Electrostatic Interactions between the Syntaxin Membrane Anchor and Neurotransmitter Passing through the Fusion Pore

Xue Han and Meyer B. Jackson
Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT Recent experiments have shown that flux through the fusion pore is sensitive to manipulations of the side-chain size of certain residues in the syntaxin (syx) membrane anchor. These residues were proposed to line the wall of the fusion pore of Ca^{2+}-triggered exocytosis. Here we continued this line of experimentation to examine possible electrostatic interactions between the pore lining residues and the neurotransmitter norepinephrine (NE). Replacing syx pore-lining residues with aspartate enhanced NE flux above that expected for the size of the aspartate side chain. In contrast, substitution with arginine reduced NE flux below that expected for the size of its side chain. Substituting aspartate and arginine into the nonpore-lining residues did not alter the fusion pore flux. Other amino acids with ionizable side chains had variable effects. These results indicate an electrostatic interaction between the pore-lining residues of syx and NE, and provide additional evidence that the syx membrane anchor is a structural component of the fusion pore.

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Address reprint requests and inquiries to Meyer Jackson, Email: mjackson@physiology.wisc.edu.

Ca^{2+}-triggered exocytosis proceeds through an intermediate structure known as the fusion pore. One hypothetical model for the fusion pore is a proteinaceous channel-like complex (1), and recent experiments indicated that the syntaxin (syx) membrane anchor could be part of this complex (2). Altering the side-chain size of residues in the syx transmembrane segment influenced the flux of the neurotransmitter norepinephrine (NE), in the same way that altering the side chains along the ion permeation pathway of channel-forming proteins alters their ionic current (3). The ion channel flux is uniquely sensitive to manipulations of the pore-lining residues of a channel forming protein (3, 4).

NE carries a positive charge at the neutral pH of the extracellular fluid or the acidic pH inside a vesicle. When passing through a fusion pore, an electrostatic interaction is possible if the pore contains a charge. We therefore introduced amino acids with ionizable side chains into the syx membrane anchor. NE release from single vesicles was detected as a spike in amperometry recordings. The ''foot'' signal preceding a spike corresponds to the leak of NE through an open fusion pore (Fig. 1) (2, 7, 8, 9). The average amplitude of the prespike foot (PSF) was used to evaluate the NE flux through fusion pores.

Three residues had been identified previously as pore-lining residues (2), and two of these residues, 276 and 283, were selected for charge substitutions. PSF amplitudes were first evaluated in plots versus amino acid side-chain volume. Linear fits for the nonpolar substitutions provide an assessment of the effect of side-chain size in the steric occlusion of fusion pores. The value for aspartate (*D*) and glutamate (*E*), which ionize to form a negative charge, fell above the linear fit for both pore-lining positions (Fig. 2, *A* and *B*), indicating enhanced NE efflux. Substituting positively charged arginine (*R*) at these sites reduced the fusion pore flux below this line, indicating reduced fusion pore flux. Lysine (*K*) and histidine (*H*) substitutions failed to alter the fusion pore flux, and this may reflect their weaker tendency to ionize.

Parallel substitutions were performed at two nonpore-lining residues, positions 275 and 280, where aspartate and arginine failed to alter the fusion pore flux (Fig. 2, *C* and *D*). This supports the hypothesis that these positions are not exposed to the neurotransmitter expulsion pathway.

Subtracting the experimentally recorded foot current from that based on the linear fits in Fig. 2, *A* and *B*, yields the ''current offset'', which represents the electrostatic contribution. The current offset varied inversely with the side-chain pK (Fig. 3). (The pK values were for ionization in an aqueous solution (5), and could be altered by the protein/membrane environment.)

These results demonstrate an electrostatic interaction between the syx membrane anchor and NE escaping from a vesicle through the fusion pore. It has been suggested that substitutions that increase the fusion pore flux would provide stronger support for a direct role of the syx membrane anchor (10). Since aspartate substitutions at positions 276 and 283 produce such an increase, this strengthens the case for a direct involvement of syx. The demonstration of two separate physical interactions, steric and electrostatic, between the syx membrane anchor and neurotransmitter indicates that this region of the protein is a structural component of the fusion pore.

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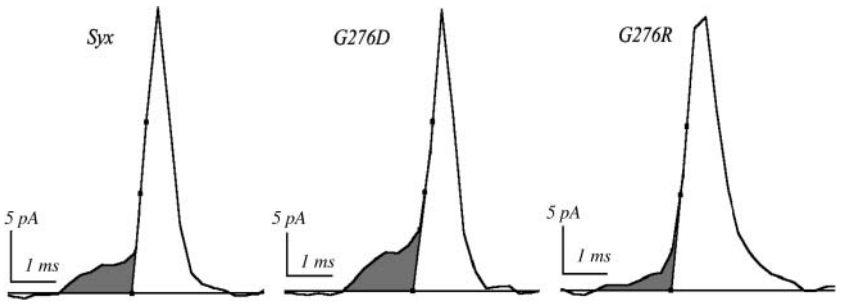

FIGURE 1 Amperometry spikes recorded from PC12 cells transfected with wild-type syx and two mutants, as indicated. The pre-spike feet are shaded.

METHODS

Molecular cloning

Syx point substitutions at residues 280 and 283 were made by a single PCR reaction with the designated mutation on one primer. Residues 275 and 276 are too far from the COOH-terminus for single PCR, so mutations were introduced by modified sequential PCR (6). Wild-type syx and mutants were subcloned into the pIRES2-EGFP vector (Clonetech, Palo Alto, CA). All mutants were confirmed by DNA sequencing.

Cell culture and amperometry

PC12 cells were cultured and transfected as previously described (2). Cells were transferred to dishes coated with collagen-I and poly-D-lysine (BD Bioscience, Bedford, MA) and incubated with 1.5 mM NE and 0.5 mM ascorbate (Sigma, St. Louis, MO) overnight. Amperometry recording was performed with 5 μm carbon fiber electrodes connecting to a VA-10 amplifier at a polarization of 650 mV (2, 7, 8). The bathing solution contained (in mM): 150 NaCl, 4.2 KCl, 1 NaH_2PO_4, 0.7 $MgCl_2$, 2 $CaCl_2$, and 10 Hepes (pH 7.4). Secretion was induced by depolarization using the same bathing solution but with elevated KCl (105 mM) and reduced NaCl (5 mM). Data were analyzed with a computer program to extract foot information (8). Feet were analyzed for spikes larger than 20 pA. The foot current was calculated as the PSF area divided by the foot duration. Feet with durations <0.75 ms (three times the sampling rate) were rejected as too brief for reliable detection.

ACKNOWLEDGMENTS

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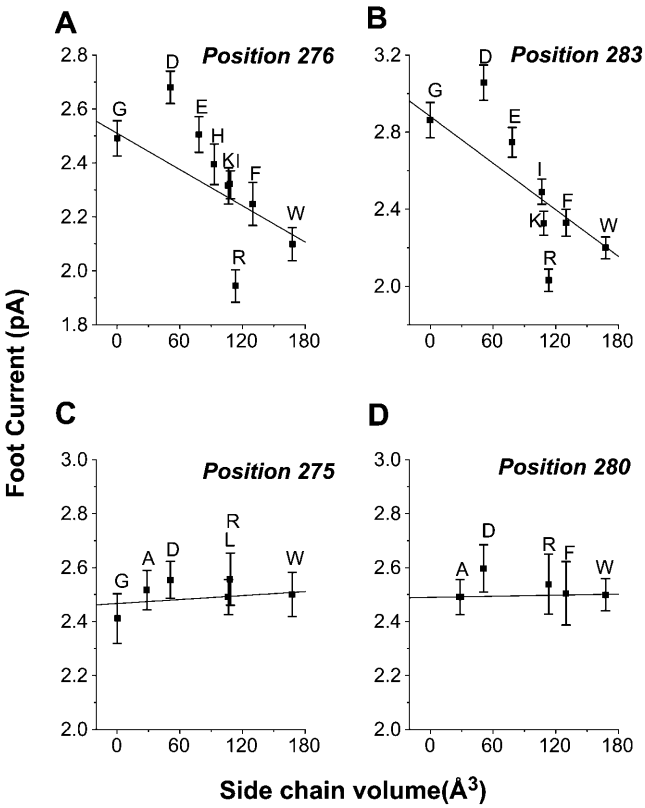

FIGURE 2 Positively charged NE electrostatically interacts with the residues residing in the syx membrane anchor. The mean foot current is plotted against the side-chain volume for residues 276 (*A*), 283 (*B*), 275 (*C*), and 280 (*D*). Data for nonpolar residues in this plot are from Fig. 3 of reference (2). The line drawn in each plot is the linear fit to the data from nonpolar residues.

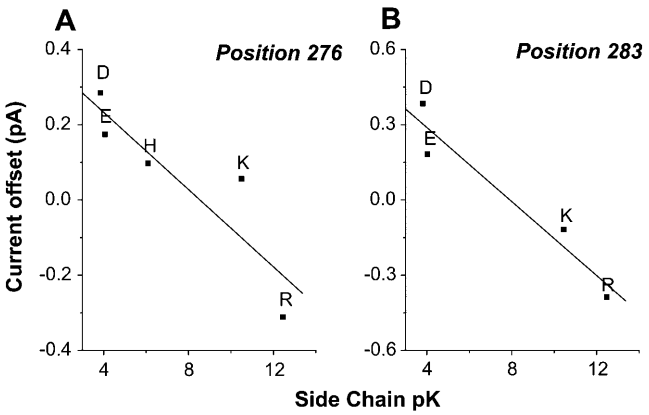

FIGURE 3 Current offset (see text) is plotted against the side-chain pK for positions 276 (*A*) and 283 (*B*). Linear fits gave $p = 0.04$ for both plots.

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