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Control of local actin assembly by membrane fusion-dependent compartment mixing

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Abstract

Local actin assembly is associated with sites of exocytosis in processes ranging from phagocytosis to compensatory endocytosis. Here, we examine whether the trigger for actin-coat assembly around exocytosing *Xenopus* egg cortical granules is ‘compartment mixing’ — the union of the contents of the plasma membrane with that of the secretory granule membrane. Consistent with this model, compartment mixing occurs on cortical granule–plasma membrane fusion and is required for actin assembly. Compartment mixing triggers actin assembly, at least in part, through diacylglycerol (DAG), which incorporates into the cortical granule membranes from the plasma membrane after cortical granule–plasma membrane fusion. DAG, in turn, directs long-term recruitment protein kinase C β (PKC β) to exocytosing cortical granules, where it is required for activation of Cdc42 localized on the cortical granules. The results demonstrate that mixing of two membrane compartments can direct local actin assembly and indicate that this process is harnessed during *Xenopus* egg cortical granule exocytosis to drive compensatory endocytosis.

Local actin assembly is associated with processes that entail insertion of new membrane into the plasma membrane in many systems, ranging from phagocytosis¹ to cell locomotion^{2, 3}. A particularly striking example of coupling between exocytosis and local actin assembly is provided by regulated exocytosis in eggs⁴ and pancreatic acinar cells⁵. In these cells, actin ‘coats’ assemble specifically around exocytosing secretory granules, where they stabilize these compartments and/or promote their retrieval^{4, 5}. Moreover, actin accumulates around exocytosing secretory granules in a variety of other cell types, suggesting that such actin coating represents a conserved mechanism for driving compensatory endocytosis⁶.

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It is not known how exocytosis is coupled to local actin assembly. One possibility is that local actin assembly has the same proximal trigger as exocytosis itself — for example, regulated exocytosis is typically triggered by an increase in intracellular free calcium. As calcium has a number of targets that are known to regulate the actin cytoskeleton (for example, gelsolin), this is an attractive possibility.

However, it is also possible that local actin polymerization is more directly entrained to exocytosis. In particular, it has been proposed that compartment mixing on fusion of the secretory granules with the plasma membrane triggers local actin assembly, that is, the admixture created by union of components of the plasma membrane and the secretory granule generates a unique membrane environment that promotes local actin assembly⁶. In this situation, an increase in intracellular free calcium is not the proximal trigger for actin assembly *per se*, but instead works indirectly, by triggering exocytosis.

Here, we have examined the compartment mixing hypothesis during regulated exocytosis of cortical granules in *Xenopus* eggs. We show that the components of the cortical granule membranes and plasma membrane mix and that this mixing is essential for assembly of actin coats. We then identify plasma membrane DAG as a key signal that incorporates specifically into the cortical granules that undergo exocytosis and show that it is crucial for actin-coat assembly. DAG directs long-term recruitment of PKC β to exocytosing cortical granules, where it is required for the activation of Cdc42 that is localized on the cortical granules. Based on these and other results, we conclude that fusion of two different membrane compartments can act as a proximal trigger for local actin assembly.

RESULTS

Plasma membrane and cortical granule membranes mix when cortical granules exocytose

To study actin-coat assembly, eggs microinjected with fluorescent actin probes were subjected to uncaging of caged inositol 1,4,5-triphosphate (InsP₃) to elevate calcium and trigger cortical-granule exocytosis during imaging⁴. Exocytosis was triggered in the presence of Texas red–dextran, which filled the cortical-granule compartments on fusion with the plasma membrane and were observed as red discs (Fig. 1a and see Supplementary Information, Movie 1), allowing visualization of exocytosing cortical granules. As previously observed⁴, actin coats assembled around cortical granules only after exocytosis (as indicated by Texas red–dextran filling; Fig. 1a and see Supplementary Information, Movie 1). *z*-views revealed that actin coats assembled specifically on cortical-granule membranes, but not on the plasma membrane immediately adjacent to exocytosing cortical granules (Fig. 1b), implying that the actin assembly signal is confined to the exocytosing cortical granule.

If the trigger for coat assembly is dependent on compartment mixing, components of the plasma membrane and cortical-granule membrane must be capable of mixing after exocytosis. To examine this prediction, exocytosis was triggered in cells expressing farnesylated-eGFP (F-eGFP), which labels the plasma membrane. F-eGFP incorporated into cortical-granule membranes well before actin-coat assembly (Fig. 1c), demonstrating that the plasma membrane contents do mix with cortical-granule membranes following

exocytosis. *z*-views further revealed that F-eGFP was incorporated into the cortical granule membranes during cortical granule–plasma membrane fusion (Fig. 1d). Importantly, this incorporation was limited to exocytosing cortical granules and occurred gradually rather than instantly (Fig. 1e), indicating that mixing of the two compartments is not completely free.

A second prediction of the compartment-mixing hypothesis is that blocking cortical granule–plasma membrane fusion should prevent actin coat assembly. To examine this prediction, botulinum toxin A (BotA), which blocks exocytosis in many different systems⁷, including *Xenopus* oocytes⁸, was used. BotA blocked cortical-granule exocytosis, as demonstrated by failure of both dextran filling and incorporation of F-eGFP into cortical granule membranes (Fig. 1f). Consistent with the compartment-mixing hypothesis, BotA also completely suppressed actin assembly in response to InsP_3 uncaging (Fig. 1g). These results were specific, in that denatured BotA did not block cortical-granule exocytosis or actin-coat assembly (see Supplementary Information, Fig. S1a). In contrast with cortical-granule exocytosis and actin-coat assembly, other events characteristic of InsP_3 uncaging occurred normally in BotA-injected cells (see below). Therefore, not only does mixing of cortical granule–plasma membrane components occur during cortical-granule exocytosis, this mixing is somehow essential for subsequent actin-coat assembly.

DAG incorporation into the cortical-granule membrane on exocytosis is crucial for actin-coat assembly

Compartment mixing-dependent coat assembly implies that one or more signals diffusing between the plasma membrane and cortical granules initiates actin polymerization on the cortical granules. Although the plasma-membrane lipid, phosphatidylinositol 4,5-bisphosphate ($\text{PtdIns}(4,5)\text{P}_2$) has been shown to regulate actin assembly⁹, perturbation of $\text{PtdIns}(4,5)\text{P}_2$ by several means did not perturb actin-coat assembly (H.E.Y., unpublished observations). Therefore, we examined another promising candidate — the plasma-membrane lipid DAG, which increases in abundance during egg activation^{10,11} and activates $\text{PKC}\beta$ ¹¹, which has been implicated in actin assembly in *Xenopus* eggs¹².

If DAG represents a key signal for coat assembly, it should incorporate into the cortical granule following exocytosis. eGFP fused to the DAG-binding C1 domain from $\text{PKC}\beta$ (eGFP–C1) was used to label DAG. *z*-views revealed that DAG was elevated in the plasma membrane on InsP_3 uncaging (Fig. 2a and see Supplementary Information, Fig. S1b), even before cortical granule exocytosis (Fig. 2a). More importantly, immediately after cortical granule–plasma membrane fusion, DAG incorporated into cortical granule membranes as observed in both *z*-views (Fig. 2a) and subcortical views (Fig. 2b and see Supplementary Information, Movie 2). To verify that the recruitment of eGFP–C1 is due to its binding to DAG, we generated a mutant eGFP–C1 (eGFP–C1^{mut}) previously shown to be incapable of DAG binding^{13,14}. eGFP–C1^{mut} was not recruited to exocytosing cortical granules (Fig. 2c and see Supplementary Information, Movie 3), indicating that eGFP–C1 recruitment is DAG specific. Furthermore, co-imaging of eGFP–C1 and actin during cortical-granule exocytosis indicated that DAG incorporation into exocytosing cortical-granule membranes occurred before actin assembly (Fig. 3a), consistent with the hypothesis that DAG incorporation is

required for actin-coat assembly. Quantification of eGFP–C1 signal intensity showed that DAG incorporation into the cortical-granule membrane continued to increase after cortical granule–plasma membrane fusion (Fig. 3b), similarly to that of F-eGFP (Fig. 1e), suggesting that DAG is incorporated from the plasma membrane into cortical-granule membranes. To further show that cortical granule–plasma membrane compartment mixing is required for DAG incorporation into the cortical granule membrane, DAG recruitment was monitored after BotA microinjection. BotA prevented incorporation of DAG into cortical-granule membranes, but not DAG elevation at the plasma membrane (Fig. 3c). Thus, DAG incorporation into the cortical-granule membrane requires cortical granule–plasma membrane compartment mixing.

If DAG incorporation into cortical-granule membranes is essential for coat assembly, perturbation of DAG should perturb actin-coat assembly. Elevation of DAG at the plasma membrane on calcium increase is dependent on phosphatidylcholine-specific phospholipase D (PLD)¹⁰. Therefore, cells were treated with a phosphatidylcholine–PLD inhibitor (C2 ceramide^{15,16}) to reduce DAG generation at the plasma membrane on calcium increase. Consistent with our prediction, C2 ceramide treatment prevented DAG elevation on the plasma membrane on calcium increase (Fig. 4a, b) and reduced the amount of DAG recruited to exocytosing cortical granules (data not shown). In addition, C2 ceramide significantly reduced actin assembly on the exocytosing cortical-granule membranes (Fig. 4c, d and see Supplementary Information, Fig. S2). To further examine whether DAG is necessary for actin-coat assembly, cells were treated with DAG kinase (DGK) inhibitor — this prevents DAG metabolism, thereby allowing more DAG to bind to its downstream target¹⁷. Thus, if DAG is required for coat assembly, inhibition of DGK should augment actin assembly. Consistent with this prediction, actin assembly was enhanced in cells treated with DGK inhibitor (Fig. 4c, d and see Supplementary Information, Fig. S2). DAG alone should also trigger actin-coat assembly. To examine this prediction, cells were treated with exogenous DAG (1,2-diocytanoyl-sn-glycerol), to trigger cortical granule exocytosis^{18,19} instead of uncaging InsP₃. Similarly to uncaging InsP₃, exogenous DAG induced actin-coat assembly around exocytosing cortical granules (Fig. 4e). Taken together, the results show that DAG incorporation into cortical-granule membranes during cortical-granule exocytosis is essential for actin-coat assembly.

Fusion with plasma membrane sustains PKC β recruitment to the cortical granules

PKCs are a major class of DAG effectors²⁰ and we examined PKC β in detail as it is activated during egg activation¹¹ and has been implicated in actin polymerization in *Xenopus* eggs¹². To monitor PKC β dynamics, eGFP-labelled PKC β was used¹². PKC β was rapidly recruited to both exocytosing and non-exocytosing cortical granules on calcium elevation (Fig. 5b and see Supplementary Information, Movie 4). However, after recruitment, two different behaviours for PKC β –eGFP were observed: PKC β –eGFP remained on cortical granules that exocytosed (Fig. 5b and see Supplementary Information, Movie 4), but was lost from cortical granules that did not exocytose (Fig. 5b, c and see Supplementary Information, Movie 4).

These results demonstrated that PKC β is targeted to the cortical granules in a two-step process. Based on previous studies^{21,22}, we hypothesized that: the initial recruitment of PKC β to all cortical granules was due to an increase in affinity of the C2 domain for phosphatidylserine on the cortical-granule membrane on calcium elevation; and that the subsequent retention of PKC β on the exocytosing cortical granules was due to DAG binding by the C1 domain. If this is how PKC β is recruited, then the C1–C2 domain of PKC β should be sufficient for correct PKC β recruitment. Consistent with this, PKC β^{C1-C2} -eGFP was recruited to cortical granules in a manner similar to PKC β -eGFP (Fig. 5f and see Supplementary Information, Movie 5). The only difference was that PKC β^{C1-C2} -eGFP was present on the plasma membrane before InsP₃ uncaging (Fig. 5e), whereas PKC β -eGFP was present on the plasma membrane only after InsP₃ uncaging (Fig. 5d). This difference is probably due to the absence of the pseudo-substrate clamp in PKC β^{C1-C2} -eGFP, which normally maintains an autoinhibitory conformation of PKC in the absence of activators, and prevents it from binding to the membrane²¹.

As a second test for the PKC recruitment model, the recruitment pattern of eGFP–C2, which lacks the C1 domain and should therefore be uninfluenced by the presence or absence of DAG, was followed. eGFP–C2 was rapidly recruited to all cortical granules, but was not retained on the cortical granules — even those that fused with the plasma membrane (Fig. 5g) and therefore acquired DAG. Similarly to PKC β -eGFP, eGFP–C2 was recruited to the plasma membrane after calcium increase (Fig. 5h).

The third test of this model involved generating a mutant form of PKC β -eGFP (PKC β^{C1mut} -eGFP) that is incapable of binding to DAG^{13,14}. Similarly to eGFP–C2, PKC β^{C1mut} -eGFP was rapidly recruited to all cortical granules, but was not retained on the cortical granules, even those that exocytosed (Fig. 5i). PKC β^{C1mut} -eGFP was also present on the plasma membrane after calcium increase (Fig. 5j). Collectively, these results show that the different domains of PKC β are responsible for its recruitment pattern, with the C2 domain responding to the calcium increase to provide rapid targeting of PKC β to all cortical granules and the plasma membrane, whereas the C1 domain directs the maintenance of PKC β selectively on those cortical granules that have undergone exocytosis. Consistent with this model, when exogenous DAG was used to trigger cortical-granule exocytosis, PKC β was recruited to the exocytosing cortical granules before actin assembly (Fig. 4e).

Perturbation of PKC β affects actin-coat assembly

The finding that PKC β is recruited to all cortical granules transiently on calcium increase, but only remains on exocytosing cortical granules where DAG was incorporated (and actin-coat assembly eventually occurred), was consistent with the hypothesis that this kinase may be a DAG target required for coat assembly. If PKC β is the key DAG target, PKC β perturbation should disrupt normal actin-coat assembly. Consistent with this prediction, overexpression of PKC β induced hyper-developed actin coats on a subset of exocytosing cortical granules (Fig. 6a and see Supplementary Information, Movie 6) in 71% of the cells ($n = 21$). Importantly, this phenotype was not observed when PKC β^{K368R} (an ATP-binding mutant^{23,24}) was overexpressed (Fig. 6a and see Supplementary Information, Movie 7), indicating that the kinase activity of PKC β is essential for coat assembly.

If actin-coat assembly is initiated by PKC β , inhibition of PKC β should inhibit coat assembly. To selectively inhibit PKC β without affecting other PKC isozymes, PKC β^{C1-C2} was expressed in *Xenopus* eggs to function as an isoform-specific dominant negative^{25–27}. PKC β^{C1-C2} was used instead of PKC β^{K368R} because higher expression level could be obtained for PKC β^{C1-C2} -eGFP as compared with PKC β^{K368R} -eGFP when equal amounts of RNA was microinjected into the cells ($194 \pm 46\%$ more PKC β^{C1-C2} -eGFP compared with PKC β^{K368R} -eGFP; three independent experiments, 18 cells per group; see Supplementary Information, Fig. S3a). Expression of PKC β^{C1-C2} significantly slowed cortical-granule membrane retrieval (Fig. 6b–d) and actin-coat assembly (Fig. 6b, e and see Supplementary Information, Fig. S3b). To confirm the specificity of this phenotype, full-length PKC β was coexpressed in eggs expressing PKC β^{C1-C2} and partially rescued the phenotype induced by PKC β^{C1-C2} expression (Fig. 6b, d, e and see Supplementary Information, Fig. S3b). Two kinetically distinct types of rescue were observed: near complete rescue in which actin accumulation began just as quickly as controls (magenta line, Fig. 6e) and less complete rescue where the onset of actin assembly was delayed relative to controls, but still occurred much sooner than in cells expressing PKC β^{C1-C2} alone (yellow line, Fig. 6e). The ability of full-length PKC β to rescue the phenotype induced by PKC β^{C1-C2} indicates that PKC β^{C1-C2} does function as a specific inhibitor of PKC β and actin-coat assembly. If, instead, it functioned by suppressing the activity of another calcium, phosphatidylserine and DAG-dependent enzyme, coexpression with full-length PKC β would have made the phenotype more pronounced, rather than less. Furthermore, expression of the kinase-truncated construct of a different PKC does not affect actin-coat assembly (H.E.Y., unpublished data), further indicating that PKC β^{C1-C2} is a specific inhibitor of PKC β . Taken together, these results indicate that PKC β is the DAG target required for actin-coat assembly.

PKC β functions upstream of Cdc42 to initiate actin-coat assembly

As actin-coat assembly is Cdc42-dependent⁴, we wanted to determine whether PKC β acted through Cdc42 to initiate actin-coat assembly. To monitor active Cdc42 recruitment, we expressed wGBD (the Cdc42-binding domain of N-WASP)-eGFP, a probe that binds specifically to active (GTP-bound) Cdc42 (ref. 28). If PKC β facilitates actin-coat assembly in a Cdc42-dependent manner, perturbation of PKC β should perturb active Cdc42 recruitment on exocytosing cortical granules. When PKC β was overexpressed, Cdc42 activation on exocytosing cortical granules was enhanced (Fig. 7a, b). In contrast, when dominant negative PKC β^{C1-C2} was expressed, Cdc42 activation was reduced (Fig. 7a, b). Thus, PKC β acts upstream of Cdc42 to initiate actin-coat assembly.

z-views indicated that, similarly to actin assembly, Cdc42 was activated only on exocytosing cortical granules (Fig. 7c). In principle, this could reflect either recruitment of active Cdc42 specifically to exocytosing cortical granules, or activation of Cdc42 that is localized to cortical granules before induction of exocytosis. To distinguish between these hypotheses, localization of Cdc42 itself was examined by immunohistochemistry. Strikingly, comparison between the distribution of Cdc42 and a cortical-granule marker revealed that, in unactivated eggs, Cdc42 was highly concentrated on cortical granules (Fig. 7d). Moreover, triple labelling of Cdc42, cortical granules and the plasma membrane showed that comparatively little Cdc42 was located on the plasma membrane itself (Fig. 7e).

Recently, Toca-1 (a protein that binds to both Cdc42 and N-WASP) was shown to function downstream of Cdc42 to activate N-WASP (a Cdc42-activated protein that promotes actin assembly) in *Xenopus* egg extracts²⁹. As active Cdc42 and N-WASP are recruited to exocytosing cortical granules for actin-coat assembly⁴, we examined whether Toca-1 is also recruited. eGFP–Toca-1 was expressed in *Xenopus* eggs and its recruitment was compared with active Cdc42. eGFP–Toca-1 was recruited to the exocytosing cortical granules 2.86 ± 0.30 s (mean $\pm$ s. e.m.; $n = 36$) after Cdc42 activation (Fig. 7f), consistent with a model where Cdc42 activation triggers recruitment of Toca-1 to the exocytosing cortical granules to activate N-WASP. Unfortunately, further attempts to determine the roles of Toca-1 in actin-coat assembly were inconclusive. Specifically, three putative Toca-1 dominant negatives (Toca-1^{W517K}, Toca-1^{105–477}, Toca-1^{245–477}) failed to be properly targeted to exocytosing cortical granules (data not shown). In addition, microinjection of a Toca-1 antibody shown to immunodeplete Toca-1 from egg extracts²⁹ did not prevent coat assembly (data not shown). Thus, further investigation is needed to clarify the exact roles of Toca-1 in actin-coat assembly.

DISCUSSION

The results of this study demonstrate that local actin assembly can be triggered by the mixing of membranes from two different compartments — cortical secretory granules and the plasma membrane. Although many of the players are distinctly different, this compartment mixing triggers the sequential recruitment of upstream proteins required to initiate actin-coat assembly in a manner reminiscent of the sequential recruitment of actin regulators during receptor-mediated endocytosis^{30,31}. As previously proposed⁶, a compartment mixing-dependent mechanism for local actin assembly not only explains F-actin localization specifically to exocytosing secretory granules in fish eggs³², frog eggs⁴, pancreatic acinar cells⁵ and alveolar epithelial cells³³, but also has a clear functional rationale — restriction of actin assembly to the surfaces of exocytosing secretory granules following a general exocytotic signal (that is, calcium increase), ensures that F-actin will not limit subsequent exocytotic events. Because F-actin is known to function as a physical barrier to membrane fusion³⁴, wholesale actin assembly in response to calcium elevation would be expected to suppress exocytosis in situations where not all secretory granules fuse with the plasma membrane simultaneously. Likewise, actin assembly that is entrained to the initial trigger for fusion would be expected to severely limit the amount of material released in systems, such as acinar cells, that exhibit both compound exocytosis and actin coating of exocytosing secretory granules. In contrast, the compartment-mixing mechanism described here would allow multiple fusion events to occur in an asynchronous manner, particularly as there is a built in ‘fuse’ — the ~12–16 s between fusion and the onset of local actin assembly.

The results also identify DAG as a key signal for compartment mixing-dependent actin assembly, and show that it directs actin assembly by promoting sustained recruitment of PKC β that, in turn, promotes local activation of Cdc42 (Fig. 8). The involvement of DAG and PKC β in Cdc42-dependent actin assembly is consistent with previous work showing that *Xenopus* egg activation entails DAG generation^{10,11} and PKC β activation¹¹, as well as the

observation that the DAG mimic, PMA (phorbol 12-myristate 13-acetate), can promote actin assembly in *Xenopus* egg extracts¹².

More generally, the results obtained from the dissection of different PKC β domains explain how the molecular properties of a conventional PKC lead to spatio-temporal control of a specific process — conventional PKCs are thought to be targeted and activated in a stepwise manner, with calcium elevation directing targeting PKC to the plasma membrane through the C2 domain, and subsequent DAG binding resulting in retention and full activation of the PKC through the C1 domain^{21,22}. Our results confirm this basic scheme and show how it promotes coat assembly: the initial recruitment event ensures rapid PKC targeting, whereas the retention event ensures local specificity of PKC-induced actin assembly.

Two additional points emerge from this work: first, the recruitment of Toca-1 to exocytosing cortical granules after Cdc42 activation, but before actin-coat assembly, provides direct *in vivo* support for a recent model of N-WASP activation²⁹; and second, although the plasma membrane provides DAG during compartment mixing, it seems that the cortical granules provide Cdc42. This explains why Cdc42 activation and the resultant actin assembly are largely confined to the exocytosing cortical granules, even though DAG and PKC β are elevated on the plasma membrane as well as the cortical granules. Inactive Cdc42 is itself concentrated on the cortical granules rather than the plasma membrane. Thus, in contrast to the canonical pathway for Rho GTPase activation, where the inactive proteins are targeted from the cytoplasm to membrane compartments after activation, the results indicate that, in this case, the GTPase is concentrated at its site of action in the inactive form and then activated there.

METHODS

Preparation of probes

Utr^{1–261}–mRFP, provided by B. Burkel (University of Wisconsin-Madison, Madison, WI), is a monomeric red fluorescent protein (mRFP) fused with the calponin homology domain of utrophin (B. Burkel & W.M.B, unpublished observations). F-eGFP was provided M. V. Danilchik (Oregon Health & Science University, Portland, OR). Myc–Cdc42 was provided by T. Gomez (University of Wisconsin–Madison, Madison, WI). *Xenopus* PKC β (PKCII in ref. 35) was cloned from cDNA obtained from stage 4 oocytes. *Xenopus* Toca-1 (BC110949) was purchased from ATCC (Manassas, VA).

All subclonings and point mutations were performed using PCR. The ATP-binding mutant of PKC β has a mutation at its putative ATP binding site, where Lys 368 was mutated to arginine^{23,24}. To generate the DAG-binding mutant of C1 domain of PKC β , the conserved cysteine residues (Cys 64, Cys 67, Cys 129 and Cys 132, where amino-acid residues are numbered with respect to full-length PKC β) that are essential for DAG binding^{13,14} were mutated to alanine.

All PKC β and Toca-1 constructs were cloned into custom pCS2 vector, pCS2–eGFP vector⁴, or pCS2–mRFP vector (mRFP was provided by R. Tsien (University of California–San Diego, San Diego, CA) and subsequently cloned into pCS2 vector). Full-length PKC β and

its C1–C2 domain were cloned upstream of the eGFP; C1 domain and C2 domain from PKC β were cloned downstream of the eGFP; Toca-1 was cloned downstream of the eGFP. Toca-1^{W517K} has a mutation at its putative N-WASP binding site, where Trp 517 was mutated to lysine (ref. 29); Toca-1^{105–477} and Toca-1^{245–477} are truncates of Toca-1 at amino acids 105–477 and 245–477 respectively, where the putative N-WASP binding domain was removed²⁹. All probes were transcribed *in vitro* into RNA using the SP6 mMessage mMachine Kit (Ambion, Austin, TX) for microinjection into oocytes for expression.

Egg procurement and microinjection

Oocytes were obtained from adult *Xenopus* females, as described previously⁴, and stored in Barth solution (87.4mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.6 mM NaNO₃, 0.7 mM CaCl₂ and 10 mM HEPES at pH 7.4), with 0.1 mg ml⁻¹ gentamycin and 6 μ g ml⁻¹ tetracycline. Microinjections were performed as described previously⁴. All oocytes were injected with NPE-caged InsP₃ (Molecular Probe, Carlsbad, CA) to a final intracellular concentration of 10 μ M if eggs were activated by uncaging InsP₃. Proteins probes or inhibitors were injected to attain final intracellular concentrations as follows: 80 μ g ml⁻¹ Alexa 488–G-actin (Molecular Probe); 5 mg ml⁻¹ Toca-1 antibody (provided by M. Kirschner, Harvard Medical School, Boston, MA); 260 μ M BotA (provided by T. Martin, University of Wisconsin–Madison, Madison, WI); or 40 μ g ml⁻¹ BotA (Sigma, St Louis, MO). Capped mRNA were injected to final intracellular concentrations as follows: 4 μ g ml⁻¹ F-eGFP; 80 μ g ml⁻¹ Utr^{1–261}–mRFP; 26.4 μ g ml⁻¹ wGBD–eGFP; 200 μ g ml⁻¹ eGFP–Toca-1; 250 μ g ml⁻¹ Myc–Cdc42; 320 μ g ml⁻¹ full-length PKC β (wild type and ATP-point mutant); 200 μ g ml⁻¹ PKC β ^{C1–C2}; 240 μ g ml⁻¹ full-length PKC β –eGFP (wild type and C1^{mut}); 240 μ g ml⁻¹ eGFP–C1 (C1 domain of PKC β , wild type and point mutant); 80 μ g ml⁻¹ eGFP–C2 (C2 domain of PKC β) and 160 μ g ml⁻¹ PKC β ^{C1–C2}–eGFP. The concentration of F-eGFP and eGFP–C1 used did not alter cortical-granule membrane retrieval (see Supplementary Information, Fig. S4). When higher concentrations of F-eGFP and PKC β ^{C1–C2}–eGFP were used, cortical-granule membrane retrieval was perturbed (data not shown). Therefore, the concentrations were titrated so that the eGFP-tagged proteins could be detected without perturbing cortical-granule membrane retrieval. When higher concentrations of PKC β ^{C1–C2} were used, cortical-granule exocytosis was often inhibited¹⁸. As PKC β ^{C1–C2}–eGFP is approximately twice the size of untagged C1–C2, the molar concentration of untagged C1–C2 injected was approximately twice that of PKC β ^{C1–C2}–eGFP, explaining why 160 μ g ml⁻¹ PKC β ^{C1–C2}–eGFP did not induce a phenotype, but 200 μ g ml⁻¹ PKC β ^{C1–C2} did. Similarly, PKC β –eGFP is approximately 1.4 times the size of untagged PKC β , thus the molar concentration of injected untagged PKC β was approximately twice PKC β –eGFP, explaining why PKC β –eGFP did not induce a phenotype, but PKC β did. All microinjections were performed at least 4 h before induction of meiotic maturation, which was triggered by incubating oocytes with 5 μ g ml⁻¹ progesterone for 8–14 h⁴.

Cell manipulation and live imaging

Single-plane, time-lapse imaging was performed using a Zeiss Axiovert 100 M microscope (Carl Zeiss, Thornwood, NY) with Bio-Rad Lasersharp Confocal software. Eggs were mounted and imaged with either neutral Texas Red–dextran (relative molecular mass (M_r)

3,000K) or Alexa 647–dextran (M_r 10,000K; Molecular Probe) to facilitate visualization of exocytosing cortical granules⁴. While imaging, egg activation was triggered by focusing UV light onto the egg cortex for 3–5 s to photolyse NPE-caged InsP_3 (Molecular Probes). When endogenous DAG (1,2-Diacytanoyl-sn-glycerol; Biomol, Plymouth Meeting, PA) was used to activate cortical-granule exocytosis, cells were mounted in the presence of Alexa 647–dextran containing a final concentration of $7 \mu\text{g ml}^{-1}$ DAG, freshly diluted from stock. When C2 ceramide (Calbiochem, San Diego, CA) was used to inhibit PLD^{15,16}, cells were incubated in $50 \mu\text{g ml}^{-1}$ C2 ceramide for 1–2 h before imaging, and mounted in the presence of Texas Red–dextran containing $8 \mu\text{g ml}^{-1}$ C2 ceramide during imaging. When the DGK inhibitor R59949 (Calbiochem) was used, cells were incubated in $80 \mu\text{M}$ R59949 for at least 1 h before imaging, and mounted in the presence of Texas Red–dextran containing $13 \mu\text{M}$ R59949 during imaging. Movies were generated with Volocity 3.1 software (Improvision, Lexington, MA). Montages were subsequently made with Adobe Photoshop 7.0. All movies and montages shown are representatives of experiments repeated at least three times.

Data quantification and statistical analysis

NIH Object-Image 2.10 or ImageJ 1.34s software was used to measure fluorescence intensities. When measuring the signal intensity of F-eGFP, eGFP–C1, active Cdc42 and actin around exocytosing cortical granules, average signal intensity was measured by first carefully outlining the circumference of the exocytosing cortical granule to define the region of interest. Background signal intensity was then subtracted from the raw signal intensity of the region of interest. The F-eGFP and eGFP–C1 signal around the cortical granules (Figs 1e and 3b) were normalized against the maximum signal intensity (defined as 100%) obtained before constriction of the exocytosing compartments. When actin intensity was measured around exocytosing cortical granules in cells where PKC β was perturbed (Fig. 6e), actin intensity was measured before constriction of the exocytosing compartments as coat constriction may result in apparent further enhancement of actin-coat intensity. To further illustrate that actin-coat assembly was perturbed in the experimental groups, another representation for the actin intensity is shown in the Supplementary Information, Fig. S3b — total actin intensity was measured in the circular area, defined as the area that covered the entire cortical granules at the beginning of cortical granule exocytosis. Average background signal intensity was subtracted from the raw signal intensity measured in the fixed area to obtain net signal intensity. The net signal intensity was then multiplied by the area of interest to give the ‘total signal’. The rationale is that this total signal intensity should be more robust against the apparent increase of signal intensity due to constriction (that is, if the increase of signal intensity on constriction is strictly due to constriction, then the total signal intensity represented here should remain unchanged). The total signal intensity shown in the Supplementary Information, Fig. S2c, was measured in similar way.

To measure plasma-membrane signal intensity of eGFP–C1 (Fig. 4b and see Supplementary Information, S1b), z-view images were collected. eGFP–C1 signals on the plasma membrane was measured and corrected with background signal intensity. In the Supplementary Information, Fig. S1b, signal intensity was normalized against the maximum signal intensity of eGFP–C1 after calcium increase (defined as 100%).

Cortical granule-membrane retrieval time was the time in which the dextran-filled compartments existed. Statistical analysis was performed using Excel. Two-tail Student's *t*-test was used to compare whether there was significant difference between two sets of data when appropriate.

Immunofluorescence staining of Cdc42

Oocytes injected with Myc-Cdc42 were induced to undergo meiotic maturation. On maturation, cell-surface proteins were biotinylated by incubating cells with 1 mg ml⁻¹ sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) in Barth solution for 1 min, then rinsed with Barth solution and then with PBS. Cells were then fixed in buffer containing 3.7% paraformaldehyde, 0.05% glutaraldehyde, 0.1% Triton X-100, 100 mM KCl, 150 mM sucrose, 3 mM MgCl₂, 10 mM HEPES for 30 min. Fixed cells were washed with PBS for 1 h, quenched in PBS containing 250 mM NaBH₄ for 1 h, washed with TBS containing 0.1% NP-40 (TBSN), bisected and blocked in TBSN containing with 5 mg ml⁻¹ BSA overnight at 4 °C. All subsequent steps were carried at 4 °C. Samples were then incubated with Alexa 680-conjugated streptavidin (Molecular Probe) for 1 h, anti-Myc 9E10 antibody (Santa Cruz, Santa Cruz, CA) and rhodamine-dolichos-biflorus-agglutinin (Vector Lab, Burlingame, CA) for a day. After a one day wash with TBSN-BSA, samples were incubated with anti-mouse Oregon green (Molecular Probe) for one day, washed with TBSN for one day. Samples were then mounted and viewed on the confocal laser-scanning microscope.

Immunoblotting

Oocytes injected with 80 µg ml⁻¹ PKCβ^{C1-C2}-eGFP or 80 µg ml⁻¹ PKCβ^{K368R}-eGFP were induced to undergo meiotic maturation. Eggs were homogenized with buffer³⁶ containing 10 mM HEPES, 1 mM EGTA, 0.25 M sucrose, 2 mM MgCl₂ at pH 7.4, supplemented with 2.5 mM DTT and protease inhibitors immediately before use at 4 °C. Homogenates were centrifuged at 14,000g for 5 min at 4 °C. Supernatant was separated on a SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed using primary anti-eGFP antibody (JL-8; Clontech, Mountain View, CA) or anti-tubulin (DM1A; Sigma), and secondary anti-mouse coupled to HRP (Promega, Madison, WI). Blots were developed using ECL western blotting substrate (Pierce).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Actin-coat assembly occurs after plasma membrane and cortical granule membrane incorporation on cortical granule exocytosis. **(a)** Subcortical view showing that actin coats (Alexa488-G-actin, arrowhead) assemble around the exocytosing cortical granules after cortical granule exocytosis (Texas Red-dextran, arrow). **(b)** z-view showing that actin (Utr¹⁻²⁶¹-mRFP) assembly occurs on cortical granule membranes (arrowhead) on cortical granule exocytosis (Alexa647-dextran) at 0 s, but not on the cortex (arrow). **(c)** Subcortical images showing that plasma membrane components (F-eGFP) are incorporated into the cortical granule membrane on cortical granule exocytosis (Alexa647-dextran, arrowhead), before actin-coat assembly (Utr¹⁻²⁶¹-mRFP, arrow). **(d)** z-view showing that plasma membrane (F-eGFP, green) incorporates into cortical granule membranes on cortical granule exocytosis (Texas Red-dextran) at 0 s. **(e)** Quantification of F-eGFP recruitment to exocytosing cortical granules ($n = 16$; 100% is maximum signal intensity before cortical granule constriction), where cortical granule exocytoses at 1.5 s. Signal intensity shown at 0

s represents basal signal intensity. The error bars represent s.e.m. (**f, g**) Subcortical images showing that BotA microinjection blocks cortical granule exocytosis (the absence of Texas Red–dextran filled compartment), and prevents (**f**) plasma membrane (F-eGFP) incorporation into the cortical granule membrane, as well as actin coat assembly (**g**; Alexa488–G-actin). InsP₃ uncaging occurred at 0 s in **a, f** and **g**. Out, outside of cell; in, inside of cell. The scale bars represent 5 μm in **a, f** and **g**, 3 μm in **b** and 2 μm in **c** and **d**.

Figure 2.

Ca^{2+} elevation triggers DAG generation on the plasma membrane, which incorporates into the cortical granule membrane on cortical granule-plasma membrane fusion. **(a)** z-view showing that DAG (eGFP-C1) is generated at the plasma membrane on Ca^{2+} increase and incorporates into the cortical granule membrane during cortical granule-plasma membrane fusion (Texas Red-dextran, arrowhead). **(b)** Subcortical view showing that DAG (eGFP-C1) is recruited to exocytosing cortical granules only (Texas Red-dextran, arrow), but not to non-exocytosed cortical granules (arrowhead). **(c)** Subcortical view showing that eGFP-C1^{mut} (non-DAG binding) is not recruited to exocytosing cortical granules (arrowhead). InsP_3 uncaging occurred at 0 s in **a**, **b** and **c**. The scale bars represent 2 μm in **a** and 4 μm in **b** and **c**.

Figure 3.

DAG incorporation into cortical granule membranes occurs before actin assembly and is dependent on cortical granule–plasma membrane compartment mixing. **(a)** Subcortical view showing that DAG (mRFP–C1, red) incorporates into the cortical granule membrane when a cortical granule exocytoses at 0 s, before actin assembly (Alexa488–G-actin, green). **(b)** Quantification of eGFP–C1 recruitment to exocytosing cortical granules ($n = 16$; 100% is maximum signal intensity before cortical granule constriction), where the cortical granule exocytoses at 1.5 s. Signal intensity shown at 0 s represents basal signal intensity. The error bars represent s.e.m. **(c)** z-view showing that DAG (eGFP–C1) levels at the plasma membrane increases on InsP_3 uncaging at 0 s, even though it fails to be incorporated into the cortical granule membrane when treated with BotA. The scale bars represent 1 μm in **a** and 2 μm in **c**.

Figure 4.

DAG is required for actin-coat assembly. **(a)** *z*-view showing that C2 ceramide treatment inhibits DAG (eGFP-C1) elevation at the plasma membrane on Ca^{2+} increase. **(b)** Quantification of DAG intensity on plasma membrane showing the percentage increase in DAG levels after Ca^{2+} increase when compared with pre-activation levels ($n = 12$; asterisk indicates $P = 0.00047$). The error bars represent s.e.m. **(c)** Subcortical images showing that C2 ceramide treatment reduces actin coat (Alexa488-G-actin) assembly around exocytosing cortical granules (Texas Red-dextran), whereas DGK inhibitor enhances actin-coat assembly. **(d)** Quantification of the intensity of actin coats at exocytosing cortical granules ($n = 20$ for control; $n = 21$ for C2 ceramide; $n = 21$ for DGK inhibitor). The error bars represent s.e.m. **(e)** Exogenous addition of DAG induces cortical granule exocytosis (Alexa647-dextran) at 0 s, where PKC β -eGFP is recruited to the exocytosing cortical granule before actin coat (Utr¹⁻²⁶¹-mRFP) assembly. The scale bars represent 1 μm in **a** and 2 μm in **c** and **e**.

Figure 5.

PKC β is transiently recruited to all cortical granules on Ca²⁺ elevation, but only remains on those that have exocytosed. **(a)** Schematic representation of the primary structure of PKC β . The regulatory portion is comprised of C1 and C2 domains, which bind to DAG and phosphatidylserine and/or Ca²⁺, respectively. The catalytic portion is comprised of C3 and C4 domains, which bind to ATP and substrate, respectively. **(b)** Subcortical views showing that PKC β -eGFP is recruited to all cortical granules on InsP₃ uncaging at 0 s, but only remains on those that have fused with plasma membrane (Texas Red-dextran, arrow), and disappears from those that have not exocytosed (arrowhead). **(c)** z-view showing that PKC β -eGFP disappears from cortical granule that has not exocytosed (the absence of a Texas Red-dextran filled compartment). **(d)** z-view showing that PKC β -eGFP is translocated to the plasma membrane on InsP₃ uncaging. **(e)** z-view showing that PKC β ^{C1-C2}-eGFP is present

on the plasma membrane before InsP_3 uncaging. **(f)** Subcortical views showing that $\text{PKC}\beta^{\text{C1-C2}}$ -eGFP is recruited to all cortical granules on InsP_3 uncaging at 0 s, but only remains on those that have fused with plasma membrane (Texas Red-dextran, arrow), and disappears from those that have not exocytosed (arrowhead). **(g)** Subcortical images showing that eGFP-C2 is recruited to cortical granules (arrowhead) on InsP_3 uncaging at 0 s before exocytosis. It subsequently disappears from cortical granules regardless of whether the cortical granules have exocytosed (Texas Red-dextran, arrow), or not (asterisk). **(h)** z -images showing that eGFP-C2 is translocated to plasma membrane on InsP_3 uncaging. **(i)** Subcortical images showing that $\text{PKC}\beta^{\text{C1mut}}$ -eGFP (non-DAG binding $\text{PKC}\beta$) is recruited to cortical granules (arrowhead) on InsP_3 uncaging at 0 s before exocytosis. However, they disappear from cortical granules at later time points regardless of whether the cortical granules have exocytosed (Texas Red-dextran, arrow), or not (asterisk). **(j)** z -images showing that $\text{PKC}\beta^{\text{C1mut}}$ -eGFP is translocated to plasma membrane on InsP_3 uncaging. The scale bars represent 4 μm in **b**, 2 μm in **c**, 5 μm in **d**, **e** and **f**, and 3 μm in **g-j**.

Figure 6.

Perturbation of PKC β affects actin-coat assembly. **(a)** Subcortical images showing that overexpression of PKC β induces formation of thicker, longer-lived actin coats (Alexa488–G-actin, arrows) on some exocytosing cortical granules (Texas Red–dextran), whereas others (arrowheads) have normal coats. In contrast, actin coats (Alexa488–G-actin) assemble normally (arrowheads) in PKC β^{K368R} -overexpressing cells. InsP $_3$ uncaging occurred at 0 s. (The background level of actin in the PKC β -overexpressing figure is intentionally reduced so that the hyperdeveloped actin coats would not seem too saturated in

green. Before adjustment, the actin background is comparable in both PKC β -overexpressing and PKC β^{K368R} -overexpressing cells.) **(b)** Subcortical images showing that exocytosing cortical granules (Texas Red–dextran) in cells expressing PKC β^{C1-C2} remain trapped at the plasma membrane over time with little or no actin coat (Alexa488–G-actin) assembly. Coexpression with full-length (FL) PKC β partially rescues the phenotype, where assembly of actin coat (Alexa488–G-actin, arrowhead) is slightly delayed compare with control (arrowhead). **(c)** *z*-view showing the exocytosing cortical granule remains trapped at the plasma membrane when expressing PKC β^{C1-C2} . **(d)** Quantification of time taken to retrieve cortical granule membranes ($n = 117$ for control; $n = 90$ for PKC β^{C1-C2} ; $n = 89$ for PKC β^{C1-C2} with full-length PKC β ; $P = 10^{-41}$ for control versus PKC β^{C1-C2} ; $P = 10^{-25}$ for PKC β^{C1-C2} versus PKC β^{C1-C2} with full-length PKC β). **(e)** Quantification of the intensity of actin coats at exocytosing cortical granules before coat compression. PKC β^{C1-C2} + FL1 and FL2 represent two levels of rescue in different cells. ($n = 18$ for control; $n = 17$ for PKC β^{C1-C2} ; $n = 12$ for PKC β^{C1-C2} with FL1; $n = 5$ for PKC β^{C1-C2} with FL2. The error bars represent s.e.m. in **d** and **e**. The scale bars represent 4 μm in **a**, 3 μm in **b**, and 2 μm in **c**.

Figure 7.

Perturbation of PKC β affects activation of Cdc42, which is localized on cortical granule membranes before egg activation. **(a)** Subcortical images showing that Cdc42 activation (wGBD-eGFP) on exocytosing cortical granules (Texas Red-dextran) is enhanced in cells overexpressing PKC β , but reduced in cells expressing PKC β^{C1-C2} . **(b)** Quantification of active Cdc42 recruitment to exocytosing cortical granules ($n = 15$ for control; $n = 14$ for PKC β overexpression; $n = 15$ for PKC β^{C1-C2}). The error bars represent s.e.m. **(c)** z-view showing that Cdc42 is activated (wGBD-eGFP) on cortical granule membrane when cortical granule exocytoses (Texas Red-dextran), but not on the cortex. **(d)** Low magnification of *en face* view showing immunofluorescence staining of Myc-Cdc42 (green) localizes on the cortical granules (red) before egg activation. **(e)** High magnification of z-view showing Myc-Cdc42 is localized only on cortical granules (red), but not on the plasma membrane (blue) before egg activation. **(f)** eGFP-Toca-1 (arrow) is recruited to the exocytosing cortical granule (Alexa647-dextran) after Cdc42 activation (wGBD-RFP, arrowhead). 0 s indicates time at which cortical granule exocytoses in **c** and **f**. The scale bars represent 1 μ m in **a**, **c** and **e**, 10 μ m in **d** and 2 μ m in **f**.

Figure 8.

Schematic representation of how compartment mixing results in actin-coat assembly. **(a)** Timeline showing the recruitment of different signalling molecules (black) with respect to the different events (red) during cortical granule (CG) exocytosis or endocytosis. **(b)** On calcium (green dot) increase, DAG (orange) is generated in the plasma membrane (PM, light-green line). Calcium binding results in the translocation of PKCβ (red) to the cortical granule (blue) and plasma membrane. On cortical granule–plasma membrane fusion, DAG from the plasma membrane incorporates into the cortical granule membranes, which sustains the recruitment of PKCβ at the cortical granule membrane. **(c)** Schematic representation summarizing how calcium elevation triggers different events that lead to actin-coat assembly (based on the results presented here and in ref. 4).