We thank the reviewers for their interest in and support of our work. We appreciate the opportunity to respond to their comments and questions, believe that we have now effectively clarified matters raised, and hope that the manuscript is now acceptable for publication.

Reviewer-1

To improve the clarity of the messages, the authors should clarify or answer the following points to strengthen the manuscript:

1. Although well presented, another round of copy-editing would be welcome. Certain words seem to be missing (eg. line 194: “essentially ….” may be missing a word?), and various sentences could be shortened or rewritten.

Response: Word “essentially” is deleted. There has also been another round of editing to polish and shorten some sentences and reduce some repetition throughout. We hope the reviewer now finds the manuscript satisfactory. If not, we would respectfully request some specific examples of what the reviewer considers issues.

2. The figure legends are very brief, do not clearly describe the meaning of the results, and miss critical information. This makes the figures difficult to understand. For instance, Fig.5A shows organic and aqueous lipid phases with no description in the figure’s legend. Readers should be able to have a general understanding of figures without having to refer to the text, so it would be beneficial to have more descriptive legends.

Response: Thank you for the suggestion. While we respectfully disagree that this is true for all the figure legends, we have made an effort to provide more detail where appropriate, including adding citations to specific references so the reader would not need to refer back to the Methods. Fig. 5A is updated showing chromatograms after CuSO₄ charring/detection and further description is added in the figure legend.

3. “Dose-dependent” should be changed to “concentration-dependent” as dose dependence relates more to oral drug administration.

Response: “Dose-dependent” is replaced with “concentration-dependent” throughout the manuscript.

4. Fig. 1B. The figure shows separation of both NBD-FFA and NBD-PA. It would be useful if the authors could clarify which protocol was used to separate those lipids. In the method section, the authors indicate that they used the neutral lipid protocol for neutral lipid separation (e.g. TAG, cholesterol, and FFA), while phospholipid separation protocol was used to separate phospholipids.
(e.g. PE, PC, PA). Then it becomes unclear how both NBD-FFA and NBD-PA were resolved on the same plate. FFAs are normally separated along with neutral lipids, while PA is resolved by using a phospholipid protocol. Also, the surface of silica gel is highly polar; thus, polar lipids in the solvent would interact strongly with the surface of the silica gel and move slowly. Lipids that are more hydrophobic interact weaker and move quicker. Thus, FFA should move to the top of the plate while PA is more polar and should be closer to the bottom of the plate if separated together. Fig.1B shows the opposite. This needs to be explained/addressed in the manuscript.

**Response:** The established neutral lipid protocol (Churchward et al. J Chem Biol. 2008) was used to resolve lipids shown in Fig. 1A, B and C; this has now been added to the figure legend. Also, note that only a critical section of the chromatogram is shown in the Fig. 1A; these were from the same HPTLC plate. Prior to the experiments discussed in the manuscript, probe experiments using neutral and phospholipid protocols were carried out to resolve NBD-PA and –ARA standards and after acceptable separation of these standards using the neutral lipid protocol, full experiments were carried out (Please refer to the TLC plate raw images at the end of the document). As expected, while the ARA standard does migrate slightly differently than the NBD- dodecanoyl species cleaved from the labelled phospholipids, these initial tests and the subsequent experiments confirm what is being resolved with this protocol.

5. **Fig.1C** shows resolved neutral lipids. No DAG is present there. Is it below detection levels? If the same plate was used for CuSO_4_ charring (**Fig.1C**) and NBD fluorescence (**Fig.1B**), then it becomes unclear how polar PA was detected on the same plate (Note: only neutral lipids are shown on **Fig.1C** and there is no PA there). The **Fig.1** legend says that CSC were resolved for lipids after labeling with NBD-PC and inhibitor treatments. It would help if PC levels after CuSO4 staining would be shown for tested conditions to assess changes. It seems logical to include a representative image of resolved phospholipids in **Fig.1C** for quantification of PC and/or PE levels. The authors need to check the data and/or give more details on the protocol used.

**Response:** Thank you for the detailed interpretation and suggestions. On some of the plates DAG is not detected (e.g. Figs 1C, 5B and supplementary S 1B, vs. supplementary S 1A (lane 2 but not in lane 3). This has been observed multiple times in separate HPTLC plates (not shown) and is consistent with previous work with this model system in which, depending upon inhibitors used, changes in certain lipids through a biosynthetic pathway are not consistently detected, thus simply indicating a lack of direct correlation with the mechanism being assessed (Abbineni et al. IJBCB 2018; Rogasevksaia et al. JBC 2015; Churchward et al. J. Cell Sci. 2005). Hence, in some cases DAG was likely converted (e.g. to TAG) during the course of the experiment.
Regarding concerns relating to FFA and PA, please refer to the explanation provided to the previous question, and again note the test separations of lipid standards attached to the end of this document. However, as the aim of this initial experiment was to follow the possible generation of NBD-FFA from the labelled exogenous phospholipid substrate, further phospholipid analysis was not done. Notably, Fig. 1C also confirms our normalization and thus the loading of equal amounts of lipids in each lane.

More detail about the protocol has now added in the Material and Methods section.

6. **Fig 5A, B.** Again FFA and phospholipids (PE) are shown on the same plate. It would help if the authors could provide details of the protocol used to allowed PE (very polar) and FFA (much more hydrophobic) to be side by side on the plate. Further, it would be very informative if the authors would be able to demonstrate resolved lipids after CuSO4 charring on the same HPTLC plate.

**Response:** We are sincerely sorry for the confusion and believe that the expanded figure legend now effectively clarifies the matter, as requested. The time-dependent changes in fluorescence intensity in NBD-PE and –PC shown in figure 5A (clearly noted as sections of the chromatograms where appropriate) are from separate HPTLC plates. The phospholipid protocol was used to separate lipids shown in 5A, while lipids shown in 5B and D were resolved using the neutral lipid protocol. After capturing NBD fluorescence, the same plates shown in the left-hand panels of 5A and B were assessed using CuSO4 and are now included as right-hand panels in the figure. The (section of the) chromatogram shown in 5A is now a new image in order to be able to show the same plate for both NBD fluorescence and CuSO4 charring. The protocols used for the lipid analyses have now been explicitly stated in the Fig. 5 legend and also in all other figure legends, including Fig. S2.

7. **Line 500-501.** In the text the authors refer to Supplementary Fig. 1A, B concerning DAG levels. However, no DAG was detected/shown on that figure.

**Response:** Apologies for not labelling the band corresponding to DAG in Supplementary Fig. 1A, B; this has been corrected.

8. **Line 489.** The authors claim that luminal sPLA cannot access exogenous NBD-PE and NBD-PC. However, NBD phospholipids are known to quickly flip-flop across the membrane (matter of minutes; Stevens et al., 2008; Sharom, 2011). It would be informative if the authors address this point.

**Response:** Indeed, flip-floppases are known to quickly translocate NBD-PC or -PE across native membranes. However, to begin with, we do not know if there are endogenous flip-floppase in CV. Nonetheless, CSC labelling was carried out for 15 min followed by 20 min inhibitor treatments. Hence, any putative flip-floppase essentially had 35 min to translocate NBD-PC or -PE from the outer to the inner CV monolayer before unbound lipids and inhibitors were washed out. Then lipids were
Labelled CSC treated with LY311727 did not differ from controls in terms of amount of NBD-FFA, whereas treating unlabeled CSC with LY311727 significantly reduced endogenous FFA, relative to the control. This indicated that either CV membrane do not contain flip-floppases or that their activity is extremely low in CV. This latter interpretation also holds true for the control shown in Fig. 6C, in which no significant change in the fluorescence intensity was observed even after 30 min incubation. This indicated that 30 min was insufficient for a putative CV flippase to translocate PED6 (modified PE) toward the CV lumen; had there been such translocation of PED6, a significant increase in fluorescence intensity should have occurred within 30 min due to luminal sPLA$_2$ activity. It is important to note that a significant increase in fluorescence intensity was observed in an overnight control, relative to the 0 min control (not shown; n=2). This may indicate a very minimal CV flip-floppase activity or simply a low spontaneous rate of transfer between the monolayers. Overall, this strongly suggests that NBD-PC or -PE were not accessible to the luminal sPLA$_2$ in intact CV. These additional interpretations are also added in the discussion.

Test TLC plates showing separation of NBD-ARA and -PA using the neutral lipid protocol.