SUPPLEMENTARY INFORMATION

GPN does not release lysosomal Ca^{2+}, but evokes Ca^{2+} release from the ER by increasing cytosolic pH independent of cathepsin C

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Abbreviations

$[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; CPA, cyclopiazonic acid; CTSC, cathepsin C; ER, endoplasmic reticuum; GPN, glycyl-L-phenylalanine 2-napthylamide (D-GPN has D-phenylalanine); IP_3R, inositol 1,4,5-trisphosphate receptor; NaP, sodium proprionate; pH_{cyt} (pH_{ly}, pH_{ER}) cytosolic (lysosomal, ER) pH; RyR, ryanodine receptor; SERCA, SR/ER Ca^{2+}-ATPase.

Table S1. Properties of GPN, fluoxetine and established lysosomotropic agents.

	[a]Estimated pK_a	[a]Estimated ACD/logP	[d]Estimated *C*logP
NH_3	8.86	-0.98	ND
LysoTracker Red	[b]7.5	ND	[b]2.10
[c]GPN	7.84	2.40	3.01
Fluoxetine	9.8	4.17	4.57
Leu-Leu-OMe	8.43	2.01	1.54

Basic pK_a refers to the pK_a of the most basic moiety within the molecule. logP is the log of the partition coefficient of the neutral species between n-octanol and water. ACD/logP and *C*log*P* are derived from different algorithms used to estimate logP values (reviewed in Mannhold et al., 2009). [a]From Chemicalize (2018) calculation module: https://chemicalize.com/ developed by ChemAxon (http://www.chemaxon.com); except for [b](Duvvuri et al., 2004). [c]For GPN, we disregard the most extremely basic moieties since their pK_a values (13.6 and 15.3) lie too far beyond the physiological pH range; pK_a values determined using the ACD/PhysChem Suite, version 12.0, Advanced Chemistry Development Inc, Toronto, Canada (http://ilab.cds.rsc.org/?cdsrdr=1). [d]From http://ilab.cds.rsc.org/?cdsrdr=1, except for [b](Duvvuri et al., 2004). Most lysosomotropic compounds have basic pK_a values >6.5 and *C*log*P* values >2 (Nadanaciva et al., 2011). ND, not determined.

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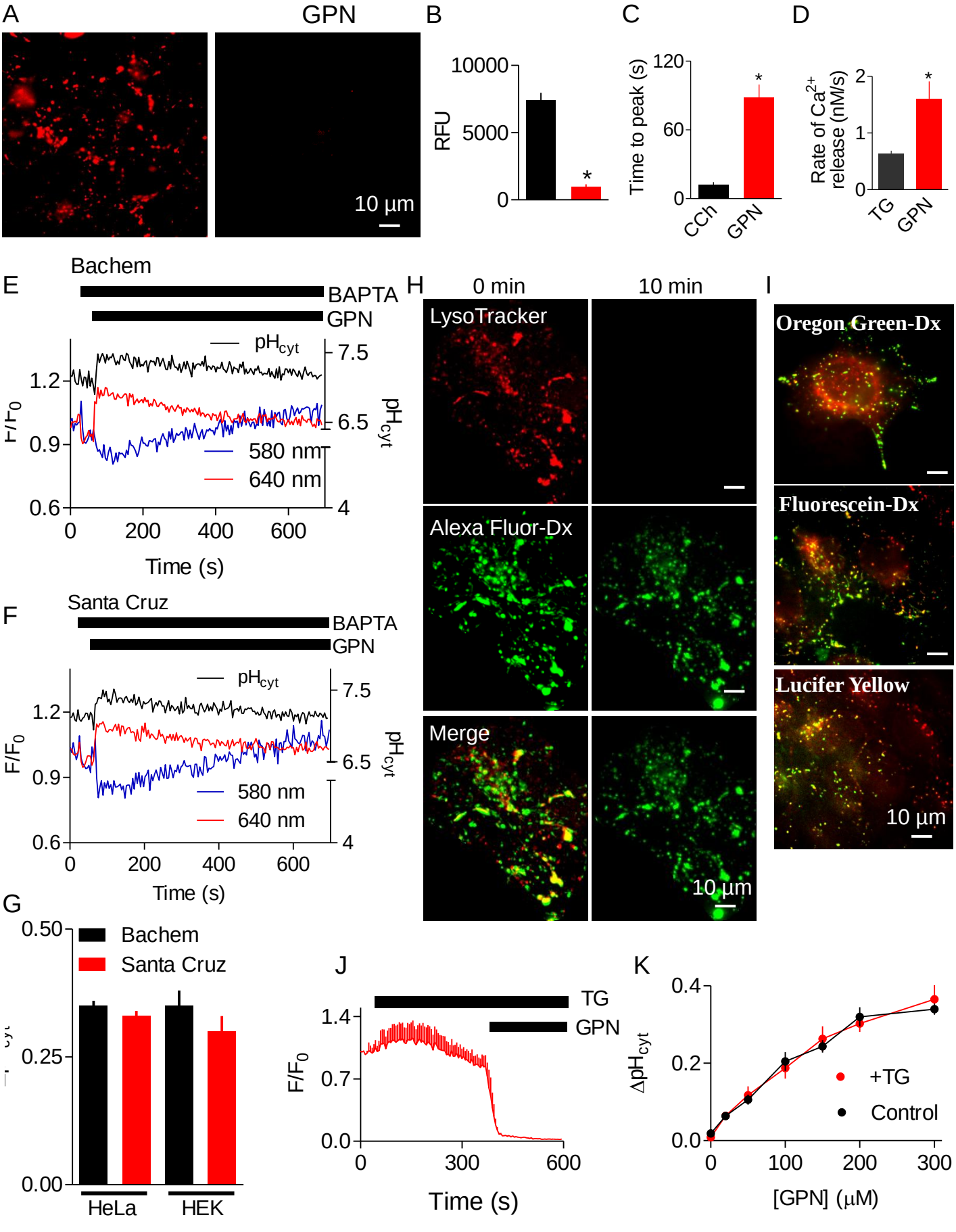

Fig. S1. Similar responses to GPN from different sources and in different cells. Figure on preceding page. (**A**) Wide-field images show LysoTracker Red staining of HEK cells with and without GPN (200 µM, 30 min). The results show that even after prolonged stimulation with GPN, pH_{ly} does not recover. (**B**) Summary results (mean ± SEM, *n* = 4 coverslips) show the intensity of LysoTracker Red staining. (**C**) From experiments similar to those in **Fig. 1G**, times to peak increase in $[Ca^{2+}]_c$ are shown (mean ± SEM, *n* = 4, with 3 replicates). $^*P < 0.05$, Student's *t*-test. (**D**) Similar analysis shows rates of rise of $[Ca^{2+}]_c$ after addition of GPN (200 µM) or thapsigargin (1 µM) in Ca^{2+}-free HBS, each measured over the same range of $[Ca^{2+}]_c$. Results show means ± SEM, *n* = 5, with 3 replicates. $^*P < 0.05$, Student's *t*-test. (**E,F**) Typical traces show the reciprocal changes in SNARF-5F fluorescence at the two emission wavelengths used (580 nm and 640 nm) and pH_{cyt} after addition of GPN (200 µM) from Bachem (E) or Santa Cruz (F). (**G**) Summary results (mean ± SEM, *n* = 3, with 3 replicates) show the effects of GPN (200 µM, from the indicated suppliers) on ΔpH_{cyt} of HEK and HeLa cells. (**H**) Simultaneous recording of LysoTracker Red and Alexa Fluor 488-Dx (M_r ~10,000) from HEK cells treated with GPN (200 µM, 10 min) showing that an increase in pH_{ly} is not accompanied by loss of large molecules from lysosomes. (**I**) Overlay images of HEK cells loaded with LysoTracker Red and either Oregon Green-Dx, Fluorescein-Dx or Lucifer Yellow. (**J**) Typical time course showing effect of GPN (200 µM) on LysoTracker Red fluorescence in HEK cells after treatment with thapsigargin (TG, 1 µM, 5 min). See **Fig. 1C** for a similar analysis without TG. (**K**) Similar analyses of the effects of the indicated concentrations of GPN in Ca^{2+}-free HBS alone or after treatment with thapsigargin (TG, 1 µM, 15 min) on ΔpH_{cyt} (mean ± SEM, *n* = 3, each with duplicate determinations).

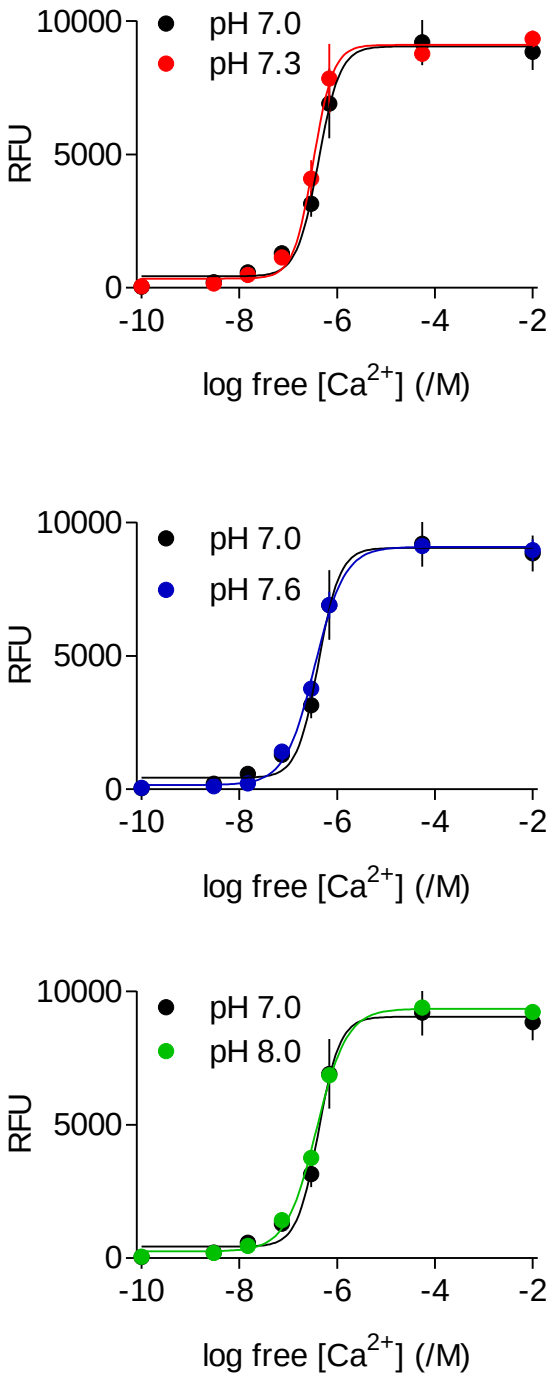

Fig. S2. The affinity of fluo 8 for Ca^{2+} is unaffected by changing pH between 7 and 8. Since the K_d of BAPTA for Ca^{2+} (160 nM in the absence of Mg^{2+}) (Pethig et al., 1989) is unaffected by pH changes between 7 and 8 (Tsien, 1980), the free [Ca^{2+}] of cytosol-like medium (CLM) containing 10 mM BAPTA, but without Mg^{2+}, was computed at the indicated pH using the same K_d for Ca^{2+} for each pH. Results (mean ± SEM, *n* = 3, each with 3 determinations) show the fluorescence recorded from fluo 8 (2 µM). RFU, relative fluorescence units. The results indicate that the K_d of fluo 8 for Ca^{2+} is the same at pH 7 (434 ± 51 nM), pH 7.3 (374 ± 43 nM), pH 7.6 (355 ± 39 nM) and pH 8 (401 ± 29 nM).

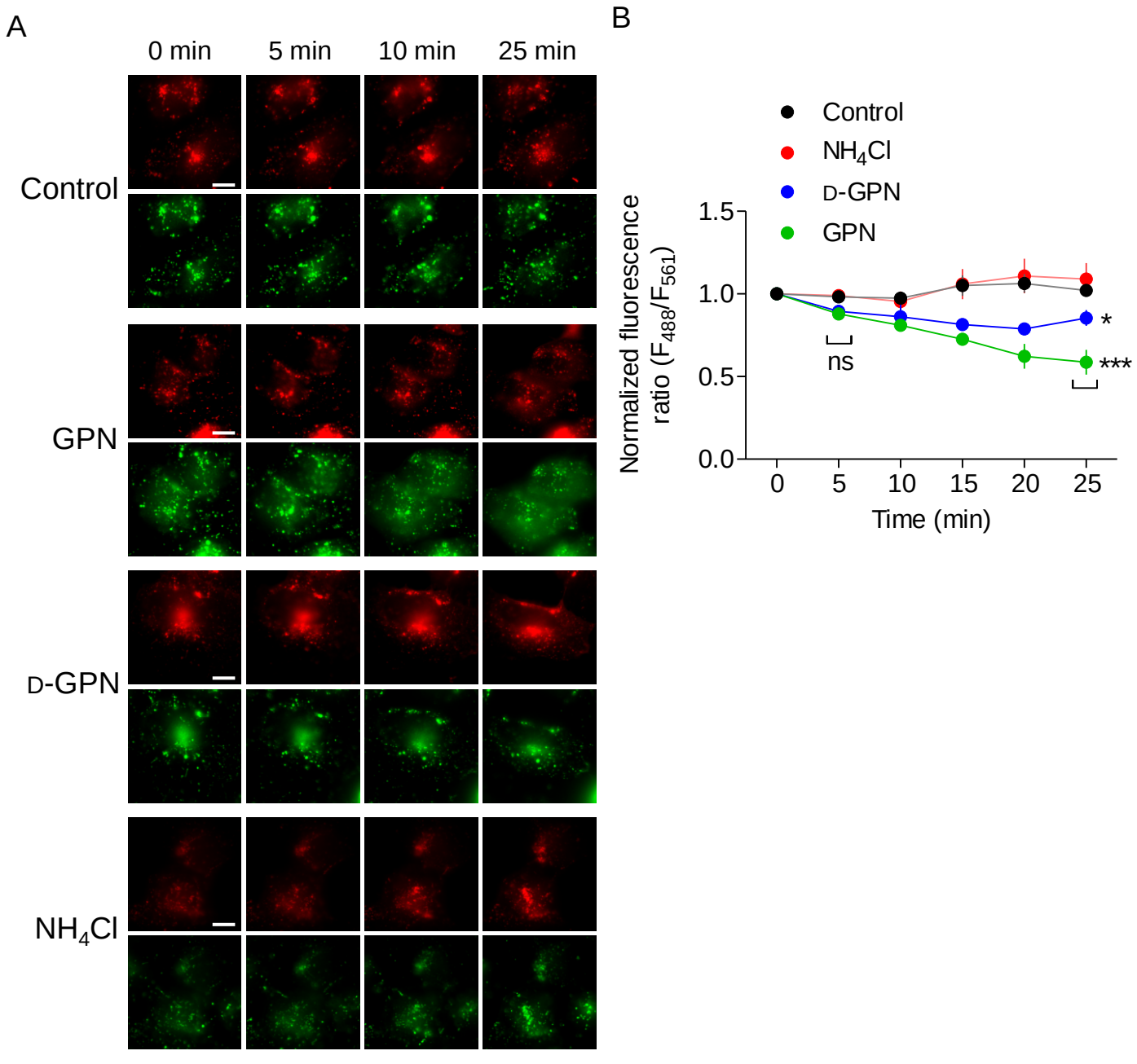

Fig. S3. Sustained exposure to GPN causes some loss of high-M_r fluorophores from lysosomes. (**A**) Typical wide-field images of HEK cells expressing LAMP1-mCherry (red) and with their lysosomes loaded with dextran-conjugated Alexa Fluor-488 (~10 kDa, green) and then treated with GPN (200 µM), D-GPN (200 µM), NH_4Cl (20 mM) or vehicle for the indicated times. Scale bars = 10 µm. Images were collected immediately before the additions and at 5-min intervals thereafter to minimize photobleaching. (**B**) Fluorescence ratios (F_{488}/F_{561}; i.e., $F_{Dextran}/F_{LAMP}$) were determined for each ROI enclosing a lysosome, and the ratios for each cell were normalized to the value immediately before treatment (t = 0). Results show mean ± SEM, n = 5 cells. $^{*}P < 0.05$, $^{***}P < 0.001$, ns, not significant, relative to time-matched control, 2-way ANOVA with Bonferonni test for the indicated comparisons only.

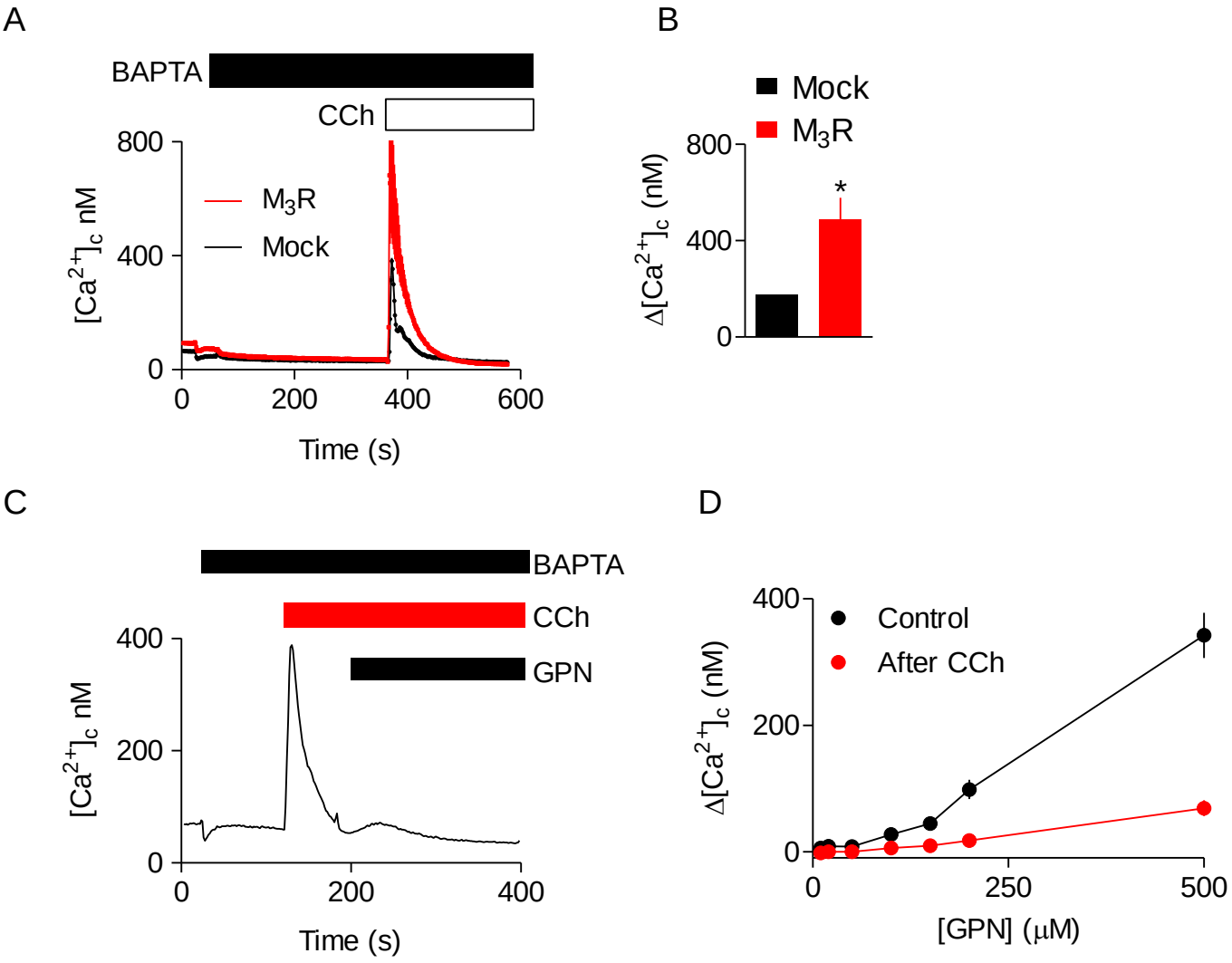

Fig. S4. Depleting ER Ca^{2+} stores by activating IP_3Rs rapidly attenuates GPN-evoked increases in $[Ca^{2+}]_c$. (**A**) HEK cells express M_3 muscarinic receptors (M_3Rs). When activated by carbachol (CCh), M_3Rs stimulate phospholipase C and thereby Ca^{2+} release from ER through IP_3Rs. However, endogenous expression of M_3 receptors is insufficient to fully deplete the ER of Ca^{2+} (Konieczny et al., 2017). We therefore used a viral vector, BacMam, to over-express M_3Rs in HEK cells. The results (mean ± SD from 2 replicates) show $[Ca^{2+}]_c$ recorded form a population of fluo 8-loaded HEK cells with or without (mock-infected) over-expressed M_3Rs and stimulated with a maximally effective concentration of CCh (1 mM). BAPTA (2.5 mM) was added before CCh to chelate extracellular Ca^{2+}. (**B**) Summary results (mean ± SEM, n = 3) show the peak increase in $[Ca^{2+}]_c$ ($\Delta[Ca^{2+}]_c$). $^*P <$ 0.05, Student's t-test. Responses to CCh were abolished in cells pre-treated with thapsigargin (1 μM, 15 min) to inhibit SERCA (results not shown). (**C**) HEK cells over-expressing M_3Rs were stimulated with CCh (1 mM) in Ca^{2+}-free HBS and then with GPN (200 μM). Results show mean of 3 replicates. (D) Summary results (mean ± SEM, n = 3) show responses to the indicated concentrations of GPN alone or after stimulation with CCh. The results demonstrate that depleting the ER of Ca^{2+} by stimulating IP_3Rs rapidly attenuates the increase in $[Ca^{2+}]_c$ evoked by GPN.

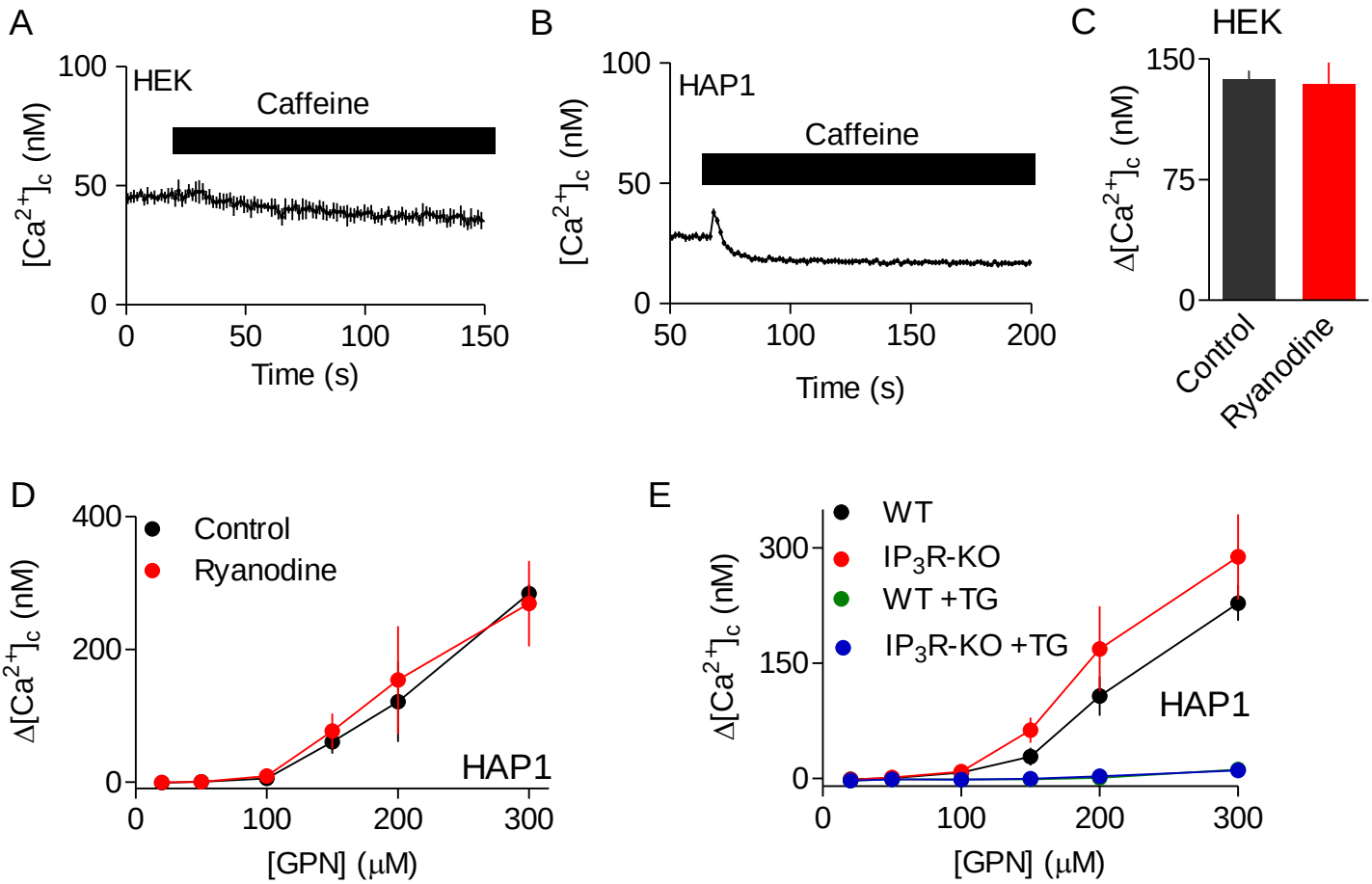

Fig. S5. Neither RyRs nor IP_3Rs contribute to cytosolic Ca^{2+} signals evoked by GPN. (**A,B**) Effects of caffeine (1 mM) in Ca^{2+}-free HBS on $[Ca^{2+}]_c$ in HEK (A) and HAP1 (B) cells. Traces show mean ± SD from 3 replicates. Similar results were obtained in 3 independent analyses. (**C,D**) Effects of GPN in Ca^{2+}-free HBS on the peak increase in $[Ca^{2+}]_c$ ($\Delta[Ca^{2+}]_c$) alone or after treatment with ryanodine (20 µM, 15 min) to inhibit RyRs. Results (mean ± SEM, *n* = 3, each with 3 replicates) are from HEK cells stimulated with 200 µM GPN (C) and HAP1 cells stimulated with the indicated concentrations of GPN (D). (E) Effects of GPN (200 µM) in Ca^{2+}-free HBS on HAP cells with (wild-type, WT) or without IP_3Rs (IP_3R-KO), alone or after treatment with thapsigargin (TG, 1 µM, 15 min). Results show mean ± SEM, *n* = 3, each with 3 replicates.

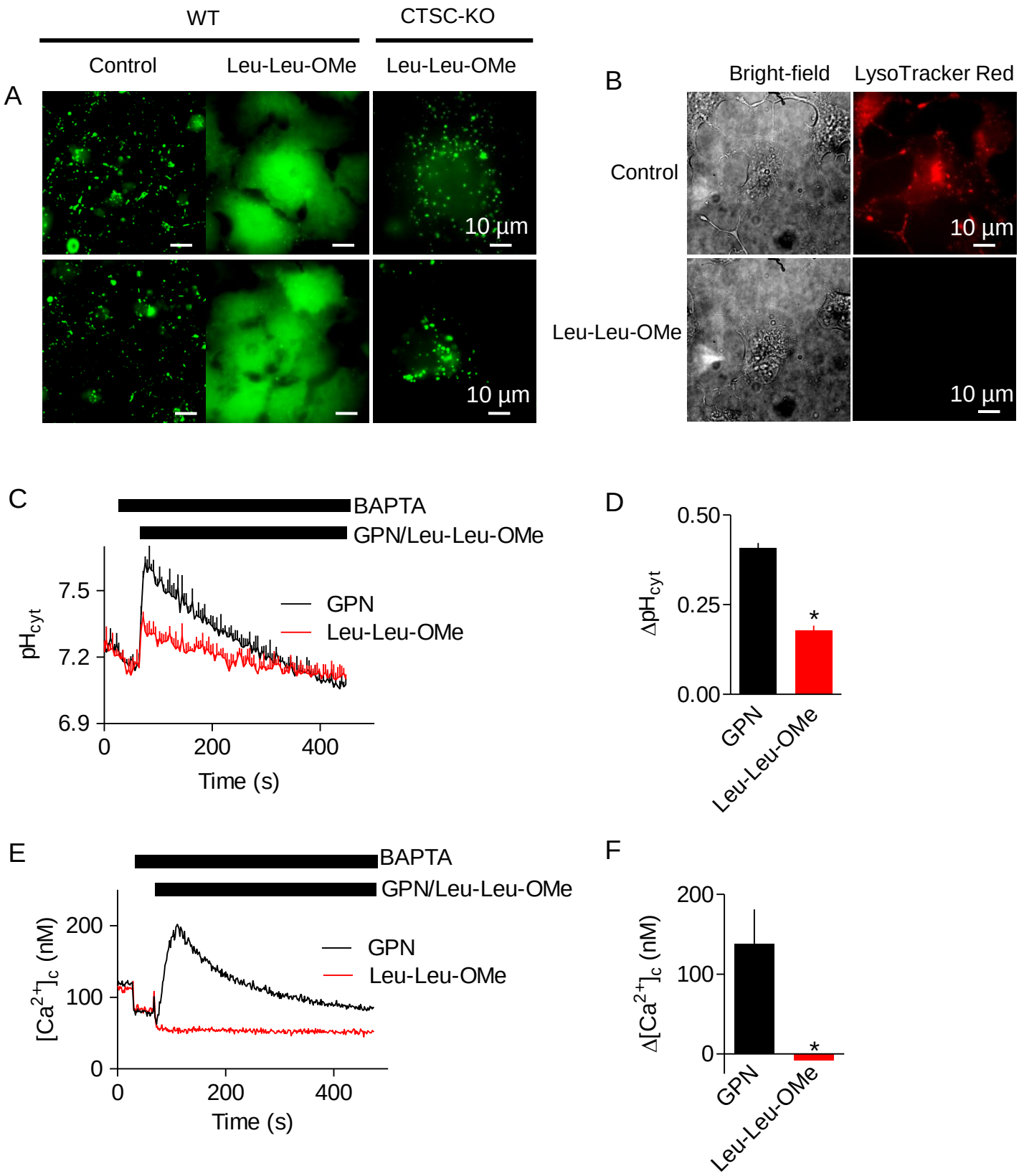

Fig. S6. Leu-Leu-OMe ruptures lysosomes in a cathepsin C-dependent manner, but it does not increase $[Ca^{2+}]_c$. Figure on preceding page. (**A**) Two representative wide-field fluorescence images show the punctate distribution of endocytosed Alexa Fluor 488-dextran (M_r ~3,000) in HEK WT cells. Treatment with Leu-Leu-OMe (1 mM, 1 h) causes loss of the puncta in WT cells, but not in CTSC-KO cells. Results are typical of 3 experiments. Scale bar = 10 µm in all images. (**B**) Bright-field and wide-field fluorescence images of HEK cells loaded with LysoTracker Red (100 nM, 20 min) with or without Leu-Leu-OMe (1 mM, 1 h). Images are typical of three experiments. (**C**) Effects of GPN (200 µM) or Leu-Leu-OMe (1 mM) on pH_{cyt} in HEK cells. Results are mean ± SD for 3 replicates. (**D**) Summary results (mean ± SEM, *n* = 4, each with 3 replicates) show peak ΔpH_{cyt}. $^{*}P < 0.05$, Student's *t*-test. (**E**) BAPTA (2.5 mM) was added to chelate extracellular Ca^{2+}, before addition of GPN (200 µM) or Leu-Leu-OMe (1 mM) to fluo 8-loaded HEK cells. Results show mean for 3 replicates. (**F**) Summary results (mean ± SEM, *n* = 4-6, each with 3 replicates) show peak $\Delta[Ca^{2+}]_c$ evoked by GPN or Leu-Leu-OMe. $^{*}P < 0.05$, Student's *t*-test.

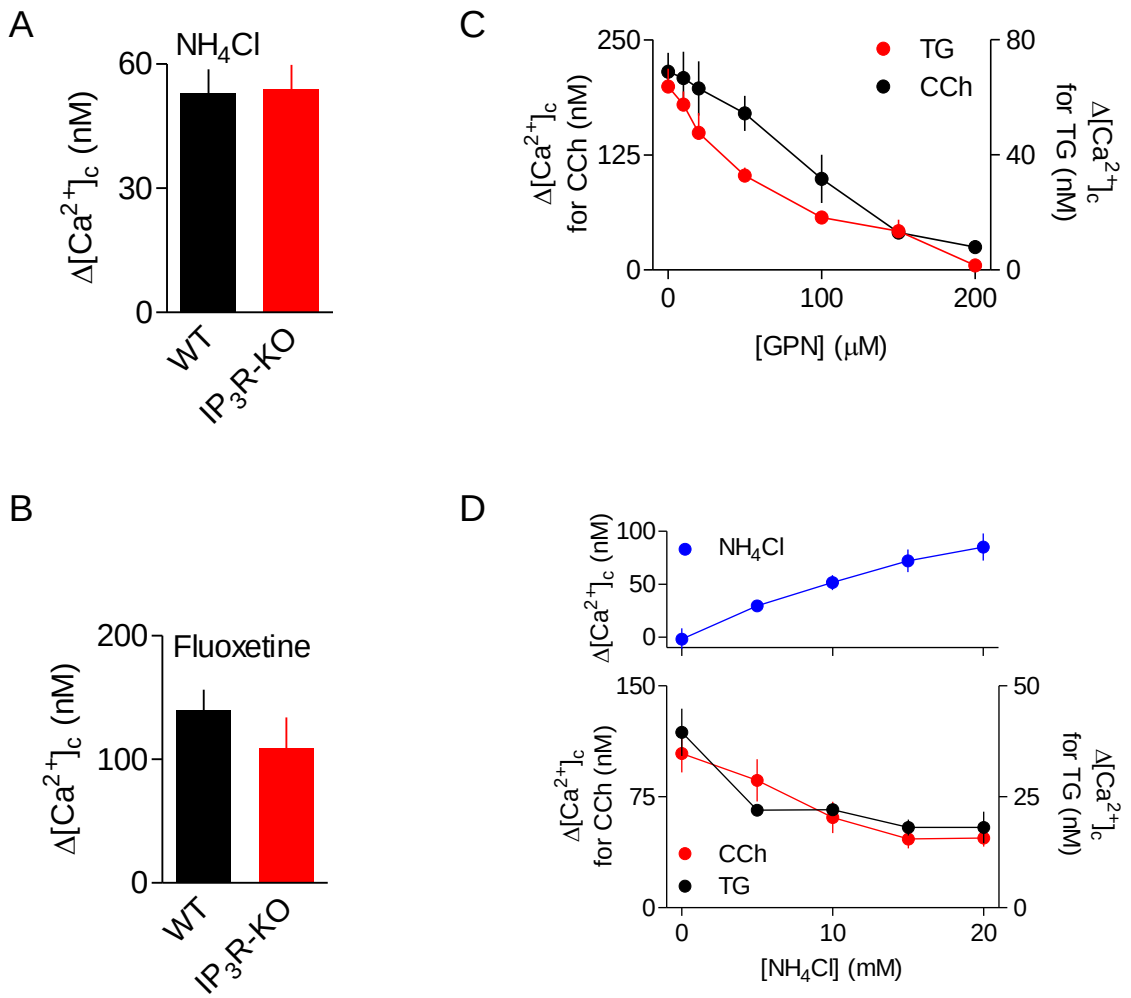

Fig. S7. Weak bases deplete the ER of Ca^{2+} independent of IP_3Rs. (**A,B**) Effects of NH_4Cl (20 mM) (A) or fluoxetine (300 µM) (B) in Ca^{2+}-free HBS on HEK cells with (WT) or without IP_3Rs (IP_3R-KO). Results show mean ± SEM, *n* = 6 (A) or *n* = 5 (B), each with 3 replicates. *P* = 0.90 (A) and *P* = 0.34 (B), Student's *t*-test. (**C**) HEK cells were stimulated with the indicated concentrations of GPN (15 min) in Ca^{2+}-free HBS before addition of carbachol (CCh, 1 mM) to stimulate IP_3 formation or thapsigargin (TG, 1 µM) to estimate the amount of Ca^{2+} remaining within the ER. Since CCh stimulates Ca^{2+} release, while TG, by inhibiting SERCA, unmasks a basal leak, the peak amplitudes of the Ca^{2+} signals are different for the two stimuli. Both, however, reveal a similar concentration-dependent decrease in ER Ca^{2+} content after GPN treatment. Results (mean ± SEM, *n* = 3, each with 3 replicates) show $\Delta[Ca^{2+}]_c$ for CCh or TG. (**D**) Similar analyses (mean ± SEM, *n* = 4-6, each with 3 replicates) show the effects of pre-treatment with NH_4Cl (15 min) on the subsequent $\Delta[Ca^{2+}]_c$ evoked by CCh or TG. The upper panel shows $\Delta[Ca^{2+}]_c$ evoked by NH_4Cl.