Identification of early endocytic structures after stimulation of pancreatic acinar cells

A. Shukla and P. Thorn, School of Biomedical Sciences, University of Queensland, QLD 4072, Australia.

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 do not collapse but persist at the plasma membrane for many minutes over which time the fusion pores remain open (Thorn *et al.*, 2004). Most recently we have suggested that exocytosis is terminated by closure of the fusion pore not granule collapse (Larina *et al.* 2007). However, the next step, that of endocytic recovery of the granule membrane, still remains unclear. Past studies of endocytosis in epithelial cells have used extended stimulus times and followed endocytosis over hours (Oliver & Hand, 1978). In contrast, to identify the earliest endocytic events we use short stimulation times and now describe for the first time the recovery of large (~450 nm diameter) vesicles.

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 Horseradish Peroxidase (HRP, 50 mg/ml) for 30 minutes. Acetylcholine (1 mM) was applied at room temperature to the tissue fragments and its action terminated 1 minute later by the application of Atropine (10 mM). At various time points after stimulation, the tissue fragments were washed at 4^{o}C in extracellular buffer and then incubated in TRIS-buffered saline at pH 7.6 that contained diaminobenzidine (DAB, 1 mg/ml), ascorbic acid (50 mM) and H_2O_2 (0.012%) on ice. This protocol has been used to specifically stain endocytic structures; any compartment that is in continuity with the extracellular environment the ascorbic acid quenches the HRP-DAB reaction (Kirkham *et al.*, 2005). After allowing 10 minutes for the HRP-DAB reaction the tissue fragments were fixed in glutaraldehyde (2.5%) and processed according to standard electron microscopy protocols. Thin (69 nm) tissue sections were then studied by transmission electron microscopy.

Results. In unstimulated tissue fragments the cells showed no evidence of granules fused with the plasma membrane. They did show some electron-dense DAB reaction product, localized to small, mulitvesicular bodies likely to be lysosomal compartments where endogenous oxidases may have reacted with the DAB. In stimulated tissue fragments, left to recover for 10 minutes prior to adding DAB, we observed unstained granules fused to the plasma membrane with open fusion pores; consistent with the protracted fusion events seen in live-cell experiments (Thorn *et al.*, 2004). Electron-dense DAB reaction product was now observed in large vesicles that were predominantly located towards the apical end of the cells. The measured diameter of these electron-dense vesicles was 450 ± 17 nm (mean diameter ± SEM, n = 44, 3 independent preparations). These labeled vesicles are statistically smaller in size compared to the total population of zymogen granules (748 ± 11 nm, n = 230, mean diameter ± SEM).

Conclusions. We here describe the primary endocytic structures formed immediately after stimulation of epithelial cells. Our technique unambiguously identifies recaptured vesicles that are no longer in continuity with the extracellular environment. The labeled structures we observe are surprisingly large and while this might suggest that whole granules are endocytically recaptured it is still unclear why they are smaller than the total population of zymogen granules.

Kirkham M, Fujita A, Chadda R, Nixon SJ, Kurzchalia TV, Sharma DK, Pagano RE, Hancock JF, Mayor S & Parton RG. (2005) *Journal of Cell Biology*, **31:** 465-76

Larina O, Bhat P, Pickett JA, Launikonis BS, Shah A, Kruger WA, Edwardson JM, & Thorn P. (2007) *Molecular Biology of the Cell*, **18:** 3502-11.

Oliver C & Hand AR. (1978) *Journal of Cell Biology*, **76:** 207-20.

Palade G. (1975) *Science* **189:** 347-68.

Thorn P, Fogarty KE & Parker I. (2004) *Proceedings of the National Academy of Science USA,* **101:** 6774-9