SUPPLEMENTARY MATERIAL

METHODS

Transgenic *UAS-ANF-Tpz* flies were generated as described previously for *UAS-ANF-GFP* (1). A second chromosome insertion of *UAS-ANF-Tpz* was crossed to the *386-GAL4* driver (2) to create a homozygous line for the ANF-Tpz experiments. *UAS-synaptophluorin* (*UAS-n-Syb-pH*) flies, kindly provided by G. W. Davis (UC San Francisco), and *UAS-cameleon* flies (3) were crossed with *386-GAL4*, and heterozygous F1 progeny were studied. In all cases 3rd instar larvae were filleted, and boutons were imaged as described previously (4). However, YFP measurements utilized a 500/20 excitation filter and a 535/25 emission filter instead of the standard fluorescein optics used with other constructs. Most experiments were performed with a Ca^{2+}-free saline (130 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 36 mM Sucrose, 5 mM Hepes, 0.5 mM EGTA). For experiments in the presence Ca^{2+}, the extracellular solution contained: 70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 115 mM Sucrose, 5 mM Trehalose, 5 mM Hepes, 10 mM $NaHCO_3$, 10 mM glutamate. In experiments in which surface fluorescence was quenched, Ca^{2+}-free extracellular solutions contained 10 mM glutamate to provide buffering capacity over a wide range of pH values. Surface fluorescence quenching was induced by pH 5.5 solution. The vesicle pH gradient was collapsed by substituting 50 mM NaCl in the extracellular solution with NH_4Cl. The pH of the latter solution was then adjusted to a basic value (9.2 or 10.5) to dequench the pH indicators. Finally, release was evoked after collapsing the pH gradient by applying a high potassium medium (45 mM NaCl, 90 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 36 mM Sucrose, 5 mM Hepes) for 5 minutes. The preparation was then returned to the standard ammonium solution to measure the decrease in peptide content.

The indicators used here have been fully characterized and validated in mammalian cells. However, because their pH sensitivities could be shifted by temperature and differences in the *Drosophila* mileu, the pK of each indicator was calibrated using the Henderson-Hasselbach equation: pH - pK= log([conjugate base] /[conjugate acid]). For the fluorescent proteins studied here, only the conjugate base is fluorescent. Furthermore, since these indicators have neutral pKs, the maximal fluorescence signal (F_{max}) will be evident at very high pH values when essentially all of the indicator is deprotonated. Finally, we made use of the ability to clamp the pH throughout the cell by bath applying buffered solutions with high concentrations of ammonium.

To measure the pK of ANF-Tpz, we used a solution containing 50 mM ammonium chloride buffered to pH 7.2 to measure $F_{7.2}$ and then a similar solution buffered to 10.5 to determine F_{max}. Subsequently, we determined that F_{max} could also be measured at pH 9.2, a condition that did not evoke the spontaneous muscle contractions seen at pH 10.5. With these measurements we can rewrite the above equation to determine the pK of the indicator: $pK=7.2 - \log(F_{7.2}/(F_{max}-F_{7.2}))$ since [conjugate acid]=[total indicator]-[conjugate base].

To use ANF-Tpz to measure vesicular pH, we compared the fluorescence of the peptide before (F_v) and after setting the pH with ammonium to 10.5 (F_{max}). The fraction of indicator in the conjugate base form before collapsing the pH is therefore F_v/F_{max}, while the fraction in the conjugate acid form is $1-(F_v/F_{max})$. Therefore, vesicular pH (pH_v) can then be determined from the previously determined pK of the indicator: $pH_v = pK + \log(F_v/F_{max}/(1-(F_v/F_{max})))$.

The pK of synaptophluorin was measured with a similar approach (i.e., based on measurements in neutral and alkaline ammonium solutions). However, synapto-phluorin is localized on the cell surface as well as in SSVs. Therefore, vesicular fluorescence (F_v) must be assayed by quenching the surface signal with a pH 5.5 solution. The difference between the initial fluorescence and the vesicle signal (F_i-F_v) equals the surface protein fluorescence at pH 7.2 (F_{surf}). We then applied an ammonium solution to set vesicular and surface pH to 7.2. Under these conditions, total fluorescence equals the unchanged F_{surf} plus the new vesicle signal at pH 7.2. Subtracting the surface signal therefore reveals the fluorescence from the vesicle after collapsing the pH. The vesicular fluorescence under control conditions and after collapsing the pH gradient, and the measured pK were used with the Henderson-Hasselbach equation to calculate the initial vesicle pH.

For cytoplasmic pH, we measured YFP fluorescence at three pH values (7.2, 7.4 and 7.8) by using ammonium solutions. The second value gave no significant change in fluorescence compared to ammonium-free saline. Therefore, interpolation was not required with the Henderson-Hasselbach equation. Because the responses to the different calibration solutions were obtained in independent preparations, a standard error for the pK value is not produced by this method.

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