Calmodulin Availability Regulates Basal Autophagy

Elizabeth McConnaha, Jennifer Giles, Eric Wauson, and Quang-Kim Tran
Department of Physiology & Pharmacology, Des Moines University College of Osteopathic Medicine

BACKGROUND

- Macroautophagy (hereafter referred to as "autophagy") is a catabolic process in which cells degrade unused or unwanted proteins and organelles. A basal level of autophagy maintains tissue homeostasis. However, autophagy is upregulated under many conditions, notably cellular nutrient depletion, or "starvation".
- The activation of autophagy requires numerous events that can be categorized into the major steps depicted in Fig. 1. Materials to be eliminated (cargos) are wrapped in a double-membrane phagophore. The autophagosome is formed as the phagophore elongate and closes around its content. The autophagosome then fuse with the lysosome, forming the autophagolysosome, in which cargos are degraded. For successful degradation cargos by the lysosomal enzymes, the lysosomes must maintain an acidic environment.

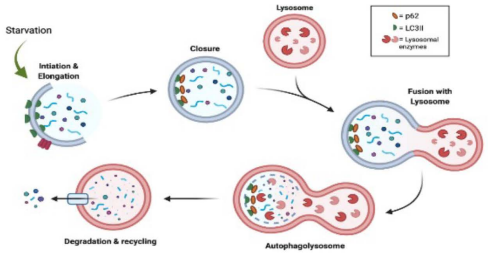

Figure 1. *The main steps of autophagy. LC3-II is an important autophagic marker that are used in autophagic assays to reflect the rate of cargo delivery to the lysosome and degradation therein.*

- Intracellular Ca^{2+} plays diverse roles in autophagy. In many cases, intracellular Ca^{2+} first binds its ubiquitous transducer, calmodulin (CaM), to form a Ca^{2+}-CaM complex, which subsequently interacts with and activates over 300 cellular proteins.
- Despite its universal requirement, CaM is not expressed sufficiently for its numerous target proteins in cells, rendering a shortage of CaM across tissues. Factors that regulate CaM availability is thus have extensive effects on tissue functions.
- Although some CaM-binding proteins have been implicated in the regulation of autophagy, it is completely unknown if the availability of CaM is an important factor regulating autophagy in basal condition, when cytosolic Ca^{2+} levels are generally low.

HYPOTHESIS

The availability of CaM is an important factor regulating basal autophagy.

METHODS & RESULTS

Enhanced CaM availability promotes autophagic flux in basal and short-term nutrient-depleted conditions

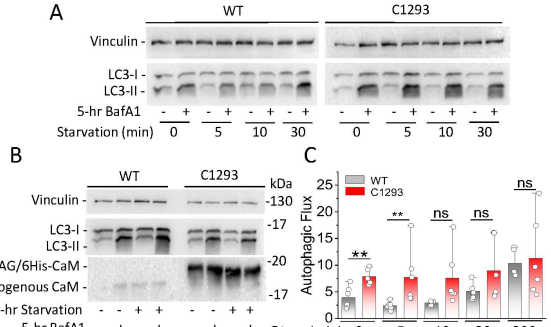

Figure 2. Autophagic flux in wild-type (WT) or CaM-overexpressing (C1293) HEK293 cells. ***A-B****, Autophagic marker LC3-II under basal conditions or after exposure to short- (A) or long-term (5 hrs) amino acid starvation (B) during treatment with or without 100 nM V-ATPase inhibitor BafA1 for 5 hrs, as specified. Vinculin was used as a loading control. Anti-CaM blot (lower vignette, B) was probed from the same samples on a separate SDS-PAGE membrane. C, Paired comparison of calculated autophagic flux from A and B. n = 6. **, p < 0.01.* ***Note*** *that in cells overexpressing CaM (C1293) basal autophagic flux is significantly higher than in wildtype cells. This increase was also observed in cells exposed to short-term amino acid starvation.*

Pharmacological inhibition of CaM decreases lysosomal acidity

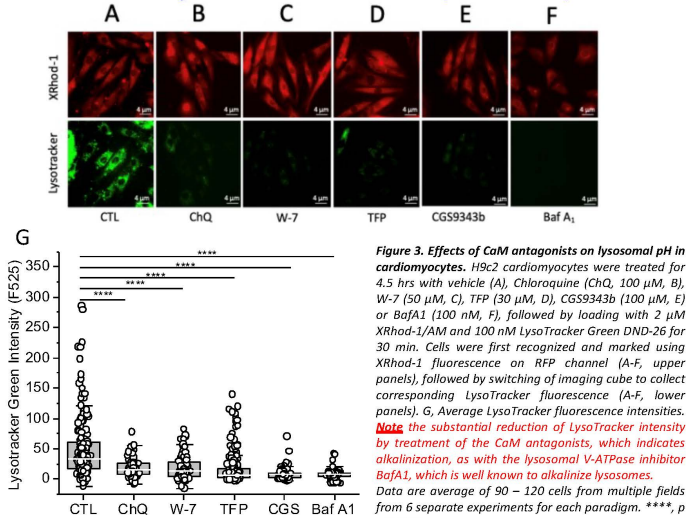

Figure 3. Effects of CaM antagonists on lysosomal pH in cardiomyocytes. *H9c2 cardiomyocytes were treated for 4.5 hrs with vehicle (A), Chloraquine (ChQ, 100 μM, B), W-7 (50 μM, C), TFP (30 μM, D), CGS9343b (100 μM, E) or BafA1 (100 nM, F), followed by loading with 2 μM XRhod-1/AM and 100 nM LysoTracker Green DND-26 for 30 min. Cells were first recognized and marked using XRhod-1 fluorescence on RFP channel (A-F, upper panels), followed by switching of imaging cube to collect corresponding LysoTracker fluorescence (A-F, lower panels). G, Average LysoTracker fluorescence intensities.* ***Note*** *the substantial reduction of LysoTracker intensity by treatment of the CaM antagonists, which indicates alkalinization, as with the lysosomal V-ATPase inhibitor BafA1, which is well known to alkalinize lysosomes. Data are average of 90 – 120 cells from multiple fields from 6 separate experiments for each paradigm. ****, p < 0.0001.*

Molecular approach to buffer free CaM in cells

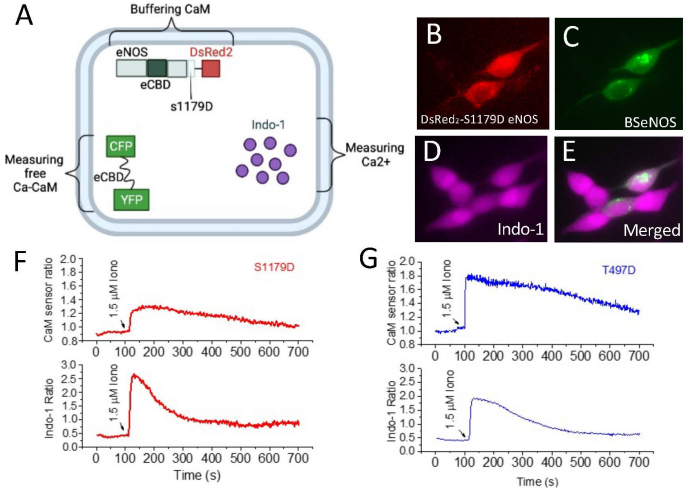

Figure 4. A, Molecular approach to buffer free CaM: principle and verification. *CaM is buffered by overexpression of DsRed2-tagged S1179D eNOS, which binds CaM with sub-nanomolar affinity (JBC 2009(284)11892-9); a control is T497D eNOS, which does not bind CaM (Circ Res 2001(11)E68-75). Cells were co-transfected with a FRET CaM biosensor (BSeNOS, built on eNOS' CaM-binding domain (eCBD)) and either DsRed2-S1179DeNOS or DsRed2-T497DeNOS, then loaded with the Ca^{2+} indicator indo-1/AM. This system allows for simultaneous measurement of free Ca^{2+}-CaM and free Ca^{2+} in the presence of a high affinity CaM-binding protein to buffer CaM or a non-binder control.* ***B****, RFP image of S1179DeNOS-DsRed2;* ***C****, EYFP image of BSeNOS in the same cells as in B;* ***D****, indo-1 fluorescence in the same microscopic field as in B and C;* ***E****, merged images.* ***F-G****, Free CaM (upper panel) and Ca^{2+} (lower panel) measured in the same cells co-expressing S1179DeNOS and BSeNOS (F) or T497DeNOS and BSeNOS (G).* ***Note*** *that cells expressing S1179DeNOS responded to ionomycin with higher free Ca^{2+} levels than cells expressing T497DeNOS (lower panels, F&G, respectively); however, cells expressing S1179DeNOS exhibited lower free CaM levels than cells expressing T497DeNOS (upper panels, F&G, respectively). These data indicate that overexpression of S1179DeNOS effectively buffers CaM in cells.*

Molecular buffering free CaM reduces lysosomal acidity

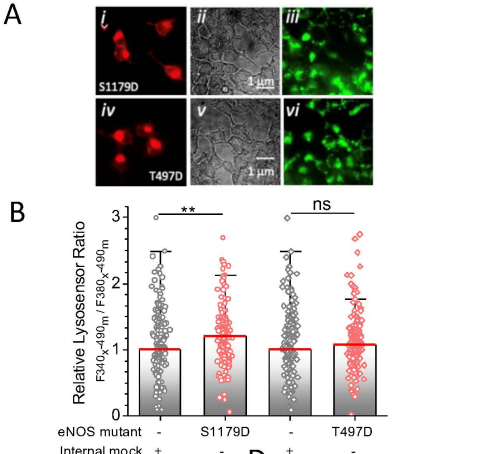

Figure 5. Effect of buffering CaM on lysosomal pH. A, *Approach; CaM was buffered by overexpression of DsRed2-S1179DeNOS (control: non-CaM binder DsRed2-T497DeNOS) for 24 hrs in the presence of 100 μM eNOS inhibitor L-NAME. Cells were then loaded with 1 μM LysoSensor Yellow/Blue for 5 minutes.* ***i and iv****, RFP images of S1179DeNOS-DsRed2 and T497DeNOS-DsRed2, respectively;* ***ii and v****, corresponding brightfield images for i and iv, respectively;* ***iii and vi****, corresponding LysoSensor signals for cells in* ***i*** *and* ***v****, respectively, under excitation at 380 nm and emission at 490 nm.* ***B****, Relative lysosensor ratios from cells expressing S1197DeNOS-DsRed2 or T497DeNOS-DsRed2 paired randomly with non-transfected cells in the same microscopic fields.* ***C****, Excitation spectra of 1 μM LysoSensor Yellow/Blue at varying pH.* ***D****, F340/F380 excitation ratios at emission 490 nm from titration in C. Arrows, pH levels predicted in non-transfected cells (black arrow) or cells overexpressing the S1179DeNOSDsRed2 plasmid (red arrow). For G-H, n = 145 – 170 cells for each condition from 15-20 repetitions; **, p < 0.01. For C-D, n = 4 independent determinations. Note that in cells in which CaM was buffered at basal condition (S1179DeNOS-expressing cells), lysosomal pH was significantly higher than the non-expressing counterparts from the same environment. This effect was not seen in cells overexpressing the non-CaM binder T497D eNOS.*

SUMMARY & CONCLUSIONS

- Increasing CaM availability promotes autophagic flux under basal condition and following short-term amino acid starvation.
- Under basal condition, pharmacological inhibition of CaM inhibits lysosomal acidity, which is a critical regulator of autophagic flux.
- Molecular buffering of intracellular CaM at basal condition also inhibits lysosomal acidity.
- These data strongly indicate that CaM availability is an important regulator of basal and stimulated autophagy. Factors that alter CaM availability therefore are predicted to affect tissue homeostasis significantly via this mechanism.

ACKNOWLEDGMENTS

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