Integrating studies of proteins and lipids: dissecting the mechanism of Ca^{2+}-triggered membrane fusion

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Ca^{2+}-triggered membrane fusion is the defining step of fast, regulated exocytosis, providing temporal and spatial control over the release of biologically active compounds. Despite recognising that the fusion machinery must include lipids and proteins working in concert, only more recently has the field begun to focus more equally on both these components. Thus, the mechanism by which Ca^{2+} triggers and modulates native membrane fusion is still poorly understood. We use a stage-specific preparation of Ca^{2+} sensitive, release-ready cortical vesicles (CV) isolated from sea urchin eggs that enables the tight coupling of quantitative functional (end-point and kinetic fusion assays) and molecular (protein and lipid) analyses necessary to dissect molecular mechanisms (Coorssen *et al*., 2003).

The stalk pore model proposes that bilayer merger proceeds rapidly *via* transient, high negative curvature intermediate membrane structures (Efrat *et al*., 2007). Consistent with this, cholesterol, a major CV membrane component, contributes to a critical local negative curvature that promotes formation of fusion intermediates (Churchward *et al*., 2005). Following depletion or sequestering of endogenous CV membrane cholesterol, structurally dissimilar lipids having intrinsic negative curvature ≥ cholesterol rescue the ability of CV to fuse but not fusion efficiency (Ca^{2+} sensitivity and kinetics; Churchward *et al*., 2008). Conversely, cholesterol- and sphingomyelin-enriched regions of the membrane regulate the efficiency of the fusion mechanism, presumably *via* spatial and functional organization of other critical lipids and proteins at the fusion site (Rogasevskaia & Coorssen, 2006). Critical proteins are thought to participate in Ca^{2+}-sensing, initiating membrane deformations and facilitating fusion pore expansion.

As an unbiased approach to identifying critical proteins, the effects of several thiol-reactive reagents on the homotypic fusion of isolated CV have been characterized - these reagents alkylate the free sulfhydryl groups on proteins and have been consistently shown to inhibit triggered fusion. We have however recently characterized an additional effect of the reagent, iodoacetamide (IA). IA treatment was found to enhance the Ca^{2+} sensitivity and kinetics of both CV-plasma membrane and CV–CV fusion (Furber *et al*., 2008). If Sr^{2+}, a weak Ca^{2+} mimetic, was used to trigger fusion the potentiation after IA treatment was even greater than that observed for Ca^{2+}; the maximal leftward shift in EC_{50} to ~600 μM $[Sr^{2+}]_{free}$ brings the triggering effect of this metal into a physiologically relevant range. This substantial effect on Sr^{2+} sensitivity is highly indicative that IA promotes fusion by acting on a thiol site that regulates a Ca^{2+}-sensing step of triggered fusion. Together with the known inhibitory roles of other thiol-reactive reagents, this implicates at least two distinct thiol sites in the fusion process: one involved in fusion competency (the ability of vesicles to fuse) and one that modulates fusion efficiency (Ca^{2+}-sensitivity and kinetics).

Capitalizing on the potentiating effect of IA, we have now identified other fluorescent thiol-reactive reagents with similar effects: treatment with Lucifer yellow iodoacetamide, monobromobimane or dibromobimane resulted in an average leftward shift in EC_{50} from 17.2±1.6μM to 8.9±1.9μM $[Ca^{2+}]_{free}$. These fluorescent reagents can be used to simultaneously enhance fusion and label proteins involved. Knowing that proteins involved in Ca^{2+}-sensing are likely to be situated in cholesterol-enriched areas of the CV membrane, we are narrowing the list of protein candidates by isolating these membrane fractions using density gradient centrifugation. 2D gel electrophoresis is then used to identify proteins potentially involved in the Ca^{2+}-triggering steps of membrane fusion.

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