Measurements of the Acidification Kinetics of Single SynaptopHluorin Vesicles

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Supporting Material

Materials and Methods

Isolation of SynaptopHluorin Vesicles

SynaptopHluorin vesicles were prepared from whole brains dissected from transgenic mice between 20-25 days old. Brains were immediately frozen and stored at -80 ^{o}C before vesicle isolation. Five to ten brains were pooled in liquid nitrogen for synaptic vesicle isolation and pulverized into a powder using a Waring commercial blender. The powder was re-suspended in homogenization buffer (0.3 M sucrose, 50 mM HEPES, pH 7.4, 2 mM EGTA) at a concentration of 8.5 ml/brain and homogenized with 10 passes in a Teflon-glass homogenizer. The homogenate was centrifuged at 37,000 rpm (MLA-80 rotor) for 28 min at 4 ºC in a Beckman-Coulter (Brea, CA) tabletop ultracentrifuge. The resulting supernatant was loaded onto a 0.6 M/1.5 M sucrose step gradient and centrifuged at 56,000 rpm for 72 min at 4 ºC. Synaptic vesicles were collected from the interface of the 0.6 M/1.5 M sucrose steps. Isolated synaptic vesicles were diluted to ~1 mg/ml as determined using Bio-Rad (Hercules, CA) protein assay kit with bovine serum albumin as a standard before being frozen and stored at -80 ºC for up to 6 months. Before use the vesicles were diluted into load buffer (20 mM HEPES, 4 mM $MgSO_2$, 4 mM KCl, 110 mM potassium acetate, pH 7.4).

Fabrication of PDMS wells

Imaging wells were generated by punching holes in a thin layer of polydimethylsiloxane (PDMS) using aluminum tubing (~5 mm diameter). The PDMS chip was sealed irreversibly to a clean borosilicate glass coverslip by oxidizing the PDMS surface and glass coverslip in oxygen plasma. Before oxidation, the glass coverslip was cleaned thoroughly by boiling for 1 hour in a 3:2:1 mixture of water, ammonium hydroxide, and 30% hydrogen peroxide, followed by thorough rinsing with ultra pure water and drying for 30 min at 60 ^{o}C. The PDMS was cleaned with a 100% ethanol wash before plasma treatment. To facilitate synaptic vesicle adsorption to the glass surface, the wells were functionalized with 3-aminopropyltriethoxysilane (ATPES) following a modified protocol from Qin, et al (*1*). The wells were filled with 2% APTES in acetone for 5 min under N_2. The wells were washed 3× with acetone followed by one rinse with DI water then allowed to dry under N_2 overnight.

Optical Setup

A home built setup (Figure 1B) was used to generate total internal reflection fluorescence by directing 488 nm light from a 20 mW solid-state laser (Coherent Inc., Santa Clara, CA) off axis, via a polychroic mirror, onto the outer edge of the back aperture of a high numerical aperture objective (1.45NA, Nikon, Tokyo, Japan), creating total internal reflection at the coverslip/water interface (Figure 1B). Molecules on the surface were excited by a ~300 nm thick evanescent field that results from the internal reflection of the laser beam. Fluorescence emission was collected through the objective and filtered by a 550/100 nm bandpass filter before being imaged onto a PhotonMax EMCCD camera (Princeton Instruments, Trenton, NJ). To remove any

potential drift due to stage vibrations, the microscope stage was fitted with a Continuous Reflective-Interface Feedback Focus System (CRIFF, ASI Imaging, Eugene, OR) that monitors the position of the focal point and holds it within 0.1 µm for several hours. SpH vesicles were loaded into the PDMS well where they immediately sat on the APTES-modified glass surface. When sufficient surface coverage was obtained, unbound or "loose" vesicles were rinsed with three applications of vesicle-free buffer. The well was then refilled with load buffer and ATP and glutamate were added simultaneously while the fluorescence signal was collected with Metamorph software (Molecular Devices Silicon Valley, CA). The fluorescence was monitored for 80 sec total with 200 msec frames collected without the use of a shutter.

Data analysis

SpH vesicles were selected using Metamorph software by defining a region of interest (ROI) that encompassed a single, diffraction limited fluorescent spot. The maximum intensity was collected for each ROI in each frame. The average background intensity for each frame was subtracted from each ROI before further analysis. The background corrected intensity stack was then imported into a MATLAB (MathWorks, Natick, MA) script file. The script averaged the first ten frames and compared that to the intensity of the following frames to determine when the vesicle intensity decreased by 20%. The initial frame of the intensity decrease was chosen as the initial decay time, t_0. The fluorescence data from t_0 to the end was selected and fit with the exponential decay curve $F/F_0 = A*\exp(-k*t)$ where *A* and *k* were the fitted parameters and *t* is the time parameter starting at the program defined t_0. The rate of decay for each vesicle was binned into a histogram and fit with the appropriate distribution to determine the average acidification rate constant for each condition tested. Error bars for acidification rate constants correspond to the standard deviation of the mean determined by the distribution fit. Comparisons between different extravesicular vesicle conditions were analyzed using unpaired *t* tests. Acidification rates in units of pH/sec were used in *t* tests.

Figures

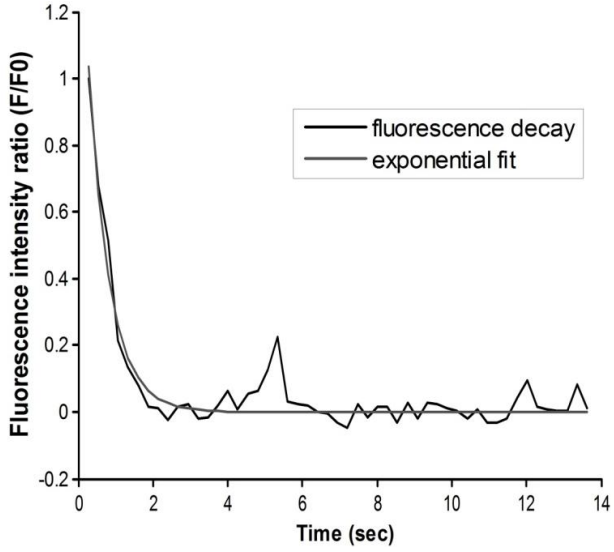

Figure S1: Fitting of fluorescence decay curve. Example showing the fit of the raw data with an exponential decay curve.

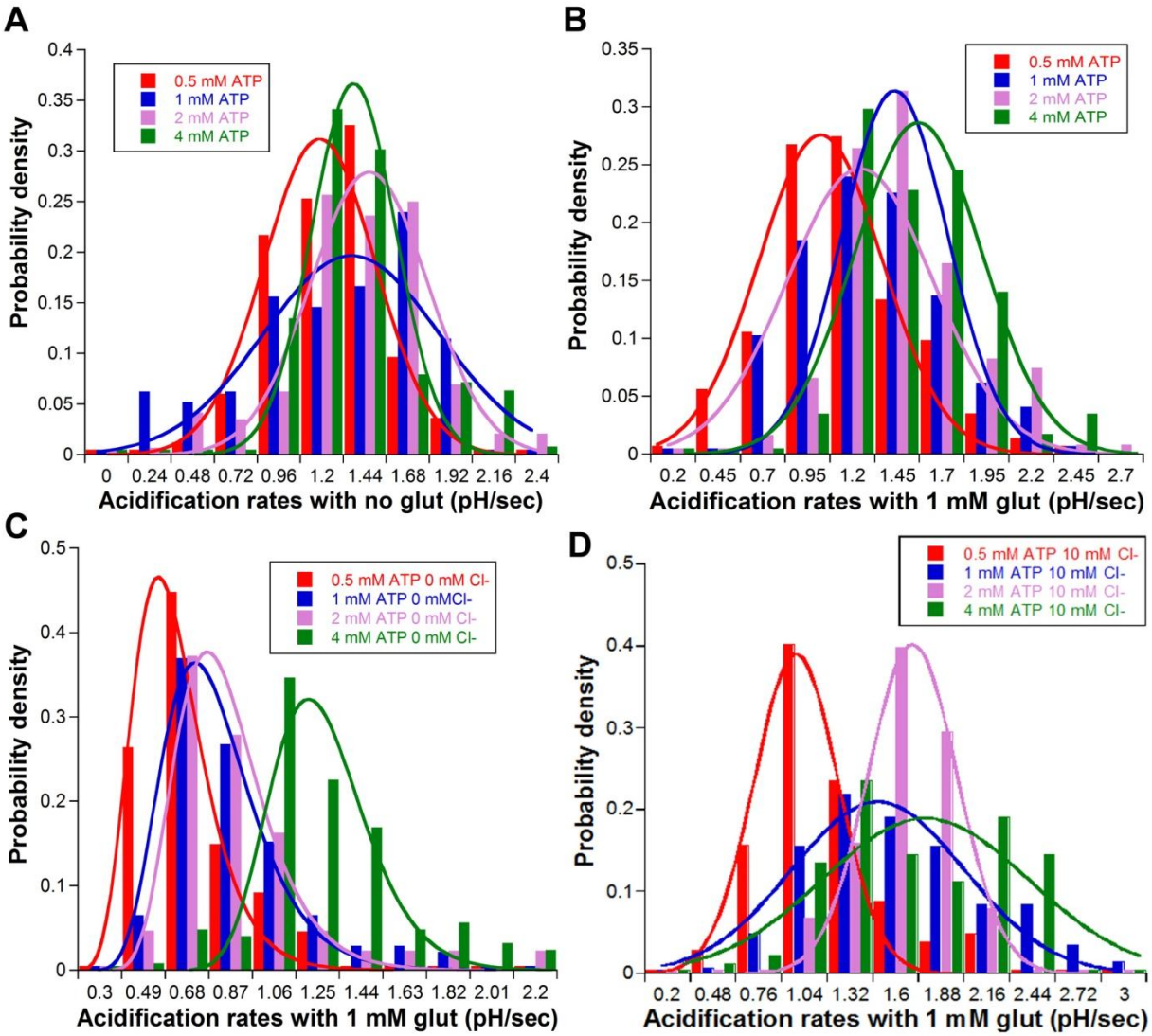

Figure S2: (A) Histogram of acidification rates determined for individual SpH vesicles in the presence of increasing ATP concentrations (no glutamate and 4 mM Cl^-). Each histogram was fit with normal distribution to determine the average acidification rate for each ATP concentration. (**B**) Distribution of the acidification rates of single SpH vesicles in the presence of glutamate. Acidification rates measured at 1 mM glutamate with varying ATP concentrations were binned then fit with normal distributions to obtain average values used in the Michaelis-Menten plots. (**C**) Rates of SpH acidification measured without Cl^- (performed in potassium gluconate buffer) at different ATP concentrations with 1 mM glutamate. Removal of chloride resulted in skewed distributions best fit by log-normal distributions. (**D**) Distribution of acidification rates measured in the presence of 10 mM Cl^- (20 mM HEPES, 104 mM potassium acetate,10 mM KCl, 4 mM $MgSO_4$, pH 7.4) at different ATP concentrations and at 1 mM glutamate. Presence of chloride gave rise to normally distributed acidification rates.

References

1. Qin, M., Hou, S., Wang, L. K., Feng, X. Z., Wang, R., Yang, Y. B., Wang, C., Yu, L., Shao, B., and Qiao, M. Q. (2007) Two methods for glass surface modification and their application in protein immobilization, *Colloid Surf. B-Biointerfaces 60*, 243-249.