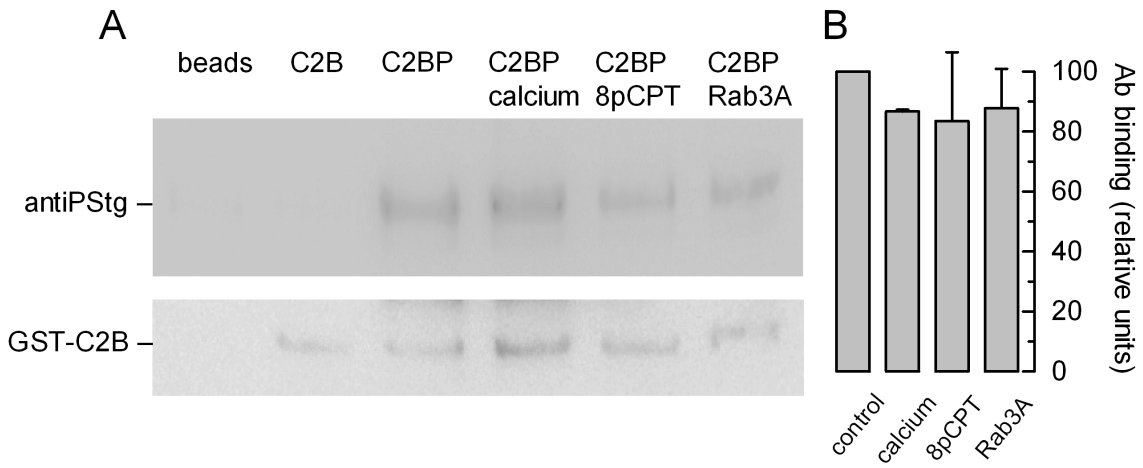

Figure S1. The binding of the antiPStg antibody to phosphorylated C2B synaptotagmin VI domain was not influenced by calcium. (A) 0.5 μM GST-C2B domain immobilized in glutathione-sepharose beads were phosphorylated as describe in experimental procedures using 100 μM ATP. The beads loaded with phosphorylated C2B domain (C2BP) were washed with PBS and incubated for 1 h at 20ºC in washing buffer (PBS, 0.2% Tween 20) supplemented with 5% BSA The beads were washed twice with PBS and resuspended in sucrose buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-K, pH 7) and incubated with 2.5 μM antiPStg antibody for 30 min. at 37°C with no addition or in the presence of 10 μM free calcium, 300 nM Rab3A, or 50 μM 8pCPT. Beads were washing four times with 50 mM Hepes, 150 mM NaCl, pH 7. As a control, the C2BP beads were substituted by uncoated beads (beads) or by beads carrying the non phosphorylated C2B domain (C2B). Proteins were resolved in 12.5 % Tris-tricine SDS-PAGE and transferred to 0.22 μm nitrocellulose membranes (Hybond, GE Healthcare). Non-specific reactivity was blocked by incubation for 2 h at room temperature with 5% skim milk dissolved in washing buffer. To detect antiPStg bound to GST-C2BP, blots were incubated for 1h at 20ºC with HRP-conjugated goat anti-rabbit-IgG (Jackson Immunochemicals Seroimmuno Diagnostics, Inc. Tucker, GA, 1:20000 in washing buffer containing 5% skim milk). To detect GST-C2B and GST-C2BP domains, blots were incubated for 1 h at 20ºC with an anti synaptotagmin antibody that recognizes phosphorylated and non-phosphorylated proteins (1)(10 nM in washing buffer containing 5% skim milk) using the same secondary antibody. Detection was accomplished with a chemiluminescence system (Western Lightning, Perkin Elmer, Migliore Laclaustra, Buenos Aires, Argentina). The images of the bands were obtained using a Luminescent Image Analyzer LAS-4000 (Fujifilm). (B) The antiPStg bound to C2BP domains from two experiments as shown in A were quantified, normalized for the C2BP content of the assays and referred to the binding observed in the sucrose buffer with no additions. Bars represent the mean ± range.

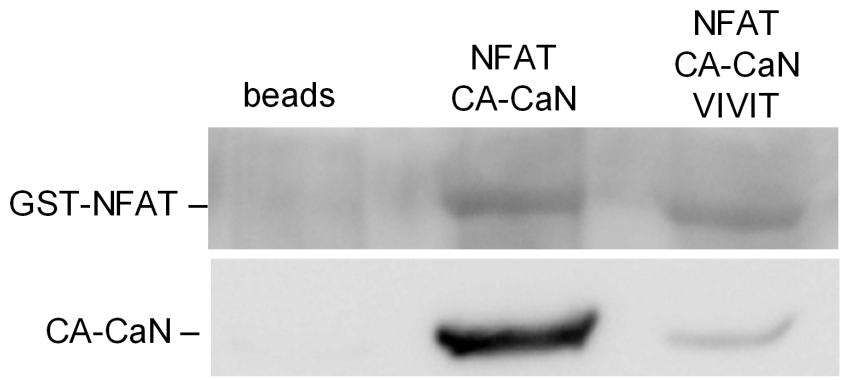

Figure S2. CA-CaN binding to NFAT is blocked by VIVIT. cDNA-encoding NFAT regulatory domain (residues 4-385) was generously provided by Dr. JM. Redondo (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain). GST-NFAT was purified as describe (2). For the binding assay, 0.6 μM GST-NFAT immobilized in glutathione-sepharose were washed with binding buffer (20 mM Tris, 100 mM NaCl, 6 mM $MgCl_2$, 1 μM DTT, 0.2% Triton X-100, pH 8, containing protease inhibitors) and then incubated with 90 nM CA-CaN in the presence or absence of 100 μM VIVIT for 30 min at 4°C. After washing five times with binding buffer, proteins were resolved in 12.5 % Tris-tricine SDS-PAGE and transferred to 0.22 μm nitrocellulose membranes (Hybond, GE Healthcare). Non-specific reactivity was blocked by incubation for 2 h at room temperature with 5% skim milk dissolved in washing buffer. To detect CA-CaN, blots were incubated with a home-made antiCA-CaN mouse antibody (1.3 nM in washing buffer/5% skim milk for 1h at RT). HRP-conjugated goat anti-mouse IgG (Kierkegaard & Perry Laboratories Inc., Gaithersburg, MD) was used as secondary antibody (0.25 μg/ml, in washing buffer/5% skim milk, 1 h at 20ºC). Finally to detect NFAT, blot were incubated with antiGST antibody (Chemicon Australia Pty. Ldt, Victoria, Australia, 0.16 nM in washing buffer/5% skim milk for 1h at RT) using HRP-conjugated goat anti-rabbit IgG as secondary antibody. Detection was accomplished as described in Figure S1

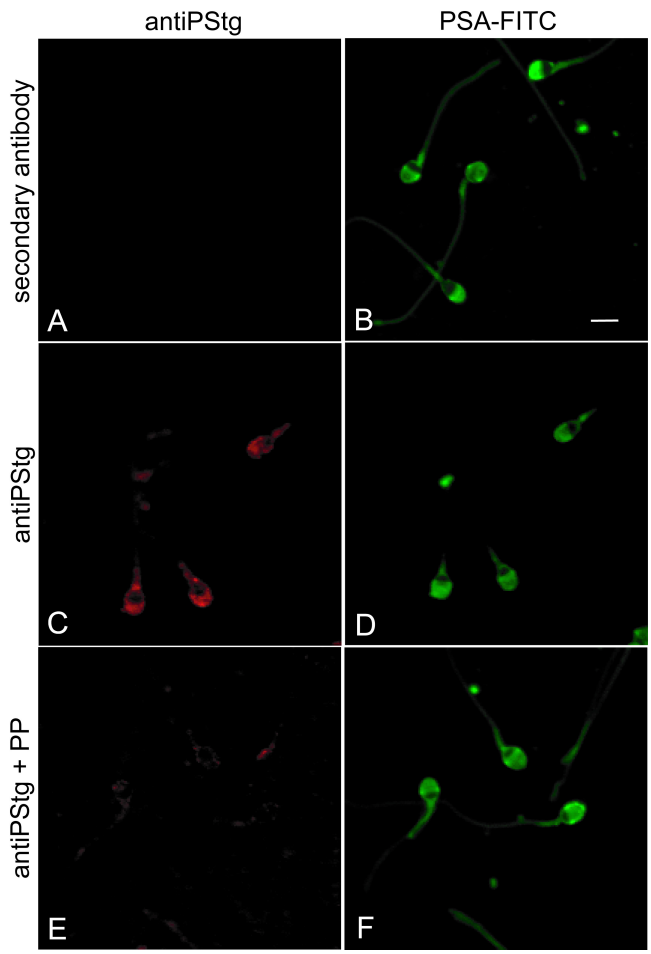

Figure S3: Specificity of the antiPStg labeling. Sperm were fixed and stained with antiPStg antibody (left panels) and PSA-FITC (right panels). In panels A and B, sperm were incubated with secondary antibody without antiPStg. In panels C and D, the antiPStg antibody was used at 135 nM. In panels E and F antiPStg was preincubated with an excess of phosphorylated peptide (PP, 1.35 µM).

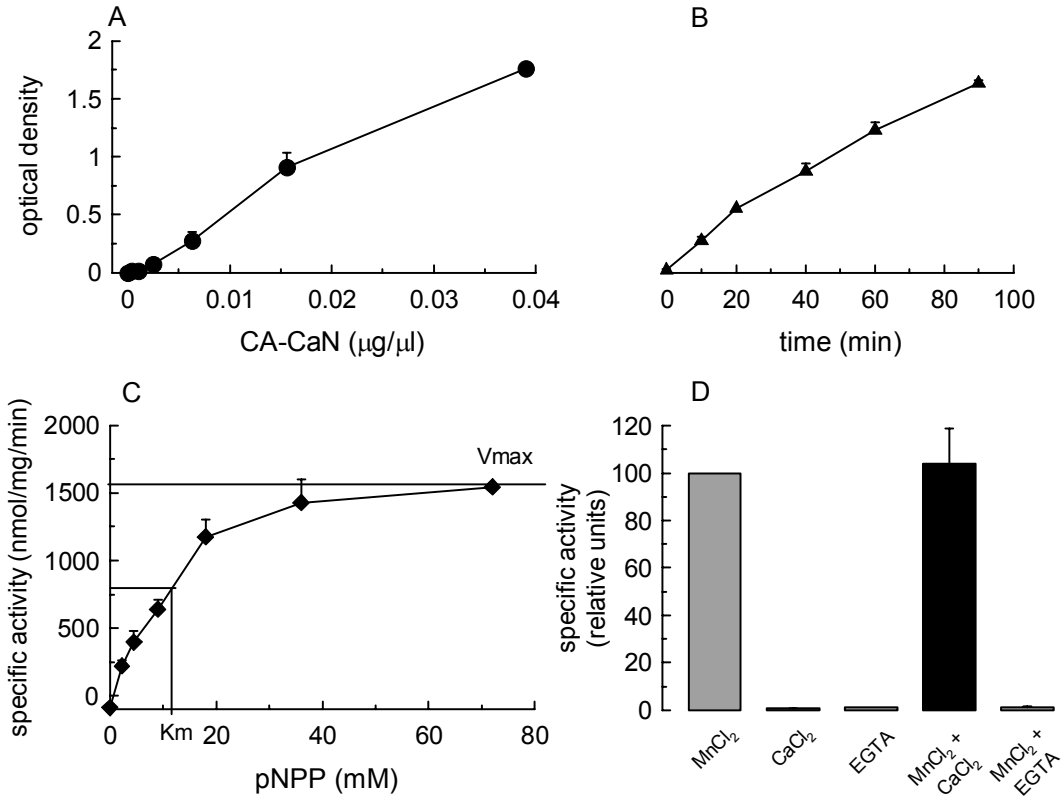

Figure S4. Phosphatase properties of recombinant CA-CaN. (A) Increasing concentrations of recombinant Ca-CaN were mixed in 45 µl phosphatase buffer (20 mM p-nitrophenylphosphate, 50 mM Tris-HCl, 0.1 mg/ml BSA, 1 mM DTT, 1 mM $MnCl_2$, pH 7.4) for 1h at 30°C. The reaction was stopped with 450 µl 0.5 M Na_2CO_3 and optical density was measured at 405 nm in a BIO-RAD microplate reader. (B) A reaction mix containing 0.015 µg/µl CA-CaN was stopped at different times and optical density was measured. (C) Reaction mixes containing 0.015 µg/µl CA-CaN and increasing concentrations of pNPP were incubated for 1 h at 30°C and the optical density was measured. Specific activity was estimated considering a 18300 p-nitrophenol molar extinction coefficient ($OD.cm^{-1}.M^{-1}$). (D) Reaction mixes containing 0.015 µg/µl CA-CaN and 1 mM $MnCl_2$, or 1 mM $CaCl_2$, or 1 mM EGTA, or 1 mM $MnCl_2$ + 1 mM $CaCl_2$, or 1 mM $MnCl_2$ + 1 mM EGTA were incubated for 1 h at 30°C and the optical density was measured. Data represent the mean and s.e.m. of at least 3 independent experiments.

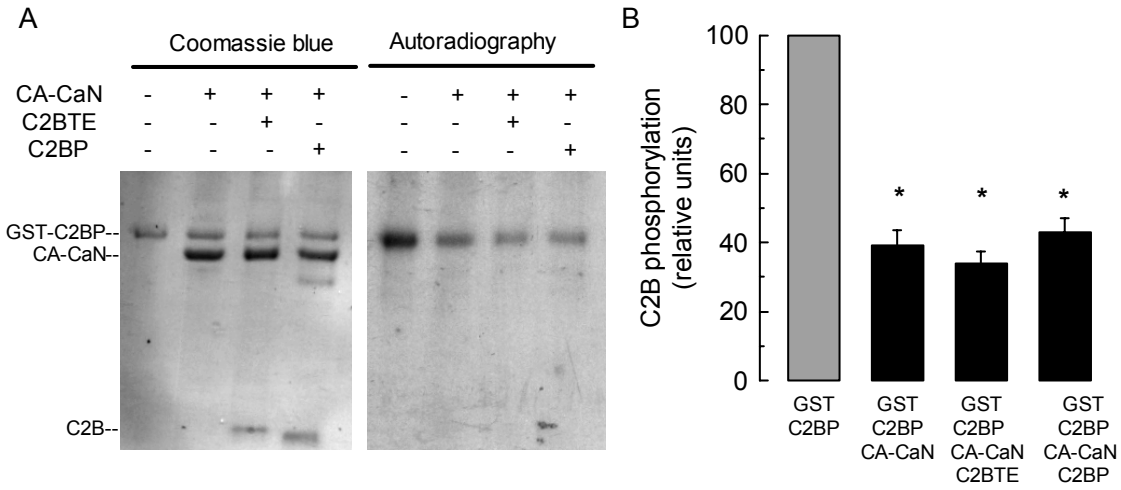

Figure S5. Recombinant proteins do not prevent synaptotagmin C2B domain dephosphorylation. (A) 1.8 μM GST-C2B domains immovilized in gluthation beads were incubated for 40 min at 37ºC with PKCβII under activating conditions in the presence of [γ^{32}P]ATP. The beads were washed and then incubated for 1h at 30ºC in the presence or absence of 1.8 μM constitutively CA-CaN, 3.6 μM C2BTE, and 1.8 μM cold phosphorylated C2B. Samples were then resolved by SDS-PAGE. Total proteins (left panels) are shown by coomassie blue stain. Phosphorylated proteins were detected by autoradiography (right panels). (B) Autoradiographies from three experiments as shown in A were quantified and normalized for the protein load. Data represent mean $\pm$ s.e.m of 3 independent experiments. Asterisks indicate significant differences from the untreated control (one way ANOVA and 95% confidence interval for each condition).

Reference List

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2. Rodriguez, A., Martinez-Martinez, S., Lopez-Maderuelo, M. D., Ortega-Perez, I., and Redondo, J. M. (2005) *J. Biol. Chem.* **280,** 9980-9984