Supplemental Online Material

Materials and Methods

Fura-2 Calcium Imaging

Coverslips containing the chromaffin cells were placed in a Petri dish containing 3 μM cell permeant Fura-2 (Molecular Probes, Invitrogen Co.) for 15 min. The cells were then gently washed twice with buffer solution such that the dye was removed and either buffer solution (control), 10 μM Blebbistatin, or 4 μM Cytochalasin-D was added onto the cells. The dishes were mounted on a custom built stage for an inverted microscope (Zeiss Axiovert 135 TV) equipped with a Zeiss FT425 dichroic filter and a Schott GG495 emission filter, and protected from light for a period of 10 min prior to imaging. The fluorescence intensity was recorded using 100 ms exposures at a rate of 0.2 Hz for a total period of 150 sec using a charge-coupled device (iXon EMCCD, Andor Technology). At each time point a pair of images was acquired, using a filter wheel (Lambda 10-2, Sutter Instruments) to switch between 358 nm and 390 nm excitation wavelengths. Images were stored as 16-bit integer matrices and were analyzed using custom written procedures in Igor Pro. The background fluorescence intensity was determined for each image at each wavelength in the sequence of images by taking the average plus three standard deviations of the intensity measured from a ~20 μm^2 square region in each image in an area which did not contain any cells or floating particles. For each image the background was subtracted from the average fluorescence intensity from a ~20 μm^2 square region within each cell. The ratio of the Ca^{2+} sensitive (390nm) over the Ca^{2+} insensitive (358nm) channel was then obtained. The response from all the cells within a treatment

group was averaged at each time point. The data is presented as MEAN ± SEM, where n = 12 cells for control, 24 cells for Blebbistatin, and 15 cells for Cytochalasin-D treated cells. Student's unpaired T-test was used to obtain the statistical significance of the measured data.

Immunofluorence microscopy detection of PKC and Myosin II

Resting chromaffin cells were treated with the different pharmacological reagents and fixed with 3.7% formaldehyde for 10 min after 30 min incubation with the drug. Cells were then permeabilized with 0.1% Triton X-100 for 5 min and labeled with an anti-PKC antibody (clone MC5, BD Biosciences) that reacts with the conventional alpha isoform (and cross-reacts with the beta isoform) or with anti-nonmuscle Myosin II (Heavy Chain A Polyclonal Antibody, Covance inc.). After washing, cells were incubated with Alexa 488 goat anti-mouse IgG1 antibody (for PKC labeled cells) or goat anti-rabbit IgG H+L (for Myosin labeled cells) and subsequently imaged using confocal microscopy.

Results

In order to confirm the specificity of the pharmacological manipulations it was necessary to confirm that there was no effect on intracellular Ca^{2+} levels at rest or Ca^{2+} influx following stimulation by ionomycin. For this purpose, chromaffin cells were labeled with the ratiometric dye Fura-2 and imaged using fluorescence microscopy. The ratio of the fluorescence intensity at 390 nm over 358 nm excitation wavelength was measured before addition of ionomycin and found to be similar between control and Blebbistatin ($p > 0.64$) and between control and Cytochalasin-D ($p > 0.65$) treated cells

(Supplemental Fig. 1A). This indicates that the pharmacological manipulations used in our study had no effect on the levels of intracellular Ca^{2+} at rest. Another possible indirect effect of the pharmacological agents would be to influence Ca^{2+} influx following stimulation by ionomycin. To test this, a glass pipette with ~5.0 µm tip diameter containing 10 µM ionomycin solution was positioned ~20 µm away from the cells and a 30-sec 1.0×10^4 Pa puff was applied to the pipette using a pressure application system (PicoSpritzer II, Parker-Hannifin/General Valve, Fairfield, NJ, USA) to promote Ca^{2+} entry into the cells. The Ca^{2+} dynamics were monitored as fluorescence intensity ratios obtained every 5 sec for a total of 150 sec (30 images). The response of all the cells per treatment group was averaged at each time point to obtain the average time course of intracellular Ca^{2+} levels for each treatment group. No significant difference was observed between control, Blebbistatin and Cytochalasin-D treated cells (Supplemental Fig. 1B). The minimum of the averaged ratios, which occurs when the intracellular Ca^{2+} amount is maximum, was similar for all treatment groups (control and Blebbistatin: $p = 0.46$, control and Cytochalasin-D: $p = 0.80$). These results thus clearly indicate that there is no difference between treatment groups in the intracellular Ca^{2+} levels at rest (Supplemental Fig. 1A) or in the amount of Ca^{2+} influx following ionomycin stimulation of chromaffin cells (Supplemental Fig. 1B).

Another factor that may contribute to our observations is the redistribution of protein kinase C (PKC) upon Cytochalasin-D or Blebbistatin treatment. Therefore, to rule out this possibility, resting chromaffin cells were labeled with a PKC antibody (clone MC5) and PKC localization was compared between control cells and cells treated with Blebbistatin or Cytochalasin-D. Again, the results indicated that neither inhibitor affected

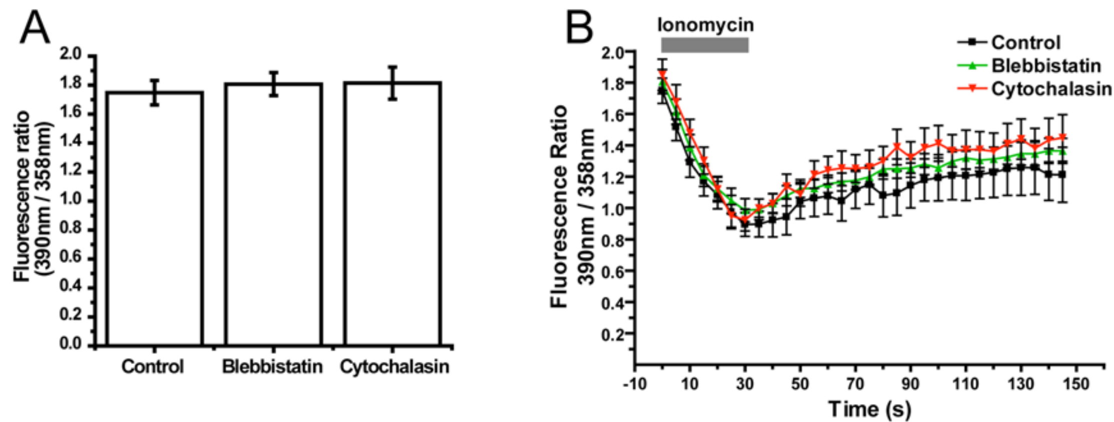
the distribution of PKC in the cells (Supplemental Fig. 2). Overall, these results confirm that Blebbistatin and Cytochalasin-D are specific and that their effects observed in our study are not due to indirect inhibition of signaling molecules like Ca^{2+} or PKC, but rather due to inhibition of myosin II or actin polymerization respectively.

To investigate the effects of drug treatment on myosin II localization, chromaffin cells were fixed and labeled with anti-myosin II after drug treatment. As shown in the Supplemental Fig. 4, treatment with Blebbistatin, cytochalasin-D or ionomycin did not cause detectable differences in myosin II localization.

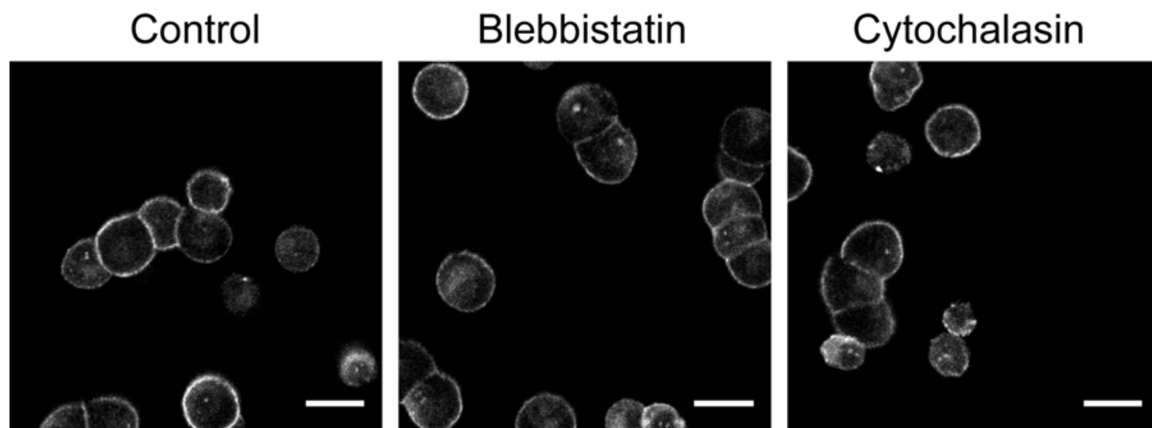
Supplemental Reference

Nüße O, Lindau M (1990) GTPγS-induced calcium transients and exocytosis in human neutrophils. Bioscience Reports 10:93-103.

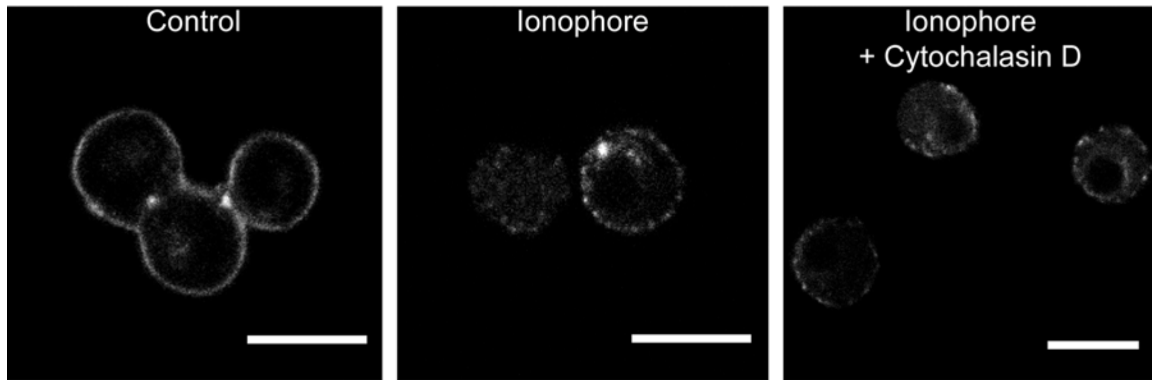
Supplemental Figures



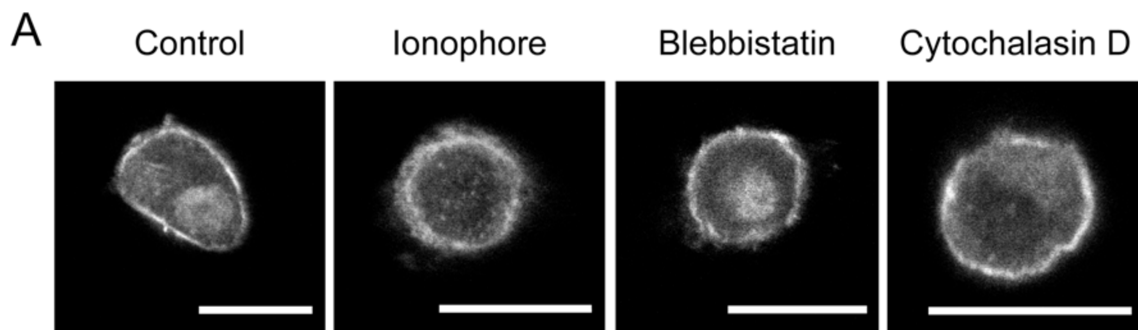
Supplemental Figure 1: Effects of Cytochalasin-D and Blebbistatin on chromaffin cell calcium influx. Ratio of fluorescence intensity excited at 390 nm over 358 nm. Response from cells at rest (A) and following addition of ionomycin (B). Data is mean ± SEM, n = 12 cells for control, 24 cells for Blebbistatin and 15 cells for Cytochalasin-D.

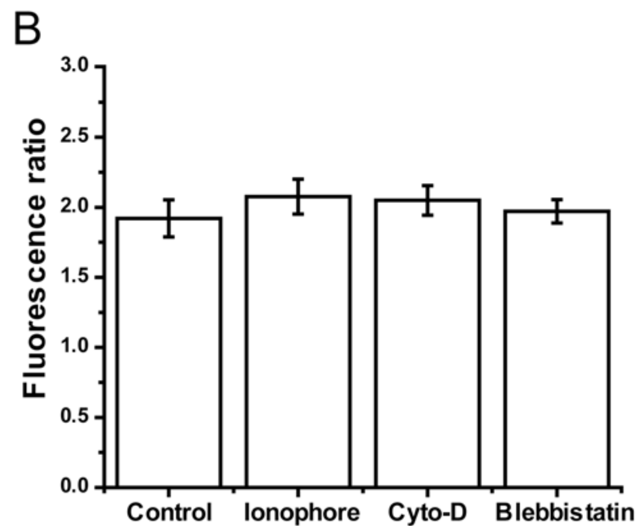


Supplemental Figure 2: Confocal micrographs of chromaffin cells labeled with an anti-PKC antibody shows both cytoplasmic and plasma membrane labeling. Data shows that PKC localization on resting cells is not affected by treatment with 4μM Cytochalasin-D or 10μM Blebbistatin. Scale bar represents 20μm.



Supplemental Figure 3: Cortical actin distribution after ionophore stimulation. Confocal images of Alexa 568 phalloidin labeled chromaffin cells. Cells were stimulated with either 10μM ionophore alone or in the presence of 4μM Cytochalasin-D for 30 min at 37°C. Control cells were incubated only in buffer. Scale bar represents 20μm.





Supplemental Figure 4: Effects of drug treatment on myosin II distribution on chromaffin cells. A) Confocal micrographs showing equatorial planes of chromaffin cells labeled with anti-myosin IIA. Resting cells were treated with either 10µM Blebbistatin, 4µM cytochalasin-D (Cyto-D) or stimulated with 10µM ionophore (ionomycin) for 30 min at 37°C. Control cells were incubated only in buffer. Scale bar represents 20µm. (B) Quantitative analysis of average peripheral/cytosolic fluorescence intensity ratio of anti-myosin IIA labeled cells show no significant differences between treatment groups. Data is shown as mean ± SEM, with n = 20 cells for each group.