The behaviour and control of post exocytic vesicles

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Introduction. Classical work suggests that granule exocytosis in epithelial cells is followed by granule collapse into the plasma membrane and endocytic recovery of small clathrin-coated vesicles (Palade, 1975). Our recent work challenges this model and shows that granules don't collapse but persist at the plasma membrane for many minutes over which time the fusion pores remain open (Thorn *et al.,* 2004, Larina *et al.,* 2007). This suggests that post-fusion behaviour of the granule may influence release of granule content. Here we present evidence that F-actin and myosin 2 act in consort to maintain an open fusion pore.

Methods. Male CD-1 mice were killed according to the approved ethical procedures of The University of Queensland. The pancreas was excised and collagenase-digested to produce fragments of pancreatic tissue (see Thorn *et al.,*, 2004 for details). The tissue fragments were bathed in extracellular fluorescent dyes and either imaged live with 2-photon microscopy or after paraformaldehyde fixation with confocal microscopy. Cell exocytic responses were stimulated with acetylcholine (1 μM) and its action terminated 1 minute later by the application of atropine (10 μM) or cholesystokinin (15 pM). Upon exocytosis the extracellular fluorescent dye enters and therefore labels the granules. Using different dyes and different times of dye addition, we have developed methods to enable positive identification of whether the fusion pores are open or closed (see Larina *et al.,* 2007 for details).

Results. We initially used immunofluorescence to identify the localization of myosin 2 isoforms in cells within pancreatic tissue fragments. Our data indicate that myosin 2b is located in the basal region and myosin 2a is located in the apical region, coincident with the apical F-actin cytoskeleton. Using Western blot, we found that agonist stimulation increased myosin 2 phosphorylation to a peak level, after 4 minutes of ~3 times background levels. This elevated phosphorylation remained for many minutes, even after stimulation is terminated. Taken together these lines of evidence show that myosin 2a is a potential candidate to regulate agonist-evoked apical exocytosis.

We then studied the possible physiological functions of myosin 2 using our dye methods for identifying open and closed fusion pores and inhibiting myosin activity with 50 μM blebbistatin (a myosin ATPase inhibitor). Drug treatment increased the proportion of granules with a closed fusion pore. In control, we measured 22% of granules with closed fusion pores 5 minutes after stimulation compared to 58% with blebbistatin (measured from 6 independent experiments). ML-9 (a myosin light chain kinase blocker) similarly increased the proportion of closed fusion pores consistent with activation of myosin 2 *via* this kinase. The negative enantomer of blebbistatin did not change the numbers of closed fusion pores. In past work we have shown similar results with Latrunculin A treatment (inhibits F-actin formation, Larina *et al.,* 2007). Together this data indicate that F-actin and myosin 2 are necessary to maintain an open fusion pore.

Finally, we directly measured fusion pore lifetimes in living cells using a photobleaching protocol (Larina *et al.,* 2007). Here we determined the fusion pore lifetime. In the presence of blebbistatin the mean value of the pore lifetime was 4.86 minutes compared to 10.44 minutes in control (n = 16 and 33 granules respectively).

Conclusions. We here describe the actions of F-actin and myosin 2, probably myosin 2a, in maintaining an open fusion pore during exocytosis in secretory epithelial cells. Our work adds to a growing body of evidence that post-fusion events are important in regulating the exocytic process and may be important in the control of granule content release.

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