Supplemental Data – Molecular Pharmacology

Inhibitors of PI(4,5)P_2 Synthesis Reveal Dynamic Regulation of IgE Receptor Signaling by Phosphoinositides in Mast Cells

Marcela de Souza Santos, Rose Mary Zumstein Georgetto Naal, Barbara Baird and David Holowka

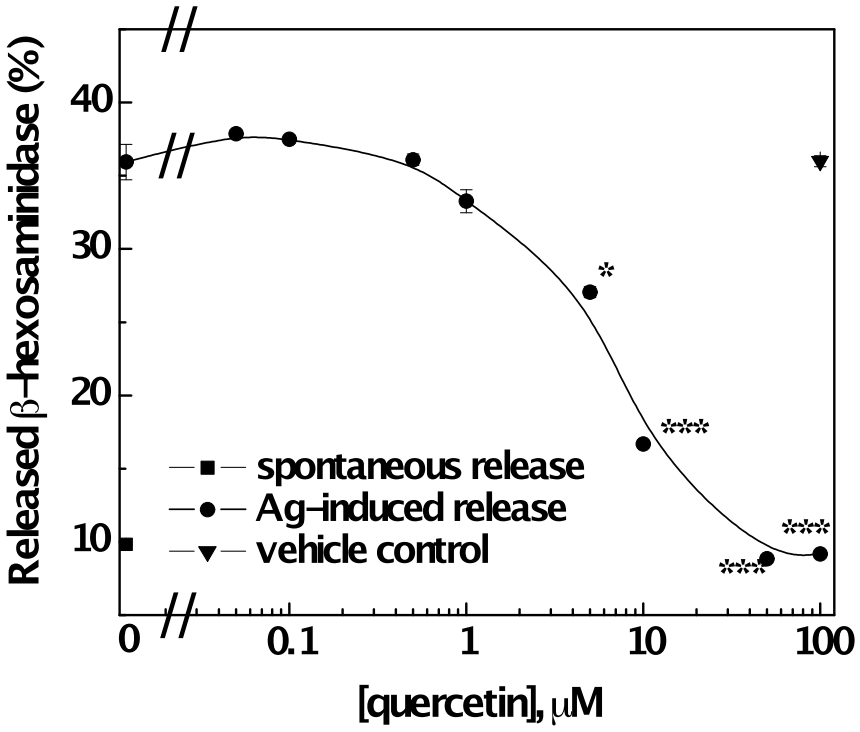

Figure S1. Inhibition of Ag-stimulated degranulation in RBL mast cells by quercetin. IgE-receptor mediated cell degranulation was assessed as described by Naal et al., 2004. Briefly, IgE-sensitized RBL cells attached to microtiter wells were pretreated with quercetin at the indicated concentrations, followed by cell stimulation with of DNP-BSA (0.1 μg/mL). The samples were then incubated for 1 h at 37 °C and degranulation was stopped by placing the cells on ice. To determine the amount of β-hexosaminidase activity released by the cells, an aliquot of supernatant was added to 1.2 mM β-hexosaminidase-substrate (4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide) in 0.05M sodium acetate buffer (pH 4.4), incubated for 30 min, at 37 ◦C, and quenched by addition of 0.1 M glycine-carbonate buffer, pH 10.0. Controls without antigen were used to measure spontaneous release, and total β-hexosaminidase levels were obtained following lysis of the cells with 0.1% Triton-X 100 prior to removal of supernatant. β-Hexosaminidase activity was quantified by measuring the fluorescence intensity of the hydrolyzed substrate in a fluorescence microplate reader (BioTek, Winooski, VT). In this representative curve for three independent experiments, error bars represent the standard deviation of triplicate samples from one experiment. **P*<0.05, ****P*<0.001.

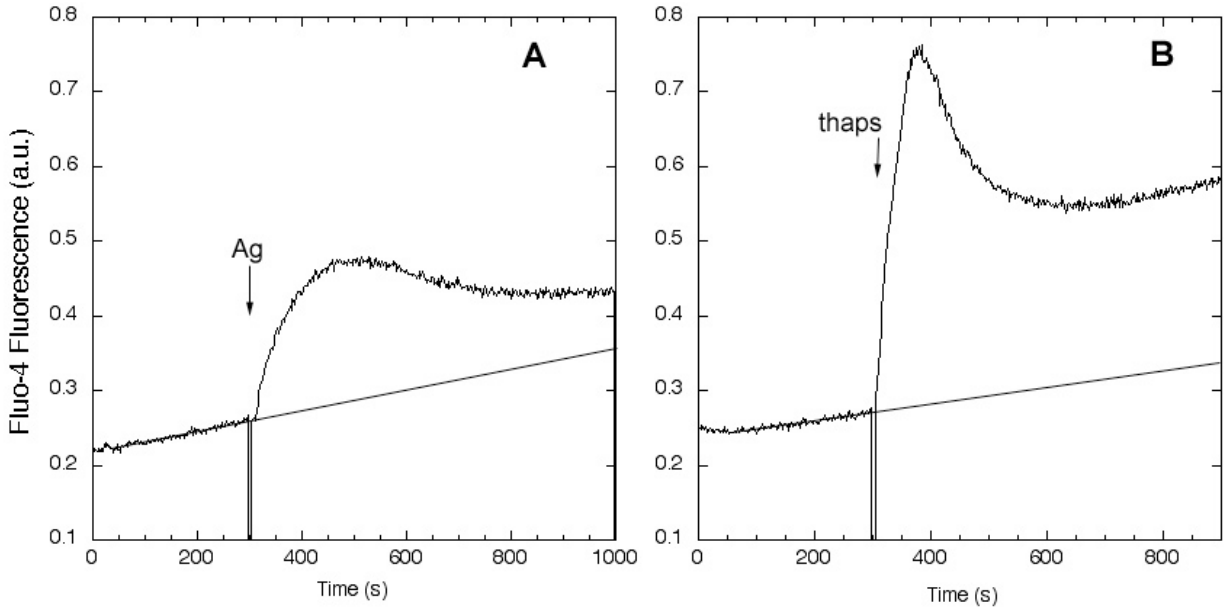

Figure S2. Stimulated Ca2+ responses to antigen (A) and thapsigargin (B) are maintained for at least 10 minutes at 37^{o}C. RBL mast cells sensitized with IgE and loaded with Fluo-4 were stimulated with 0.4 μg/ml DNP-BSA (A) or 0.2 μM thapsigargin (B) and monitored for ≥10 min as controls for responses in Figure 1 in which inhibitors were added several hundred seconds after stimulation was initiated.

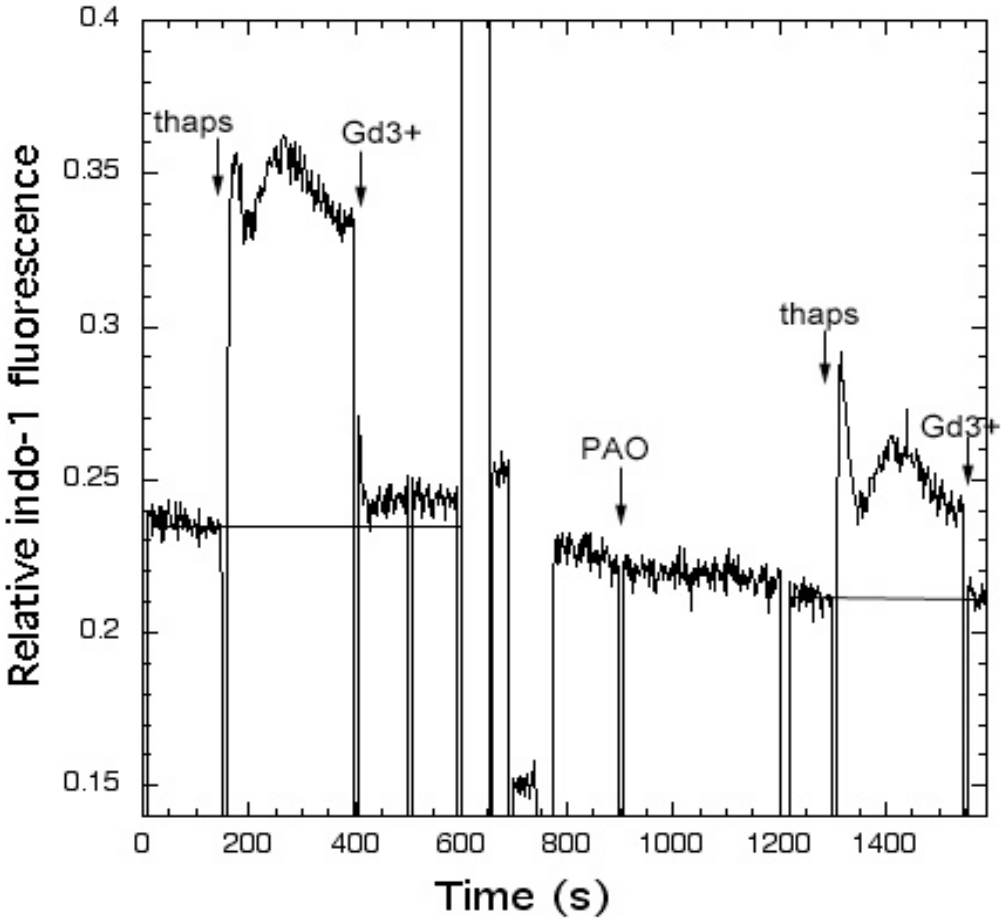

Figure S3. Preincubation of RBL cells with PAO for 10 min at 37°C causes substantial inhibition of thapsgargin-stimulated SOCE. RBL cells loaded with indo-1 AM were stimulated with 0.2 uM thapsigargin either before (left side) or after (right side) treatment with 1 μM PAO for 10 min prior to stimulation. $Gd^{3+}Cl_3$ (1 μM) was added 250 sec after stimulation to assess specificity of CRAC channel activation. Pretreatment of cells with PAO caused 48% inhibition of the peak SOCE response to thapsigargin, assessed 150 sec after addition, and normalized to the total indo-1 fluorescence following cell lysis by TX-100. Representative of two independent experiments.

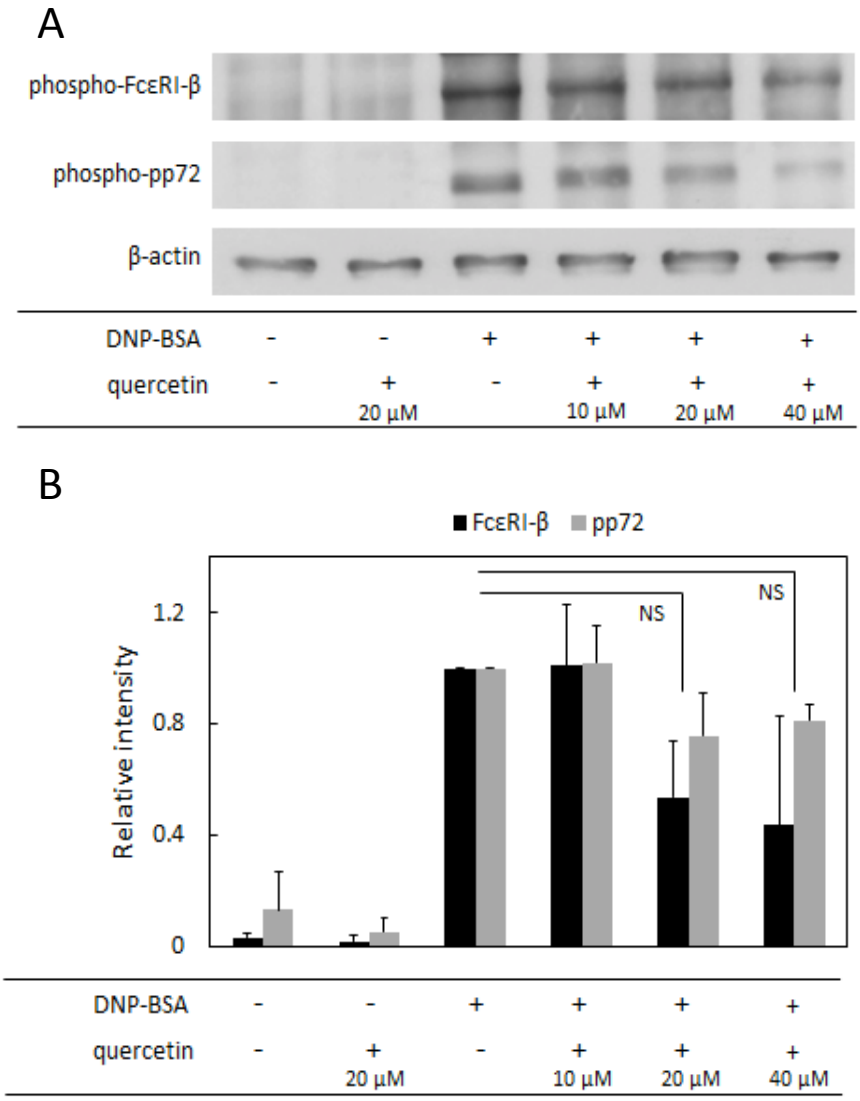

Figure S4. Effects of quercetin on antigen-stimulated tyrosine phosphorylation of pp72-Syk substrate and FcεRI β. (A) RBL cells ($4x10^6$ cel/mL) were sensitized overnight with anti-DNP IgE, quercetin was added as indicated, and tyrosine phosphorylation was stimulated for 3 min at 37°C with 0.1 μg/mL DNP-BSA. Whole cell lysates were resolved by electrophoresis on 12.5% polyacrylamide SDS gels and electrotransferred to nitrocellulose membranes. After blocking non-specific binding with 5% fish gelatin, membranes were probed with anti-phosphotyrosine 4G10 mAb conjugated with horseradish peroxidase (1:2000 dilution, Millipore, Billerica, MA). Immunoreactivity was detected using an enhanced chemiluminescence detection kit (AMRESCO, Solon, OH). Cellular actin was re-probed as a loading control. **(B)** Quantification for three independent experiments is expressed as the normalized intensities relative to stimulated control samples. Pixel intensities of the bands were determined by densitometric analysis and normalized by the intensity of β-actin using ImageJ. Error bars represent s.d. of three independent experiments and ANOVA statistical analysis followed by Dunnett's test revealed no statistical significance between quercetin-treated and control (+ antigen) samples.

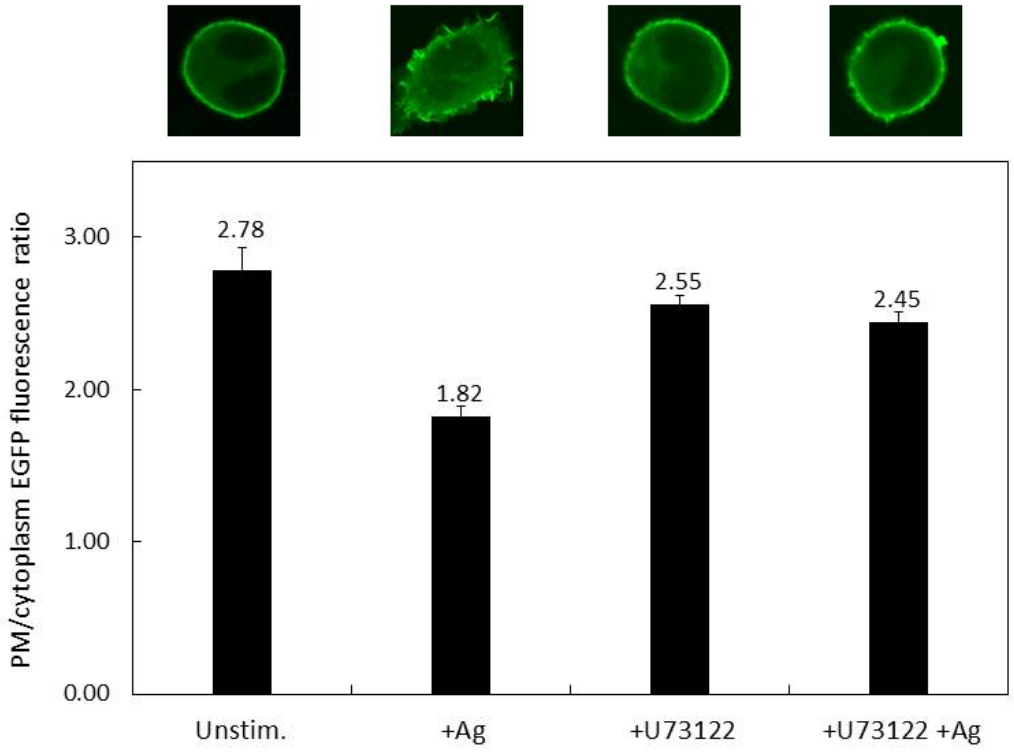

Figure S5. The PLC inhibitior U73122 reduces antigen-stimulated loss of $PI(4,5)P_2$ from the plasma membrane, but does not reveal stimulated net synthesis of $PI(4,5)P_2$ at the plasma membrane. PLCδ PH-EGFP expressed in RBL-2H3 cells was used to monitor the ratio of $PI(4,5)P_2$ at the plasma membrane to that in the cytoplasm as described in Materials and Methods. Changes in this ratio due to stimulation by 0.1 μg/ml antigen for 5 min at 37°C in the presence and absence of 2 μM U73122 were monitored for 20 cells for each sample in a single representative experiment. Error bars show SEM and images above histograms show representative cells under each condition of measurement.

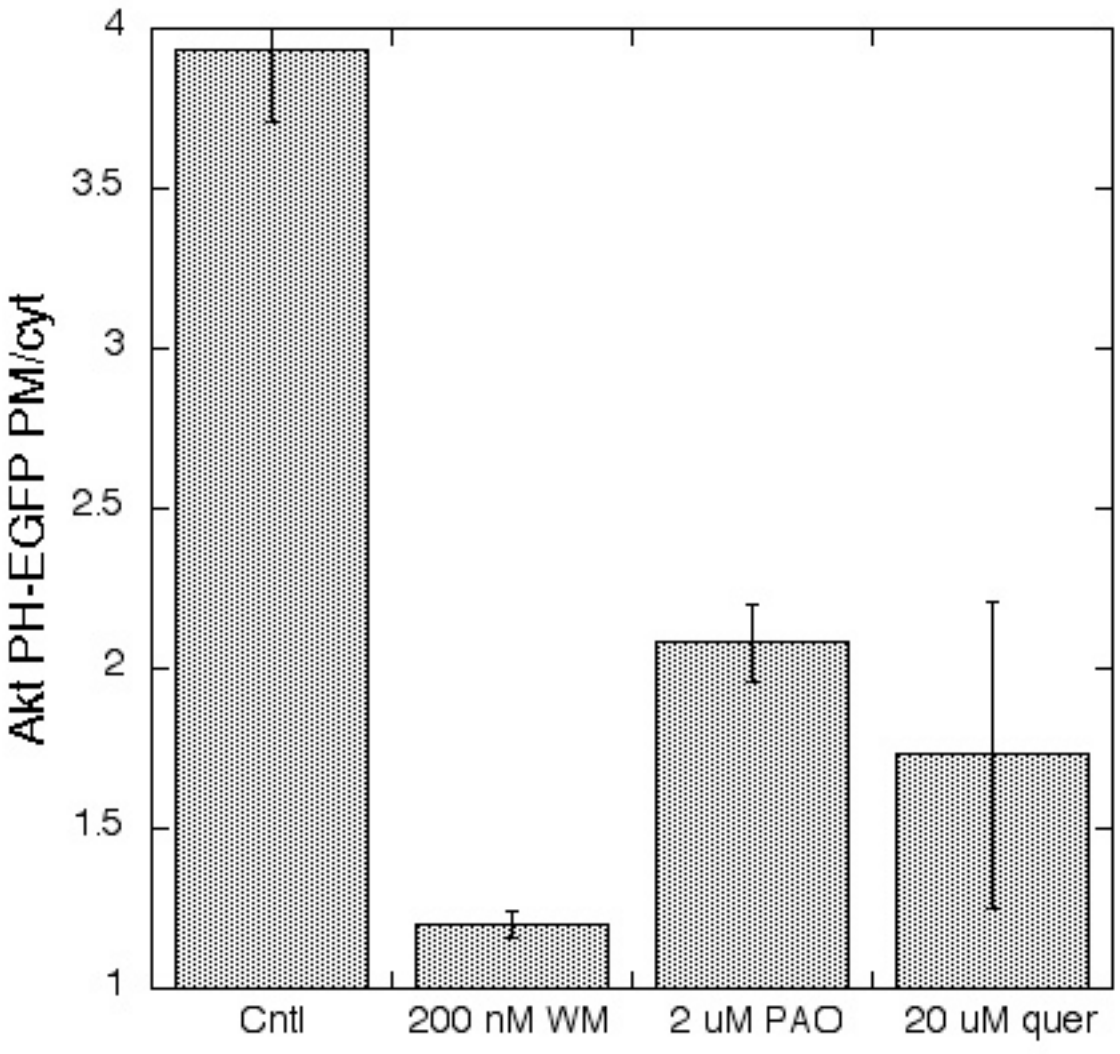

Figure S6. PM levels of PI(3,4,5)P3 detected by Akt PH-EGFP are less effectively reduced by PAO or quercetin than by wortmannin. To assess the effectiveness of 2 μM PAO and 20 μM quercetin to inhibit PI3-kinase activity, we compared the ratio of PM to cytoplasmic (cyt) Akt PH-EGFP fluorescence as described for PLCδ PH-EGFP in Materials and Methods. RBL cells expressing Akt PH-EGFP and untreated or treated for 5 min at 37^{o}C with 200 nM wortmannin (WM), 2 μM PAO, or 20 μM quercetin were fixed as described in Materials and Methods and imaged by confocal microscopy. Errors bars show SEM for 12-15 cells for each sample from one representative experiment.

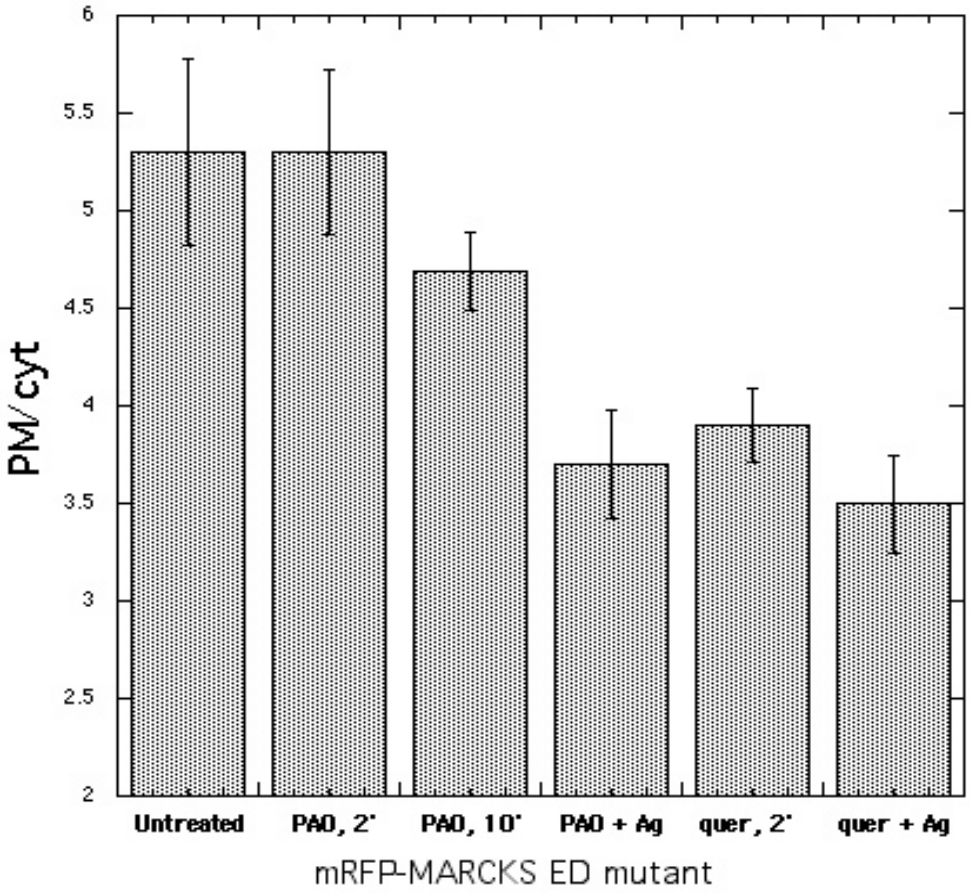

Figure S7. PAO and quercetin cause displacement of the MARCKS effector domain from its binding to phosphoinositides at the PM. IgE-sensitized RBL cells transiently transfected with mRFP-labeled MARCKS effector domain (MARCKS ED) that was mutated to prevent displacement by PKC phosphorylation (Gadi et al., 2011) exhibit dissociation of MARCKS ED from the PM following incubation with 2 μM PAO for 10 min (but not 2 min), 20 μM quercetin for 2 min, or PAO (2 μM) + antigen (0.8 μg/ml DNP-BSA) or quercetin (20 μM) + antigen for a total of 5 min each. Assessment of displacement from the PM (decrease in the PM/cytoplasmic ratio) was determined as previously described (Smith et al., 2010). Error bars show SEM for > 15 cells per sample from one of two consistent experiments.

References:

Naal RMZG, Tabb J, Holowka D, and Baird B (2004) In situ measurement of degranulation as a biosensor based on RBL-2H3 mast cells. *Biosens Bioelectron* **20:** 791-796.