Calcium Flashes Orchestrate the Wound Inflammatory Response through DUOX Activation and Hydrogen Peroxide Release

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Summary

A crucial early wound response is the recruitment of inflammatory cells drawn by danger cues released by the damaged tissue. Hydrogen peroxide (H₂O₂) has recently been identified as the earliest wound attractant in Drosophila embryos and zebrafish larvae [1, 2]. The H₂O₂ signal is generated by activation of an NADPH oxidase, DUOX, and as a consequence, the first inflammatory cells are recruited to the wound within minutes. To date, nothing is known about how wounding activates DUOX. Here, we show that laser wounding of the Drosophila embryo epidermis triggers an instantaneous calcium flash, which travels as a wave via gap junctions several cell rows back from the wound edge. Blocking this calcium flash inhibits H₂O₂ release at the wound site and leads to a reduction in the number of immune cells migrating to the wound. We suggest that the wound-induced calcium flash activates DUOX via an EF hand calcium-binding motif and thus triggers the production of the attractant damage cue H₂O₂. Therefore, calcium represents the earliest signal in the wound inflammatory response.

Results and Discussion

Wounding the Drosophila Embryo Epidermis Results in an Immediate Calcium Wave

Drosophila embryos are able to heal laser-induced epithelial wounds [3] and in parallel mount a robust inflammatory response with the rapid recruitment of embryonic macrophages, called hemocytes [4, 5]. In Drosophila embryos and zebrafish larvae, hydrogen peroxide (H₂O₂) synthesized by the NADPH oxidase (NOX) enzyme DUOX appears to be pivotal in the stimulation of this inflammatory response [1, 2], but precisely how DUOX is activated by tissue damage remains unknown. However, DUOX possesses two canonical EF hands on an intracellular loop, which implicates cytosolic calcium as a potential regulator of H₂O₂ production.

To test for calcium signals in cells at the wound edge—signals similar to those observed in wounded epithelial cells in vitro [6–10], the C. elegans embryo epidermis, [11] or zebrafish larval tissues [12, 13]—we expressed the intracellular calcium reporter GCaMP3 [14] specifically in the epidermis via the GAL4-UAS system [15] by using the e22c-Gal4 driver [16]. We coexpressed mCherry-moesin to visualize cortical actin in epithelial cells.

The ventral epithelium of stage 15 embryos was laser wounded and imaged by spinning-disk microscopy. Prior to wounding, GCaMP3 fluorescence, and hence cytosolic calcium levels, did not alter in the epithelial cells; however, laser wounding resulted in a rapid calcium flash extending outward from the point of wounding as a wave to an average maximum distance of 39 ± 4.8 μm from the wound margin (SD, n = 6 movies) at a speed of 6.9 ± 2.5 μm/s (SD, n = 7 movies) across multiple cells (Figure 1A and Movie S1, available online). Ventral epithelial cells are elongated along the dorsoventral axis such that the calcium wave is propagated in a stereotypical ellipsoidal shape. Closer inspection revealed the wave to be traveling through individual cells before activating calcium release in neighboring cells (Figure S1Ai). Plotting the intensity of the GCaMP3 fluorescence in cells at varying distances from the wound edge over time showed that each cell was consecutively activated but that calcium response levels were reduced stepwise in successive rows of cells as cell distance from the wound edge increased (Figure S1Aii) until a threshold such that the wave could not travel further. This threshold was not absolute, given that larger wounds activated coordinately larger calcium waves (Figures S1B–S1D).

Previous scratch-wound analyses in cultured cells loaded with calcium reporter dyes showed that similar calcium waves depend upon extracellular diffusible mediators [8, 17]. However, in vivo, the spread of calcium is unlikely to be propagated in this manner, given that wounding the epidermis adjacent to the zippering seam of the dorsal embryonic hole, where cells are yet to be junctionally linked, resulted in immediate termination of the wave (Figure 1Bii), suggesting that cells must be intimately connected for transfer of the calcium wave. Gap junctions allow calcium waves to spread via diffusion of IP3 from cell to cell [18]. Drosophila gap junctions are thought to be composed of innexins, which are analogous to vertebrate connexins; therefore, to determine whether gap junctions are important for the wound-induced calcium wave to spread, we used two innexin 2 (Inx2)-null alleles, Inx200308 and Inx200306 [19], because Inx2 is highly expressed in the embryonic epidermis [20]. Compared to the controls, both alleles showed a significantly reduced spread of the calcium wave (Figures 1Bii and 1Biii), and the calcium influx was often restricted to the front row of cells at the wound edge. These alleles, however, did not affect the intensity of the initial calcium flash (Figure 1Biv), suggesting that innexins allow the calcium wave to propagate or maintain the calcium signal as it spreads but are not involved in its initiation.

After the rapid elevation of intracellular calcium, this signal subsequently decays to background levels of GCaMP3 fluorescence after approximately 15 min (830 ± 360 s, n = 9 wounds; Figure 1C and Movie S2), many minutes prior to the completion of wound closure. Resolution commences in a distal-to-proximal direction, and wound margin cells return to basal levels of calcium last of all (Figure 1C and Movie S2). During the resolution period, around 40% of cells within the...
calcium flash zone exhibited calcium oscillations (approximately 30% oscillated only once), and no cell underwent greater than three oscillations. Cells rapidly reset their calcium machinery because subsequent wounding (within 20 min of the original wound) again elicited an identical calcium response (Figure S1E).

Reducing the Calcium Flash Impairs the Inflammatory Response of Hemocytes
This calcium flash represents the earliest identified signal following tissue damage and might therefore orchestrate the rapid recruitment of immune cells. To assess the impact of a reduced calcium flash upon the inflammatory response, we monitored the numbers of hemocytes drawn to laser-induced epithelial wounds under a range of pharmacological and genetic treatments.

calcium-blocking pharmacological treatments, and the number of hemocytes present at ventral epidermal wounds was assessed after 20 min. Thapsigargin or EGTA treatments reduced the average hemocyte response to 55% or 72%, respectively, of that of negative controls (Figure 2B). Live imaging revealed no difference in the basal developmental migration of hemocytes posttreatment (Figure S2B), suggesting that the reduced recruitment was due to a failure by hemocytes to detect wounds rather than a general migration defect.

Similarly, loss-of-function mutant embryos of the TRPM channel (trpm2), whose C. elegans ortholog is important in wound-induced calcium responses [11] and is expressed across epithelial tissues in the embryo [22], displayed both a reduced calcium response after epidermal wounding (Figure 2C and Figure S2C) and reduced hemocyte migration to wounds (Figure 2D). Importantly, loss of TRPM did not perturb
numbers of red-stinger-labeled hemocytes recruited to wounds in wave (n > 12 embryos per genotype).

controls, indicating an epithelial role for TRPM in the generation of a calcium wave immediately after wounding of trpm2

The mean integrated density of GCaMP3 fluorescence per embryo depicts 20

TRPM RNAi specifically in the epithelium is lower than that for wild-type trpm2

mutants (n > 10 embryos per genotype).

TRPM channel opens at 37°C and induces calcium influx into cells [29], which we confirmed by coexpressing TRPA and GCaMP3 in the epidermis (Figure S3C). Treatment of TRPA-expressing embryos for 30 min at 37°C increased the levels of H2O2, as assayed via Amplex Ultrade, fluorescence, suggesting that calcium influx is sufficient for the production of H2O2 in the epithelium (Figure S3D). Furthermore, overexpression of TRPA in spiracle bottle cells was sufficient to stimulate hemocyte recruitment to these sites when embryos were shifted to 37°C (Figure S3E).

To directly assess the role of calcium in the activation of DUOX, we knocked down DUOX by using RNAi and then coexpressed either full-length DUOX or a DUOX mutant lacking the
calcium responses displayed impaired H\textsubscript{2}O\textsubscript{2} production via Amplex Ultrared.

tous expression of dDUOX RNAi restored wound-induced H\textsubscript{2}O\textsubscript{2} production.

(C and D) Re-expression of full-length dDUOX, but not a truncated form (H\textsubscript{2}O\textsubscript{2}, red) show that embryos ubiquitously expressing DUOX RNAi and

6 show mean (see Experimental Procedures for details of F-F\textsubscript{0} quantification). The graphs

m via Amplex Ultrared fluorescence. The scale bars represent 20 µm.

Figure 3. DUOX Interprets the Calcium Wave via Its EF Hand Domain to Drive H\textsubscript{2}O\textsubscript{2} Production and Recruitment of Macrophages.

(A) Single confocal slices depicting GMA (actin, green) and Amplex Ultrared (H\textsubscript{2}O\textsubscript{2}, red) show that embryos ubiquitously expressing DUOX RNAI and GMA produced less H\textsubscript{2}O\textsubscript{2} at wounds than did wild-type controls, as assayed via Amplex Ultrared fluorescence. The scale bars represent 20 µm.

(B) tprm\textsuperscript{2} and inx2 mutant embryos with a reduction in their wound-induced calcium responses displayed impaired H\textsubscript{2}O\textsubscript{2} production via Amplex Ultrared (see Experimental Procedures for details of F-F\textsubscript{0} quantification). The graphs show mean ± SEM of at least 20 (tprm\textsuperscript{2}) and 7 (inx2) embryos per genotype. (C and D) Re-expression of full-length dDUOX, but not a truncated form specifically lacking the EF hand motif (dDUOX\textsubscript{EF}), in embryos with ubiquitous expression of dDUOX RNAI restored wound-induced H\textsubscript{2}O\textsubscript{2} production (C) and hemocyte recruitment (D) to the levels of wild-type controls. The graphs show mean ± SEM of at least 21 (C) and 11 (D) embryos per genotype.

See Movie S3 for a typical example of this data. Hemocyte numbers at wounds were determined from images of hemocytes labeled independently of GAl4 with srg–GMA. The p values were generated with a Student’s t test at the final time point (B [tprm\textsuperscript{2}], a two-way ANOVA with a Bonferroni posttest (B [inx2] and C), or a one-way ANOVA with a Bonferroni posttest (D). Asterisks denote p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***) and ns = not significant.

degraded and both displayed wild-type localization within cells (Figure S3F). Taken together, these results suggest that the calcium-binding EF hands of DUOX function as a fundamental link to couple wound-induced calcium signals to the activation of DUOX and to subsequent H\textsubscript{2}O\textsubscript{2}-mediated attraction of hemocytes to wounds.

DUOX Activity at the Wound Site Is Dependent on a DUOX Maturation Factor, NIP.

In mammalian cells, the maturation factor DUOXA2/NIP is necessary for DUOX2’s folding and translocation to the plasma membrane [31]. The Drosophila ortholog nip/DUOXA/mol is expressed throughout embryogenesis and localizes to the plasma membrane during cellularization [32]. Interestingly, expression of an RNAi targeting NIP transcripts developed fragile wings highly reminiscent of those observed from overexpression of DUOX RNAI in the wing [30].

to determine whether production of the wound cue is similarly dependent upon NIP, we analyzed the inflammatory response in a previously characterized nip-null mutant [32]. In nip mutant embryos, hemocytes migrated to their appropriate developmental locations at stage 15 and lay along the midline and lateral lines of the ventral nerve cord (Figure S4A). Furthermore, hemocytes exhibited the same levels of motility as wild-type controls (Figure S4B), suggesting that they were not intrinsically affected by loss of NIP. However, live imaging revealed that hemocytes in nip mutant embryos often ignored laser-induced epithelial wounds (Figures 4A and 4B) such that
20 min after wounding, there were many fewer hemocytes at wounds in nip mutants than in wild-type embryos (Figures 4A and 4B). To test how the loss of nip affects DUOX function, we imaged H$_2$O$_2$ production in nip mutant embryos by using the Amplex Ultradure assay. These mutants showed a reduced H$_2$O$_2$ response (Figure 4C) but a normal calcium flash on wounding (Figure S4C), suggesting that NIP is critical for the wound-associated H$_2$O$_2$ signal. Again, we could not completely block the H$_2$O$_2$ signal, suggesting a contribution from maternal protein or that there are additional alternative pathways at play in the generation of H$_2$O$_2$ at wounds.

The importance of DUOX has been previously implicated in the synthesis of the H$_2$O$_2$ signal that draws immune cells to wounds [1, 2]. However, how DUOX is activated upon wounding (Figure S4 C), suggesting that NIP is critical for the synthesis of the H$_2$O$_2$ signal that draws immune cells to wounds. We have shown that a calcium wave, induced upon wounding the Drosophila embryo epidermis, leads to activation of DUOX via its canonical EF hands wave, induced upon wounding the animal [1, 2]. However, how DUOX is activated upon wound-

Experimental Procedures
Fly Lines and Genetics
Flies were raised and embryos were imaged at room temperature. Embryos for experiments involving RNA interference were raised at 29°C. Genotypes used are outlined in the Supplemental Experimental Procedures and Table S1.

Imaging and Wounding
Embryos collected from overnight apple juice agar plates were dechorio-
nated in bleach and mounted ventral side up (they were mounted dorsal side up when they were wounded near the dorsal hole) on Greiner Lumox gas-permeable culture dishes (Sigma) in halocarbon oil 700 (Sigma) or glass slides with double-sided sticky tape with vol fate oil (WVR International); see Evans et al. [33] for a video protocol. Calcium and Amplex Ultradure (Invitro-
gen) imaging was performed on a Zeiss LSM510 confocal microscope or a Leica DMi6000B Ultraview Vox spinning-disk system (Perkin Elmer) fitted with Micropoint nitrogen ablation lasers (Andor) for wounding; red-stinger-

Drug and Amplex Ultradure Treatments
Dechorionated stage 15 embryos were incubated in a mixture of 1:1 heptane:drug (or Amplex Ultradure) solution in a glass vial for 30 min on a shaker. For TRPA overexpression experiments, embryos were incubated at 37°C in water prior to further treatments. Drug or Amplex Ultradure solu-
tions consisted of Drosophila Ringer solution, composed of 128 mM NaCl (Fisher Scientific), 2 mM KOI (Sigma), 35.5 mM sucrose (Fisher Scientific), 5 mM HEPES (Sigma), and 4 mM MgCl$_2$ (Fisher Scientific), as well as 5 mM thapsigargin (1 mM stock dissolved in DMSO; Sigma), or 50 μM Amplex Ultradure. DMSO (Sigma) was added to Ringers at a 1:1,000 dilution as a negative control for thapsigargin treatments. After incubation, embryos were transferred from the heptane-aqueous interface to halocarbon oil 700 and were then mounted and imaged as above.

Supplemental Information
Supplemental Information includes four figures, one table, Supplemental Experimental Procedures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.01.058.

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