**Figure EV1.** PKCB and PKCA regulate mast cell secretion.

A  β-hexosaminidase release in wild-type RBL cells induced by PDBu (100 nM) and ionomycin (100 nM). Dose-dependent inhibition of secretion with PKCB inhibitor, ruboxistaurin (n = 10).

Values are mean ± SEM.

B  PKCB knockout in RBL-2H3 cells, achieved with genome editing using TALENs. Genomic DNA sequences targeted by TALENs had deletions in all three alleles (three copies of chromosome 1; the 2-nt and 5-nt deletions cause a frameshift, and the 54-nt deletion is across an exon/intron boundary).

C  IgE-induced β-hexosaminidase release in RBL cells. Wild-type cells (in pink) compared to PKCB knockout cells (in blue). siRNA knockdown of PKCA in PKCB knockout cells further reduced secretion in PKCB knockout cells (n = 10). Values are mean ± SEM; *P < 0.05, **P < 0.001.

D  Representative single-cell Ca²⁺ recordings in wild-type RBL cells stimulated with antigen (n = 3).

E  Single-cell Ca²⁺ recordings in PKCB knockout cells stimulated with antigen (n = 3).

**Figure EV2.** Proteomics: identification of secretion-related phosphorylation targets of PKCB.

A  Schematic of three different conditions of cell stimulation for the phosphoproteomics experiments.

B  Number of mass spectrometry runs where each phosphopeptide was detected. Maximum number of times a phosphopeptide could be detected was nine times (three conditions with three biological replicas for each condition). Around half of all phosphopeptides were detected in all nine runs.

C  Four phosphopeptides were identified from VAMP8. The identified phosphopeptides had each a single phosphorylated residue at S17, T47, T53, and S54, respectively.
Figure EV3. Evolutionarily conserved phosphorylation sites in v-SNAREs.

A. Comparison of the sequences for SNARE domains of different v-SNAREs for rat. Phosphorylation sites found in our phosphoproteomics data (as well as in PhosphoSitePlus) are highlighted with a red box and phosphorylation sites reported in PhosphoSitePlus (but not found in our data) are highlighted with a black box. The 16 conserved SNARE domain layers are highlighted in yellow and numbered below, from −7 to +8, with arginine (R) at layer zero.

B. Comparison of 3,790 eukaryotic sequences for the different v-SNAREs. Serine or threonine residues are shown in red, alanine in blue, and all other in gray. Species category for each sequence is shown to the right with mammals in cyan, other vertebrates in pink, other animals in green, fungi in orange, and other eukaryotes in white.
Figure EV4. Evolutionarily conserved phosphorylation sites in SNARE-Qa, SNARE-Qb, and SNARE-Qc proteins.

A. Comparison of 4,564 eukaryotic sequences of the SNARE domain for SNARE-Qa proteins. SNARE layer +4 has been reported in PhosphoSitePlus to be phosphorylated for STX.

B. Comparison of 3,675 eukaryotic sequences of the SNARE domain for SNARE-Qb proteins. SNARE layer +4 has been reported in PhosphoSitePlus to be phosphorylated for VTI1A and SNARE layer +6 has been reported for GOSR2.

C. Comparison of 3,375 eukaryotic sequences of the SNARE domain for SNARE-Qc proteins. These sites have not yet been reported in PhosphoSitePlus.

Data information: Serine or threonine residues are shown in red, alanine in blue, and any other amino acid in gray. Species category for each sequence shown to the right in each panel.
**Figure EV5.** SNARE proteins in mast cell secretion.

A SNARE protein reconstitution was analyzed by SDS–PAGE of proteoliposomes containing t-SNAREs (SNAP23 and STX3), and the v-SNARE VAMP8 or VAMP8Glu (T47E, T53E, S54E).

B In vitro ensemble lipid-mixing assay. v-SNARE lipids labeled with acceptor dye (DiD) reconstituted with VAMP8 and t-SNARE lipids labeled with donor dye (DiI) reconstituted with SNAP23 and STX3. v-SNARE VAMP8Glu reduced the kinetics of lipid-mixing compared to wild-type VAMP8 (n = 3).

C SNARE complex formation of VAMP8, STX3, and SNAP23 is resistant to SDS. Half the samples were subjected to 5-min boiling prior to SDS–PAGE. On the left, each purified protein was analyzed individually, and on the right, SDS-resistant SNARE complex formation was assessed. There were no detectable differences between the SNARE complex formation of VAMP8 and VAMP8Ala, whereas VAMP8Glu was less efficient since free STX3 was observed in the non-boiled sample.

D Verification of siRNA knockdown of VAMP8 in RBL cells. qPCR was used to quantify mRNA for VAMP8 (n = 3). Values are mean ± SEM.

E Protein expression levels of VAMP8 mutants in RBL cells by Western blotting. siRNA knockdown of VAMP8 in combination with stable expression of VAMP8, VAMP8Ala, or VAMP8Glu, compared to endogenous expression of VAMP8. All VAMP8 constructs were resistant to siVAMP8 and untagged.

F Secretion of β-hexosaminidase triggered by antigen in RBL cells with siRNA knockdown of endogenous VAMP8. Four different conditions: wild-type RBL cells, stable overexpression of VAMP3, VAMP4, or VAMP7 (n = 6). Values are mean ± SEM.