dt = ~1 sec), scale bar 5 mm.

**Movie S4** F-Actin and N-WASP oscillations. High resolution (60x) TIRF timelapse of F-Actin (left), N-WASP (middle) and membrane marker Lyn. Bottom row shows the temporal derivative. Movie is sped up x35 times (10 fps imaging dt = ~3.5 sec), scale bar 5 mm.

**Movie S5** F-Actin and PH-plcd oscillations. High resolution (60x) TIRF timelapse of F-Actin (left), and PH-plcd (right) Bottom row shows the temporal derivative. Movie is sped up x60 times (15 fps imaging dt = ~4 sec), scale bar 5 mm.

**Movie S6** Calcium oscillation. TIRF imaging of calcium dynamics at the bottom of cells using Flou-4 calcium sensitive dye.

**Movie S7** Exocytosis after physiological stimulation. Low resolution (10x) TIRF/Epi imaging of Fura-2 (left, pseudo-colored) and dextran-FITC (right). Scale bar 32 mm.

**Supplementary Note.** Mathematical model. Description of the mathematical model that analyzes the effect of oscillatory Ca2+ and F-actin dynamic on secretory granule secretion.

**Supplementary Data.** Code implementing the mathematical model. Matlab code that implements the model described in the Supp. Note.

**Supplementary Table 1.** Raw data for all TRIF time series presented in figures 3,4,5,6. Raw data is shown after correction for photo bleaching.