Tetanus Toxin Is Transported in a Novel Neuronal Compartment Characterized by a Specialized pH Regulation*5

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Tetanus toxin binds specifically to motor neurons at the neuromuscular junction. There, it is internalized into vesicular carriers undergoing fast retrograde transport to the spinal cord. Despite the importance of this axonal transport pathway in health and disease, its molecular and biophysical characterization is presently lacking. We sought to fill this gap by determining the pH regulation of this compartment in living motor neurons using a chimera of the tetanus toxin binding fragment (TeNT HC) and a pH-sensitive variant of the green fluorescent protein (ratimetric pHluorin). We have demonstrated that moving retrograde carriers display a narrow range of neutral pH values, which is kept constant during transport. Stationary TeNT HC-positive organelles instead exhibit a wide spectrum of pH values, ranging from acidic to neutral. This distinct pH regulation is due to a differential targeting of the vacuolar (H+)-ATPase, which is not present on moving TeNT HC compartments. Accordingly, inhibition of the vacuolar (H+)-ATPase under conditions that completely abolish the intracellular accumulation of acidotrophic dyes does not affect axonal retrograde transport of TeNT HC. However, a functional vacuolar (H+)-ATPase is required for early steps of TeNT HC trafficking following endocytosis, and it is localized to axonal vesicles containing TeNT HC. Altogether, these findings indicate that the vacuolar (H+)-ATPase plays a specific role in early sorting events directing TeNT HC to axonal carriers but not in their subsequent progression along the retrograde transport route, which escapes acidification and targeting to degradative organelles.

The clostridial neurotoxin family is formed by tetanus (TeNT)2 and seven serotypes of botulinum neurotoxins (BoNTs, named A–G). They all share an identical structural organization, comprising a 100-kDa heavy (H) chain and a 50-kDa light (L) chain linked via a disulfide bond and other noncovalent interactions (1, 2) (Fig. 1a). The carboxyl-terminal part of the heavy chain (H50, 50 kDa) is responsible for membrane binding and internalization, whereas the amino-terminal domain (H50, 50 kDa) mediates membrane translocation of the L chain into the cytosol (1, 3). The L chain is a highly specific zinc endopeptidase, which is responsible for the cleavage of synaptic SNARE proteins necessary for neurotransmitter release (2). TeNT binds to motor neurons (MNs) at the neuromuscular junction. Upon internalization, TeNT is retrogradely transported toward the MN cell body, where it is released into the extracellular medium and enters adjacent inhibitory interneurons (4). In these cells, TeNT blocks the release of inhibitory neurotransmitters by cleaving VAMP/synaptobrevin, a member of the SNARE superfamily (1, 3).

We have recently developed an assay to follow the retrograde transport of TeNT in MNs using fluorescently tagged versions of the non-toxic TeNT HC binding fragment (5). In these cells, TeNT and TeNT HC are internalized and transported in morphologically identical organelles with overlapping speed distributions (6). These carriers are powered for their retrograde movement by distinct molecular motors, including cytoplasmic dynein and myosin Va (6–8). TeNT HC shares this compartment with the nerve growth factor and the low affinity neurotrophin receptor p75NTR (9). These findings validate TeNT HC as an ideal tool for dissecting the molecular machinery controlling axonal retrograde transport and the trafficking of neurotrophin receptors and their ligands in living MNs.

To date, the precise characterization of the intraluminal pH of this axonal retrograde pathway and its relevance in the regulation of transport in MNs remain unclear. These features are particularly important, because acidic pH triggers a conformational change in TeNT and other CNTs, allowing the active subunit to translocate through the endosomal membrane into the cytosol (9, 10). Moreover, acidic pH is known to drive the dissociations of ligands, including growth factors, from their receptors and influence their signaling and intracellular targeting (11, 12). We sought to address these important questions by preparing chimeras of TeNT HC and ratimetric pHluorin, a pH-sensitive green fluorescent protein mutant (13). Several pH-sensitive variants of green fluorescent protein have been developed by independent mutagenesis approaches and used as pH reporters to follow vesicular transport dynamics (14, 15). Whereas the wild-type green fluorescent protein has a largely unaltered spectrum between pH 5.5 and 10, the excitation spectrum of the ratiometric pHluorin shows two main peaks at 395 and 475 nm, the intensity of which reciprocally varies as a function of pH because of the protonation of its chromophore (13).

In this study, we demonstrated that moving axonal retrograde TeNT HC carriers display a neutral pH, which is kept constant during transport. In contrast, stationary TeNT HC organelles located in axons and in somas exhibit a wide range of pH values. The distinct pH regulation of these compartments is due to a differential targeting of the vacuolar (H+)-ATPase (vATPase), which activity is required for early events in the formation and/or sorting of axonal TeNT HC vesicles but not for their subsequent axonal transport.

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2 The abbreviations used are: TeNT, tetanus toxin; BafA1, bafilomycin A1, ConA, concanavalin A; DAMP, [3-(2,4-dinitroanilino)-3-aminotriazin-2-yl]acetic acid; GTP, guanosine 5′-triphosphate; G418, G418 resistance; IF, immunofluorescence; MN, motor neuron; PBS, phosphate-buffered saline; TeNT HC, carboxyl-terminal 50-kDa domain of tetanus toxin; vATPase, vacuolar (H+)-ATPase; VSV-G, vesicular stomatitis virus G protein; WB, Western blot.

* The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Videos 1–7.

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for 1 h in PBS containing 5% skimmed milk, the membranes were incubated with primary antibody for 1 h in the same buffer, washed with PBS, and incubated with horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was detected using enhanced chemiluminescence (ECL, Amersham Biosciences).

**Endocytosis Assays**—MN s were treated with 0.5 nM BafA1 for 15 min at 37°C and then incubated for 30 min with VSV-G-Kin-TeNT Hc (20 nM) or TeNT Hc labeled with disulfide-linked biotin (30 nM). After VSV-G-Kin-TeNT Hc incubation, the cells were fixed in 4% paraformaldehyde in PBS and blocked with 2% embryo-tested bovine serum albumin, 0.25% porcine skin gelatin, 0.2% glycerine, and 15% goat serum in PBS. The rabbit α-VSV-G tag antibody (BioGenes, Berlin, Germany) diluted 1:800 in blocking solution was incubated with the fixed cells for 30 min at room temperature. The cells were washed twice with PBS and then permeabilized with 0.1% Triton X-100 in blocking solution and blocked for a further 30 min. MNs were incubated with a primary mouse α-VSV-G (Cancer Research UK, London, UK) for 30 min, washed twice with PBS, incubated for 30 min with AlexaFluor goat α-mouse and α-rabbit IgG (Molecular Probes), and then incubated in 1:300 blocking solution. After extensive washing with PBS, coverslips were mounted with Mowiol 4–88. Alternatively, MNs incubated with TeNT Hc-biotin were cooled on ice and then treated three times with ice-cold 20 mM sodium 2-mercaptoethanesulfonate in neurobasal medium (Invitrogen), pH 8.3, for 15 min to cleave the biotin moiety from surface-bound TeNT Hc. The cells were washed three times in neurobasal medium, pH 8.3, once in PBS fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 and then blocked in solution blocking. TeNT Hc-biotin was revealed using streptavidin-AlexaFluor-488 1:500 in blocking solution. The MNs were extensively washed in PBS and mounted in Mowiol 4–88.

**Fluorescence Excitation Spectra of pHluorin-TeNT Hc in Vitro and in Vivo**—Samples containing 0.5 μM pHluorin-TeNT Hc or GST-pHluorin in 50 mM sodium cacodylate, 100 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2 were adjusted to the indicated pH values with NaOH or HCl. Excitation spectra (350–500 nm) were acquired using a 710 Photomultiplier detection system (Photon Technology International) at a fixed emission of 508 nm. As expected, the fluorescence excitation spectrum of GST-pHluorin was identical to that of pHluorin-TeNT Hc.

MN s were incubated with 40 nM pHluorin-TeNT Hc in neurobasal medium for 30 min at 37°C and 7.5% CO2. The medium was then replaced with a buffer containing 120 mM KCl, 20 mM NaCl, 0.5 mM CaCl2, 0.5 mM MgSO4, 20 mM HEPES, adjusted to a pH between 5.0 and 8.0 with NaOH or HCl. 20 min before imaging, the ionophores monensin and nigericin (both 10 μg/ml; Calbiochem), were added to equilibrate the intracellular pH to that of the external buffer (19). The emission intensities of pHluorin-TeNT Hc compartments in axons and soma were measured for each pH at 508 nm upon excitation at 405 and 488 nm. The background was then subtracted, and the corrected emission values for the 405 nm excitation were divided by the corresponding emission intensities obtained by exciting at 488 nm (R405/488).

**Microscopy and Data Quantification**—To monitor fluid phase uptake, MN s were incubated with 6 μM GST-pHluorin in neurobasal medium (Invitrogen) for 1 h at 37°C and 7.5% CO2, washed with nonfluorescent Dulbecco’s minimum essential medium (without phenol red), riboflavin, folic acid, penicillin/streptomycin, and supplemented with 30 mM HEPES-NaOH, pH 7.3 (DMEM), and immediately imaged by low light time lapse microscopy. Alternatively, MN s were transferred to neurobasal medium, incubated for a further 30 min, washed, and then imaged.
MNs were incubated with 40 nM pHluorin-TeNT Hc, or 40 nM TeNT Hc-AlexaFluor-488 in complete medium for 30 min at 37 °C, followed by three washes with DMEM. The cells were placed in a humidified chamber maintained at 37 °C and after 20 min, imaged every 5 s. In selected samples, where axonal transport of TeNT Hc-AlexaFluor-488 was observed, concanamycin A (ConA) (Alexis Biochemicals) or bafilomycin A1 (BafA1) (Alexis Biochemicals) were added at a final concentration of 10 and 0.5 nM, respectively, and the same cell was imaged after 5, 15, and 30 min of incubation. The ConA analysis shown in Fig. 4 is representative of five independent experiments using MNs from two different preparations, whereas the BafA1 effects have been evaluated in three independent experiments using MNs isolated from the same preparations. The functionality of the proton pump inhibitors was tested in three independent experiments using MNs from two different preparations, whereas the BafA1 effects have been evaluated in five independent experiments using MNs from two different preparations, whereas the BafA1 effects have been evaluated in three independent experiments using MNs isolated from the same preparations.

RESULTS

Axonal TeNT Hc carriers have recently been shown to avoid accumulating the acidotropic probe Lysotracker Red DND-99 in their lumen, suggesting that these organelles escape acidification (5). However, no conclusions could be drawn from previous work about the pH dynamics of the retrograde transport compartment or its regulation in the axon and soma. To address this issue, we chose a strategy based on bacterially expressed ratiometric pHluorin fused with TeNT Hc. First of all, we tested whether pHluorin still retains its described optical properties (13) once tagged to TeNT Hc by diluting it with buffers ranging from pH 5.0 to 8.0 and monitoring its excitation spectrum at an emission wavelength of 508 nm (Fig. 1a). As expected, the fluorescence spectrum of pHluorin-TeNT Hc shows two peaks at 395 and 475 nm. The

FIGURE 1. Spectral and biophysical characterization of pHluorin-TeNT Hc. a, scheme of clostridial neurotoxins and the ratiometric pHluorin-TeNT Hc fusion protein. This chimera is expressed as recombinant GST fusion protein containing a thrombin cleavage site (arrow), L, light chain; H, heavy chain. b, pH dependence on the fluorescence excitation spectra of pHluorin-TeNT Hc in vitro. Samples containing 0.5 μM pHluorin-TeNT Hc at the indicated pH values were excited with wavelengths ranging from 350 to 500 nm, and emission was monitored at 508 nm. c, calibration curve of pHluorin-TeNT Hc in MNs. MNs were incubated with 40 nM pHluorin-TeNT Hc in neurobasal medium for 30 min at 37 °C. The medium was then exchanged with buffers at the indicated pH values prior to the addition of mimosin and nigericin. Ratiometric images were taken for every pH and processed as described under Materials and Methods. d, 405/488 ratios (R405/488; ± SEM) of pHluorin-TeNT Hc compartments (n = 24 to 52) were averaged for each pH. The resulting calibration curve is best described by the trend line y = −0.0006x3 + 0.0088x2 − 0.013x + 0.1163 (R² = 0.999). d, GST-pHluorin is a functional probe to monitor the pH of endosomal compartments in MNs. MNs were incubated with 6 μM GST-pHluorin in neurobasal medium for 1 h at 37 °C. Cells were washed and immediately imaged as described under Materials and Methods (filled bars). Alternatively, the incubation medium was replaced by neurobasal medium and incubated for other 30 min (empty bars) prior to imaging. The endocytic compartments detected by fluid phase uptake of GST-pHluorin displayed a broad range of pH values, whereas upon 30 min of chase, the probe was shifted to acidic compartments.
FIGURE 2. pHluorin-TeNT Hc is retrogradely transported along axons in MNs. a, MNs were incubated with 40 nM pHluorin-TeNT Hc for 30 min at 37 °C, washed, and imaged by low light microscopy. Two representative 488- and 405-nm time series of a MN axon and its ratio (R405/488) are shown. 405-nm frames have been inverted to improve their contrast. The cell body is located out of view at the bottom of the image. Intervals between single frames are 5 s. A retrograde carrier (arrowheads) and a stationary compartment (asterisks) are indicated. The single frame image size is 2.6 × 1.4 μm. See also supplemental Video 1, a–c. Scale bar, 2 μm. b, the pH of pHluorin-TeNT Hc carriers remains constant during transport. The 405/488 ratios of a representative pHluorin-TeNT Hc-positive round vesicle and tubule were measured every 5 s, converted to pH (right axis) and plotted as a function of time. c, moving TeNT Hc carriers display an overall neutral pH. Shown is the speed and frequency of single movements (n = 736) of pHluorin-TeNT Hc carriers and their corresponding pH values. Neutral carriers (pH ≈ 7.0) account for the large majority of the total. d, comparison between the pH distribution of stationary organelles containing pHluorin-TeNT Hc and axonal retrograde compartments. The stationary organelles (white bars) include compartments in both axons and soma (n = 171), whereas the carriers (black bars) include axonal round vesicles and tubules (n = 67).

395-nm peak was more intense at a neutral pH and decreased at acidic pH values, whereas the 475-nm peak showed an opposite behavior. To establish a pH calibration curve in this system, MNs were pretreated with 40 nM pHluorin-TeNT Hc at 37 °C for 30 min, washed, and then incubated with buffers at different pH values containing monensin and nigericin. Following treatment with these ionophores, all cellular compartments have been observed to re-equilibrate to the pH of the external medium (19). Ratiometric images were taken, and the 405/488 ratios for each pH, averaged from randomly chosen pHluorin-TeNT Hc-positive organelles in axons and somas (n = 24 to 52), were used to build a cellular calibration curve for pHluorin-TeNT Hc (Fig. 1c). To test the ability of pHluorin to monitor pH in living cells, we incubated MNs with an excess of GST-pHluorin (6 μM) under conditions allowing the labeling of all endocytic compartments by fluid phase uptake. Following incubation for 1 h at 37 °C, MNs were washed and imaged immediately or after a 30-min chase. In the absence of chase, the labeled organelles (n = 119) showed a broad pH spectrum, ranging from acidic to neutral (Fig. 1d), which was in agreement with the pH heterogeneity observed experimentally in the endosomal pathway (20). This distribution drastically changed after a 30-min chase, when mainly acidic structures were observed (n = 96). Approximately 70% of the organelles had a luminal pH below 5.5 (Fig. 1f), which reflects the accumulation of GST-pHluorin in late endosomes and lysosomes (20).

These combined results indicate that pHluorin can be used as a functional pH reporter to assess the pH of axonal retrograde carriers in our cellular system. To this end, we incubated rat MNs with 40 nM pHluorin-TeNT Hc at 37 °C for 30 min. Binding experiments confirmed that pHluorin-TeNT Hc binds specifically to the MN surface (data not shown), as reported for untagged TeNT Hc (21). The cells were then washed, and after 20 min, low light time lapse imaging started in the 488-nm channel, followed by the 405-nm channel. Ratiometric images (R405/488) were obtained from these two time series (Fig. 2a) (see also supplemental Video 1, a–c). The movement of a retrograde pHluorin-TeNT Hc carrier is indicated by arrowheads, whereas a stationary compartment is labeled with asterisks (Fig. 2a). TeNT and TeNT Hc have been shown to undergo axonal retrograde transport with a similar speed in vivo (0.8–3.6 μm/s) (4) and in vitro (0.2–3.6 μm/s) (5, 6). To investigate whether pHluorin-TeNT Hc carriers are transported at the same rate as the TeNT compartments, we determined their speed distribution profile. Both the average speed and the overall curve shape observed for pHluorin-TeNT Hc carriers fit well with the previously reported data (5, 6). In particular, the average speed observed for round vesicles (n = 33, 402 movements) was 0.82 ± 0.25 μm/s, whereas tubules (n = 34, 334 movements) had an average speed of 1.18 ± 0.29 μm/s (supplemental Fig. S1).

The pH dynamics of axonal retrograde carriers were then followed by monitoring the 405/488 emission ratio of TeNT Hc compartments during transport. As shown in Fig. 2b, the pH of both types of carriers remained relatively constant during transport. Based on this finding, we then measured the pH of these organelles by ratiometric imaging only in the first
frame of the videos, thus avoiding possible artifacts because of phototoxicity and photobleaching. To unravel a possible correlation between intracellular pH and the average speed of axonal pHluorin-TeNT HC compartments, the velocity of the single movements \( n = 11005 \) and 735 (67 carriers) was plotted against their corresponding pH (Fig. 2c). The large majority of moving organelles was neutral, their pH falling in the 7.0–7.5 (42% of the total) or 7.5–8.0 range (26% of the total). This neutral population of axonal carriers included both round vesicles and tubules and presented a speed distribution profile overlapping with that observed previously (compare Fig. 2c with supplemental Fig. S1) (5, 6). Only a few pHluorin-TeNT HC-positive compartments displayed a pH below 7.0 (14% of the total), whereas no moving carriers having an acidic pH (pH < 6.0) were detected. In sharp contrast, stationary pHluorin-TeNT HC-positive organelles in somas and axons displayed a wide range of pH values, from acidic to neutral (Fig. 2d). This result was confirmed by the partial co-localization of these stationary compartments in the soma with Lysotracker Red DND-99 using time lapse confocal microscopy (supplemental Fig. S2). Organelle acidification in the cell body has also been observed in real time, although with extremely low frequency (data not shown).

What is the cause of the different pH distribution in moving and stationary pHluorin-TeNT HC-positive organelles? At least three formal explanations might account for this phenomenon: (i) the acidification machinery is excluded from or not acquired by transported organelles, (ii) its activity is specifically inhibited, or (iii) the axonal retrograde carriers containing TeNT HC rapidly dissipate the pH gradient due to a high permeability to protons. A major cellular complex involved in organelle acidification is the vATPase. vATPases are found on a variety of intracellular compartments, including clathrin-coated vesicles, synaptic vesicles, and secretory granules (22) and are responsible for the acidification of degradative organelles, such as lysosomes and phagosomes (22, 23). We tested the presence of the vATPase on axonal and somatic organelles containing TeNT HC using antibodies against subunits B and E in the V1 domain of the vATPase and the a subunit in the V0 domain (Fig. 3a).

FIGURE 3. The vATPase is excluded from axonal retrograde carriers containing TeNT HC-AlexaFluor-488. a, schematic structure of the vATPase (45). The antibodies used for the WB analysis and in IF experiments were raised against the subunits B and E of the V1 domain and the a subunit of the V0 domain. b, the antibodies recognized single bands (α 31-kDa, α 60-kDa, and α 116-kDa) and a doublet (α 75-kDa) at their expected molecular weights in unboiled rat brain extracts. IF showed very limited co-localization between vATPase (red) and TeNT HC-positive organelles (green) in MN axons (c) and soma (d). Note the different morphology of vATPase and TeNT HC compartments. Scale bars, 5 μm.
and b), and with the synaptic vesicle protein VAMP1 (supplemental Fig. S3c), thus confirming that these antibodies are able to detect functional vATPase on acidic organelles in neurons (22).

A major caveat of an experimental approach based on immunolocalization is the limit imposed by the sensitivity of the staining antibodies. To exclude a role for the vATPase activity in axonal TeNT transport, we specifically blocked its catalytic function using ConA and BafA1. Both drugs bind with high affinity to the subunit c of the vATPase V0 domain (IC50 = 10 nM for ConA and 0.5 nM for BafA1) (24). At these concentrations, ConA and BafA1 are effective in blocking Lysotracker Red DND-99 accumulation in acidic organelles in MNs (Fig. 4a), demonstrating that the activity of the vATPase was inhibited under these experimental conditions. However, direct comparison of the speed distribution profile of TeNT HC carriers in untreated MNs (Fig. 4, b and c, solid lines) with those of treated MNs at different time points (Fig. 4, b and c, dotted lines) indicates that neither of the drugs alter axonal retrograde transport (see also supplemental Video 2, a e and Fig. S4a). TeNT Hc transport was also unaltered by long term incubation with ConA (90 min). In treated MNs, we observed a slight reduction in speed values and a decrease in carrier frequency at later time points (Fig. 4, b and c, empty circles). We attribute these minor variations to the repetitive, long term imaging of the same axon, because untreated MNs showed the same overall alterations of AlexaFluor488–TeNT Hc transport (supplemental Fig. S4b).

To test whether the vATPase is involved in an early step of TeNT Hc trafficking, we modified our experimental protocol by pretreating MNs for 15 min with BafA1 and ConA before adding AlexaFluor-488–TeNT HC (Fig. 5a). Imaging started 20 min after removal of the fluorescent TeNT HC (t = 0 min). Only a faint, rather homogeneous membrane staining of MN axons was observed at t = 30 min (Fig. 5b). As shown by the kymographs in Fig. 5b, no moving TeNT HC carriers or stationary TeNT HC-positive compartments were detected, strongly indicating that this probe was not sorted to the retrograde transport pathway. In contrast, kymographs of MNs treated with vATPase inhibitors after endocytosis and sorting of TeNT HC following the protocol described in Fig. 4b (t = 30 min) displayed several progressing carriers (Fig. 5c, arrowheads) and stationary compartments (Fig. 5c, asterisks). Interestingly, we were able to observe a significant overlap between AlexaFluor-488–TeNT Hc and vATPase at early time points of internalization (t = 2 min) (Fig. 5, d and e), in sharp contrast to that observed at late time points (t = 45 min) (Fig. 5e).

Is TeNT Hc internalization impaired by treatment with vATPase inhibitors? Two independent lines of evidence shown in Fig. 6 argue
against this possibility. Treatment with 0.5 nM BafA1 prior to incubation with VSV-G-tagged TeNT Hc followed by sequential staining in the absence and the presence of permeabilization (Fig. 6a) revealed that a pool of TeNT Hc is internalized under these conditions. Furthermore, BafA1 application did not impair the entry of biotinylated TeNT Hc into MNs (Fig. 6b). These findings indicate that a functional vATPase is not required for TeNT Hc endocytosis but plays a specific role in an early sorting step(s) targeting TeNT Hc to axonal transport carriers.

DISCUSSION

In this study, we monitored the pH dynamics of TeNT Hc-positive compartments in real time using a pHluorin-TeNT Hc fusion protein. Chimeras of pHluorin have been used as pH probes in a variety of biological systems. In neurons, pHluorin-based sensors have been exploited to follow the presynaptic activity within neuronal networks (14) to report synaptic vesicle fusion (25) and to assess the molecular identity of different synaptic compartments during synaptic vesicle recycling (26). On this basis, we chose a novel fusion protein between ratiometric pHluorin and TeNT Hc as a noninvasive method to investigate the pH of the fast axonal retrograde compartment in MNs. Here, we showed that these endocytic carriers have a neutral pH, which is kept constant during axonal movement. This finding is in contrast with the pH dynamics described for the classical endosomal pathway, which undergoes a rapid acidification upon internalization (20). The entry of TeNT into a neutral endocytic compartment has important mechanical effects on the pathogenesis of tetanus. In fact, acidic pH triggers a conformational change of TeNT, which allows its membrane insertion and the translocation of the L chain into the cytosol, where it cleaves VAMP (2). This phenomenon occurs in hippocampal neurons in vitro (27) and in spinal cord inhibitory interneurons in vivo (1, 28). In artificial liposomes, the pH initiating the membrane insertion of TeNT is ~5.0 (29), which is within the pH range of the endosomal pathway. By dem-

FIGURE 5. A functional vATPase is required for TeNT Hc sorting to the retrograde transport route. a, schematic time scale of the experiment. MNs were treated with 10 nM ConA or 0.5 nM BafA1 and then incubated with 40 nM TeNT Hc-AlexaFluor-488, washed, and imaged by low light time lapse microscopy. b, epifluorescence of MN axons at t = 30 min of imaging. The corresponding kymographs are shown below. c, kymographs of MN axons treated as described in the legend to Fig. 4c (t = 30 min). Arrowheads indicate retrograde TeNT Hc-AlexaFluor-488 carriers, whereas asterisks mark stationary organelles. In b and c, the MN soma is located out of view on the right. d, at early time points of TeNT Hc-AlexaFluor-488 internalization (t = 2 min), a partial overlap between TeNT Hc-AlexaFluor-488 (green) and vATPase (116-kDa subunit; red) can be detected. e, relative colocalization between the vATPase stained with an antibody against the 116-kDa subunit and TeNT Hc at early (t = 2 min) and late (t = 45 min) internalization points in axons and soma (n = 6 to 8). Vertical bars, 40 s (b and c); 5 μm (d); horizontal bars, 5 μm (b and c).
onstrating that the fast axonal retrograde compartment in MNs displays a neutral pH, we provide an explanation of the entrapment of TeNT in the lumen of the retrograde carrier during axonal transport to the soma, a journey shared with neurotrophins and their receptors (5). The lack of acidification of the fast retrograde transport carriers might contribute to the low degradative capability of this compartment, which ensures the integrity of its endogenous and exogenous cargoes. This is in agreement with the long half-life of internalized TeNT \textit{in vivo} (28).

Several pathogens and virulence factors have been shown to either transit or accumulate in nonacidic cellular compartments. This is the case for shiga-like (SLT-1B) and cholera toxin (CT-B) B subunits, which have been extensively used as probes to uncover Golgi-dependent and -independent pathways from the plasma membrane to the endoplasmic reticulum (30, 31). \textit{Chlamydia pneumoniae}, an intracellular parasite, inhabits a nonacidic vacuole, which is distinct from late endosomes and lysosomes (32). Furthermore, echovirus 1 and simian virus 40 (SV40) enter a nonacidic compartment after leaving the plasma membrane in caveolin-enriched structures termed "caveosomes," which lack markers for endosomes, lysosomes, endoplasmic reticulum, or Golgi apparatus (33–35). In contrast with the retrograde transport carriers, stationary TeNT H\textsubscript{C}-positive structures, which are distributed in soma and neurites, have a much broader pH range spanning from pH 5.0–7.5. This observation is in agreement with the reported trimodal frequency distribution of the endocytic organelle pH in axon shafts of sympathetic neurons (36). Furthermore, it suggests that the machinery determining the intraluminal pH of these axonal compartments is strictly regulated and coordinated to the activity of the motor complexes responsible for their retrograde movement (37).

What is the mechanistic basis of this differential pH regulation? A likely possibility is that this is due to the sorting of the vATPase away from this compartment. Acidification is essential for diverse cellular processes, such as protein targeting along the secretory pathway, recycling of receptors to the plasma membrane, and protein degradation in the endomembrane lumen (22, 38). Inhibition of the vATPase activity perturbs some, if not all of these functions (39, 40). Consequently, we
tested for the presence of the vATPase by using several antibodies against different subunits of its V_{1} and V_{0} domains. The experiments revealed very little colocalization between TeNT H_{c}- and vATPase-positive organelles, suggesting that the lack of acidification is due to the absence of the vATPase from the retrograde carriers and not to an altered protoxide permeability of their membrane bilayer (41). In contrast, the vATPase is likely to be present on stationary organelles, which can be acidified. An inducible, organelle-specific vATPase sorting has been previously demonstrated for phagosomes containing Mycobacterium avium, which fail to acidify due to the exclusion of vATPase from their delimiting membranes (42). The notion that vATPase activity is dispensable for fast retrograde transport in MNs has been confirmed by the lack of effect on this process of vATPase inhibitors used at concentrations abolishing the accumulation of acidotrophic dyes. In contrast, pretreatment of MNs with vATPase inhibitors blocked TeNT accumulations abolishing the accumulation of acidotrophic dyes. An inducible, organelle-specific vATPase sorting has been observed for fast retrograde transport in MNs. The experiments tested for the presence of the vATPase by using several antibodies against different subunits of its V_{1} and V_{0} domains. The experiments revealed very little colocalization between TeNT H_{c}- and vATPase-positive organelles, suggesting that the lack of acidification is due to the absence of the vATPase from the retrograde carriers and not to an altered protoxide permeability of their membrane bilayer (41). In contrast, the vATPase is likely to be present on stationary organelles, which can be acidified. An inducible, organelle-specific vATPase sorting has been previously demonstrated for phagosomes containing Mycobacterium avium, which fail to acidify due to the exclusion of vATPase from their delimiting membranes (42). The notion that vATPase activity is dispensable for fast retrograde transport in MNs has been confirmed by the lack of effect on this process of vATPase inhibitors used at concentrations abolishing the accumulation of acidotrophic dyes. In contrast, pretreatment of MNs with vATPase inhibitors blocked TeNT accumulations abolishing the accumulation of acidotrophic dyes. An inducible, organelle-specific vATPase sorting has been observed for fast retrograde transport in MNs.

Acknowledgments—We thank Prof. G. Miesenbock for the ratiometric pH-regulated TeNT HC- probes, which may coincide with the stationing of axonal carriers and other early endocytic organelles during transport. Mild acidic pH values might play a role in the early steps of the endocytic pathway of TeNT by inducing a conformational change, which, although not sufficient to trigger the insertion of the molecule in the inner core of the lipid bilayer, might promote an enhanced interaction with the membrane surface and facilitate the initial sorting of TeNT H_{c}.

In conclusion, we demonstrated that a functional vATPase is transiently required for the initial sorting of TeNT H_{c} to its retrograde transport route, and it is then sorted away from the axonal carriers at later stages. As a consequence, these retrograde organelles display a neutral luminal pH, which might protect the native conformation of acid-labile cargoes and stabilize receptor-ligand interactions. In particular, these conditions might allow the sustained interaction of some neurotrophin receptors (such as nerve growth factor), which has been found to enter TeNT H_{c}- carriers (5), and its receptors, providing additional basis for the long range axonal neurotrophin signaling (44). Future experiments will directly address the molecular composition of these retrograde carriers and the activation status of neurotrophin-receptor cargo complexes during transport.

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