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Supplemental materials and methods

Detection of CCT isoform mRNAs by real-time PCR

Total RNA was isolated from cells using the Absolutely RNA RT-PCR kit (Stratagene) and subjected to DNase I digestion to remove any contaminating genomic DNA. Isolated RNA was resuspended in nuclease-free water, reprecipitated in ethanol, and stored at −20°C. Reverse transcription was performed using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen), the RNA template, and random primers to make the corresponding cDNAs. Quantitative real-time PCR of the *CCTα*, *β2*, and *β3* isoform cDNAs was done to measure the relative levels of expression using the ABI Prism 7700 Sequence Detection System, and the primers and probes were listed in Table S1 (Karim et al., 2003). The Taqman Rodent GAPDH Control Reagent (Applied Biosystems) was the source of the primers and probes for quantitating the control *GAPDH* mRNA. The real-time values for each cell sample were averaged and compared using the C_T method, where the amount of target RNA ($2^{-\Delta\Delta CT}$) was normalized to the endogenous *GAPDH* reference (ΔC_T) and related to the amount of target *CCTα* in NIH-3T3 cells, which was set as the calibrator at 1.0.

Northern hybridization probes

Probes are as follows: *γ-actin*, a 1.55-kb XhoI insert from pHF-1 (Dr. R. Corley, Boston University Medical Center, Boston, MA); human *ERdj3*, a 1.7-kb XhoI–HindIII insert from pT7T3-hERdj3 (Dr. L. Hendershot, St. Jude Children's Research Hospital, Memphis, TN); mouse *CEPT1*, a 946-bp HindIII fragment from pCMV-Sport6-mCEPT1 (GenBank accession no. BC023783; obtained from the American Type Culture Collection, no. 7062235); mouse *CPT1*, a 1231-bp PCR fragment amplified from mouse RNA using the primer pair 5′-AGGCAGCCATGGCGGCGG-3′ and 5′-TTCTCCTCTTTTCCTTGGGCT-3′ designed based on the sequence of GenBank accession no. AY445814.

Choline kinase and choline cytidylyltransferase assays

2×10^7 pelleted frozen cells were resuspended in 75 μl of ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 1 mM PMSF, 1 μg/ml leupeptin, and 2 μg/ml aprotinin) and incubated on ice for 60 min to allow cell swelling before sonication six times for 30 s at 4°C in a cuphorn Sonicator 3000 (Misonix). The lysate was centrifuged at 5,000 *g* for 5 min at 4°C to pellet large cellular debris and unbroken cells. Choline kinase enzymatic assays in a final volume of 50 μl contained 100 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 10 mM adenosine triphosphate, pH 7.0, and 0–200 μg of lysate protein. Assays were started by addition of 2 nmol (1 μl) of 1-2[^{14}C]choline chloride (sp. act. 50 mCi/mmol; 0.1 mCi/ml exchanged into water; American Radiolabeled Chemicals, Inc.). Choline kinase assays were incubated at 37°C for 30 min. Choline cytidylyltransferase enzymatic assays in a final volume of 50 μl contained 150 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM cytosine triphosphate, pH 7.0, 500 μM lipid vesicles (dioleylphosphatidylcholine/oleic acid, 1:1 molar ratio), and 0–200 μg lysate protein. Assays were started by addition of 50 nmol phosphocholine (10 μl; 0.16 μCi phosphoryl *methyl*[^{14}C]choline, sp. act. 55 mCi/mmol [Amersham Biosciences], plus nonradioactive phosphocholine). Cytidylyltransferase assays were incubated at 37°C for 15 min. For both assays, reactions were stopped by adding 5 μl of 0.5 M Na_3EDTA, mixing, and transferring the tubes to ice. A 40-μl aliquot was spotted onto a preadsorbent silica gel G thin-layer plate (Analtech), and the plate was developed in 2% ammonium hydroxide/95% ethanol (5:1, vol/vol) to separate the substrate from the product. The radiolabeled phosphocholine or cytidine diphosphocholine products from either the choline kinase or the choline cytidylyltransferase assays, respectively, were identified by comigration with authentic standards, and the percent conversion was quantified using an imaging scanner (model AR-2000; BioScan). The assays were linear through the time of incubation at all protein concentrations. Lysate protein concentration was determined using the Bradford method (Bradford, 1976) and with gammaglobulin as the standard.

Choline phosphotransferase assay

2×10^7 frozen cell pellets were resuspended in 0.5 ml of ice-cold lysis buffer (10 mM Hepes-HCl, pH 7.4, 50 mM KCl, 1 mM EDTA, 2% aprotinin, and 1 mM PMSF) and then were passed through a 23-gauge needle 20 times to lyse the cells. The lysate was centrifuged at 15,000 *g* for 30 s at 4°C to pellet unbroken cells and nuclei. The supernatant was then centrifuged at 100,000 *g* for 60 min at 4°C to pellet microsomes. Microsomes were resuspended in 50 μl of 50 mM Tris-HCl, pH 8.0, and 20 mM $MgCl_2$, and were kept on ice until assay the same day. Enzymatic assays in a final volume of 50 μl contained 50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 1 mM EGTA, 100 μM dioleyl-*sn*-glycerol in 0.02% Tween 20, and 0–80 μg of microsomal protein. Assays were started by

addition of 0.5 nmol (0.5 μl) of [^{14}C]cytidine-diphosphocholine (American Radiolabeled Chemicals, Inc.; sp. act. 55 mCi/mmol; 0.1 mCi/ml). After incubation at 37°C for 40 min, reactions were stopped by adding 180 μl chloroform/methanol/HCl (1:2:0.02, vol/vol/vol), followed by 60 μl chloroform and 60 μl 2 M KCl. Samples were mixed well and separated into two phases by low speed centrifugation. An 80-μl aliquot of the lower organic phase was removed and quantified by scintillation counting in 3 ml of ScintiSafe (Fisher Scientific) after evaporation of the solvent. The assay was linear throughout the time of incubation at all protein concentrations. Microsomal protein concentration was determined using the Bradford method (Bradford, 1976) and with gamma-globulin as the standard.

Phospholipid mass determinations

Frozen cell pellets (2×10^7 cells) were thawed on ice and resuspended in 1 ml of water. The total volume was measured and a 100-μl aliquot was removed for protein determination. A 900-μl aliquot was extracted by the method of Bligh and Dyer (1959) using 2.4 ml acidic methanol and 1 ml chloroform in the first step, followed by 1.5 ml chloroform and 1.2 ml water in the second step to yield two phases. The lower organic phase containing lipid was concentrated under nitrogen and resuspended in 100 μl chloroform/methanol (2:1, vol/vol). A 1-μl aliquot was loaded onto a thin-layer silica gel rod and developed first in ether, dried, and then developed in chloroform/methanol/acetic acid/water (50:25:8:2, vol/vol/vol/vol). Lipids were detected by flame ionization using an Iatroscan instrument (Iatron Laboratories) with PEAK SIMPLE software (SRI Instruments). Peaks were identified by comigration with authentic standards. Phosphatidylcholine, phosphatidylethanolamine, or cholesterol mass was calculated using standard curves for each prepared with either the polar lipid (no. 1127) or the neutral lipid (no. 1129) mixture from Matreya, Inc.

References

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