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New transport assay demonstrates vesicular acetylcholine transporter has many alternative substrates

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Abstract

The acetylcholine-binding site in vesicular acetylcholine transporter faces predominantly toward the outside of the vesicle when resting but predominantly toward the inside when transporting. Transport-related reorientation is detected by an ATP-induced decrease in the ability of saturating substrate to displace allosterically bound [^{3}H]vesamicol. The assay was used here to determine whether structurally diverse compounds are transported by rat VAChT expressed in PC12$^{A123.7}$ cells. Competition by ethidium, tetraphenylphosphonium and other monovalent organic cations with [^{3}H]vesamicol is decreased when ATP is added, and the effect depends on proton-motive force. The results indicate that many organic molecules carrying +1 charge are transported, even though the compounds do not resemble acetylcholine in structural details.

Keywords: Vesicular acetylcholine transporter; Vesamicol; Tetraphenylphosphonium; Ethidium; Transport assay; Acetylcholine-binding site; PC12$^{A123.7}$ cells

1. Introduction

Vesicular acetylcholine transporter (VAChT, SLC18A3) exchanges luminal protons for cytoplasmic acetylcholine (ACh)[1] to drive storage of ACh. Protons are pumped into vesicles by V-ATPase. Radiolabeled analogues of ACh have been used in assays that test for accumulation of radioactivity to demonstrate that many of the analogues are transported (Clarkson et al., 1992). Nonlabeled analogues have been used in assays that test for tighter apparent affinity under transport conditions compared to equilibrium conditions to demonstrate that many of the analogues are transported (Rogers and Parsons, 1989; Bahr et al., 1992). The results of such structure–activity studies indicated that VAChT has low selectivity among a range of ACh analogues.

Bravo et al. (2004a) recently developed another type of transport assay applicable to unlabeled compounds. The assay uses a trace concentration of the allosteric ligand [^{3}H]vesamicol [(−)-*trans*-2-(4-phenylpiperdino)cyclohexanol] as an indicator. The ACh-binding site switches from predominantly outside orientation in resting or energized vesicles to predominantly inside orientation in transporting vesicles (Nguyen et al., 1998). Vesamicol cannot bind if ACh is bound to externally oriented VAChT (Bahr et al., 1992). When a VAChT molecule in an energized vesicle, not bound by a trace concentration of [^{3}H]vesamicol, binds and transports ACh, the ACh-binding site *in that molecule of VAChT* reorients toward the inside of the vesicle. A molecule of [^{3}H]vesamicol then can bind to the allosteric site, as it probably stays on the outside. For as long as the ACh-binding site is oriented toward the inside, a *second* molecule of external ACh cannot bind. The result is an increase in the amount of [^{3}H]vesamicol binding. Thus, addition of ATP to vesicles resting in saturating ACh increases the amount of bound [^{3}H]vesamicol by decreasing the ability of ACh to compete with the indicator.

Abbreviations: ACh, acetylcholine; VAChT, vesicular acetylcholine transporter; vesamicol [(−)-*trans*-2-(4-phenylpiperdino)cyclohexanol]; TPP, tetraphenylphosphonium; Eth, ethidium; BFA_1, bafilomycin A_1

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This reorientation assay was used by Bravo et al. (2004b) to demonstrate that choline and tetramethylammonium are transported by VAChT. The result was confirmed with radiolabeled choline. Development and validation of the assay has led us in the current work to test whether molecules even more distant in structure from ACh are transported.

2. Materials and methods

2.1. Materials

Tetraphenylphosphonium (TPP) chloride, cetyltrimethylammonium chloride, *N*-methylpyridinium-2-aldoxime chloride, trigonelline hydrochloride, betaine, and ethidium (Eth) bromide were obtained from Fisher Scientific (Fair Lawn, NJ). Succinylcholine chloride, reserpine, ACh chloride, vesamicol·HCl, ATP magnesium salt, and bafilomycin A_1 were obtained from Sigma Chemical Corp. (St. Louis, MO). [^{3}H]Vesamicol (20 Ci/mmol) was obtained from Perkin-Elmer Life Sciences Inc. (Boston, MA). Rat VAChT was transiently expressed in PC12$^{A123.7}$ cells, and post-nuclear supernatant containing synaptic-like microvesicles was prepared in 0.32 M sucrose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid adjusted to pH 7.4 with KOH and containing protease inhibitors and 100 μM diethyl-*p*-nitrophenyl phosphate to inhibit ACh esterase as described (Ojeda et al., 2003). Post-nuclear supernatant was stored at −80 °C until used. Other materials were obtained from usual commercial sources.

2.2. Competition with [^{3}H]vesamicol binding

Solutions were warmed to 37 °C shortly before use. Post-nuclear supernatant (50 μL) containing 200–400 μg protein was mixed with 100 μL uptake binding buffer (110 mM potassium tartrate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM ascorbic acid, 100 μM diethyl-*p*-nitrophenyl phosphate, adjusted to pH 7.4 with KOH; UBB) containing twice the indicated final concentration of unlabeled compound and, if present, 100 nM bafilomycin A_1. Either 50 μL UBB containing 20 nM [^{3}H]vesamicol, or 50 μL UBB containing 20 nM [^{3}H]vesamicol and 20 mM ATP plus 8 mM $MgCl_2$, was added to determine total binding. The resulting 5 nM [^{3}H]vesamicol is 4-fold less than the dissociation constant of 20 nM. This concentration accordingly is ''trace'', yet it produces a very good binding signal. The suspension was mixed well and allowed to incubate for 10 min at 37 °C. Nonspecific binding was determined in like manner with 4 μM of unlabeled vesamicol present during incubations.

2.3. Filtration of microvesicles

A 90-μL portion of the vesicle suspension was mixed with a 1-mL portion of ice-cold UBB. The diluted suspension was rapidly and quantitatively filtered with vacuum assistance through a polyethylenimine-coated glass microfiber filter circle GF/F (1.3 cm) pre-wetted with UBB, after which the filter immediately was washed with vacuum assistance by three 1-mL portions of ice-cold UBB. Damp filters were transferred to a liquid scintillation vial. Scintillation cocktail (3.5 mL) was added and radioactivity was determined by liquid scintillation counting. Eth in incubations was shown not to affect scintillation counting rates. Specific binding (total binding − nonspecific binding) is reported.

3. Results

3.1. Effects of ATP on competition with [^{3}H]vesamicol

Preliminary titrations of structurally diverse compounds against binding of trace [^{3}H]vesamicol to resting vesicles were carried out in the absence of ATP (not shown). For each compound that displaced [^{3}H]vesamicol, a minimal concentration giving good displacement was chosen to test for transport. For each compound that displaced poorly, the highest achievable concentration that did not overtly damage vesicles was chosen.

The ability of ATP to protect bound, trace [^{3}H]vesamicol from displacement by the test compound then was determined (Table 1). We first tested ACh itself as the standard compound that undergoes good transport. In the absence of ATP, VAChT bound 1.9 pmol [^{3}H]vesamicol/mg in the absence of ACh or test compound, but it bound only 0.15 pmol [^{3}H]vesamicol/mg in 200 mM ACh. In the presence of ATP, VAChT bound 2.2 pmol [^{3}H]vesamicol/mg in the absence of ACh or test compound, and it bound 0.65 pmol [^{3}H]vesamicol/mg in 200 mM ACh. Thus, ATP caused a 17% increase of binding in the absence of test compound, but it caused a 330% increase of binding in the presence of ACh. Similarly, ATP also caused substantial increases in binding of trace [^{3}H]vesamicol in the test compounds Eth (2300%), TPP (350%), *N*-(4′-pentanonyl)-4-(4″-dimethylaminostyryl)pyridinium (280%) and *N*-methylpyridinium-2-aldoxime (280%). The increase in the absence of ACh or test compound is due to a small increase in affinity of vesamicol due to proton-motive force (Bravo et al., 2004a). It does not account for the large ATP-dependent increases observed in the presence of ACh, Eth, TPP, *N*-(4′-pentanonyl)-4-(4″-dimethylaminostyryl)pyridinium and *N*-methylpyridinium-2-aldoxime.

The molecules succinylcholine and *N*-(2′-acetoxyethyl)-3-aminopyridinium exhibited intermediate behavior. High concentrations of these test compounds inhibited binding of [^{3}H]vesamicol by ∼one-half, and addition of ATP increased binding of [^{3}H]vesamicol by 40% and 30%, respectively. These increases are larger than the 17% increase due to proton-motive force in the absence of transport. The maximal ATP-dependent increase in these cases could have