Supporting Information

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SI Materials and Methods

Gene Construction. Wild-type apo-aequorin gene was amplified by PCR using the following primers: forward 5′-ttggtaccGG-TAAACTTACATCAGACTTC-3′, (*KpnI* site is underlined); reversed 5′-ccgaattcTTAGGGGACAGCTCCACCG-3′ (*EcoRI* site is underlined). The product was digested with *KpnI* and *EcoRI* and cloned into a pcDNA3 vector cleaved with *KpnI* and *EcoRI*. Next, the GFPuv gene was amplified by PCR from the vector pBAD–GFPuv (Clontech) with the following primers: forward 5′-ccaaagcttGCCACCATGGCTAGCAAAGGA-3′ (*HindIII* site is underlined) and reversed 5′-ttggtaccCGGTTTGTAGAG-CTCAT-3′ (*KpnI* site is underlined) in which the final Stop codon was removed and fused in frame with apoaequorin in the *KpnI* site. Finally, complementary oligonucleotides 5′-AGCGACCCCAG-CAACCACGCCGACCACTGCACCGACCGCGGGTAC-3′ and 5′-CCGCGGTCGGTGCAGTGGTCGGCGTGGTTGCTGGG-GTCGCTGTAC-3′ which codify for the TATPATTPTTAPT-AGT linker were inserted at the *KpnI* site between GFP and aequorin. All mutations were introduced into GAP (GFP-Aequorin protein) by site-directed mutagenesis using the QuikChange Site-directed mutagenesis kit (Stratagene).

To express the genes in bacteria, GAP and GAP1 were amplified by PCR and cloned in frame into the *EcoRI* site of the pET28A plasmid. The different organelle-targeted GAP constructs were generated by in-frame fusion of the *HindIII–HindIII* fragments of either the *Xenopus* nucleoplasmin (nucleus), luciferase (cytosol), or the 34 first residues of cytochrome *c* oxidase VIII (mitochondria) to the 5′-end of GAP cDNA (1). GAP was targeted to ER by adding the calreticulin signal peptide and the sequence encoding the ER retention KDEL to the 5′- and 3′-end of GAP, respectively (2). Targeting to the Golgi apparatus was made by fusing the 5′-end of GAP1 to the N-terminal 81 amino acids of the galactosyltransferase (3). erGAP1 was subcloned into the pCAG-GS vector with a CAG promoter (CMV enhancer, β-actin promoter) and regulatory elements from the woodchuck hepatitis virus (WPRE) for making transgenic mice. The integrity of all constructs was verified by sequencing.

Protein Purification and Ca^{2+} Calibration. The plasmids pET28A–GAP or pET28A–GAP1 were transformed in *Escherichia coli* BL21 (Stratagene). For protein expression, bacteria were grown at 37° to A_{600} of >0.6 in LB containing 40 mg/L kanamycin, then induced with 0.5 mM isopropyl β-D-thiogalactoside for 6 h at 25°. The cells were then pelleted by centrifugation and sonicated in a buffer containing (in mM): NaCl, 250; EDTA, 5; PMSF, 0.5; DTT, 2; Tris–HCl, 50, pH 8.8. The bacterial lysate was centrifuged at 30,000 g for 15 min. Most of the protein was present in the inclusion bodies as an insoluble material which could be solubilized in 8 M urea, 5 mM DTT, 50 mM Tris–HCl, pH 8.8, by rocking at 4° overnight. The insoluble protein was removed by centrifugation at 30,000 × *g* for 15 min, and the supernatant was subjected to dialysis against 50 mM Tris–HCl buffer, pH 8.8 for 8 h. Then, 5 mM DTT was added. The protein was further purified with Ni^{2+}–Sepharose beads (GE Healthcare) following the manufacturer's protocol and eluted with 500 mM imidazol.

Fluorescence spectra were measured without Ca^{2+} (0.1 mM EGTA) and with excess (1 mM) Ca^{2+} in a solution containing 140 mM KCl, 1 mM $MgCl_2$, and 20 mM MOPS/Tris, pH 7.2.

Calcium calibrations of GAP were performed using a Tecan Genios Pro Basic 96-well plate reader. GAP protein (3 μg) was added to each well containing 200 μL of 140 mM KCl, 1 mM $MgCl_2$, and 20 mM MOPS/Tris, pH 7.2 and various buffered Ca^{2+} concentrations (4, 5) (see figure legends for details) and either X-Rhod-5F (K_d = 1,6 μM) or Rhod FF (K_d = 17 μM). Traces of Ca^{2+} in the solutions were removed by pretreatment with Chelex resin (Bio-Rad; before Mg^{2+} addition) followed by Calcium Sponge (Molecular Probes). GAP fluorescence was measured in triplicates using the 390 and 485 excitation filters and 535-nm emission filter, and the values were used to calculate the F_{485}/F_{390} ratio (very similar to the F_{470}/F_{403} measured in the spectrofluorimeter). The Rhod fluorescence was measured simultaneously in the red channel (Ex: 535; Em: 590). Titration curves were obtained by fitting the data to a sigmoidal curve.

Cell Culture and Expression in Mammalian Cells. HeLa and HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin. For imaging experiments, 3×10^4 cells were seeded on poly-L-lysine–coated 12-mm–diameter coverslips and transfected with 0.1–0.5 μg of the adequate pcDNA3 GAP variant using lipofectamine 2000 (Invitrogen). For mitochondria colocalization analysis, mitGAP was cotransfected together with pDsRed2–mito (Clontech) (1:1 mix of DNA with the GAP plasmid). Stable HeLa clones expressing erGAP1 or goGAP1 were obtained by electroporation or lipofectamine transfection, respectively. In the case of goGAP1, the population of GFP-positive cells was enriched by several rounds of cell sorting. Single-cell clones were selected in culture medium containing 0.8 mg/mL G-418 by limited dilution and maintained in 0.1 mg/mL G-418.

Immunofluorescence. SERCA2 immunofluorescence was performed by using a monoclonal anti-Serca IID8 antibody (1:200; Santa Cruz). Mouse motor neurons were identified by staining with a monoclonal anti–SMI-32 antibody (1:200, Abcam). Trans-Golgi network (TGN) was selectively stained with an anti-TGN64 antibody (1:200, AbD Serotec). As secondary antibodies, Alexa Fluor 568-conjugated anti-mouse and anti-sheep antibodies were used. Nuclei were stained with DAPI. For ER colocalization studies, living HeLa cells were incubated with 400 nM ER-Tracker (Molecular Probes) for 30 min in culture medium at 37 °C, and then fixed with 4% paraformaldehyde. Cells were mounted with Vectashield^R (Vector) and observed with an Achroplan 20× water-immersion objective, an EC–Plan–Neofluor 40×, or a C-Apochromat 63× water-immersion objective (NA, 1.20) in a Zeiss Axioplan Z microscope. Green (Ex, 470/40; Em, 540/50 nm), red (Ex, 560/40; Em, 605/50 nm), or blue (Ex, 390/22 nm; Em, 460/50 nm) fluorescences were imaged in fixed cells. The Zeiss ApoTome system was used for optical sectioning.

Primary Neuronal Cultures. Animal procedures were approved by the Valladolid Medical School Animal Ethics Committee. Dorsal root ganglion (DRG) neurons were isolated from neonatal (P0–P5) mice as described previously (6). Briefly, ganglia were removed in Ca^{2+}/Mg^{2+}-free HBSS at 4 °C and digested with trypsin–EDTA (0.25%, Gibco) and DNase (0.1 mg/mL) for 20 min at 37 °C. Neurons were dissociated from digested ganglia by manual trituration and resuspended in Neurobasal-A medium (Invitrogen) supplemented with 1% B-27 (Invitrogen), 0.5 mM glutamax, 50 ng/mL NGF, 100 μg/mL streptomycin, and 100 U/mL penicillin and plated on 12-mm–diameter glass coverslips coated with laminin (10 μg/mL) and poly-D-Lysine (100 μg/mL) at a density of 30,000 cells/coverslip. The experiments were performed after 6–8 d in vitro.

Motor neurons cultures were prepared from E12–13 mice embryos as described previously (7). Briefly, cells were dissoci-

ated mechanically from the ventral horns of the spinal cords after trypsin (0.025%) treatment. The largest cells were isolated by iodixanol density gradient centrifugation. The 3×10^4 motor neurons were plated onto poly-ornithine-laminin–coated 12-mm glass coverslips in Neurobasal-A medium supplemented with 2% horse serum, 1% B-27, 0.05 mM L-glutamine, 25 μM β-mercaptoethanol, and neurotrophic factors [1 ng/mL BDNF, 10 ng/mL glial cell-derived neurotrophic factor (GDNF), 10 ng/mL hepatocyte growth factor (HGF), 10 ng/mL cardiotrophin-1 (CT-1), and 10 ng/mL ciliary neurotrophic factor (CNTF)].

Hippocampal neurons were isolated from P0-3 mice. Hippocampi were dissected in cold HBSS and digested with papain (0.5 mg/mL) and DNase (0.04 mg/mL) dissolved in a Ca^{2+}- and Mg^{2+}-free HBSS containing 1 mg/mL BSA and 10 mM glucose at 37 °C for 30 min. The papain solution was replaced with 1 mL of Neurobasal-A medium supplemented with 2% B-27, 2 mM glutamax, 100 μg/mL streptomycin, and 100 U/mL penicillin, and 10% FBS. Cells were seeded onto poly-DL-lysine–coated 12-mm–diameter glass coverslips (30,000 cells/coverslip) at a final density of 1000 cells/mm^2 in the center of the coverslip. The cells were maintained in the medium described above containing 2.5% FBS for 1 wk before recordings.

Virus Production. erGAP1 cDNA was cleaved from pcDNA3 as a HindIII/EcoRI fragment and cloned into the Herpes Simplex Virus pHSVpUC plasmid. Packaging and titration were performed as earlier reported (1, 4). The $3–5 \times 10^4$ neurons (DRG, hippocampal, or motor neurons) were infected with a multiplicity of infection (moi) ranging between 0.01 and 0.1. Neurons were used for imaging 24–48 h later.

Ca^{2+} Imaging. Fluorescence was imaged on a Nikon Diaphot inverted microscope using a 20× PlanApoUV (NA, 0.7) objective (Olympus). Test solutions were applied by perfusion at 2–3 mL/min. GAP was monitored using the two excitation filters, 403/12 DF and 470/25 DF and a 400-nM dichroic mirror. Cells were alternately epi-illuminated at 403 and 470 nm, and light emitted above 520 nm was recorded using a Hamamatsu C4742-98 camera handled by the Simple PCI 6.6 Hamamatsu software. For imaging GAP in combination with fura-2 ($[Ca^{2+}]_C$) the cells were first incubated with fura-2-acetoxymethyl ester (2–4 μM, Molecular Probes) for 60 min at 22 °C. Then, cells were excited sequentially with 340/10, 380/10, and 470/25 excitation filters, and fluorescence was read at >520 nm, as above. At 380 nm the interference of GAP signal with fura-2 fluorescence was estimated to be less than 10%, and fura-2 did not interfere with the GAP fluorescence excited at 470 nm. Output was background-substracted and ratioed pixel-to-pixel using Image J software. The ratio F_{340}/F_{380} was used as an index of $[Ca^{2+}]_C$ and F_{470} as an index of $[Ca^{2+}]_{ER}$.

The in situ calibration of GAP1 was performed in the erGAP1–HeLa clone permeabilized by a 1-min treatment with 60 μM digitonin. The following ionophores were added to the internal medium used for calibration to assure complete collapse of ionic gradients: 10 μM nigericin, 20 μM monensin, 10 μM 4-BrA23187, 1 μM gramicidine, and 2 μM CCCP. Internal-like medium had the following composition (in mM): MOPS–Na, 20; KCl, 140; $MgCl_2$, 1; pH 7.2. To avoid calcium contaminations, the medium was first run through a Chelex column to remove divalent cations, and then, after addition of Mg^{2+}, through a calcium sponge column (Molecular Probes) to remove calcium traces. The final medium contained 1 mM $MgCl_2$ and either 0.1 mM EGTA (Ca^{2+}-free medium), nominally Ca^{2+}-free internal medium or solutions containing increasing Ca^{2+} concentrations (5–1000 μM). Ca^{2+} concentrations in the range 1–100 μM were made by blending titrated solutions containing hydroxyethylenediaminetriacetic acid (HEDTA) and $CaCl_2$ in the required amounts, calculated using the program MaxChelator (5). Great care was taken to maintain constant pH and $[Mg^{2+}]$.

Experiments to compare D1ER and erGAP1 were performed in lipofectamine-transfected HeLa cells or hippocampal neurons. Fluorescence was imaged on a Zeiss Axiovert 200M inverted microscope using a 20× Plan-Apochromat Zeiss objective (NA, 0.8). D1ER was excited at 436 nm reflected by a 450-nm dichroic mirror, and emission was recorded at 470 and 520 nm. erGAP1 was excited at 405 and 485 nm (530-nm emission).

Generation of Transgenic Mice. The erGAP1 fragment containing the calreticulin/KDEL signal peptides and the mutations D117A, D119A, and D163A was cloned into the pCAG-GS vector (CMV enhancer, β-actin promoter, and regulatory element from the WPRE), obtained from L. Looger (Howard Hughes Medical Institute, Ashburn, VA). The 5-kb transgene was excised with *SspI/BsaBI*, gel-purified, and injected into B6CBAF2 oocytes using standard techniques. Thirty mice were screened for GAP sequences by routine PCR of genomic DNA obtained from tail biopsies using the primers #50 (forward, 5′-GATGGATCCG-TTCAACTAGCAGACC-3′) and #201 (reverse, 5′- GTACCCG-CGGTCGGTGCAGTGGTC-3′). The specific PCR product had 255 bp. We found 12 transgenic founders for which we analyzed the copy number of the transgene by quantitative-PCR (Q-PCR) with the same primers as for routine PCR, using 20 ng of mouse genomic DNA and SYBRGreen. As housekeeping gene we used the mGAPDH, whose 274-pb product was amplified with the following primers: forward: 5′-TCCTGCACCACCAACTGCTT; reversed: 5′-GTGGCAGTGATGGCATGGAC. We selected transgenic founders with high copy numbers of the transgene and crossed them with wild-type C57BL6 mice. Among them, we focused on three lines (L11, L20, L30) with erGAP1 expression in neural tissues, and all of the experiments were done in these lines.

Histology. Animals were anesthetized and fixed by intracardiac perfusion with 4% paraformaldehyde at 4 °C. Tissues were cryoprotected in 30% sucrose overnight, embedded in Tissue-Tek optimum cutting temperature (O.C.T) (Sakura) before cutting them in 30-μm sections using a microtome (Leica). Images were taken with a Nikon Eclipse 80i microscope using Nikon PlanApo 4×0.2 and 20×0.75 objectives with a 450–490 excitation filter.

Calcium Imaging of Hippocampal Slices. Acute hippocampal slices were prepared (8) from 2-to-4-wk–old transgenic mice (lines L11, L20, or L30). The hippocampus was dissected out along with the cortex and sliced into 350–400-μm–thick sections with a McILwain Tissue Chopper. Slices were quickly transferred to a fine-meshed membrane filter and maintained in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 125; KCl, 2.5; $MgCl_2$, 1; NaH_2PO_4, 1.25; $NaHCO_3$, 26; $CaCl_2$, 1; glucose, 10; 7.4 pH, continuously bubbled with a 95% O_2/5% CO_2 gas mixture at 25 °C. Slices were mounted onto the stage of a Zeiss axioplan upright microscope equipped with a 20× objective (W-Achroplan, Zeiss; NA = 0.5) or 63× (W-Plan-Apochromat, Zeiss; NA = 1) and a Zeiss AxioCam camera MRm (12 bit) connected through a software interface (Axiovision, Zeiss) to a Xenon fluorescent excitation source and a filter wheel. erGAP1 calcium probe was excited at 405/470 nm, and the emission fluorescence was acquired at 535 nm. All of the experiments were performed in ACSF at pH 7.4, in a custom-made chamber of 42-μL volume under constant perfusion (3 mL/min).

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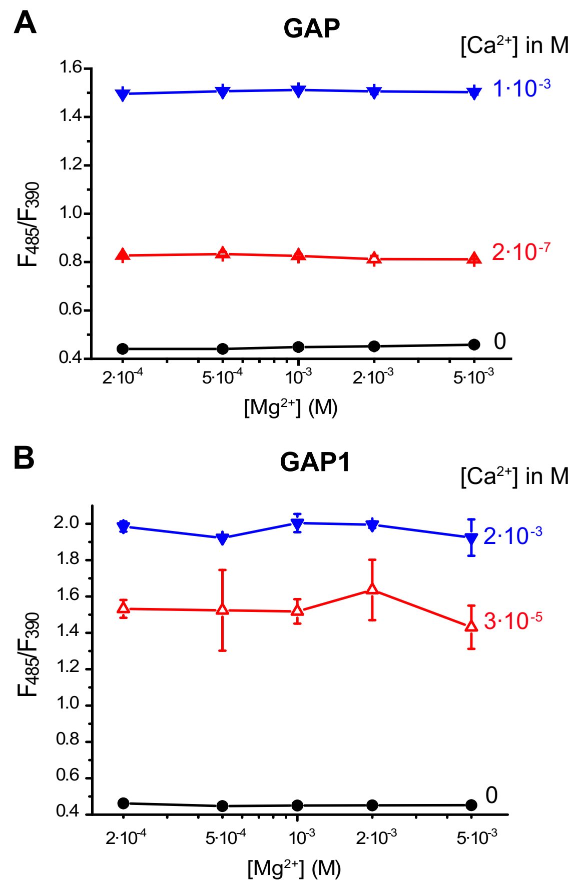

Fig. S1. GAP is not sensitive to Mg^{2+}. Data are given as ratio of fluorescences excited at 485 and 390 nm, measured in a fluorescence plate reader with 3 μg protein/well of GAP (*A*) or GAP1 (*B*). All of the measurements were performed at 25 °C in medium containing 140 mM KCl and 20 mM MOPS–Tris, 7.2 pH. $[Mg^{2+}]$ was adjusted to 0.2, 0.5, 1, 2, or 5 mM by adding $MgCl_2$. $[Ca^{2+}] = 0$ was made by adding 0.2 mM EGTA; $[Ca^{2+}] = 2{\cdot}10^{-7}$ M was made with a EGTA/Ca^{2+} buffer; $[Ca^{2+}] = 1$ or 2×10^{-3} M solutions were made by adding $CaCl_2$; $[Ca^{2+}] = 3 \times 10^{-5}$ M was the estimated contaminant $[Ca^{2+}]$ in a nominally Ca^{2+}-free solution . Each point represents the mean ± SD of three measurements.

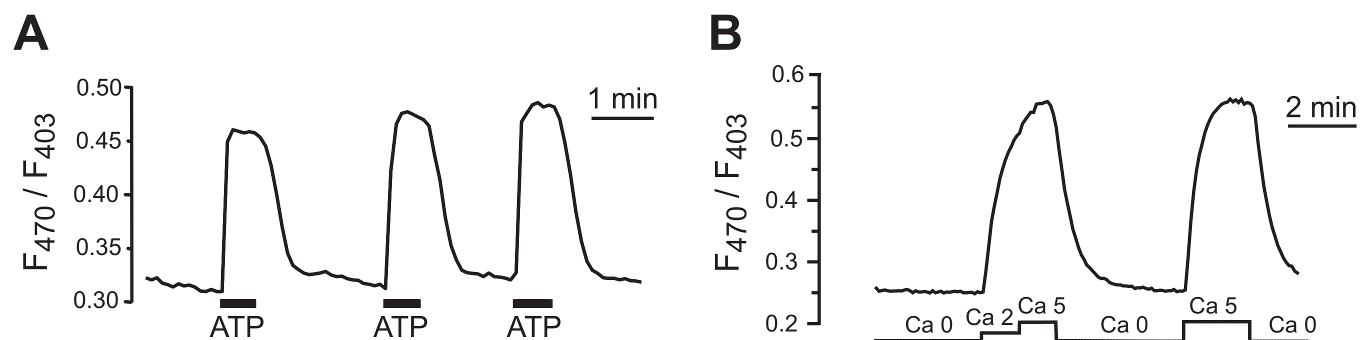

Fig. S2. Intracellular Ca^{2+} dynamics in GAP-transfected HEK 293 cells. (*A*) Cells were maximally stimulated with ATP + carbachol (100 μM each; labeled as ATP). The trace is the average of nine cells. (*B*) Capacitative calcium entry [store-operated Ca^{2+} entry (SOCE)]. Cells were previously treated with thapsigargin (1 μM) in 0.1 mM EGTA for 10 min to empty the ER calcium stores. At the times shown, cells were perfused with external solutions containing either no Ca^{2+} (0.1 mM EGTA; Ca0), 2 mM (Ca2), or 5 mM Ca^{2+} (Ca5). The trace is the average of 27 cells.

Fig. S3. Design and calibration of low-affinity GAP (GAP1). (*A*) Scheme of mutations in the EF hands within the aequorin moiety of GAP. The screening of the mutants was directly performed in transfected HeLa cells through a functional assay comparing the ratio decrease challenged by ATP plus carbachol (100 μM each). In general, the substitutions in the EF1-hand site produced more dramatic effects than those in the other two sites, with either very noisy signals (D24A) or loss of fluorescence (D24A + D119A). By contrast, the substitutions in the EF3 hand led to milder effects. (*B*) Typical in situ Ca^{2+} calibration in erGAP1 stably expressing HeLa cells. The trace shown is the average of seven cells present in the same microscopic field. Cells were permeabilized with 60 μM digitonin (DIG) and incubated with either HEDTA/calcium buffers or with no calcium buffers. Further details can be found in *SI Materials and Methods*. (*C*) Ca^{2+} calibration curve of erGAP1 at pH 7.2 obtained in several experiments performed as in *B*. Each data point represents the mean ± SEM of three to nine measurements obtained in three to six different experiments. Data obtained with and without Ca^{2+} buffers were averaged together and expressed as Normalized Ratio (F_{470}/F_{403}), computed in each experiment as: $(R - R_{min})/(R_{max} - R_{min})$. The line represents the best-fitting curve using the equation $R = (V_{max} \times [Ca^{2+}])/(K_m + [Ca^{2+}])$ where $V_{max} = 1$ and $K_m = 12 \times 10^{-6}$ M. When the Hill equation was used, a similar fitting of the curve was obtained ($n = 0.86 \pm 0.24$).

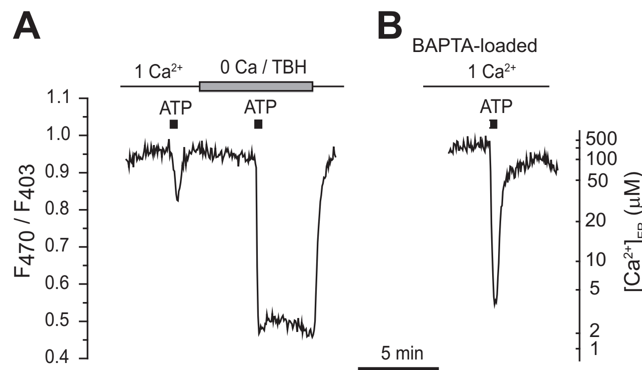

Fig. S4. $[Ca^{2+}]_{ER}$ transients in erGAP1-expressing HeLa cells. (*A*) Representative $[Ca^{2+}]_{ER}$ transients (average of 61 cells) in erGAP1-expressing HeLa cells stimulated with ATP + carbachol (100 μM each; labeled as ATP) in 1 mM Ca^{2+} or in 0.5 mM EGTA plus 10 μM tert-butyl-hydroquinone (labeled as 0 Ca/TBH). (*B*) $[Ca^{2+}]_{ER}$ transient (average of 60 cells) in HeLa cells preincubated with bis-(alpha-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA/AM) (10 μM) for 60 min.

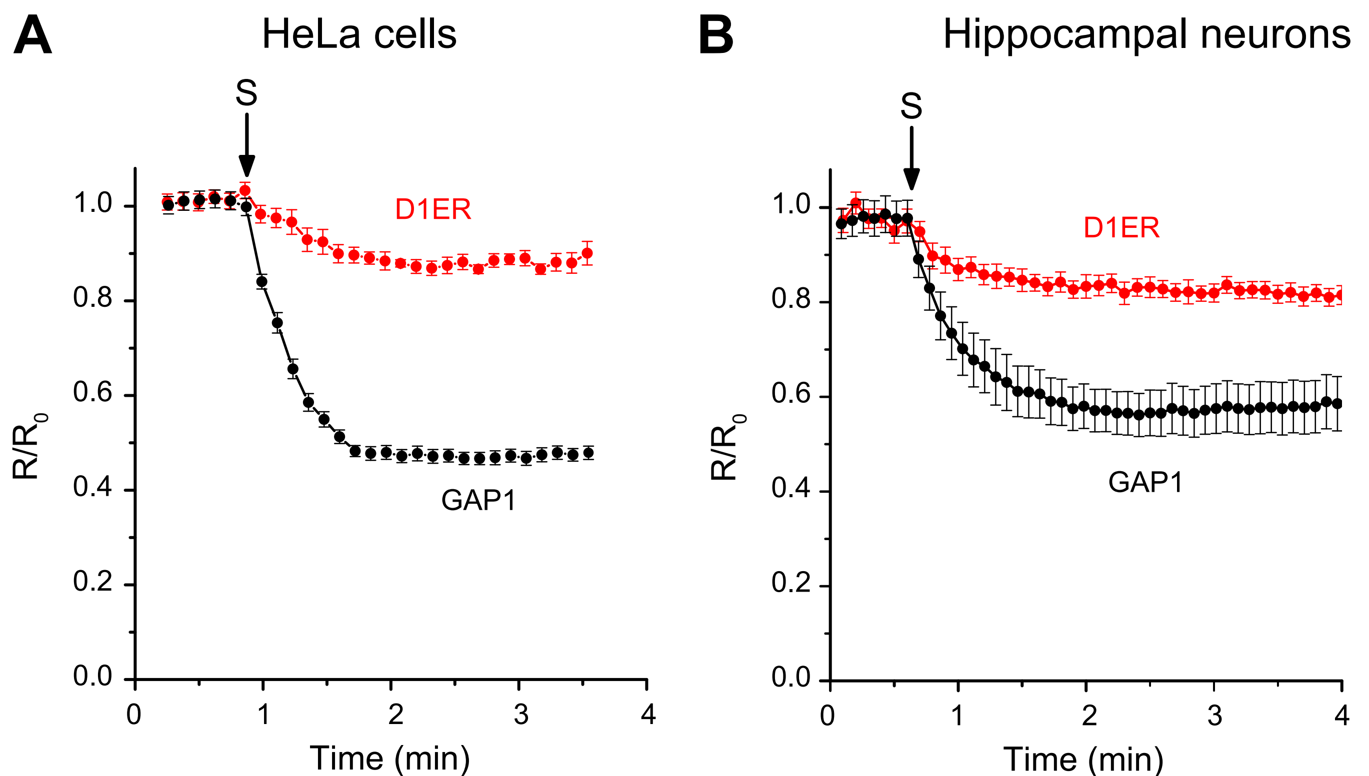

Fig. S5. Comparison between erGAP1 and D1ER. (*A*) Lipofectamine-transfected HeLa cells with pcDNA–D1ER (1 μg/well) or stably erGAP1-expressing HeLa cells were stimulated with Histamine + ATP (100 μM each), labeled as "S" (stimulus). Each point is the mean ± SD (*n* = 7 for D1ER; *n* = 28 for erGAP1). Only the responding cells are included. (*B*) Dissociated hippocampal neurons were transfected with 1–2 μg of D1ER or erGAP1, 2 d before measurements. Stimulus (S) was acetylcholine (1mM) + ATP (100 μM) + TBH (10 μM). Traces show the mean ± SD of eight cells for each sensor.

Fig. S6. GAP1 fluorescence is weakly sensitive to pH. Data given as ratio of fluorescences excited at 485 and 390 nm, measured in the fluorescence plate reader (see *SI Materials and Methods*). All measurements were performed with GAP1 (3 μg) at 25 °C in a solution containing 140 mM KCl and 20 mM MOPS–Tris in the absence of Ca^{2+} (0.1 mM EGTA + 0.8 mM $MgCl_2$) or at different Ca^{2+} concentrations: 3×10^{-6} M (1 mM EDTA + 0.21 mM $CaCl_2$ + 1.6 mM $MgCl_2$) and 1×10^{-3} M (1 mM $CaCl_2$ + 0.8 mM $MgCl_2$) and three different pHs (6.2, 7.2 and 8.2). Note that free Mg^{2+} concentration was 0.8 mM in all cases. Each point represents the mean ± SD of three measurements.

Fig. S7. Ca^{2+} dynamics in the Golgi apparatus recorded by goGAP1. (*A*) Colocalization of transfected goGAP1 (green fluorescence) and TGN46 (red fluorescence), a marker of TGN. Nuclei were stained with DAPI (blue fluorescence). (*B*) Response to stimulation with ATP + histamine (100 μM each) in a representative goGAP1-expressing HeLa cell. Medium contained 1 mM Ca^{2+} and 10 μM TBH as indicated. (*C*) F_{470}/F_{403} ratio image of the microscope field taken at rest and during stimulation. Note that most of the cells responded to the stimuli with a decrease of ratio.

Fig. S8. ER calcium transients induced by caffeine in DRG and spinal cord motor neurons isolated from erGAP1 transgenic mice. (*A*) A representative DRG neuron isolated from neonatal transgenic mice (L11) expressing erGAP1 and cultured for 4 d in vitro challenged with 50 mM caffeine. (*B*) Individual (in colors) and average (in black) traces from 10 spinal cord motor neurons isolated from transgenic E13 embryos (L30) and cultured for 4 d in vitro challenged with 25 mM caffeine.

Movie S1. Real-time monitoring of $[Ca^{2+}]_{ER}$ in DRG neurons using GAP. ER Ca^{2+} dynamics in erGAP1 virally transduced DRG neurons. Stimulation with caffeine (20 mM) either in 1 mM $CaCl_2$ or in 0.2 mM EGTA together with TBH, 10 μM. ER Ca^{2+} is monitorized as the Ratio F_{470}/F_{403}, codified in pseudocolor. Trace is the average of the seven neurons present in the field.

Movie S1

Movie S2. Real-time monitoring of $[Ca^{2+}]_{ER}$ in hippocampal neurons using GAP. A representative hippocampal neuron dissociated from neonatal erGAP1 transgenic mice was stimulated with glutamate (20 μM) or caffeine (20 and 50 mM). ER Ca^{2+} is represented as Ratio F_{470}/F_{403} codified in pseudocolor.

Movie S2