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Preview

Phospholipase A_2-based probes to study vesicle trafficking

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Vesicle exo- and endocytosis mediate important biological functions, including synaptic transmission. In this issue of *Cell Reports Methods*, Seong J. An et al. found that the fluorescently tagged C2 domain of phospholipase A_2 binds to membrane phosphatidylcholine and thus labels vesicle membrane, allowing for super-resolution and electron microscopic visualization of vesicle trafficking.

Vesicle exocytosis releases vesicular contents, including neurotransmitters and hormones, to mediate many important functions, such as synaptic transmission, immune responses, and "fight or flight" responses (Sharma and Lindau, 2018; Wu et al., 2014). After exocytosis, vesicle endocytosis recycles vesicles and thus maintains a pool of vesicles to sustain exocytosis, including synaptic transmission in neurons (Chanaday et al., 2019; Wu et al., 2014). Vesicle exocytosis must involve vesicle movement toward the plasma membrane fusion site, docking and priming at the fusion site, and fusion that opens a fusion pore to release vesicular contents, whereas vesicle recycling involves vesicle reformation from the plasma membrane and vesicle movement toward the release site. To understand vesicle exocytosis and endocytosis, various techniques have been developed to label vesicles, which have significantly improved our understanding of vesicle trafficking (Kavalali and Jorgensen, 2014).

These techniques can be roughly divided into three categories. First, styryl dyes, such as FM dyes, are used to label cell plasma membrane, which are taken up during endocytosis to label recycled vesicles (Betz and Bewick, 1992; Cochilla et al., 1999). Second, genetically encoded synaptic vesicle proteins are tagged with fluorescent proteins, particularly pH-sensitive pHluorin, which can indicate exo- and endocytosis because their fluorescence intensity changes due to vesicular lumen pH changes during exo- and endocytosis (Kavalali and Jorgensen, 2014; Sankaranarayanan and Ryan, 2000). Third, antibodies or nanobodies targeting synaptic vesicle proteins may also be used to label vesicles (Hua et al., 2011). Although extremely powerful, each of these techniques has its drawbacks. For example, FM dye labeling may show some background fluorescence after labeling and have low brightness and photostability that limit their use for super-resolution imaging (Betz and Bewick, 1992; Revelo et al., 2014). pH-sensitive pHluorin may reveal its location only after vesicle fusion and before vesicle reacidification (Kavalali and Jorgensen, 2014; Sankaranarayanan and Ryan, 2000); its performance on super-resolution imaging is not ideal. The antibody or nanobody labeling approach may have a higher background fluorescence because the irreversible binding to target molecules makes the surface-bound ones difficult to be washed out (Kavalali and Jorgensen, 2014).

An et al. (2022) has developed a new probe that may complement these existing methods and overcome some of these drawbacks. The probe is based on the binding between C2 domain of phospholipase A_2 (PLA_2-C2) in the extracellular space and the extracellular-facing phosphatidylcholine (PC) at the plasma membrane (Figure 1A). In PC12 cells, the binding is triggered by a low concentration of Ca^{2+} (with an EC50 value of 1 μM) and can be washed out to below 5% by removing Ca^{2+}, suggesting that the binding is reversible. To label synaptic vesicles, Texas red-conjugated PLA_2-C2 (C2-TR) (Figure 1B) was extracellularly applied to cultured hippocampal neurons expressing pHluorin attached to vesicular glutamate transporter 1 (VGlut1-pHluorin). Neurons were then subjected to 90 s high K^+ (40 mM) stimulation to induce vesicle exocytosis and subsequent vesicle endocytosis that takes up C2-TR and thus labels the endocytosed vesicles (Figure 1C). Confocal microscopy showed that C2-TR localized with VGlut1-pHluorin fluorescent puncta that indicate clusters of synaptic vesicles. Reapplication of high K^+ solution induced exocytosis of C2-TR-containing vesicles, resulting in reduction of C2-TR fluorescence. In addition to using high potassium solution to stimulate and load C2-TR, hypertonic sucrose solution could also be used to load vesicles with C2-TR and to estimate the size of the recycling vesicle pool and the readily releasable pool of vesicles. In addition to confocal microscopy, An et al. (2022) showed that total internal reflection fluorescence microscopy (TIRFM) can be used to observe vesicles.

Knowing that C2-TR can label endocytosed vesicles for confocal microscopy, the authors used the same principle to label vesicles, but changed the conjugated Texas red to a different fluorescent probe suitable for a specific microscopic method. The fluorescent probes being conjugated included (1) horseradish peroxidase suitable for electron microscopy of ultrastructure of the labeled vesicles; (2) ATTO647N, a highly photostable dye suitable for super-resolution stimulated emission depletion (STED) microscopy for visualizing synaptic vesicles in

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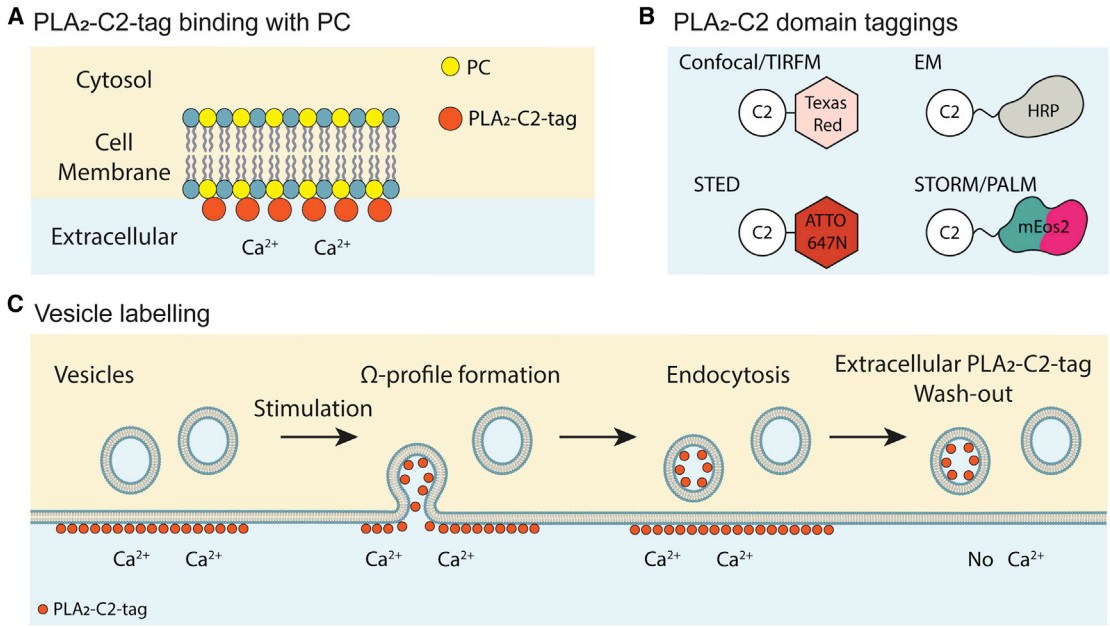

Figure 1. A phospholipase A_2-based probe for labeling synaptic vesicles

(A) The C2 domain of phospholipase A_2 (PLA_2-C2) in extracellular solution binds with phosphatidylcholine (PC) in the outer leaflet of plasma membrane in the presence of Ca^{2+}.

(B) Different conjugated C2 probes adapted for confocal and super-resolution microscopies.

(C) The strategy of PLA_2-C2 probe labeling a synaptic vesicle. PLA_2-C2 probes bind to plasma membrane depending on Ca^{2+} and are taken up into a recycling vesicle upon stimulation-induced endocytosis. The uninternalized probes could be washed out by Ca^{2+} chelator buffer.

living neurons; and (3) monomeric Eos2, a green-to-red photoconvertible fluorescent protein suitable for localization-based super-resolution techniques, such as stochastic optical reconstruction microscopy (STORM) or photoactivated localization microscopy (PALM) (McKinney et al., 2009) (Figure 1B). An et al. (2022) demonstrated the observation of vesicles loaded with these different probes with electron microscopy, STED microscopy, and STROM/PALM microscopy, respectively.

Compared to styryl FM dyes, PLA_2-C2-conjugated probes can be brighter with lower toxicity. Due to its specific binding with phospholipid molecules in cell membrane and the relatively clean washout, PLA_2-C2-conjugated probes may have less background signals originated from non-specific bindings. Compared to pHluorin, PLA_2-C2-conjugated probes are more suitable for super-resolution microscopy and electron microscopy. Compared to antibody or nanobody staining of vesicles, PLA_2-C2-conjugated probes offer more clean backgrounds and likely much brighter due to abundant PC molecules taken up by the endocytosed vesicles. PLA_2-C2-conjugated probes offer a single type of probe that could be adapted to multiple imaging systems, including confocal microscopy, TIRFM, STED microscopy, STORM/PALM, and electron microscopy.

Recently, expression of phospholipase C delta PH domain attached with mNeonGreen (PH-mNeonGreen), which binds to $PtdIns(4,5)P_2$ and thus labels the plasma membrane cytosol-facing leaflet, has been used to label the plasma membrane, vesicles fused at the plasma membrane, and vesicles endocytosed from the plasma membrane in endocrine chromaffin cells (Shin et al., 2018, 2021; Zhao et al., 2016). This method has detected hemi-fusion; hemi-fission; fusion pore opening, expansion, and constriction; sequential compound fusion; and dynamic flat-to-round vesicle formation in live cells with confocal and super-resolution STED microscopy (Ge et al., 2021; Shin et al., 2018, 2021; Zhao et al., 2016). Simultaneous labeling of the plasma membrane and vesicle membrane with PH-mNeonGreen and PLA_2-C2-conjugated probes, respectively, may offer super-resolution imaging between vesicle membrane and the plasma membrane, which may give further insights into the membrane dynamics of fusion and endocytosis in the future.

While PLA_2-C2-conjugated probes offer study of vesicle trafficking with multiple imaging systems, the vesicle loading of the probes is based on a calcium-dependent binding between the extracellularly applied probe and the extracellular-facing PC at the plasma membrane, endocytic uptake of the binding complex, and wash out of the non-endocytosed probes in a solution without calcium (Figure 1). Such a loading procedure is not as convenient as other probes that do not involve a calcium-dependent loading and washout and can be a concern when removing extracellular calcium that is not allowed in some experimental conditions, such as calcium-triggered endocytosis.

PLA_2-C2-conjugated probes resemble membrane-binding fluorophore-cysteine-lysine-palmitoyl group (mCLING), a probe reported to label synaptic vesicles several years ago (Revelo et al., 2014). mCLING is an octapeptide that consists of one cysteine and seven lysines, with

palmitoylation in one of the lysines to increase its stability of binding with membrane. Like PLA_2-C2-conjugated probes, mCLING is also suitable for super-resolution imaging (Revelo et al., 2014). It would be of interest to compare these mCLING with PLA_2-C2-conjugated probes to determine which probe is more suitable for super-resolution microscopy.

In summary, the probe developed here may enrich the tools for the study of synaptic transmission as well as the processes of endocytosis and exocytosis in many other secretory cells. We look forward to seeing its application that may provide further insights into vesicle trafficking and vesicular membrane dynamics in the future.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

An, S.J., Stagi, M., Gould, T.J., Wu, Y., Mlodzianoski, M., Rivera-Molina, F., Toomre, D., Strittmatter, S.M., De Camilli, P., Bewersdorf, J., and Zenisek, D. (2022). Multimodal imaging of synaptic vesicles with a single probe. Cell Reports Methods *2*, 100199-1–100199-12.

Betz, W.J., and Bewick, G.S. (1992). Optical Analysis of synaptic vesicle recycling at the Frog Neuromuscular Junction. Science *255*, 200–203.

Chanaday, N.L., Cousin, M.A., Milosevic, I., Watanabe, S., and Morgan, J.R. (2019). The synaptic vesicle Cycle Revisited: new insights into the modes and mechanisms. J. Neurosci. *39*, 8209–8216. https://doi.org/10.1523/JNEUROSCI.1158-19.2019.

Cochilla, A.J., Angleson, J.K., and Betz, W.J. (1999). Monitoring secretory membrane with FM1-43 fluorescence. Annu. Rev. Neurosci. *22*, 1–10. https://doi.org/10.1146/annurev.neuro.22.1.1.

Ge, L., Shin, W., and Wu, L.-G. (2021). Visualizing sequential compound fusion and kiss-and-run in live excitable cells. Preprint at bioRxiv. https://doi.org/10.1101/2021.06.21.449230.

Hua, Y., Sinha, R., Thiel, C.S., Schmidt, R., Huve, J., Martens, H., Hell, S.W., Egner, A., and Klingauf, J. (2011). A readily retrievable pool of synaptic vesicles. Nat. Neurosci. *14*, 833–839. https://doi.org/10.1038/nn.2838.

Kavalali, E.T., and Jorgensen, E.M. (2014). Visualizing presynaptic function. Nat. Neurosci. *17*, 10–16. https://doi.org/10.1038/nn.3578.

McKinney, S.A., Murphy, C.S., Hazelwood, K.L., Davidson, M.W., and Looger, L.L. (2009). A bright and photostable photoconvertible fluorescent protein. Nat. Methods *6*, 131–133. https://doi.org/10.1038/nmeth.1296.

Revelo, N.H., Kamin, D., Truckenbrodt, S., Wong, A.B., Reuter-Jessen, K., Reisinger, E., Moser, T., and Rizzoli, S.O. (2014). A new probe for super-resolution imaging of membranes elucidates trafficking pathways. J. Cell Biol *205*, 591–606. https://doi.org/10.1083/jcb.201402066.

Sankaranarayanan, S., and Ryan, T.A. (2000). Real-time measurements of vesicle-SNARE recycling in synapses of the central nervous system. Nat. Cell Biol *2*, 197–204. https://doi.org/10.1038/35008615.

Sharma, S., and Lindau, M. (2018). The fusion pore, 60 years after the first cartoon. FEBS Lett. *592*, 3542–3562. https://doi.org/10.1002/1873-3468.13160.

Shin, W., Ge, L., Arpino, G., Villarreal, S.A., Hamid, E., Liu, H., Zhao, W.D., Wen, P.J., Chiang, H.C., and Wu, L.G. (2018). Visualization of membrane pore in live cells reveals a dynamic-pore theory Governing fusion and endocytosis. Cell *173*, 934–945.e12. https://doi.org/10.1016/j.cell.2018.02.062.

Shin, W., Wei, L., Arpino, G., Ge, L., Guo, X., Chan, C.Y., Hamid, E., Shupliakov, O., Bleck, C.K.E., and Wu, L.G. (2021). Preformed Omega-profile closure and kiss-and-run mediate endocytosis and diverse endocytic modes in neuroendocrine chromaffin cells. Neuron *109*, 3119–3134.e5. https://doi.org/10.1016/j.neuron.2021.07.019.

Wu, L.G., Hamid, E., Shin, W., and Chiang, H.C. (2014). Exocytosis and endocytosis: modes, functions, and coupling mechanisms. Annu. Rev. Physiol. *76*, 301–331. https://doi.org/10.1146/annurev-physiol-021113-170305.

Zhao, W.D., Hamid, E., Shin, W., Wen, P.J., Krystofiak, E.S., Villarreal, S.A., Chiang, H.C., Kachar, B., and Wu, L.G. (2016). Hemi-fused structure mediates and controls fusion and fission in live cells. Nature *534*, 548–552. https://doi.org/10.1038/nature18598.