Thiol-reactivity: A small molecule approach to identifying proteins involved in regulating the calcium sensing steps of native membrane fusion

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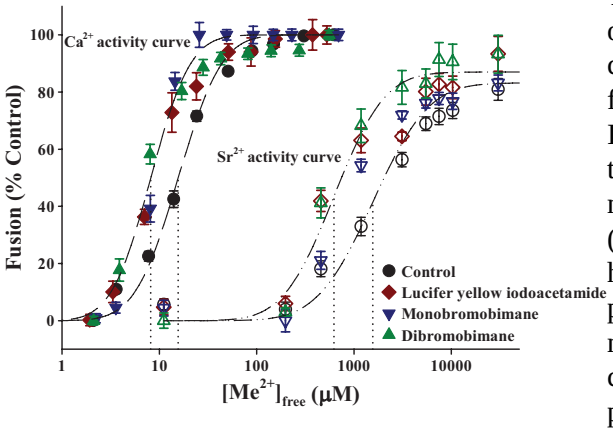
In specialized cells, fusion of secretory vesicles with the plasma membrane enables release of biologically active molecules; this process is tightly regulated by intracellular Ca^{2+}. However, the identity of critical proteins involved in Ca^{2+} sensing and triggering of native membrane fusion remain speculative. Unlike other types of secretory vesicles, isolated cortical vesicles (CV) from unfertilized urchin eggs remain fully primed and fusion-ready providing a stage-specific preparation to quantitatively assess the native, Ca^{2+}-triggered fusion mechanism (Zimmerberg *et al.*, 2000). Thiol-reactivity offers an unbiased approach in studying proteins involved in Ca^{2+}-triggered membrane fusion. Furthermore, alkylating reagents are highly selective for, and bind irreversibly to, cysteine residues, providing important information about function while also tagging the proteins involved.

Previous work indicates that multiple thiol sites differentially regulate the ability of CV to fuse as well as the efficiency of fusion (*i.e.* Ca^{2+} sensitivity and kinetics) (Furber, Brandman & Coorssen, 2009; Furber, Dean & Coorssen, 2010). Iodoacetamides and bimanes have the unique ability to access a novel thiol site that potentiates the efficiency of fusion, presumably by altering the Me^{2+} sensing mechanism (Figure).



This may be due to a direct effect on a Ca^{2+} binding protein or disruption of a regulatory interaction in the fusion complex, yet delineating the exact nature of this mechanism first requires identification of the protein(s) being targeting. Initial studies coupling Lucifer yellow iodoacetamide treatment with 2-dimensional electrophoresis of CV membranes revealed that numerous proteins become labeled (Furber, Dean & Coorssen, 2010). Thus, several strategies have been employed to better focus on the labeled spots pertinent to mechanism: (i) comparison of labeling across multiple reagents and concentrations; (ii) prefractionation of cholesterol-enriched membranes to isolate proteins preferentially located at/near the fusion site; and (iii) third-dimension electrophoretic separations for improved resolution.

Labeled proteins thus far identified include a variety of metabolic enzymes, cystoskeletal components (actin and tubulin), and several isoforms of Rab GTPases. Both the cystoskeleton and Rab proteins have defined roles in vesicle mobility and trafficking, but these data raise the possibility they may also act in later stages of exocytosis to regulate Ca^{2+} sensitivity and kinetics of secretion. Pharmacological experiments using cytoskeletal (de)stabilizing reagents in the stage-specific CV preparation rule out a direct role for actin (Hibbert, Butt & Coorssen, 2006) and tubulin (Furber *et al.*, unpublished) in the Ca^{2+}-triggering steps of membrane fusion. Nonetheless, there is evidence indicating a role for cytoskeletal components in fusion pore expansion and thus the kinetics of the release process (Berberian *et al.*, 2009; Doreian, Fulop & Smith, 2008; Larina *et al.*, 2007; Miklavc *et al.*, 2009). We are currently focusing on Rab proteins as prime candidates involved in the regulation of fusion efficiency, perhaps reminiscent of the synergistic effects between Ca^{2+} (C_E) and GTP (G_E) on secretion in other cell types (Coorssen, Davidson & Haslam, 1990; Howell, Cockcroft & Gomperts, 1987).

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