Nearly Neutral Secretory Vesicles in *Drosophila* Nerve Terminals

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ABSTRACT The acidity of mammalian secretory vesicles drives concentration and processing of their contents. Here, pH-sensitive green fluorescent protein (GFP) variants show that the ≥30-fold (H^+) difference between secretory vesicles (pH ≤ 5.7) and the cytoplasm (pH = 7.2) in mammalian cells is not present in peptidergic and small synaptic vesicles of the *Drosophila* neuromuscular junction. First, we find that fluorescence from Topaz-tagged atrial natriuretic factor, a peptidergic vesicle pH indicator, is only modestly affected by collapsing the H^+ gradient in type III synaptic boutons. Quantitation shows that peptidergic vesicles are nearly neutral (pH = 6.74 ± 0.05), even when temperature is elevated. Furthermore, small synaptic vesicles in glutamatergic synaptic boutons, studied with synaptophluorin, are as alkaline as peptidergic vesicles. Finally, yellow fluorescent protein measurements show that cytoplasmic pH is only slightly different than in mammals (pH = 7.4). Thus, the marked acidity of mammalian secretory vesicles is not conserved in evolution, and a modest vesicular H^+ gradient is sufficient for supporting neurotransmission.

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A major characteristic of mammalian secretory vesicles is that they are very acidic (i.e., luminal pH = 5.0–5.7) relative to the cytoplasm (pH = 7.2) (1). This large H^+ concentration gradient is present in large dense core vesicles that contain hormones and neuropeptides and in small synaptic vesicles (SSVs) that contain small molecule neurotransmitters. Collapsing the secretory vesicle pH gradient disrupts aggregation, enzymatic processing, and packaging of peptides and eliminates the electrochemical driving force for vesicular uptake of small molecules (1). Therefore, the large vesicular pH gradient is essential for regulated secretion in mammalian neurons and endocrine cells. This has led to the assumption that a large vesicular H^+ gradient is conserved in evolution. However, here we conduct in vivo imaging of pH-sensitive green fluorescent protein (GFP) variants transgenically targeted to peptidergic vesicles and SSVs to show that *Drosophila* neuromuscular junction secretory vesicles are only slightly acidic.

We began our studies by examining a neuropeptide/hormone called atrial natriuretic factor (ANF) tagged with the Topaz variant of green fluorescent protein (ANF-Tpz) in type III peptidergic neuromuscular junction boutons. Topaz fluorescence is quenched by acid with a neutral pK, and so collapsing the pH gradient in peptidergic vesicles of mammalian neuroendocrine cells produces a dramatic increase in ANF-Tpz fluorescence (2,3). To collapse the vesicular pH gradient, we applied an ammonium solution as described previously for *Drosophila* neuromuscular junctions (4). Ammonium and ammonia are in equilibrium (i.e., $NH_4^+ \rightleftarrows NH_3 + H^+$). Since ammonia is uncharged, it crosses membranes and binds protons until there is no pH gradient between the vesicle lumen and the extracellular solution. Setting the pH inside type III bouton vesicles to 7.2 in Ca^{2+}-free saline, which prevents activity-dependent release, only increased peptide fluorescence by 113 ± 7% ($n = 5$) (Fig. 1 *A*, *panels 1* and *2*), instead of the ≥10-fold effect expected.

We considered explanations for this small response that do not involve an alkaline vesicular lumen. First, we showed that this is not due to ANF-Tpz on the extracellular surface of terminals because application of pH 5.5 medium did not reduce the peptide signal: fluorescence changed by 1.9 ± 5.1% ($n = 4$). Thus, ANF-Tpz was only present inside the nerve terminals. Second, we tested whether sufficient ammonium was applied to collapse the vesicular pH gradient. Ammonium dose-response results showed that 50 mM ammonium, which is effective in larval neuromuscular junction (4), gave a maximal response (data not shown). Hence, ammonium was not limiting. Third, we investigated whether the pK of the fluorescent protein might be perturbed dramatically by some difference in the *Drosophila* milieu (e.g., lower ionic strength). Intravesicular pH was varied by applying ammonium solutions set at different pH values (e.g., Fig. 1 *A*, *panels 2* and *3*). Using the Henderson-Hasselbach equation, we calculated the pK of ANF-Tpz in *Drosophila* peptidergic vesicles to be 7.29 ± 0.04 ($n = 5$), a value only slightly different than in mammalian cells (3). Thus, the modest response to collapsing the pH gradient could not be attributed to insensitivity of the pH indicator. Fourth, we showed that the absence of extracellular Ca^{2+} in Fig. 1 *A* to prevent muscle contraction was not relevant: the fluorescence increase induced by collapsing the vesicular pH gradient was statistically identical in Ca^{2+}-containing saline (119 ± 15%, $n = 4$). Fifth, we verified that ANF-Tpz, like ANF-GFP (5–8), is targeted to secretory vesicles in *Drosophila*. ANF-Tpz fluorescence paralleled the known distribution

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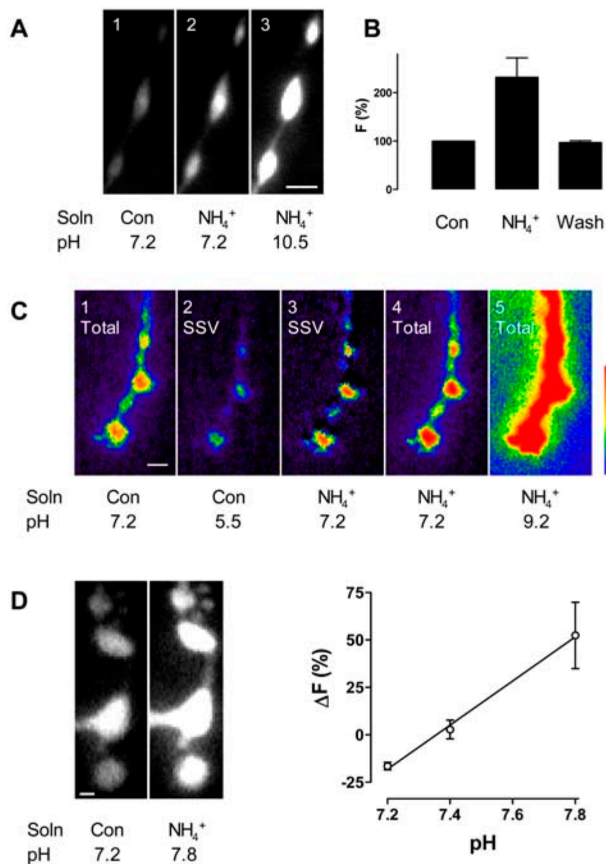

FIGURE 1 **(*A*) ANF-Tpz responses in type III boutons. (*1*) Peptide fluorescence under control conditions in Ca^{2+}-free saline; (*2*) approximate doubling of fluorescence after collapsing the vesicular pH gradient at pH 7.2; (*3*) increase in signal after setting the vesicular pH to 10.5. Bar is 2 μm. (*B*) The ammonium effect is reversible at pH 7.2 (n = 4). (*C*) Synaptophluorin responses in type I boutons. (*1*) Pseudo-color representation of synaptophluorin fluorescence under control conditions, which includes the vesicular and surface signals; (*2*) vesicular signal revealed after quenching surface fluorescence with pH 5.5 medium; (*3*) vesicular signal at pH 7.2. Obtained by subtracting surface signal (*1* – *2*) from total signal after setting the pH to 7.2 in all compartments with ammonium (*4*). (*5*) Total synaptophluorin signal after setting the pH to 9.2, which was used with *4* to calculate the pK of the indicator. Bar is 2 μm. (*D*) Cytoplasmic YFP responses in type I boutons. (*Left*) Fluorescence increase after setting the pH to 7.8. Bar is 1 μm. (*Right*) Change in YFP fluorescence versus cytoplasmic pH; n = 4 for pH 7.2, 4 for pH 7.4, and 3 for pH 7.8.**

of peptidergic vesicles: it was high in type III boutons and low in type I boutons, even though the UAS driver used here induces expression in both of these nerve terminals. Also, depolarizing terminals for 5 min after collapsing the pH gradient induced release of 65% ± 5% of the ANF-Tpz (n = 3), a robust response for neuronal peptide secretion. Hence, secretory vesicles must have contained ANF-Tpz, as had been found in mammalian cells (3). Sixth, the ammonium effect was reversible, showing that release and photobleaching did not affect our pH assay (Fig. 1 *B*). Thus, we were left to conclude that the small response to collapsing the vesicular pH gradient must reflect a limited vesicular H^+ gradient. The effect of collapsing the pH gradient combined with the calibrated indicator pK showed that the pH in peptidergic vesicles is 6.74 ± 0.05 (n = 5) (Table 1).

TABLE 1 pH of *Drosophila* peptidergic vesicles, SSVs, and nerve terminal cytoplasm

	Indicator	°C	pH
Peptidergic vesicle	ANF-Tpz	22	6.74 ± 0.05 (n = 5)
		37	6.64 ± 0.04 (n = 4)
SSV	Synaptophluorin	22	6.63 ± 0.13 (n = 5)
Cytoplasm	YFP	22	7.4 (n = 4)*

*The method used for cytoplasmic pH does not yield a standard error.

One potential basis for the difference between warm-blooded mammals and cold-blooded invertebrates such as *Drosophila* is physiological temperature. Since the vesicular pH gradient is generated by an enzyme, the vacuolar ATPase, it was conceivable that luminal acidity could increase with temperature. Therefore, to test whether temperature is a significant factor, neuromuscular junction preparations were maintained at 37°C for 30 min. Under these conditions, we first determined the pK of ANF-Tpz (pK (37°C) = 6.81 ± 0.08 (n = 4)). We then used this value along with the effect of collapsing the vesicular H^+ gradient at 37°C to determine the temperature dependence of luminal pH. As shown in Table 1, this change in temperature did not significantly alter vesicle pH. Results acquired at 30°C also supported this conclusion (data not shown). Therefore, the nearly neutral pH of *Drosophila* peptidergic vesicles cannot be attributed to temperature.

Previous studies of the *Drosophila* neuromuscular junction have utilized synaptophluorin to study SSV exocytosis and endocytosis (4,9). At glutamatergic type I boutons, much of the synaptophluorin signal originates from the cell surface, but the fluorescence from SSVs can be measured by quenching surface fluorescence with a pH 5.5 solution. Our finding that a pH 5.5 extracellular solution did not quench luminal protein fluorescence in *Drosophila* peptidergic vesicles validates this approach (see above). Synaptophluorin fluorescence is increased by collapsing the pH gradient in fly SSVs (4), showing that these organelles are more acidic than the extracellular medium. However, acid quenching of surface signal and collapsing the vesicular H^+ gradient were not combined on the same preparation, and the pK of synaptophluorin was not measured in *Drosophila*. Therefore, it is not possible to determine the luminal pH of *Drosophila* SSVs from prior studies.

Quenching the surface synaptophluorin signal (Fig. 1 *C*, *panel 2*) and applying ammonium at pH 7.2 (Fig. 1 *C*, *panel 4*) revealed the change in SSV synaptophluorin signal upon collapsing pH gradients (Fig. 1 *C*, *panel 3*). Then we varied pH after collapsing the H^+ gradient with ammonium (Fig. 1 *C*, *panels 4* and *5*) to determine that the pK of synapto-

phluorin in type I boutons is 7.55 ± 0.06 ($n = 5$). Finally, combining these measurements allowed us to calculate that the pH in SSVs is 6.63 ± 0.13 ($n = 5$) (Table 1). This is statistically identical to the results found with ANF-Tpz in type III boutons. Thus, as expected from mammalian studies, peptidergic vesicles and SSVs maintain the same luminal pH. However, in contrast to mammalian secretory vesicles, these organelles are nearly neutral in *Drosophila*.

The driving force for vesicular uptake of neurotransmitters is generated by the H^+ electrochemical gradient, which depends on the difference in pH between vesicles and the cytoplasm. However, cytoplasmic pH has not been measured in *Drosophila* nerve terminals. We reasoned that a cytoplasmic fluorescent protein with a neutral pK would be an appropriate indicator. Therefore, we used flies expressing cameleon, a construct that contains cyan and yellow fluorescent proteins (CFP and YFP) (10). Although CFP in insensitive to physiological pH, YFP's fluorescence spectra and pH sensitivity are similar to Topaz fluorescent protein (2). Typically, cameleon is used by exciting CFP (e.g., with 440 nm light), which then undergoes fluorescence resonance energy transfer with YFP that depends on Ca^{2+}. However, this fluorescence resonance energy transfer (and its Ca^{2+} dependence) can be precluded by directly exciting YFP with longer wavelengths of excitation (e.g., 500 nm). Therefore, we directly excited YFP and used this fluorescence as a cytoplasmic pH indicator.

For these experiments, we collapsed the pH gradient with ammonium solutions set at varying pH values. When pH was clamped to 7.8, cytoplasmic YFP fluorescence increased (Fig. 1 *D*, *left panels*). Thus, the cytoplasm was more acidic than 7.8. Furthermore, setting the pH to 7.2 decreased the YFP signal, showing that cytoplasmic pH was more basic than 7.2. Finally, the change evoked by setting the pH to 7.4 was not significant ($n = 4$) (Fig. 1 *D*, *right graph*). A cytoplasmic pH of 7.4 implies that there is only a fivefold H^+ concentration gradient in *Drosophila* neuromuscular junction secretory vesicles, rather than the ≥30-fold gradient found in mammals.

Our studies of *Drosophila* peptidergic vesicles and SSVs demonstrate that the extremely acidic pH that is characteristic of mammalian secretory vesicles is not evolutionarily conserved and so must not be essential for neurotransmission. Fully collapsing the vesicular pH gradient in mammalian cells disrupts condensation, packaging, and processing of secreted peptides and uptake of small molecule neurotransmitters. However, the effect of maintaining a smaller H^+ gradient has never been tested. Here modest luminal acidification was found at the *Drosophila* neuromuscular junction, a synapse that is essential for survival of the animal. In fact, our results clarify why synaptophluorin responses are so small in this preparation (4,9) and luminal processing enzymes have less acidic pH optima in fruit flies than in mammals (11). More generally, we have shown that a very acidic pH is not a defining characteristic for neurosecretory vesicles. Rather, previously reported large vesicular H^+ gradients may represent a feature associated with the evolution of vertebrates in general or mammals specifically.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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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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