

Supplementary Materials for

Agonist-Driven Maturation and Plasma Membrane Insertion of Calcium-Sensing Receptors Dynamically Control Signal Amplitude

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- Fig. S1. Agonist-driven insertion of CaSRs but not AT1Rs.
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- Fig. S4. Endogenous CaSRs in HUVECs undergo ADIS.
- Fig. S5. CaSR interactions with 14-3-3 proteins are modulated by C-terminal mutations and encompass different 14-3-3 subtypes.

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencesignaling.org/cgi/content/full/4/200/ra78/DC1)

Movie S1 (.avi format). TIRFM of BTx-A594-labeled BSEP-CaSR stimulated with extracellular Ca²⁺.

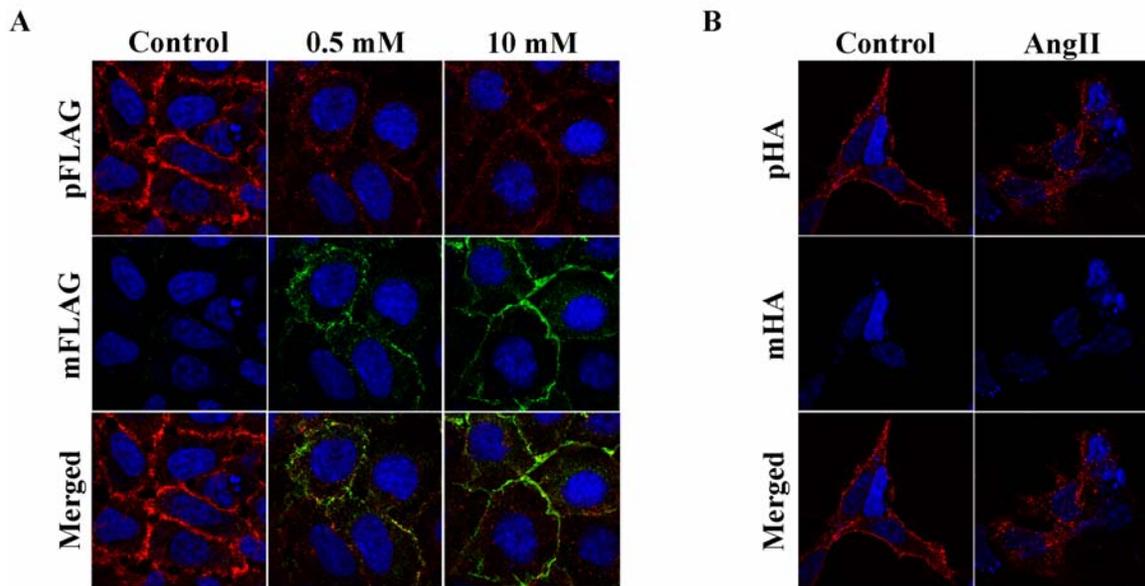


Figure S1. Agonist-driven insertion of CaSRs but not AT1Rs. (A) HEK293 cells stably expressing FLAG-CaSR were labeled with polyclonal anti-FLAG antibody (pFLAG) for 1 hour (4°C), then incubated in 0.5 or 10 mM Ca²⁺ bath for 10 min (37°C), fixed, and labeled with monoclonal anti-FLAG antibody (mFLAG) for 1 hour (room temperature). Cells were permeabilized and incubated with goat anti-rabbit Alexa568- and goat anti-mouse Alexa488-conjugated secondary antibodies. Incubation in 10 mM Ca²⁺ increased binding of monoclonal anti-FLAG antibody (mFLAG) to CaSR, demonstrating ADIS. Images shown are representative of *N* = 30 cells. (B) HEK293 cells transiently expressing human HA-tagged AT1R were labeled with polyclonal anti-HA antibody (pHA) for 1 hour (4°C), then incubated with 100 nM AngII for 15 min (37°C) followed by fixation and monoclonal anti-HA antibody (mHA) for 1 hr (RT). Cells were permeabilized and incubated with secondary antibodies as in (A). Agonist stimulation did not induce binding of the second antibody to AT1R. Images shown are representative of *N* = 10 cells. Images were collected on a Leica DM IRE2 confocal laser scanning microscope.

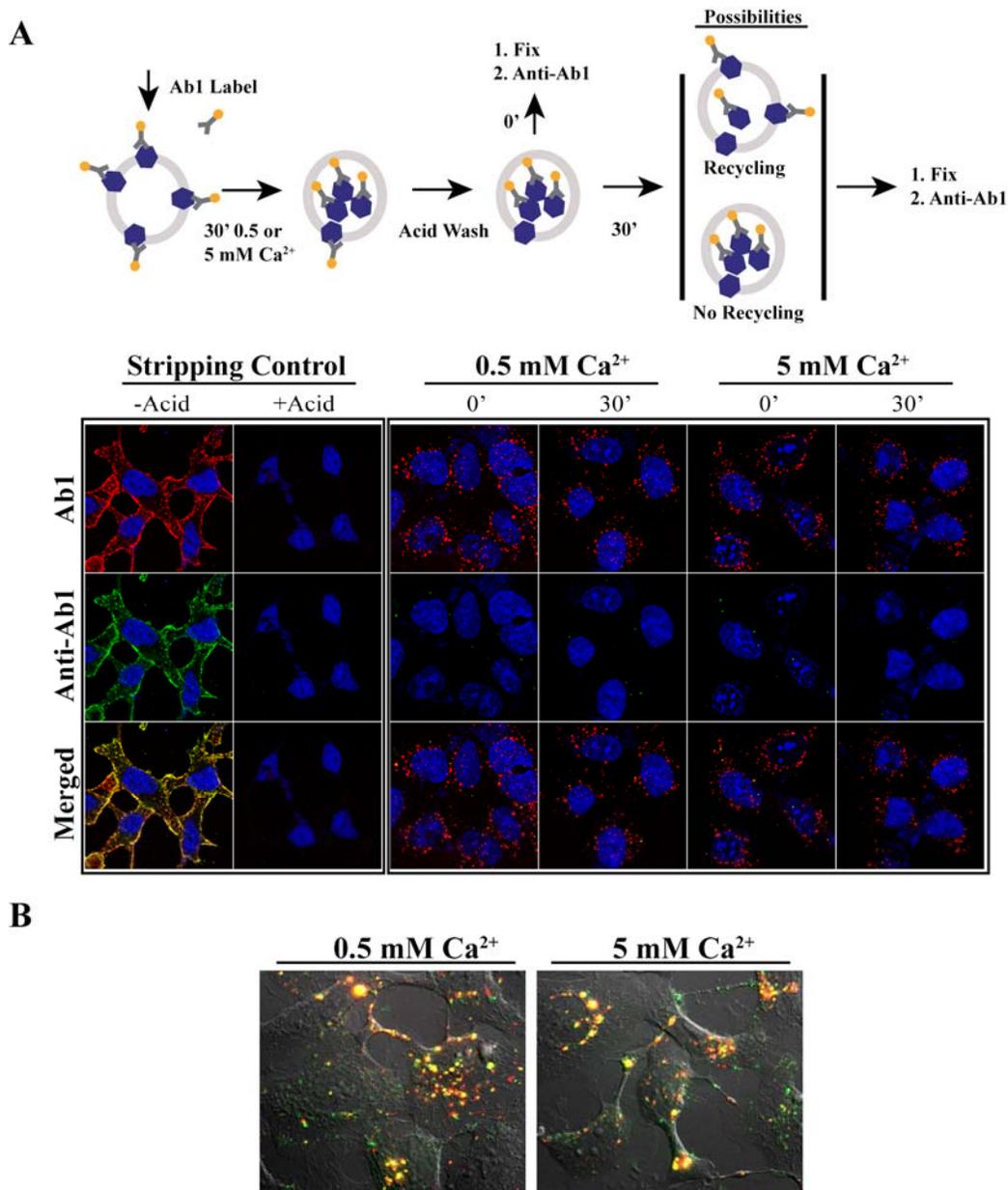


Figure S2. CaSRs do not recycle to the PM after stimulation but are targeted to the lysosome for degradation. (A) HEK293 cells stably expressing FLAG-CaSR were labeled with polyclonal anti-FLAG antibody (Ab1) for 1 hour in 0.5 mM Ca²⁺ bath (4°C) followed by goat anti-rabbit Alexa568-conjugated secondary antibody for 30 min (4°C). Cells were then incubated in 0.5 mM or 5 mM Ca²⁺ bath for 30 min (37°C) followed by an acid wash (2

min, pH 2.5). Cells were incubated for 0 or 30 min in 0.5 mM Ca^{2+} followed by fixation and donkey anti-goat Alexa488-conjugated secondary antibody (anti-Ab1). No detectable labeling by anti-Ab1 was observed for CaSR, indicating that anti-FLAG-bound CaSR did not recycle to the plasma membrane during the second incubation in 0.5 mM Ca^{2+} . Images were collected on a Leica DM IRE2 confocal laser scanning microscope. (B) HEK 293 cells stably expressing FLAG-CaSR were incubated with monoclonal anti-FLAG antibody conjugated to Alexa488 (green) and LysoTracker Red DND-99 (red) (Invitrogen, Carlsbad, CA) in 0.5 mM or 5 mM bath Ca^{2+} for 30 min (37°C) prior to live cell imaging on an Olympus IX81 spinning-disc confocal microscope using a 60x objective.

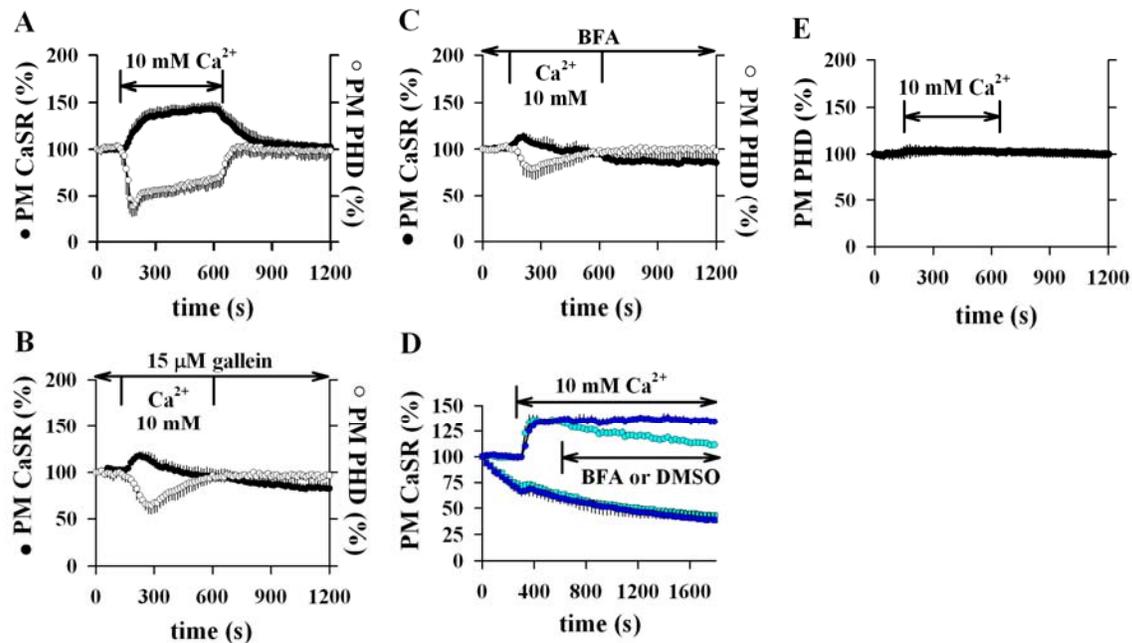


Figure S3. ADIS requires signaling by PM CaSR and release from secretory

compartments. (A) Stimulation of cells expressing BSEP-CaSR plus PHD results in a prolonged increase in steady state plasma membrane abundance of BSEP-CaSRs (black) accompanied by steady state PIP₂ (white) hydrolysis. Mean \pm S.D. $N = 8$ cells. (B) Cells expressing BSEP-CaSR plus PHD were preincubated with 15 μ M gallein (15 min) prior to initiation of experiment and throughout. 10 mM Ca²⁺ caused a transient increase in PM BSEP-CaSR (black) and translocation of PHD (white). Mean \pm S.D. $N = 6$ cells. (C) Cells expressing BSEP-CaSR and PHD were preincubated with 100 ng/ml BFA (30 min) and the experiment was performed in the continued presence of BFA. Addition of 10 mM Ca²⁺ induced a transient increase in net plasma membrane abundance of BSEP-CaSR (black) and a transient reduction in membrane PHD (white). Mean \pm S.D. $N = 5$ cells. (D) Cells labeled with BTx-A594 were perfused in 0.5 mM Ca²⁺ bath followed by 10 mM Ca²⁺ for 5 min. BFA (100 ng/ml) or DMSO were added in the continued presence of 10 mM Ca²⁺. Plotted are mean \pm S.D. for net BSEP-CaSR (circles) and endocytosis of BTx-A594 (squares) for cells treated with BFA (cyan) or

DMSO (blue). $N=5$ cells. **(E)** There was no PHD translocation in response to extracellular Ca^{2+} in the absence of CaSR. Cells were transfected with PHD and treated with 10 mM Ca^{2+} as indicated. Mean \pm S.D. $N=6$ cells.

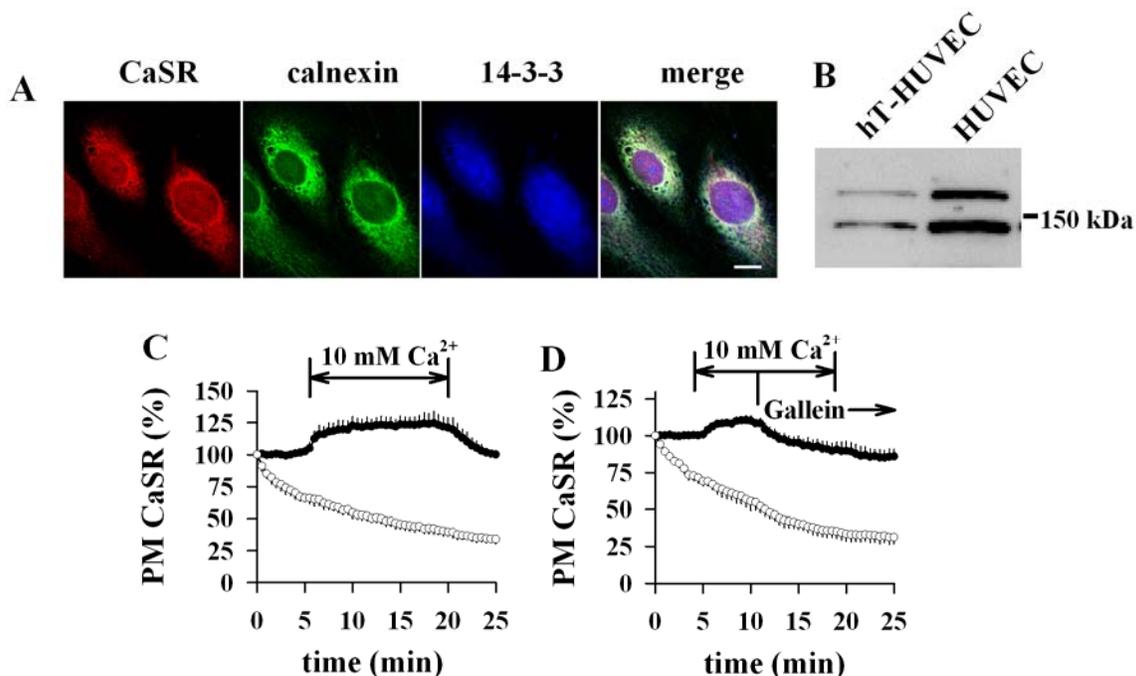


Figure S4. Endogenous CaSRs in HUVECs undergo ADIS. (A) Confocal images of HUVECs showing colocalization of endogenous CaSR (red), calnexin (green) and 14-3-3 proteins (blue). Scale bar, 10 μm . (B) HUVECs or hT-HUVECs were harvested from 5 T75 flasks, pelleted and lysed, spun at 10,000 g (10 min), and the supernatant was spun for 1 hour at 100,000g. Membrane pellets were resuspended in lysis buffer plus 1.5% Triton-X-100. 10 μg crude membrane protein was electrophoresed on 7.5% SDS gel and blot probed with anti-CaSR monoclonal antibody (Sigma). (C) HUVECs expressing human BSEP-CaSR were labeled with BTx-A594 (5 min at room temperature) then monitored by TIRFM. Cells were stimulated with 10 mM Ca^{2+} as indicated. Net plasma membrane abundance of BSEP-CaSR (black) and endocytosis of BTx-A594 (white) were plotted as mean \pm S.D. $N = 10$ cells. (D) HUVECs expressing human BSEP-CaSR labeled with BTx-A594 were exposed to 10 mM Ca^{2+} and gallein (15 μM) as indicated by arrows. Normalized net BSEP-CaSR (black) and BTx-A594 (white) were plotted as mean \pm S.D. $N = 8$ cells.

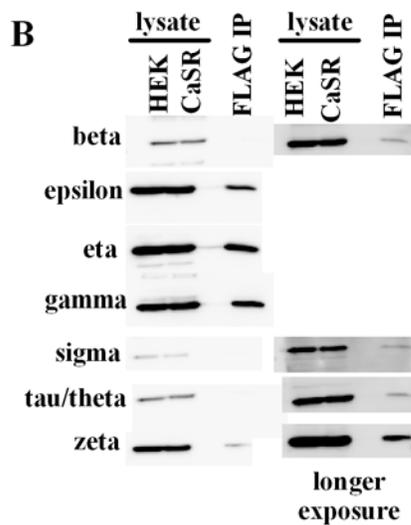
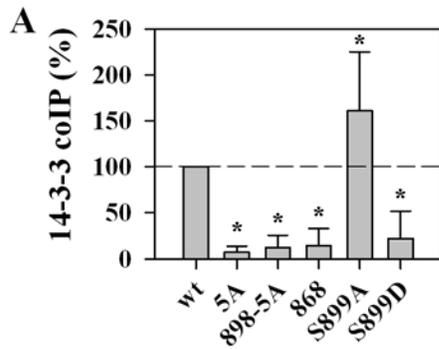


Figure S5. CaSR interactions with 14-3-3 proteins are modulated by C-terminal mutations and encompass different 14-3-3 subtypes. (A) HEK293 cells expressing the CaSR mutants were lysed and samples split for immunoprecipitation with either 14-3-3 or FLAG-specific antibody. Blots were probed with anti-CaSR antibody and bands were quantified. For each experiment, the 14-3-3 bands were normalized to the corresponding FLAG band, and then plotted as % of WT 14-3-3 coimmunoprecipitations, averaged over 4-5 independent transfections. *, $P < 0.05$ by ANOVA followed by post-hoc Dunnett's test. (B) HEK293 cells transfected with FLAG-CaSR were lysed and immunoprecipitated with anti-FLAG antibody. Blots of immunoprecipitates or lysates were probed with anti-14-3-3 polyclonal antibodies for specific isoforms.

Movie S1. TIRFM of BTx-A594-labeled BSEP-CaSR stimulated with extracellular Ca²⁺.

Live –cell TIRFM of a HEK293 cell expressing BSEP-CaSR labeled with BTx-A594 and exposed to 0.5 mM Ca²⁺ or 10 mM Ca²⁺ bath as indicated. Green fluorescence (SEP) shows net plasma membrane CaSR and red fluorescence indicates BTx-A594-bound BSEP-CaSR. SEP fluorescence increases upon addition of 10 mM Ca²⁺, whereas BTx-A594 fluorescence decreases throughout the experiment.