

# Interplay of the $\text{Ca}^{2+}$ -binding Protein DREAM with Presenilin in Neuronal $\text{Ca}^{2+}$ Signaling\*

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The  $\text{Ca}^{2+}$ -binding protein DREAM regulates gene transcription and Kv potassium channels in neurons but has also been claimed to interact with presenilins, which are involved in the generation of  $\beta$ -amyloid and in the regulation of the  $\text{Ca}^{2+}$  content in the endoplasmic reticulum. The role of DREAM in  $\text{Ca}^{2+}$  homeostasis was thus explored in SH-SY5Y cells stably or transiently overexpressing DREAM or a  $\text{Ca}^{2+}$ -insensitive mutant of it. The overexpression of DREAM had transcriptional and post-transcriptional effects. Endoplasmic reticulum  $\text{Ca}^{2+}$  and capacitative  $\text{Ca}^{2+}$  influx were reduced in stably expressing cells. The previously shown down-regulation of  $\text{Na}^+/\text{Ca}^{2+}$  exchanger 3 expression was confirmed; it could cause a local increase of sub-plasma membrane  $\text{Ca}^{2+}$  and thus inhibit capacitative  $\text{Ca}^{2+}$  influx. DREAM up-regulated the expression of the inositol 1,4,5-trisphosphate receptor and could thus increase the unstimulated release of  $\text{Ca}^{2+}$  through it. The transient coexpression of DREAM and presenilin potentiated the decrease of endoplasmic reticulum  $\text{Ca}^{2+}$  observed in presenilin-overexpressing cells. This could be due to a direct effect of DREAM on presenilin as the two proteins interacted in a  $\text{Ca}^{2+}$ -independent fashion.

DREAM was originally identified as calsenilin, a  $\text{Ca}^{2+}$ -binding protein belonging to the family of neuronal calcium sensor proteins (1). Shortly thereafter, it was found to be identical to the  $\text{Ca}^{2+}$ -dependent gene silencer DREAM (downstream regulatory element antagonist modulator) (2) and to one of the interacting proteins (KChIPs) of the voltage-gated Kv channels, KChIP3 (3). The three proteins are the products of a single gene, their function being specified by their cellular location. They contain four EF-hands, of which at least three are operational. As all neuronal calcium sensors, they process the  $\text{Ca}^{2+}$  signal by undergoing conformational changes upon  $\text{Ca}^{2+}$  binding and upon interacting with target proteins (4). The targets of the protein in the cytoplasm have been claimed to be the endoplasmic reticulum (ER)<sup>2</sup> proteins presenilins (PSs) (1), which

are related to familial Alzheimer disease (5). Since the three names above have been used to refer to the same protein, hereafter we will only use DREAM.

Only few reports have explored the possible roles of DREAM in the regulation of neuronal  $\text{Ca}^{2+}$  signals. In the cytoplasm, DREAM has been claimed to counteract the potentially pathogenic effects of mutated PSs (6), which has been proposed to be due to the potentiation of the inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ )-mediated  $\text{Ca}^{2+}$  release from the ER (7, 8). DREAM was also claimed to increase the ER  $\text{Ca}^{2+}$  content in neuroglioma cells (9); however, the increase had only been inferred from the larger amount of  $\text{Ca}^{2+}$  that appeared in the cytoplasm by exposing DREAM-expressing cells to thapsigargin.

The effect of DREAM on neuronal  $\text{Ca}^{2+}$  could have important implications, given the recent findings of increased DREAM expression in brain samples of Alzheimer disease patients and in neuronal cultures exposed to the amyloid  $\beta$  peptide A $\beta$ 42 (10). It could be related to previous findings showing that the protein contributed to the production of the A $\beta$ 42 peptide and increased neuronal susceptibility to  $\text{Ca}^{2+}$ -dependent apoptosis (11). However, DREAM has also been associated with the antiapoptotic function of interleukin-3-dependent hematopoietic progenitor cells (12).

In this study, the effect of DREAM on  $\text{Ca}^{2+}$  signaling has been investigated in a human neuroblastoma cell line (SH-SY5Y) stably or transiently expressing wild type (WT) DREAM or a  $\text{Ca}^{2+}$ -insensitive mutated version of it (EFmDREAM), which silences DREAM-sensitive genes permanently. DREAM has been coexpressed together with PSs, and  $\text{Ca}^{2+}$  signaling has been compared with that in cells only overexpressing DREAM.  $\text{Ca}^{2+}$  was monitored in the cytosolic compartment (13), in the lumen of the ER (14), and in the space beneath the plasma membrane (15).

The results have revealed a pleiotropic role of DREAM on the homeostasis of  $\text{Ca}^{2+}$ . As previously found (16), DREAM reduced the expression of one of the major neuronal plasma membrane  $\text{Ca}^{2+}$  extrusion systems, NCX3 ( $\text{Na}^+/\text{Ca}^{2+}$  exchanger 3), thus elevating  $\text{Ca}^{2+}$  in the sub-plasma membrane space and inhibiting capacitative  $\text{Ca}^{2+}$  influx. As a consequence, the refilling of the ER stores with  $\text{Ca}^{2+}$  was also inhibited.

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<sup>2</sup> The abbreviations used are: ER, endoplasmic reticulum; PS, presenilin;  $\text{InsP}_3$ , inositol 1,4,5-trisphosphate; AEQ, aequorin; PBS, phosphate-buffered

saline; erAEQ, endoplasmic reticulum-targeted AEQ; pmAEQ, plasma membrane-targeted AEQ; KRB, Krebs-Ringer buffer; tBuBHQ, 2,5-di-tert-butylhydroquinone; cytAEQ, cytosolic AEQ; BK, bradykinin; CE, 5-(and 6-)carboxyeosin diacetate succinimidyl ester; GST, glutathione S-transferase; RT, reverse transcription;  $\text{InsP}_3\text{R}$ ,  $\text{InsP}_3$  receptor; CTF, C-terminal fragment;  $[\text{Ca}^{2+}]_{\text{er}}$ , endoplasmic reticulum  $[\text{Ca}^{2+}]$ ;  $[\text{Ca}^{2+}]_{\text{c}}$ , cytosolic  $[\text{Ca}^{2+}]$ ; WT, wild type.

ited. In addition, DREAM up-regulated the  $\text{InsP}_3\text{R}$  transcript levels, possibly increasing the  $\text{Ca}^{2+}$  leak through the receptor. These effects were transcriptional. However, DREAM also had post-transcriptional effects. It interacted directly with PS and potentiated the PS-promoted efflux of  $\text{Ca}^{2+}$  from the ER in transient coexpression experiments. The PS/DREAM interaction was  $\text{Ca}^{2+}$ -independent.

## EXPERIMENTAL PROCEDURES

**Cell Cultures and Transfection**—SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, in 75-cm<sup>2</sup> flasks; before transfection, they were seeded onto 13-mm glass coverslips and allowed to grow to 80% confluence. Transfection with 0.7  $\mu\text{g}$  of plasmid DNA (or 0.5  $\mu\text{g}$  of each plasmid in cotransfections) was carried out using TransFectin Lipid Reagent (Bio-Rad) according to the manufacturer's instructions. Aequorin (AEQ) measurements were performed 36 h later. Cells plated for Western blotting were collected 24–36 h after transfection. Stable WT DREAM and EFmDREAM clones were generated by transfecting the mammalian expression plasmid pcDNA3 (Invitrogen) containing the WT or EFmDREAM cDNA and were selected in Dulbecco's modified Eagle's medium with 1 mM G418. The same plasmids were used for transient expressions. PS1/pEF6/V5-His-TOPO and PS2/pcDNA3 plasmids were used for PSs transient expression.

**Immunocytochemistry**—For immunofluorescence, SH-SY5Y cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS; 140 mM NaCl, 2 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4) for 20 min, washed three times with PBS, and then incubated for 10 min in PBS supplemented with 50 mM  $\text{NH}_4\text{Cl}$ . Membranes were permeabilized with a 5-min incubation with 0.1% Triton X-100 in PBS, followed by a 1-h wash with 1% gelatin (type IV, from calf skin) in PBS. The coverslip was processed for the DREAM staining with specific rabbit polyclonal antibody (sc-9142; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:100 dilution in PBS. Staining was carried out with AlexaFluor589 secondary antibody (1:100 dilution in PBS; Invitrogen). Fluorescence was analyzed with a Zeiss Axiovert microscope equipped with a 12-bit digital cooled camera (Micromax-1300Y; Princeton Instruments Inc., Trenton, NJ). Images were acquired using Metamorph software (Universal Imaging Corp., West Chester, PA).

**Aequorin Measurements**—ER  $\text{Ca}^{2+}$  content had to be drastically reduced before the reconstitution of functional low affinity recombinant targeted aequorin (erAEQ). To this end, the cells were incubated for 1 h at 4 °C in Krebs Ringer modified buffer (KRB; 125 mM NaCl, 5 mM KCl, 1 mM  $\text{Na}_3\text{PO}_4$ , 1 mM  $\text{MgSO}_4$ , 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37 °C), supplemented with 5  $\mu\text{M}$  coelenterazine n (Invitrogen), the SERCA pump inhibitor 2,5-di-*tert*-butylhydroquinone (tBuBHQ; 10  $\mu\text{M}$ ), and 600  $\mu\text{M}$  EGTA. After this incubation, the cells were washed extensively with KRB supplemented with 2% bovine serum albumin and 1 mM EGTA and transferred to the chamber of a purpose-built luminometer. Transfected cytosolic AEQ (cytAEQ) was reconstituted by incubating the cells for 3 h with 5  $\mu\text{M}$  coelenterazine WT (Invitrogen) in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum at 37 °C

in a 5%  $\text{CO}_2$  atmosphere. Low affinity plasma membrane-targeted AEQ (pmAEQ) was reconstituted by incubating the cells for 1–2 h with coelenterazine WT in KRB supplemented with 100  $\mu\text{M}$  EGTA. The procedure was necessary to increase the efficiency of reconstitution of pmAEQ. The additions to the KRB (1 mM  $\text{CaCl}_2$ , 100 nM bradykinin (BK), 100  $\mu\text{M}$  KB-R7943, 20  $\mu\text{M}$  tBuBHQ, and 20  $\mu\text{M}$  5-(and 6-)carboxyeosin diacetate succinimidyl ester (CE)) were made as specified in the figure legends. The experiments were terminated by lysing the cells with 100  $\mu\text{M}$  digitonin in a hypotonic  $\text{Ca}^{2+}$ -rich solution (10 mM  $\text{CaCl}_2$  in  $\text{H}_2\text{O}$ ) to discharge the remaining AEQ pool. The light signal was collected and calibrated off-line into  $\text{Ca}^{2+}$  concentration values, using a computer algorithm based on the  $\text{Ca}^{2+}$  response curve of WT and mutant AEQs as previously described (13, 17). In brief, a 13-mm round coverslip with the transfected cells was placed in a perfused, thermostated chamber placed in close proximity to a low noise photomultiplier, with a built-in amplifier discriminator. The output of the discriminator was captured by a Thorn-EMI photon counting board and stored for further analyses.

**Fura-2 Measurements**—Fura-2 loading was performed as previously described (18); the coverslip was placed on the stage of an inverted fluorescence microscope (Zeiss Axiovert 100TV) connected to a cooled charge-coupled device camera (Micro-max 1300Y; Princeton Instruments, Inc.). The sample was illuminated alternately at 340/380 nm, and the emitted light (filtered with an interference filter centered at 510 nm) was collected by the camera. Images were acquired using Metafluor software (Universal Imaging Corp.). The ratio values (1 ratio image/s) were calculated off-line after background subtraction from each single image.

**DNA Constructs**—The plasmids coding for the glutathione *S*-transferase (GST)/DREAM fusion proteins were constructed including the cDNA of WT DREAM or EFmDREAM in the pGEX4T1 vector (GE Healthcare). The coding regions were amplified by PCR using appropriate pairs of forward 5'-CGG AAT TCC GGC TTG CTC TAG ACA CCA TGG-3' and reverse 5'-GCC TCG AGC TAG ATG ACA TTC TCA AAC-3' primers and subsequently cloned into the EcoRI-XhoI restriction site of pGEX4T1. All constructs were completely sequenced.

**GST Pull-down Assay**—GST-WT DREAM and GST-EFmDREAM fusion proteins and GST alone were produced in *Escherichia coli* (BL21). Protein expression was induced by adding 0.8 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside to the growing culture ( $A_{600} = 0.6$ ), and the cells were incubated at 30 °C for 3 h. Cells were centrifuged at  $13,200 \times g$  for 15 min, resuspended in ice-cold lysis buffer (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride), and disrupted using a sonicator. Cells were then incubated with 1% Triton X-100 for 30 min at 4 °C and centrifuged at  $15,700 \times g$  for 30 min. The GST, GST-WT DREAM, and GST-EFmDREAM recombinant proteins were purified by incubating with glutathione-Sepharose 4B at 4 °C for 2 h. The supernatant was removed, and the glutathione-Sepharose 4B pellet was washed three times with ice-cold PBS. SH-SY5Y cells were transfected with PS2/pcDNA3 plasmid. 24–36 h after transfection, a cell extract was prepared by lysing cells in Tris-

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EDTA buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 5  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml pepstatin). Lysis was performed by three cycles of freeze and thaw ( $-70^{\circ}\text{C}/37^{\circ}\text{C}$ ), and lysates were cleared by collecting the supernatants after spinning. Loading of the samples was normalized for the total content of cellular proteins determined by the Bradford assay (Sigma). Different amounts of cell lysate (0.8–2 mg) were added to about 20  $\mu$ g of GST-beads and mixed by gentle rotation at  $4^{\circ}\text{C}$  for 2 h. For pull-down in the presence of  $\text{Ca}^{2+}$ , 2 mM  $\text{CaCl}_2$  was added. The beads were recovered by centrifugation at  $500 \times g$  for 5 min, washed five times with ice-cold PBS, and eluted three times by gentle rotation at  $4^{\circ}\text{C}$  for 20 min in elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) followed by centrifugation at  $500 \times g$  for 5 min.

**Western Blotting Analysis**—SH-SY5Y cells were washed twice with PBS and harvested from the culture plates in ice-cold Tris/EDTA buffer. Lysis was performed by three cycles of freeze and thaw ( $-70^{\circ}\text{C}/37^{\circ}\text{C}$ ). Loading of the samples was normalized for the total content of cellular proteins determined by the Bradford assay. Samples were run on a 12% SDS-PAGE Tris/HCl gel and then blotted onto nitrocellulose membrane (GE Healthcare). Western blottings were performed using the polyclonal antibody anti-DREAM (sc-9142; Santa Cruz Biotechnology), the mouse monoclonal antibody anti-GST (sc-138; Santa Cruz Biotechnology), the polyclonal antibody PC235 (Oncogene) that recognizes the PS2 full-length protein of 54 kDa and the C-terminal fragment (CTF) of 20 kDa, the polyclonal antibody PC267 (Oncogene) that recognizes the PS1 CTF of 18 kDa, the mouse monoclonal anti- $\beta$ -tubulin (D-10, Santa Cruz Biotechnology), and in the mouse monoclonal anti- $\beta$ -actin (Sigma). Detection was carried out by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) for 1 h and 30 min. The proteins were visualized by the chemiluminescent reagent ImmunoStar horseradish peroxidase (Bio-Rad). Densitometric analyses were performed by using the Kodak1D image analysis software (Kodak Scientific Imaging Systems, New Haven, CT).

Means of densitometric measurements of independent experiments, normalized by the endogenous  $\beta$ -actin or  $\beta$ -tubulin values, were compared by Student's *t* test. The results shown in the figures are representative of at least three separate experiments.

**RT-PCR and Quantitative RT-PCR Analysis**—Total RNA from neuroblastoma cell culture was prepared using TRIzol reagent (Invitrogen). PCR assays were performed using specific primers designed using Primer3 software.

RT-PCR cycling parameters were as follows:  $95^{\circ}\text{C}$  for 5 min, 35 cycles of  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 15 s. The reaction was performed with PlatinumTaq DNA polymerase (Invitrogen) in the presence of 5% dimethyl sulfoxide. For NCX3, the primers used for amplification were as follows: forward, 5'-GAC AGT AGA AGG GAC AGC CA-3'; reverse, 5'-CTA GTT TGG GGT GTT CAC CC-3'. The results were normalized as shown by parallel amplification of the glyceraldehyde-3-phosphate dehydrogenase cDNA. Glyceraldehyde-3-phosphate dehydrogenase primers used were as follows: forward, 5'-CAA GGT CAT CCA TGA CAA CTT TG-3'; reverse, 5'-GGG CCA TCC ACA GTC TTC TG-3'.

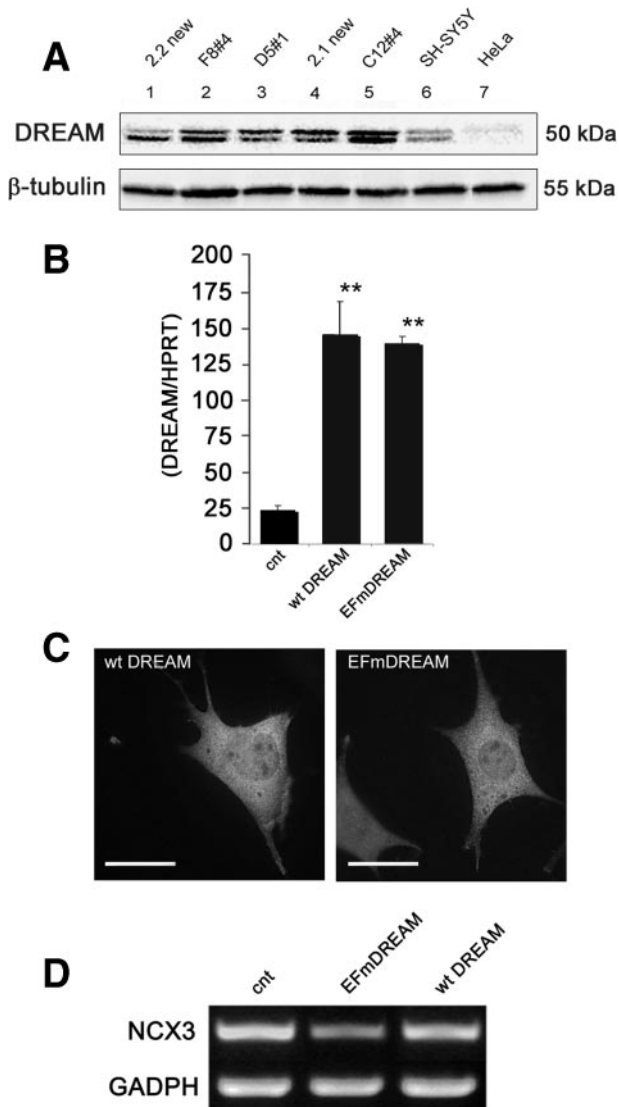
Quantitative RT-PCR was performed on a Rotor-Gene 3000 platform (Corbet Research, Sydney, Australia). The PCR cycling parameters were as follows:  $94^{\circ}\text{C}$  for 7 min, 45 cycles of  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 15 s. An amount of cDNA corresponding to 1–10 ng of total RNA was amplified in 25  $\mu$ l of a mixture containing 12.5  $\mu$ l of Platinum SYBR-Green qPCR SuperMix-UGD (Invitrogen) and a 2- $\mu$ l primer mixture (2.5  $\mu$ M each) for each sample. The primers used were as follows: for DREAM, 5'-CAC CTA TGC ACA CTT CCT CTT CA-3' (forward) and 5'-ACC ACA AAG TCC TCA AAG TGG A-3' (reverse); for InsP<sub>3</sub>R2, 5'-GGA CAT CGT GTC CCT GTA CG-3' (forward) and 5'-TGA ACT TCT TGG GAG GGT TG-3' (reverse); for InsP<sub>3</sub>R3, 5'-GGA CAT CGT CTC CCT GTA CG-3' (forward) and 5'-CAC CAC ACA GCG GTC ATC-3' (reverse); for SERCA2b, 5'-CCT CTA TGT CGA ACC CTT GC-3' (forward) and 5'-GCA GGC TGC ACA CAC TCT T-3' (reverse); for calreticulin, 5'-TCA AGG AGC AGT TTC TGG AC-3' (forward) and 5'-GTG CAT CCT GGC TTG TCT G-3' (reverse); for calnexin, 5'-GCT AAG AGG CCA GAT GCA GA-3' (forward) and 5'-TCA TGT CAT TGA GCA GAT TTC C-3' (reverse); for Grp78/BiP, 5'-TGT CCC CTT ACA CTT GGT ATT G-3' (forward) and 5'-CAA ATG TAC CCA GAA GAT GAT TG-3' (reverse).

The relative amount of amplified DNA was calculated as described (19) using hypoxanthine-guanine phosphoribosyltransferase cDNA as endogenous control. The hypoxanthine-guanine phosphoribosyltransferase primers used were as follows: forward, 5'-TTG GAT ACA GGC CAG ACT TTG TT-3'; reverse, 5'-CTG AAG TAC TCA TTA TAG TCA AGG GCA TA-3'.

**Statistical Analysis**—Data are reported as means  $\pm$  S.D. Statistical differences were evaluated by Student's two-tailed *t* test for impaired samples, with *p* value 0.01 being considered statistically significant.

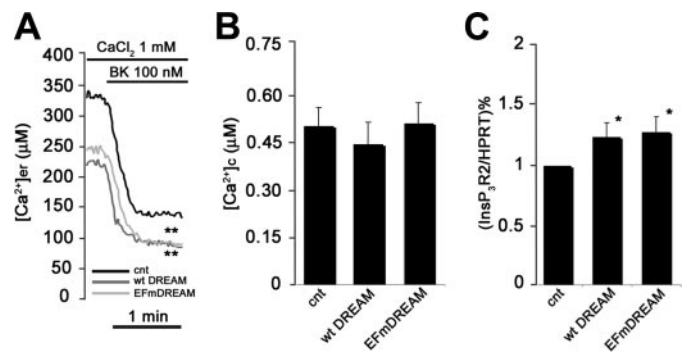
## RESULTS

**Generation of Stable Clones of SH-SY5Y Cells Expressing WT and EFmDREAM**—Expression vectors for WT DREAM and EFmDREAM were transfected into SH-SY5Y cells. A number of stable clones were obtained following G418 selection. The expression level of DREAM was verified in all selected clones by Western blotting on total cell lysates. Fig. 1A shows blots of representative DREAM-expressing clones. The specific DREAM antibody recognized a doublet of  $\sim 50$  kDa, corresponding to a dimer of the DREAM protein. Untransfected HeLa and untransfected SH-SY5Y cell lysates were used as negative and positive controls, respectively (endogenous DREAM is present in neuroblastoma cells but not in HeLa cells). A quantitative estimate by densitometric analysis of the doublet showed that the overexpressed DREAM was 2–4-fold higher than the endogenous DREAM. C12#4 (EFm) and D5#1 (WT) clones were selected for  $\text{Ca}^{2+}$  measurements, but similar results were obtained on two other independent clones for each cell type. Quantitative RT-PCR was also carried out. Fig. 1B shows the quantification, indicating that the DREAM mRNA rose about 6–7-fold above the endogenous content. Fig. 1C shows the immunolocalization of overexpressed WT and mutated DREAM; in both cases, a clear cytosolic and reticular distribution pattern was evident.



**FIGURE 1. Analysis of stable clones of SH-SY5Y cells expressing WT and mutated DREAM.** *A*, Western blots of cell extracts prepared from five different SH-SY5Y clones overexpressing EFmDREAM (lanes 1 and 5) and DREAM WT (lanes 2–4). SH-SY5Y and HeLa cells were used as positive and negative controls, respectively. The membrane was probed with a polyclonal anti-DREAM antibody, which recognized the protein in the dimer form (~50 kDa). An antibody against  $\beta$ -tubulin (55 kDa) was used for the normalization of protein amount. C12#4 (EFmDREAM) and D5#1 (WT DREAM) clones were selected for further experiments. *B*, quantitative RT-PCR showing the transcription levels of endogenous DREAM (cnt), overexpressed WT DREAM (clone D5#1), and mutant EFmDREAM (clone C12#4). Values were normalized by the content of hypoxanthine-guanine phosphoribosyltransferase mRNA. Results are the mean  $\pm$  S.D. of three experiments; \*\*,  $p < 0.001$ . *C*, immunocytochemical analysis of SH-SY5Y cells overexpressing WT DREAM and EFmDREAM. Bars, 15  $\mu$ m. *D*, RT-PCR analysis showing the levels of NCX3 mRNA in control cells (cnt), the EFmDREAM clone, and WT DREAM clone. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

The overexpression of a Ca<sup>2+</sup>-insensitive EFmDREAM mutant in the cerebellum of transgenic mice had been previously found to significantly reduce NCX3 mRNA and protein levels, increasing the basal concentration of Ca<sup>2+</sup> in cultured cerebellar granules (16). It was thus decided to investigate whether DREAM influenced the transcription of NCX3 also in SH-SY5Y cells we used. RT-PCR analysis indicated a reduction of about 25% in the transcript of NCX3 in the EFmDREAM cell clone, but no changes were detected in the WT clone (Fig. 1D).

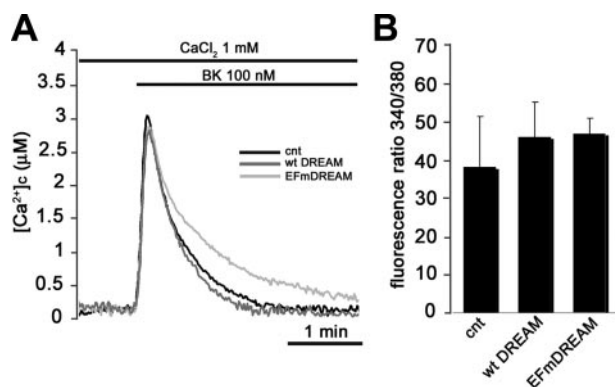


**FIGURE 2. DREAM overexpression affects ER Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>er</sub>) but not ER Ca<sup>2+</sup> release.** *A*, control cells and cells stably overexpressing WT DREAM or EFmDREAM were transiently transfected with erAEQ. Where indicated, 100 nM BK, an InsP<sub>3</sub>-generating agonist, was used to produce ER Ca<sup>2+</sup> release. The traces are representative of at least nine experiments. \*\*,  $p < 0.001$ . *B*, control cells and cells stably overexpressing WT DREAM or EFmDREAM were transiently transfected with cytAEQ. Shown are mean values of cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>cyt</sub>) peaks after application of the SERCA inhibitor (20  $\mu$ M tBuBHQ in KRB supplemented with 1 mM CaCl<sub>2</sub>). Results are the mean  $\pm$  S.D. of at least four experiments. *C*, quantitative RT-PCR showing the levels of InsP<sub>3</sub>R2 mRNA in control cells and in WT or EFmDREAM clones. Values were normalized by the content of HPRT mRNA. Results are expressed as a percentage with respect to control cells (mean  $\pm$  S.D.). At least three independent experiments were performed. \*,  $p < 0.01$ .

Values were normalized by the content of glyceraldehyde-3-phosphate dehydrogenase mRNA. EFmDREAM acts as a constitutive repressor of transcription (*i.e.* as a dominant active mutant), since its inability to bind Ca<sup>2+</sup> does not permit its detachment from the promoter region of the gene. For this reason, its effects on gene transcription are expected to be more marked and evident than those of the WT DREAM.

*The Overexpression of WT DREAM and EFmDREAM Decreases the Resting ER Ca<sup>2+</sup> Content but Not the Agonist-stimulated Ca<sup>2+</sup> Release*—Since no work has so far directly analyzed ER free Ca<sup>2+</sup> in neuroblastoma cells overexpressing DREAM, it was decided to directly monitor it with erAEQ (14) under resting conditions and following cell stimulation with an agonist coupled to the generation of InsP<sub>3</sub>. Fig. 2A shows that unstimulated cells overexpressing the two DREAM variants had significantly lower resting ER Ca<sup>2+</sup> than control cells. The reduction was about 25% in both clones: 317  $\pm$  32  $\mu$ M ( $n = 30$ ) in control cells, 241  $\pm$  19  $\mu$ M ( $n = 26$ ) in the EFmDREAM cell clone, and 232  $\pm$  32  $\mu$ M ( $n = 9$ ) in WT DREAM clone ( $p < 0.001$ ). After BK stimulation, the release of Ca<sup>2+</sup> set the ER Ca<sup>2+</sup> content at about 100  $\mu$ M in the clones expressing the two DREAM variants and to about 150  $\mu$ M in control cells (144  $\pm$  16  $\mu$ M ( $n = 9$ ) in control cells, 98  $\pm$  26  $\mu$ M ( $n = 11$ ) in EFmDREAM clone, and 98  $\pm$  3  $\mu$ M ( $n = 4$ ) in WT DREAM clone;  $p < 0.001$ ). Thus, considering the different starting levels of ER Ca<sup>2+</sup>, the net amount of Ca<sup>2+</sup> released from the ER store by InsP<sub>3</sub> remained essentially constant in the three cell types.

It was considered important to clarify the mechanism by which the overexpression of WT and EFmDREAM reduced the basal amount of ER Ca<sup>2+</sup>. The release of Ca<sup>2+</sup> through passive leak channels (*i.e.* in the absence of agonists that would open the InsP<sub>3</sub> receptor (InsP<sub>3</sub>R)) was evaluated after blocking Ca<sup>2+</sup> uptake by the sarcoendoplasmic Ca<sup>2+</sup> ATPase (SERCA pump). Cytosolic Ca<sup>2+</sup> elevations were monitored in KRB supplemented with 1 mM CaCl<sub>2</sub> using cytAEQ (13), after adding the



**FIGURE 3. Monitoring of  $[Ca^{2+}]_c$  transients and  $[Ca^{2+}]_c$  resting levels in control cells and in DREAM clones.** *A*, controls and cells stably overexpressing WT DREAM and EFmDREAM were transiently transfected with cytAEQ. Where indicated, cells were stimulated with 100 nM BK. The traces are representative of at least eight experiments. *B*, cells were loaded with 5 mM fura-2. Mean values of resting  $[Ca^{2+}]_c$  are indicated by fura-2 fluorescence ratios in nonstimulated control cells and cells stably overexpressing WT or EFmDREAM. Results are representative of at least 50 cells for each batch in three independent experiments (mean  $\pm$  S.D.).

SERCA pump inhibitor tBuBHQ (20). The increase of cytosolic  $Ca^{2+}$  due to the enhanced  $Ca^{2+}$  leak from the ER was the same in control cells, in the WT DREAM, and in EFmDREAM clones (Fig. 2*B*).

To investigate whether the changes in ER  $Ca^{2+}$  content were related to the activity of DREAM as transcriptional repressor, quantitative analysis of the transcripts of ER proteins, such as the SERCA pump, the  $InsP_3R$ , and the  $Ca^{2+}$ -dependent chaperones calnexin and calreticulin, was performed. In addition to buffering  $Ca^{2+}$  in the ER lumen (21), the last two proteins also regulate  $InsP_3R$ s activity (22). Another  $Ca^{2+}$  storage-related protein, Grp78/BiP (23), which is involved in the  $Ca^{2+}$ -activated ER stress response (24), was also investigated. Quantitative RT-PCR on total RNA extracted from the three different batches of cells using the primers indicated under "Experimental Procedures" only showed differences for the transcript of the  $InsP_3R2$ , which was increased by about 25% in the cells expressing WT and EFmDREAM (Fig. 2*C*). This finding was not surprising, since in some cases, DREAM has been described to activate transcription, rather than inhibit it, by acting on the promoters of certain genes (25).

The cytosolic transients generated by the release of  $Ca^{2+}$  through the opening of the  $InsP_3R$  were then analyzed using cytAEQ or the fluorescent  $Ca^{2+}$  indicator fura-2. The two probes yielded similar results. In agreement with the findings that the amount of  $Ca^{2+}$  released by  $InsP_3R$  was the same in the controls and in the two DREAM-expressing clones, the heights of the BK-induced cytosolic  $Ca^{2+}$  transients were about the same in the three cell types. The peaks of the transients were  $2.95 \pm 0.32 \mu M$  ( $n = 8$ ) in control cells,  $2.85 \pm 0.12 \mu M$  ( $n = 10$ ) in the EFmDREAM clone, and  $2.78 \pm 0.24 \mu M$  ( $n = 8$ ) in the WT DREAM clone (Fig. 3*A*). Previous work on cerebellar granules from EFmDREAM transgenic mice, which expressed reduced amounts of NCX3, had shown slower kinetics of the post-transient decline of the  $Ca^{2+}$  traces (16). A similar effect was found in the SH-SY5Y clones overexpressing EFmDREAM (*i.e.* a slower return of the postpeak  $Ca^{2+}$  trace to basal level); the  $t/2$  decay of the peak was  $13.9 \pm 1.8$  s ( $n = 6$ ) in control cells

and  $17.6 \pm 1.6$  s ( $n = 9$ ) in the EFmDREAM clone ( $p < 0.001$ ). However, the WT DREAM clone behaved like control cells ( $14.5 \pm 1.8$  s,  $n = 8$ ), possibly because the reduction of NCX3 in the plasma membrane was below detection level in the WT DREAM clone (see Fig. 1*D*).

In principle, the overexpressed DREAM could have buffered cytosolic  $Ca^{2+}$ , decreasing the amount available to the SERCA pump and thus the ER  $Ca^{2+}$  content. Fura-2 was used to evaluate the resting cytosolic  $Ca^{2+}$ , since AEQ is inadequate to monitor  $Ca^{2+}$  at the low nanomolar level. Fura-2 signals (ratio of fluorescence emitted by illuminating cells at 340 and 380 nm) detected in DREAM clones were similar to those in control cells, suggesting that the  $Ca^{2+}$  levels were similar in all three cell batches (Fig. 3*B*) (*i.e.* the differences in the ER  $Ca^{2+}$  content were not due to the  $Ca^{2+}$  buffering effect of overexpressed DREAM).

The effects of DREAM were analyzed in more detail by monitoring cytosolic  $Ca^{2+}$  in the presence of specific inhibitors of the three different  $Ca^{2+}$  transporter proteins that have roles in the reestablishment of the post-transient cytosolic  $Ca^{2+}$  conditions: tBuBHQ as a SERCA inhibitor, CE as a plasma membrane  $Ca^{2+}$ -ATPase inhibitor (26), and KB-R7943 as an NCX inhibitor (27). Fig. 4*A* indicates that NCX apparently had the major role in the reestablishment of the basal  $Ca^{2+}$  level after BK stimulation in SH-SY5Y cells; the kinetics of the  $Ca^{2+}$  transient was only slightly affected by tBuBHQ or CE but was markedly affected by KB-R7943. When the same inhibition protocol was applied to WT (Fig. 4*B*) and EFmDREAM (Fig. 4*C*) clones, the effect of SERCA and plasma membrane  $Ca^{2+}$ -ATPase inhibition was higher than that observed in control cells, and KB-R7943 was much more effective on DREAM clones than in control cells. These data suggest that the  $Ca^{2+}$  extrusion ability was compromised in DREAM clones, probably because of the decreased level of expression of NCX3.

*The Expression of WT DREAM and EFmDREAM Impairs the Capacitative  $Ca^{2+}$  Influx into SH-SY5Y Cells*—To further explore the dynamics of the cytosolic  $Ca^{2+}$  response, the  $InsP_3$ -generated  $Ca^{2+}$  transient was dissected into its two components: that related to the release of  $Ca^{2+}$  from the ER and that related to the influx of  $Ca^{2+}$  from the extracellular space (Fig. 5*A*). The  $Ca^{2+}$  response to BK was monitored in the absence of extracellular  $Ca^{2+}$  (*i.e.* under conditions of no  $Ca^{2+}$  influx). After the internal store had been depleted,  $Ca^{2+}$  readdition to the medium induced capacitative influx. The measurements indicated that the  $Ca^{2+}$  release from the ER store was unaffected by the overexpression of WT and EFmDREAM, whereas the influx through the plasma membrane capacitative channels was significantly impaired. The reduction was about 25% in both clones; the average  $Ca^{2+}$  peak height was  $1.21 \pm 0.95 \mu M$  ( $n = 7$ ) in control cells,  $0.96 \pm 0.12 \mu M$  ( $n = 5$ ) in the WT DREAM clone, and  $0.82 \pm 0.10 \mu M$  ( $n = 9$ ) in the EFmDREAM clone ( $p < 0.001$ ).

It was then decided to monitor  $Ca^{2+}$  directly in the restricted cytosolic space beneath the plasma membrane using pmAEQ (15).  $Ca^{2+}$  depletion of the stores was induced during AEQ reconstitution in KRB buffer supplemented with 100  $\mu M$  EGTA. The readdition of  $Ca^{2+}$  promoted its influx through the capacitative channels, generating a transient  $Ca^{2+}$  rise (Fig. 5*B*),

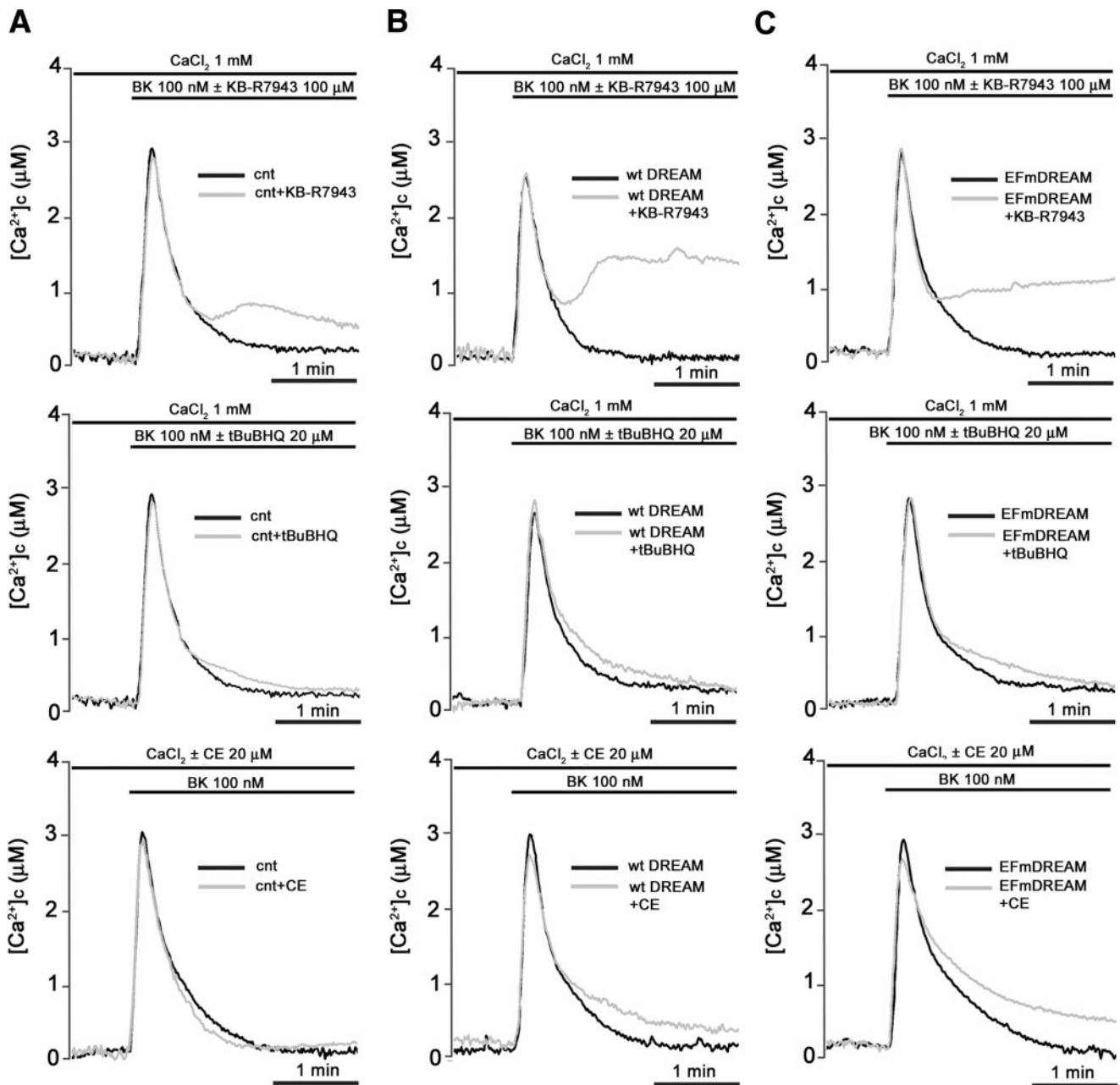


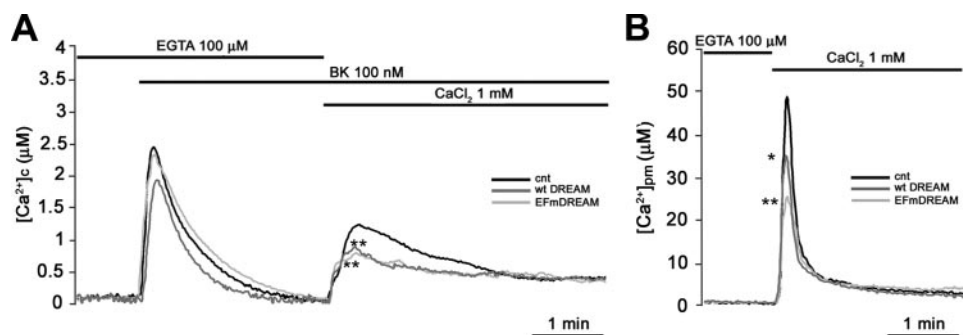
FIGURE 4.  $\text{Ca}^{2+}$  extrusion ability was impaired in DREAM clones. Control SH-SY5Y cells (A) and cells stably overexpressing WT DREAM (B) and EFmDREAM (C) transiently overexpressing cytAEQ were stimulated with 100 nM BK in the presence of 100  $\mu\text{M}$  KB-R7943, 20  $\mu\text{M}$  tBuBHQ, or 20  $\mu\text{M}$  CE. The traces are representative of at least four experiments.

which peaked at  $39.44 \pm 9.80 \mu\text{M}$  ( $n = 10$ ) in control cells, at  $28.33 \pm 7.48 \mu\text{M}$  ( $n = 11$ ) in WT DREAM clone ( $p < 0.01$ ), and at  $20.86 \pm 5.29 \mu\text{M}$  ( $n = 11$ ) in the EFmDREAM clone ( $p < 0.001$ ). These data confirm that the capacitative  $\text{Ca}^{2+}$  influx was impaired by the overexpression of WT DREAM and EFmDREAM. After the transient rise, resting  $\text{Ca}^{2+}$  conditions were reestablished in  $\sim 1$  min, after which time the  $\text{Ca}^{2+}$  level in the subplasma membrane was maintained at a plateau of about  $2.88 \pm 0.38 \mu\text{M}$  ( $n = 8$ ) in control cells and at a slightly (but not significantly) higher level in the WT DREAM ( $3.18 \pm 0.88 \mu\text{M}$ ,  $n = 8$ ) and EFmDREAM clones ( $3.41 \pm 0.92 \mu\text{M}$ ,  $n = 8$ ).

*DREAM and PS Cooperate in the Modulation of ER  $\text{Ca}^{2+}$  Content but Not of  $\text{Ca}^{2+}$  Influx from the Extracellular*

*Medium*—To further dissect the mechanism through which DREAM modulated ER  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  influx from the external medium, DREAM cell clones (WT and EFm) and control cells were co-transfected with PS1 or PS2 and erAEQ expression plasmids. This was done because the overexpression of PSs has been proposed to decrease ER  $\text{Ca}^{2+}$  in SH-SY5Y and HeLa cells (28) due to the formation of ER  $\text{Ca}^{2+}$  leak channels (29) or because their interaction with the  $\text{InsP}_3\text{R}$  enhanced the  $\text{InsP}_3$ -mediated ER  $\text{Ca}^{2+}$  permeability (30). We have found that the overexpression of PS2 (but not of PS1) indeed reduced the size of the ER  $\text{Ca}^{2+}$  pool by about 50% in SH-SY5Y cells ( $317 \pm 32 \mu\text{M}$  ( $n = 30$ ) in control cells;  $161 \pm 19 \mu\text{M}$  ( $n = 6$ ) in PS2-expressing cells ( $p < 0.001$ ); and  $299 \pm 5 \mu\text{M}$  ( $n = 4$ ) in PS1-

## Neuronal Calcium Homeostasis and DREAM



**FIGURE 5. Reduction of capacitative  $\text{Ca}^{2+}$  influx in WT DREAM and EFmDREAM clones.** *A*, control cells and cells stably overexpressing WT DREAM and EFmDREAM were transiently transfected with cytAEQ. Cells were stimulated with 100 nM BK in the absence of external  $\text{Ca}^{2+}$  (100  $\mu\text{M}$  EGTA), and after the  $\text{Ca}^{2+}$  trace was restored to base-line level, they were perfused with KRB supplemented with 1 mM  $\text{CaCl}_2$  to allow the influx of  $\text{Ca}^{2+}$ . *B*, monitoring of  $\text{Ca}^{2+}$  concentration beneath the plasma membrane ( $[\text{Ca}^{2+}]_{\text{pm}}$ ) in control cells and cells stably overexpressing WT DREAM and EFmDREAM transiently transfected with pmAEQ. Shown are effects of the  $\text{CaCl}_2$  readdition (1 mM) to cells maintained in a  $\text{Ca}^{2+}$ -free medium. The traces are representative of at least five experiments. \*\*,  $p < 0.001$ ; \*,  $p < 0.01$ .

expressing cells). Interestingly, when PS2 was expressed in DREAM cell clones, the ER  $\text{Ca}^{2+}$  content was further reduced to  $146 \pm 28 \mu\text{M}$  ( $n = 7$ ) in clones coexpressing PS2 and WT DREAM ( $p < 0.001$ ) and to  $118 \pm 19 \mu\text{M}$  ( $n = 7$ ) in those coexpressing PS2 and EFmDREAM ( $p < 0.001$ ). The effect of PS1 on ER  $\text{Ca}^{2+}$  was instead not significantly affected by the coexpression of DREAM. Fig. 6A shows the results as mean values expressed as percentage of control cells. The overexpression levels of PS1 and PS2 were checked by Western blotting, quantified by densitometric analysis normalized on  $\beta$ -actin content, and found to be comparable in all batches of cells (see the bottom panel of Fig. 6A).

It was also decided to investigate whether the level of PS2 expression had any effect on the capacitative  $\text{Ca}^{2+}$  influx by monitoring  $\text{Ca}^{2+}$  in the subplasma membrane space with pmAEQ. The PS2 expression plasmid was transiently transfected in SH-SY5Y alone or with WT and EFmDREAM plasmids.  $\text{Ca}^{2+}$  influx was also monitored in cells transiently expressing the DREAM plasmids alone. The  $\text{Ca}^{2+}$  estimates showed that the expression of PS2, as well as that of DREAM, or their coexpression failed to affect  $\text{Ca}^{2+}$  influx (data not shown).

Fig. 6A had shown that the overexpression of PS2 in the clones stably overexpressing DREAM further reduced the ER  $\text{Ca}^{2+}$  content. It was decided to clarify whether the effect was the sum of independent effects of DREAM and PS2 or whether, instead, DREAM modulated the PS-promoted  $\text{Ca}^{2+}$  release from the ER by a post-transcriptional mechanism. To rule out the transcriptional effects of DREAM protein, the measurements of ER  $\text{Ca}^{2+}$  were repeated in transient coexpression experiments, where DREAM and PS2 plasmids were cotransfected at the same time. Under these conditions, it was assumed that DREAM would have no time to modify the transcriptional pathway of the genes. As a control, ER  $\text{Ca}^{2+}$  was also monitored in SH-SY5Y cells transiently transfected only with WT or EFmDREAM plasmids (Fig. 6B). The expression level of PS2 was quantified by densitometric Western blotting analysis and found to be the same in SH-SY5Y cells transfected with PS2 alone or co-transfected with PS2 and DREAM plasmids (data not shown). The results showed that ER  $\text{Ca}^{2+}$  was not modified by the transient expression of WT and EFmDREAM, suggest-

ing that under these conditions, no transcriptional regulation had occurred on the NCX3 and  $\text{InsP}_3\text{R}$  genes. But the coexpression of DREAM with PS2 potentiated the effect of the latter on the decrease of ER  $\text{Ca}^{2+}$  content. The decrease was about 70% in cells coexpressing the DREAMs and PS2, whereas it was about 50% in cells only expressing PS2 (Fig. 6B). The effect was more striking in the case of the EFmDREAM mutant ( $83 \pm 14 \mu\text{M}$  ( $n = 5$ ) in PS2 + EFmDREAM-expressing cells ( $p < 0.001$ ),  $102 \pm 21 \mu\text{M}$  ( $n = 6$ ) in PS2 + WT DREAM-expressing cells ( $p < 0.01$ ),  $317 \pm 32 \mu\text{M}$  ( $n = 30$ ) in control SH-SY5Y cells, and  $161 \pm 19 \mu\text{M}$  ( $n = 6$ ) in PS2-expressing cells).

At this point, it was considered important to carry out additional control work on DREAM cell clones to better evaluate the transcriptional contribution of DREAM in the control of ER  $\text{Ca}^{2+}$  (i.e. to evaluate whether DREAM regulated PSs levels). The amount of endogenous PSs was estimated in the DREAM stable cell clones by Western blotting. The analysis showed that the amount of full-length PS2, which is the variant of the protein that is proposed to form the ER  $\text{Ca}^{2+}$  leak channel, was about the same in control cells and in cells expressing WT DREAM and EFmDREAM (Fig. 6C). A dramatic down-regulation affected instead the CTF of PS2 (but not of PS1) in the EFmDREAM-expressing cell clone. Thus, a nontranscriptional modulation of PS by DREAM appears to be at work.

The effects in transient coexpression experiments suggest a direct interaction of DREAM with PS, as had been claimed by others based on yeast two-hybrid work and on co-immunoprecipitation experiments (1, 31, 32). Therefore, it was decided to reinvestigate the problem using GST pull-down technology with the entire PS2 protein transfected in SH-SY5Y cells. In the cell model used, both GST-WT DREAM and GST-EFmDREAM fusion proteins interacted with PS2 (Fig. 7). However, since the experimental conditions demanded the presence of EDTA in the medium, WT DREAM was in effect a  $\text{Ca}^{2+}$ -free apoprotein. It was thus decided to repeat the experiment in the presence of  $\text{Ca}^{2+}$  to establish whether the interaction of PS with DREAM occurred with and without  $\text{Ca}^{2+}$  or was obligatorily  $\text{Ca}^{2+}$ -independent. The results have shown that the interaction occurred in both conditions. In some experiments (e.g. in that shown in Fig. 7), the intensity of the band was slightly increased in the presence of  $\text{Ca}^{2+}$ . However, this was not consistently observed.

## DISCUSSION

The precise control of  $\text{Ca}^{2+}$  homeostasis and, thus, of  $\text{Ca}^{2+}$  signaling is essential for neuronal development and function; abnormalities in the signaling operation are commonly involved in the origin of neurodegenerative disorders (33, 34).  $\text{Ca}^{2+}$  homeostasis and, thus,  $\text{Ca}^{2+}$  signaling require the concerted action of specific transport systems in the plasma mem-

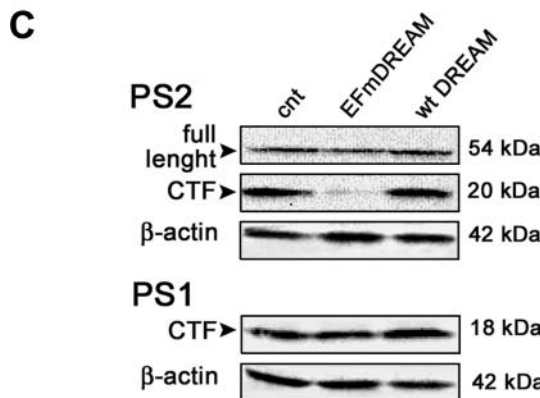
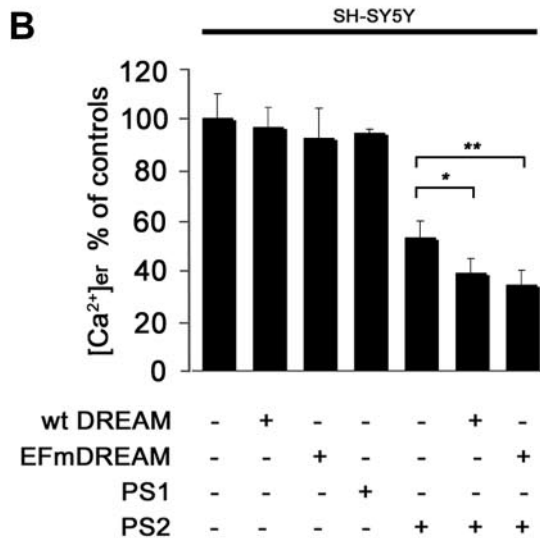
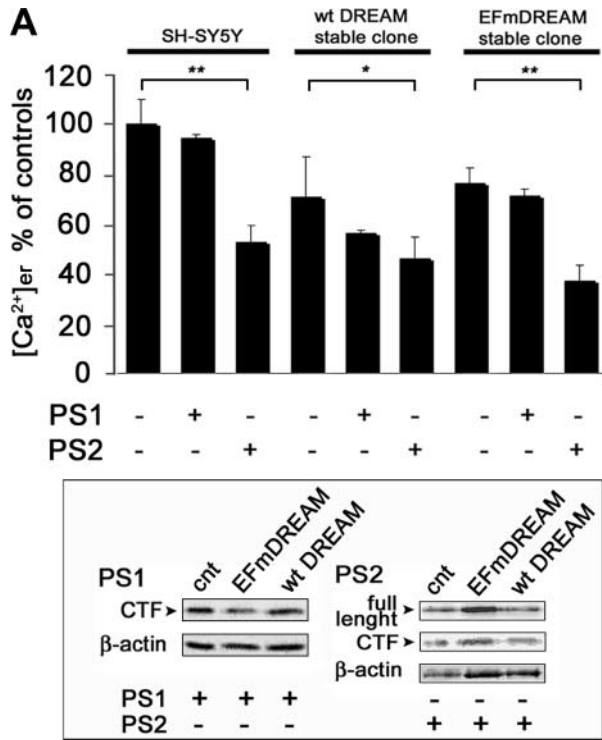


FIGURE 6. DREAM and PS cooperate in controlling the ER  $Ca^{2+}$  content. *A*, top, mean values (percentage of controls) of steady state  $[Ca^{2+}]_{er}$  in control and WT DREAM or EFmDREAM clones transiently co-transfected with PS1 or

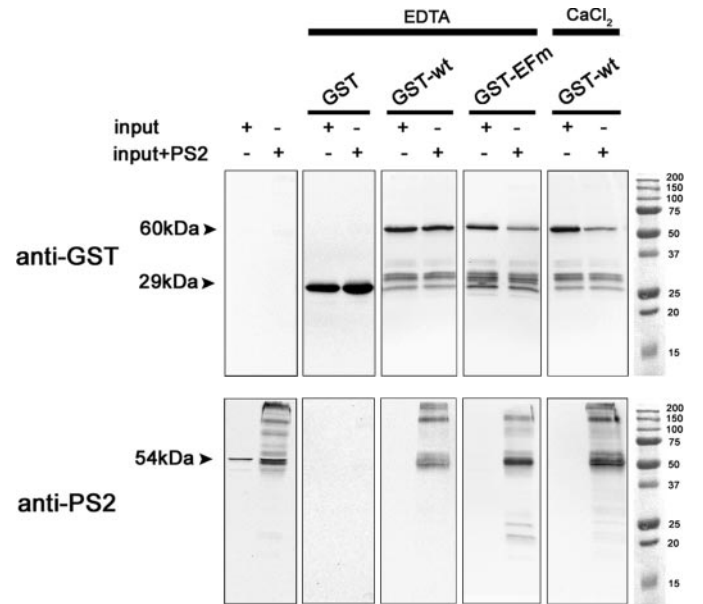


FIGURE 7. DREAM interacts with PS2 in a  $Ca^{2+}$ -independent manner. GST, GST-WT DREAM (GST-wt) and GST-EFmDREAM (GST-EFm) on glutathione-Sepharose beads were incubated with lysates from SH-SY5Y-untransfected cells (input) or from SH-SY5Y cells transfected with PS2 (input + PS2) in the absence or in the presence of  $Ca^{2+}$ . Pull-down reactions were analyzed by anti-PS2 and anti-GST antibodies in Western blotting.

brane and in intracellular organelles. However, nonmembrane proteins may also play roles. One of them, the multifunctional  $Ca^{2+}$ -binding protein DREAM, is abundantly expressed in neurons and is likely to be involved in the regulation of membrane  $Ca^{2+}$  fluxes. Previous observations in cultured cerebellar granules had shown that DREAM induced the down-regulation of the important plasma membrane  $Ca^{2+}$  extrusion system NCX3, increasing the vulnerability of cultured neurons to  $Ca^{2+}$  (16).

The work described here has shown that DREAM indeed had a role in the control of  $Ca^{2+}$  homeostasis in the cell line used. As could be expected of a multifunctional protein, DREAM appears to act by more than one mechanism. One is the transcriptional mode; the down-regulation of the NCX3 would in principle lead to  $Ca^{2+}$  overloading (35) but would at the same time reduce capacitative  $Ca^{2+}$  influx and ER  $Ca^{2+}$  content. The experiments described here have demonstrated, by measuring it directly, that ER  $Ca^{2+}$  and capacitative  $Ca^{2+}$  influx were reduced in the WT and EFmDREAM clones. Interestingly, however, the net amount of  $Ca^{2+}$  released from the ER by  $InsP_3$  did not decrease with respect to controls, even if the total

PS2 plasmids. The results are representative of at least four experiments (mean  $\pm$  S.D.). \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ , calculated on the respective PS-untransfected control. *Bottom*, Western blotting analysis of the level of transient PS1 and PS2 overexpression in control cells (cnt) and in DREAM clones. The membranes were probed with an antibody that recognizes the PS1 CTF of 18 kDa or with a polyclonal antibody that recognizes the PS2 CTF of 20 kDa and the full-length protein of 54 kDa. An antibody against  $\beta$ -actin (42 kDa) was used for the normalization of protein amount. *B*, mean values (percentage of controls) of steady state  $[Ca^{2+}]_{er}$  in SH-SY5Y cells transiently overexpressing WT DREAM, EFmDREAM, PS1, and PS2 plasmids. The results are representative of at least five experiments (mean  $\pm$  S.D.). \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ , calculated with respect to PS2-transfected cells. *C*, Western blotting analysis of the endogenous PS1 and PS2 content in control cells (cnt) and in DREAM cell clones.

amount of  $\text{Ca}^{2+}$  in the ER was reduced. The correct signaling by  $\text{Ca}^{2+}$  was thus guaranteed. The up-regulation of the  $\text{InsP}_3\text{R}$  transcripts in the cell clones overexpressing WT and EFmDREAM could explain the lower  $\text{Ca}^{2+}$  content in the ER, since an increased  $\text{Ca}^{2+}$  leak could occur through the unstimulated receptor (36–38). The possibility that DREAM could modify the plasma membrane potential (by acting on A type current Kv channels (3)) and thus influence the rate and the extent of the capacitative  $\text{Ca}^{2+}$  entry (39), appears remote, since the modulation of the Kv channel by KCHIP is  $\text{Ca}^{2+}$ -dependent (3), whereas the effect observed here was the same for WT and EFmDREAM.

The data have indicated that DREAM controlled ER  $\text{Ca}^{2+}$  content also through a nontranscriptional mechanism. The transient overexpression of PS2 (but not of PS1) still reduced the free  $\text{Ca}^{2+}$  level in the ER. The transient coexpression of WT and mutated DREAM with PS2, but not with PS1, has shown that DREAM potentiated the ability of PS2 to reduce the ER  $\text{Ca}^{2+}$ . Thus, DREAM would play a dual role in the control of ER  $\text{Ca}^{2+}$  content. One role is probably long term, involving changes in the transcription of  $\text{NCX3}$  and of the  $\text{InsP}_3\text{R}$ . The other would be short term and could involve the interplay between DREAM and PS at the protein level, as suggested by earlier work by others (6). Interestingly, as previously suggested (31), the interaction between DREAM and PS was  $\text{Ca}^{2+}$ -independent, and it tolerated EF-hand mutations. Apparently, then, DREAM acts via  $\text{Ca}^{2+}$  when interacting with DNA in the nucleus (2) and when interacting with the Kv channel in the plasma membrane (3) but independently of it in the control of  $\text{Ca}^{2+}$  signaling when interacting with PS in the ER membrane.

A relationship has been frequently suggested between the dysregulation of ER  $\text{Ca}^{2+}$  content and of capacitative  $\text{Ca}^{2+}$  entry and the etiology of familial Alzheimer disease. PSs have been claimed to play a regulatory role in capacitative  $\text{Ca}^{2+}$  entry (40–43) and in ER  $\text{Ca}^{2+}$  homeostasis (28, 29). Possibly, PSs interfere with the process of cellular  $\text{Ca}^{2+}$  homeostasis in more than one way; very recently, PSs have been shown to directly interact with SERCA pump and to regulate its activity (44). The work on the capacitative  $\text{Ca}^{2+}$  influx described here has failed to provide a role of PSs in the  $\text{Ca}^{2+}$  entry pathway, at least for PS2. It has shown, however, that both WT and EFmDREAM markedly down-regulated the influx of  $\text{Ca}^{2+}$  through the capacitative channels in stable cell clones.

The difference between the effects of the two PSs on ER  $\text{Ca}^{2+}$  seems at first glance to be at variance with recent findings showing that both PSs can function as ER  $\text{Ca}^{2+}$  leak channels (29). However, the apparent discrepancy could be rationalized by considering that most of the PS1 that accumulates *in vivo* (45) and corresponds to that found in the cells used here (both endogenous and exogenous) would not be the full-length PS but rather its N-terminal fragment and CTF, which are not involved in the formation of the putative ER  $\text{Ca}^{2+}$  leak channels.

In addition to acting directly on the PS2 protein modulating its function on ER  $\text{Ca}^{2+}$ , DREAM also acted on the PS2 CTF protein amount; its EF-hand mutant reduced it. The finding was of particular interest, since previous reports had shown that the expression of exogenous PS1 or PS2 in stably trans-

ected cells was accompanied by a compensatory decrease in the steady state levels of the endogenous PS proteins and that the levels of PS CTF were regulated by limiting cellular factors (45). DREAM could interfere with this compensatory regulation, finely adjusting the level of PSs to modulate their activity. The dramatic down-regulation of the CTF of PS2 was unexpected, also considering previous findings that the amount of an alternate PS2 CTF increased in parallel with the time course of DREAM expression (1). The portion of the PS2 protein down-regulated in the present experiments was slightly longer than the antiapoptotic protein ALG-3 (47), which is a truncated product of the PS2 gene (48, 49), but ALG-3 could perhaps still have been the strongly down-regulated PS2 peptide. However, the epitope of the anti-PS2 antibody used here lay in a region of the PS2 protein not included in the sequence of ALG-3 and would thus not be recognized by it. Therefore, the down-regulated fragment observed here appears to have been a proteolytic product of PS2 and not ALG-3.

The amount of the full-length form of the PS2 protein was similar in all three cells' batches studied, whereas the endoproteolytic CTF was only markedly reduced in the EFmDREAM clone. The result further supports the conclusion that the reduction of ER  $\text{Ca}^{2+}$  found in both the WT and EFmDREAM clones was not dependent on the different levels of endogenous PS2, since the CTF of PSs is not involved in the formation of the proposed leak channel. The result would be compatible with the suggestion that DREAM could affect the processing of PS (*i.e.* of PS2). Unfortunately, no conclusion about the PS1 protein is possible, since the antibody available for its detection only revealed the proteolytic CTF. However, at variance with PS2, the amount of the CTF was equivalent in all three cell batches.

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