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Polyamine Transport Systems in Mammalian Cells and Tissues

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Abstract

Polyamine transport plays an important role in the homeostatic regulation of the polyamine levels. In animals, dietary polyamines are absorbed efficiently in the intestinal tract. In the colon, luminal bacterial derived polyamines are important contributors to cellular polyamine contents. Polyamine transport involves unique uptake and export mechanisms. The amino acid transporter SLC3A2 acts as a polyamine exporter in colon cancer-derived cells. Polyamine uptake is mediated by caveolin-1 dependent endocytosis. The K-RAS oncogene signals increased polyamine uptake and decreased polyamine export. Here, we describe the methods of polyamine transport analysis in the colon and the small intestine using membrane vesicles, culture cells, and mouse models.

Keywords

Polyamine; Putrescine; Spermidine; Spermine; Plasma membrane vesicles; Culture cells; Mouse tissues; Transport; Colon; Small intestine

1. Introduction

Cellular polyamines are derived from intracellular biosynthesis and uptake from extracellular sources. The major sources of exogenous polyamines come from the diet and luminal bacteria (1). It has been shown that dietary polyamines were taken up by the intestinal tract and enter the systemic circulation (2–4). Treatment with antibiotics to remove microbial flora activity (5) or a polyamine-free diet (6) increases the polyamine depleting effect of the polyamine biosynthetic enzyme inhibitor α -difluoromethylornithine (DFMO). The export of polyamines from cells is also important for the maintenance of cellular polyamine levels.

Recently, we identified the amino acid transporter SLC3A2 as a polyamine export protein in colon cancer-derived cells (7). SLC3A2 catalyzes the export of acetylated polyamines by a polyamine/arginine exchange reaction. We and others have reported that polyamine uptake is mediated by caveolae-dependent endocytosis in colon cancer-derived cells (8, 9). Knocking down caveolin-1 (CAV-1) using antisense RNA increases polyamine uptake activity in colon cancer-derived HCT116 cells. Since CAV-1 inhibits caveolar endocytosis, these results suggest that polyamine uptake occurs via a caveolar endocytotic pathway.

Polyamine transport is well characterized in *Escherichia coli* and *Saccharomyces cerevisiae* (10–14). There are several polyamine transport protein families including ATP-binding cassette transporters and proton potential-dependent solute carriers. In animals, the molecular mechanisms of polyamine transport appear to be somewhat different from those in simpler organisms.

In this chapter, we describe the methods for polyamine transport analysis in the gastrointestinal system using mouse models, cultured cells, and plasma membrane-derived vesicles.

2. Materials

2.1. Cell Culture

1. Dulbecco's Modified Eagle's Medium (DMEM) and Modified Essential Medium (MEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories).
2. Saline: 0.91% NaCl, autoclaved (see Note 1).
3. Puromycin Ready Made Solution (10 mg/mL, Sigma).
4. Trypsin solution (Mediatech, Manassas, VA).

2.2. Animals

1. Wild-type B6129SF2/J and CAV-1 knockout STOCK *Cav1^{tm1Mls}/J* mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in the University of Arizona's Animal Care Facility in accordance with The University of Arizona Institutional Animal Care and Use Committee guidelines.
2. Defined synthetic diet AIN93G (Harlan Teklad, Indianapolis, IN).
3. One percent putrescine prepared in water (see Note 2).
4. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Sodium Phosphate dibasic, and 2 mM potassium phosphate monobasic, pH 7.4.

2.3. Preparation of Inside-Out Membrane Vesicles

1. Putrescine · 2HCl (Calbiochem).
2. EDTA-PBS: 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS.
3. Hypotonic buffer: 0.5 mM sodium phosphate, pH 7.0, 0.1 mM ethyleneglycol bis(2-aminoethyl ether)-tetraacetic acid (EGTA). Add 0.1 mM phenylmethylsulfonyl fluoride (PMSF) before use. Stored at 4°C.
4. TS buffer: 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 50 mM NaCl. Stored at 4°C.
5. Thirty-eight percent sucrose.
6. Fifteen-milliliters Potter-Elvehjem homogenizer.
7. Beckman 50.2 Ti fixed-angle rotor (Beckman Coulter).
8. Beckman SW28 swinging bucket rotor (Beckman Coulter).
9. Twenty-seven-gauge needle with a syringe.
10. BCA Protein Assay Kit (Pierce).

¹Unless stated otherwise, all solutions should be prepared in Milli-Q generated water. This is referred to as "water" in this chapter.

²Food coloring dye can be used for putrescine solution. This may avoid using the wrong solution.

2.4. Putrescine Uptake by Inside-Out Membrane Vesicles

1. Assay buffer: 1 mM ATP, 10 mM MgCl₂, 0.2 mM CaCl₂, 10 mM dithiothreitol, 10 mM creatine phosphate, 100 µg/mL creatine kinase, 250 mM sucrose, 10 mM Tris-HCl, pH 7.4. Prepared before use.
2. Stop buffer: 250 mM sucrose, 150 mM NaCl, 10 mM MES-NaOH, pH 5.5. Stored at 4°C.
3. [H³]putrescine-2HCl (GE Healthcare).
4. Millipore HAWP 0.45-µm filter (Millipore).
5. Sampling manifold (Millipore).
6. CytoScint (MP, Irvine, CA).
7. Liquid scintillation counter LS 5000 TD (Beckman Coulter).

2.5. Polyamine Transport Assay in Cells

1. Assay buffer: 5 mM Hepes-NaOH, pH 7.4, 145 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose. Stored at 4°C.
2. Plastic container large enough to place 6-well plate in it.
3. Trichloric acid (TCA) solution (5% in water).
4. NaOH solution (0.5 N in water).

2.6. Polyamine Determination by HPLC

1. Perchloric acid (PCA) solution (0.2 N in water).
2. Teflon pestle (Daigger, Vernon Hills, IL).
3. Buffer A: 0.1 M sodium acetate, 10 mM octane sulfonate. Adjust to pH 4.5 with HPLC grade glacial acetate. Filter before use.
4. HPLC grade acetonitrile.
5. OPA buffer stock: 24.7 g/L boric acid, 20 g/L KOH, 0.1% Brij-35.
6. *o*-phthalaldehyde (OPA).
7. β -mercaptoethanol.
8. Methanol.
9. Standard solution: 2.5 µM each of putrescine, spermidine, spermine, and diaminoheptane in 0.2 N PCA solution.
10. Diaminoheptane solution for internal standard (250 µM).
11. µBondapak C18 10 µm 125A 3.9 × 300 mm column (Waters, Milford, MA).
12. Waters HPLC system with Multi λ Fluorescence Detector (Waters).

3. Methods

Polyamine transport activity can be measured in membrane vesicles, culture cells, and animal model systems. Plasma membrane vesicles are used for short time transport analysis, on the order of seconds to minutes. This system is suitable for the direct measurement of polyamine transport activity of the plasma membrane. Other factors influencing polyamine transport activity, such as metabolism and binding to the macromolecules, are eliminated in

this system. The limitation to this system is that long-term transport activity cannot be measured since polyamine uptake into the vesicles is saturated within 1 h. In addition, a large amount of cells (10^9 cells) is required for the preparation of membrane vesicles.

The culture cell system is used for relatively short to long term (minutes to days) of polyamine transport activity. The polyamine transport activity can be measured in a relatively small amount of cells. This system represents many physiological features of cells. Polyamine metabolism and binding to the cellular components should be considered. The results are the sum of uptake and export.

We analyze the polyamine transport in vivo by measuring the polyamine contents in tissues after oral administration of polyamines. The results represent all physiological activities including uptake and export, metabolism, circulation, and distribution between organs. The use of genetically engineered mouse (GEM) models can provide insight regarding specific mechanisms.

3.1. Preparation of Inside-Out Membrane Vesicles

1. Putrescine tolerant and normal CHO cells are cultured in MEM. For maintenance, supply 15 mM putrescine in putrescine-tolerant CHO cell culture media. Prepare 30 of 100-mm culture dishes for preculture. Trypsinize cells and combine when cultures reach 70–80% confluent. Inoculate cells in six roller bottles and culture for 3 days (see Note 3). Supply 30 mM putrescine in the culture of putrescine tolerant cells and 10 mM putrescine in normal cells.
2. Place 50.2 Ti rotor and SW28 rotor in cold room on the day before harvest of cells. Check rotors are in good status. Apply grease if needed.
3. Weigh an empty 500-mL centrifuge tube. Collect cells by treating cells with EDTA-PBS at 37°C for 10 min to detach cells and centrifugation at $2,000 \times g$ for 5 min at 4°C. Discard the supernatant and the cell pellet with 100 mL of saline. Collect cells by centrifugation at $2,000 \times g$ for 5 min at 4°C and discard the supernatant. Determine the wet weight of cells.
4. Suspend cells in 15 volumes of ice-cold hypotonic buffer. Stir slowly at 4°C for 16 h (see Note 4).
5. Remove unbroken cells by centrifugation at $3,500 \times g$ for 5 min at 4°C. Transfer supernatant to a 30 mL ultracentrifuge tube (see Note 5) and centrifuge at $100,000 \times g$ (37,500 rpm in 50.2 Ti rotor) for 45 min at 4°C.
6. Collect white fluffy material around the yellowish pellet into 10 mL of hypotonic buffer. Homogenize with 20 strokes in a 15 mL Potter-Elvehjem homogenizer on ice.
7. Layer the homogenate onto 14 mL of 38% sucrose and centrifuge at $100,000 \times g$ (25,000 rpm in SW28 rotor) for 45 min at 4°C. Use hypotonic buffer for balancing tubes (see Note 6).

³This procedure is modified from the methods described by Schaub et al. (15) and Saxena and Henderson (16). This protocol can be adopted for other cell culture systems including suspension-growing cells.

⁴It is important that cells are completely separated from each other. Cells can be suspended in a small volume of hypotonic buffer by repeated pipetting and the rest of buffer is added.

⁵The supernatant can be easily transferred using a 10 mL pipette, and prevent the contamination of unbroken cells.

⁶The homogenate can be layered using 200 μ L Pipettman tip. Contact the tip with the surface of sucrose and slowly push the homogenate.

8. Collect the turbid layer at the interface between the sample and the sucrose into 17 mL of TS buffer (see Note 7). Pass the suspension through a 27-gauge needle with a syringe. Centrifuge at $100,000 \times g$ (37,500 rpm in 50.2 Ti rotor) for 45 min at 4°C. Remove the supernatant.
9. Suspend the pellet in 50 μ L of TS buffer. Determine the protein amount by the BCA Protein Assay Kit according to the manufacturer's protocol. Membrane vesicles can be stored at -80°C until use.

3.2. Putrescine Uptake by Inside-Out Membrane Vesicles

1. Prepare the stop buffer and substrate mixture. The substrate mixture contains 10 μM [H^3]putrescine (37 MBq/mmol) in the assay buffer (see Note 8). Place stop buffer on ice.
2. Set the temperature of water bath to 37°C . Place Millipore 0.45- μm filters in a 100-mm culture dish with assay buffer.
3. Construct the assay mixture containing membrane vesicles (100 μg of protein in 90 μL assay buffer) in a glass test tube on ice. Prepare one tube for each time point.
4. Set up filters in the sampling manifold (see Note 9).
5. Incubate the assay mixture at 37°C for 5 min. Start transport reaction by adding 10 μL of the substrate mixture.
6. After incubation for 5, 10, 20, 30, and 40 min, terminate reaction by placing the reaction mixture on ice and diluting with 1 mL of ice-cold stop buffer. Collect vesicles by rapid filtration through premoistened Millipore 0.45- μm filter using the sampling manifold. Wash the filter three times with ice-cold stop buffer (see Note 10).
7. Dry filters and place in scintillation vials with 4 mL of CytoScint. Count the radioactivity on the filter in a liquid scintillation counter LS 5000 TD (see Note 11).

3.3. Putrescine Transport Assay in Cells

1. HCT116 cells stably transfected with mock or caveolin-1 antisense siRNA expressing vector are maintained in DMEM supplemented with 0.5 $\mu\text{g}/\text{mL}$ of puromycin. Seed one million cells per well of 6-well plates. Cells reach 80–90% confluent after 2 days (see Note 12). Each well corresponds to one time point.
2. Prepare assay buffer, substrate mixture, and wash buffer. Substrate mixture contains 2 mM [H^3]putrescine (37 MBq/mmol) in assay buffer. Wash buffer contains 20 mM cold putrescine in assay buffer. Place wash buffer on ice.
3. Set the temperature of water bath to 37°C . Place the plastic container with water-wet paper in the water bath. Place assay buffer and substrate mixture in water bath.

⁷To collect the turbid layer, take out upper TS buffer layer first using 1 mL Pipetteman tip. It is important to take out upper layer carefully and slowly.

⁸The substrate mixture contains tenfold putrescine of final concentration of reaction mixture.

⁹Make sure that there are no holes and leaks in all filters. Leaks can be tested by applying 1 mL of assay buffer onto filters. Under optimal vacuum condition, 1 mL assay buffer goes through the filter in approximately 2 s.

¹⁰For washing, apply ice-cold assay buffer to the same test tube and pour onto the filter. This ensures all vesicles go through the filter.

¹¹It is necessary to count the radioactivity in the substrate mixture to calculate the amount of transported putrescine. The portion of substrate mixture can be directly added to CytoScint in scintillation vial.

¹²The CAV-1 protein level in antisense transfected cells is decreased to less than 10% of mock transfected cells after 2 days.

4. Place 6-well plate that cells were inoculated in the plastic container.
5. Aspirate culture medium. Wash cells twice with 1 mL of assay buffer (see Note 13).
6. Start transport reaction by applying 1 mL of substrate mixture.
7. After incubation for 5, 10, 15, 20, and 25 min, aspirate substrate mixture and wash cells twice with ice-cold wash buffer.
8. Treat cells with 0.2 mL of 5% TCA solution at room temperature for 30 min with gentle shaking. Add 0.2 mL of 0.5 N NaOH and incubate at room temperature with gentle shaking until cell lysate becomes clear.
9. Take 30 μ L of cell lysate and determine the protein amount by the BCA Protein Assay Kit according to the manufacturer's protocol.
10. Transfer 300 μ L of cell lysate to the scintillation vial containing 4 mL CytoScint, vortex and count the incorporated radioactivity by a liquid scintillation counter.

3.4. Measuring Putrescine Transport in Small Intestine and Colon in Mice

1. Wild-type B6129SF2/J, CAV-1 knockout STOCK *Cav1^{tm1Mls}/J* are raised in cages under nonsterile microisolator conditions and fed with AIN-93G diet.
2. Supply 1% putrescine in drinking water for 2 weeks (see Note 14).
3. After 2 weeks of treatment, sacrifice mice by CO₂ inhalation and spine displacement.
4. Remove intestinal tract, open and flush with PBS. Take approximately 2.5 cm portion of small intestine and colon (see Note 15). Tissues are rapidly frozen in liquid nitrogen and stored at -30°C .
5. Take 20 mg of tissues and cut into small pieces. Transfer tissues to microcentrifuge tubes and homogenize in 0.35 mL of 0.2 N PCA solution using a teflon pestle. Mix homogenates by vortexing and stand at room temperature for 1 min. Repeat five times. Stand homogenates at 4°C for 16 h.
6. Centrifuge the homogenate at 14,000 rpm for 10 min.
7. Remove supernatant and store at 4°C for polyamine determination by HPLC.
8. Suspend pellet in 0.5 mL of NaOH and stand at room temperature for overnight.
9. Centrifuge 14,000 rpm for 10 min. Determine the protein amount in the supernatant by the BCA Protein Assay Kit according to the manufacturer's protocol.

3.5. Polyamine Determination by HPLC

1. Prepare buffer A. Add 27.2 g of sodium acetate and 4.32 g of octane sulfonate to 550 mL of water. Stir until dissolved. Adjust pH to 4.5 with HPLC grade glacial acetic acid (less than 15 mL). Bring volume to 2 L with water. Filter before use.
2. Prepare buffer B. Mix 1 L of buffer A with 282 mL of HPLC grade acetonitrile just before use. Filter.

¹³It is important to remove and apply assay buffer carefully. The rough operation causes detachment of cells. The poly-l-lysine coated plate can be used for more strong attachment of cells.

¹⁴Sometimes mice do not drink the putrescine solution as much as normal water. Supplementation of 2% sugar may increase consumption.

¹⁵It is important to take the same portion of tissues in every mouse.

3. Prepare OPA buffer. In a 15 mL conical tube, add 0.56 g of OPA and 10 mL methanol. Vortex until OPA is completely dissolved. Add 2 mL of β -mercaptoethanol and OPA solution to 1 L of OPA buffer stock. Stir and filtrate before use. Use brown bottle or aluminum foil to protect from light.
4. Set up HPLC system. Place buffer A, buffer B, and OPA buffer, and flush to remove air bubbles in all tubes (see Note 16). Set flow rates for buffer A and OPA buffer to 1.5 mL/min. The pump for buffer B is stopped. The buffer A went through the column is mixed with OPA buffer and fluorescence is detected using Multi λ Fluorescence detector. Excitation and emission were performed at 340 and 455 nm, respectively.
5. Prepare samples for HPLC injection. Add 2 μ L of 250 μ M diaminoheptane solution to 198 μ L of sample.
6. Equilibrate μ Bondapak column with buffer A until the base line becomes stable. It usually takes up to 1 h.
7. Perform the run with 200 μ L injection of 0.2 N PCA without any amines to see the base line is stable (see Note 17). The program starts with 20 min of 100% buffer A at 1.5 mL/min followed by a gradual increase of buffer B from 0 to 100% for 30 min. The program is finished with 5 min of 100% buffer A.
8. Inject and run 200 μ L of standard mixture under the same condition. Standard solution contains 500 pmol of each amine in 200 μ L.
9. Run samples. Perform the standard run after every five sample runs.
10. Calculate polyamine contents (see Note 18).

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¹⁶To remove air bubbles, place all buffers lower than the pump, flush at the maximum speed and flick tubes. Flushing all tube systems with methanol before set up buffers makes it easier to remove air bubbles.

¹⁷Air bubbles and trace amount of contaminated amines and ions make the baseline unstable. Connecting the column in a reverse direction may help to stabilize the base line. Sometimes making new buffer is a faster way to stabilize the baseline.

¹⁸Correct the variability of injection volume between samples using the signal of diaminoheptane as an internal standard.

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