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ClC-3—A Granular Anion Transporter Involved in Insulin Secretion?

Dear Editor,

Recent reports published in *Cell Metabolism* (Deriy et al., 2009; Li et al., 2009) suggest a role for the anion transporter ClC-3 in the secretion of insulin in pancreatic β cells by assisting acidification of large dense core vesicles (LDCVs). However, these reports contradict each other and our own recent work (Maritzen et al., 2008) in many ways and the mechanism by which ClC-3 influences LDCV exocytosis remains, in our eyes, unclear. Whereas the physiological importance of ClC-3 is obvious from the severe neurodegeneration resulting from its disruption (Stobrawa et al., 2001), its function and localization are subject to considerable controversy. ClC-3 most likely operates as a Cl^-/H^+-exchanger, but some groups maintain that it is a Cl^- channel. ClC-3 resides on endosomes, synaptic vesicles, and synaptic-like microvesicles (SLMVs), where it may lead to luminal acidification and chloride accumulation (Maritzen et al., 2008; Salazar et al., 2004; Stobrawa et al., 2001). The postulated localization of ClC-3 to the plasma membrane and LDCVs like those containing insulin, however, is questionable. Rather than resulting from impaired LDCV acidification, the variable effect on insulin secretion of the ClC-3 KO might be owed to altered endosomal trafficking or changes in other hormones.

Let us first address the differences between the two papers in question published by Deborah Nelson and coworkers (Deriy et al., 2009) and Erik Renström and colleagues (Li et al., 2009). Deriy et al. find more than three-fold lower resting serum insulin concentrations in ClC-3 KO mice, whereas Li et al. report unchanged resting insulin levels. Deriy et al. find impaired in vitro insulin secretion only in response to glucose and not potassium chloride, whereas Li et al. report an almost abolished response to either stimulus in the KO. The lack of potassium response in Deriy et al. is confusing given that these authors report smaller depolarization-induced capacitance jumps in the KO. We find lower basal serum insulin in these KO mice and increased leptin (Maritzen et al., 2008). As leptin negatively impinges on β cell insulin secretion (Morioka et al., 2007; Seufert, 2004), this may explain the observed decrease in serum insulin. Unchanged serum leptin and insulin concentrations in β cell-specific ClC-3 KO mice consolidate this as a valid explanation (data not shown). It is important to note that in both studies WT and KO mice display identical changes in plasma glucose levels in response to a glucose load (Deriy et al., 2009; Li et al., 2009).

While the above aspects of both papers are problematic, they are not the major focus of our letter. Our findings in chromaffin cells agree in principle that a loss of ClC-3 expression can result in reduced exocytosis and release from LDCVs (Maritzen et al., 2008). Given the identical intracellular localization of ClC-3 in chromaffin and β cells (Maritzen et al., 2008), one could assume a similar effect in β cells as was identified (Deriy et al., 2009; Li et al., 2009). The main point of our letter, however, concerns the proposed mechanism presented in both papers claiming that ClC-3 is significantly expressed on LDCVs and that loss of ClC-3 reduces insulin secretion by directly reducing LDCV acidification and exocytosis. Both papers lack appropriate and essential controls required to validate the localization data.

Localization of ClC-3 on LDCVs in Deriy et al. is based on immunogold, with no KO staining performed to control for antibody specificity. These gold particles are found everywhere (Figure 3A, Deriy et al.), including but not exclusive to LDCVs. Preabsorption with excess insulin is insufficient to establish specificity, and moreover would be unnecessary if the proper KO controls had been carried out and shown no labeling.

The evidence for ClC-3 localization on LDCVs (Figures 3C–3E, Li et al.) is also unconvincing. These authors show enrichment of ClC-3 in a ‘‘secretory granule’’ fraction without stating how much protein has been loaded in either lane. However, this apparent enrichment might be entirely due to comparing postnuclear supernatant (which includes cytosolic proteins) with spun-down membranes (which are reported to be further purified by ‘‘FACS’’ (as stated in legend) or by immunoprecipitation (as stated in text on page 313)). The lower lanes in this figure contain no band whatsoever in the ‘‘SG fraction’’ and therefore fail to prove enrichment of LDCV proteins. Figure 3D of Li et al. quite remarkably shows only a single vesicle. Li et al. state that >95% of vesicles, preselected as positive for both ClC-3 and phogrin (n = 26 vesicles), are also positive for insulin. This statement seemingly provides statistical support for a localization of ClC-3 on LDCVs, a major conclusion of this and previously published work (Barg et al., 2001). However, such a finding is trivial because phogrin-eGFP and insulin should colocalize to more than 90% if the phogrin labeling is LDCV specific. The authors should rather have stated the percentage of LDCVs containing ClC-3, or of ClC-3 positive vesicles (not just the fraction sorted for phogrin) containing insulin or phogrin. The single vesicle shown has adjacent, not overlapping, staining for ClC-3 with either insulin or phogrin. This becomes relevant when one studies their immuno-EM data containing one large vesicle attached to several smaller vesicles (Figure 3E, Li et al.). Thus, ClC-3 may be expressed on the small vesicles adjacent to the large vesicle, potentially explaining the strange fluorescence localization data in Figure 3D in Li et al. (2009). The fact that several vesicles are attached to each other invalidates their approach(es) used to ‘‘purify’’ LDCVs. This immuno-EM data allegedly used both 25 nm (ClC-3) and 5 nm (insulin) diameter gold particles, but black particles (of unknown size due to lack of scale reference) of one only size can be identified. Therefore, this picture fails to provide evidence for any colocalization.

No KO controls are shown for their immuno-EM, and the antibody used is not specified. These authors have previously published work (Barg et al., 2001) using the Nelson group ClC-3 antibody that also stained KO tissue in our hands (Maritzen et al., 2008). Li et al. also had access to our own KO-controlled antibody (Maritzen et al., 2008). However, this antibody has not been tested by us for suitability in immuno-EM experiments, and Li et al. provide no evidence suggesting these authors have either. Their analysis of the gold particle density (Figure S3, Li et al.)

reveals a strong presence of gold particles also on mitochondria, which do not express ClC-3, arguing against the specificity of their labeling.

Given that both groups had ClC-3 KO mice, we wonder why the simple but entirely essential KO controls of antibody experiments were not reported. Neither publication (Deriy et al., 2009; Li et al., 2009) provides, in our view, clear and convincing evidence for a localization of ClC-3 on LDCVs. This is a major point of both papers and the basis for their hypotheses on how loss of ClC-3 affects insulin secretion. An alternative mechanism has not yet been identified but the hypothesis remains that the lack of ClC-3 directly or indirectly affects other trafficking and sorting events in these cells. We show with isolated islets and INS-1 cells that ClC-3 does not copurify with insulin or other LDCV markers, but localizes to SLMVs and endosomes in these cells and in chromaffin and PC12 cells (Maritzen et al., 2008).

We write this letter in the hope of identifying potential problems with the hypothesis put forward by these authors (Deriy et al., 2009; Li et al., 2009) that ClC-3 directly acts on LDCVs to affect insulin secretion. Our wish here is to at least draw attention to insufficient evidence supporting such a hypothesis.

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