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Differentiated Human NT2-N Neurons Possess a High Intracellular Content of *myo*-Inositol

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Abstract: *myo*-Inositol plays a key role in signal transduction and osmotic regulation events in the CNS. Despite the known high concentrations of inositol in the human CNS, relatively little is known about its distribution within the different cell types. In this report, inositol homeostasis was studied in NT2-N cells, a unique cell culture model of human CNS neurons. Differentiation of precursor NT2 teratocarcinoma cells into NT2-N neurons by means of retinoic acid treatment resulted in an increase in inositol concentration from 24 to 195 nmol/mg of protein. After measurement of intracellular water spaces, inositol concentrations of 1.6 and 17.4 m*M* were calculated for NT2 and NT2-N cells, respectively. The high concentrations of inositol in NT2-N neurons could be explained by (1) an increased uptake of inositol (3.7 vs. 1.6 nmol/mg of protein/h, for NT2-N and NT2 cells, respectively) and (2) a decreased efflux of inositol (1.7%/h for NT2-N neurons vs. 9.0%/h for NT2 cells). Activity of inositol synthase, which mediates de novo synthesis of inositol, was not detected in either cell type. The observation that CNS neurons maintain a high intracellular concentration of inositol may be relevant to the regulation of both phosphoinositide signaling and osmotic stress events in the CNS. **Key Words:** Neuronal differentiation—Inositol efflux—Osmotic stress—Phlorizin—Inositol uptake—Inositol synthase.
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myo-Inositol serves at least two major physiological roles in the CNS. First, it is a precursor of phospholipids such as the 3-phosphoinositides, which have been implicated in adhesion, growth, vesicular trafficking, and cell survival (Toker and Cantley, 1997), as well as the quantitatively major polyphosphoinositides, including phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, which mediate signal transduction (Berridge and Irvine, 1989) and cytoskeletal rearrangement (Carpenter, 1996). Second, free inositol is involved in volume regulation during persistent osmotic stress (Nakanishi et al., 1989; Isaacks et al., 1994; Strange et al., 1994; Reeves and Cammarata, 1996). Whereas acute osmotic regulation is mediated by changes in electrolyte balance, chronic osmotic stress is countered by changes in the concentration of organic osmolytes such as inositol (Gullans and Verbalis, 1993).

Disorders of neuronal inositol metabolism are believed to lead to neurological and psychiatric disease. For example, the pathogenesis of Down syndrome could be related to the expression of multiple gene copies of the Na^+/*myo*-inositol transporter and a high concentration of inositol in the fetal CSF (Berry et al., 1995; Acevedo et al., 1997). Increased cerebral inositol concentrations also result from persistent hypernatremia (Lee et al., 1994), the rapid correction of which can lead to fatal cerebral edema (Gullans and Verbalis, 1993). Conversely, conditions such as diabetic peripheral neuropathy (Yorek et al., 1994; Karihaloo et al., 1997; Sima et al., 1997) and some cases of neural tube defect (Cockroft, 1988; Greene and Copp, 1997) have been linked to a deficiency of inositol and an attenuation of phosphoinositide signaling. It has been speculated that the therapeutic effectiveness of Li^+ in the treatment of bipolar disorder derives from its ability to deplete inositol stores and consequently to inhibit inositol lipid synthesis in those neurons that exhibit abnormally high rates of phosphoinositide turnover (Berridge et al., 1989; Einat et al., 1998).

Despite the clinical significance of neuronal inositol homeostasis, the concentration of inositol in CNS neurons remains uncertain. Relatively high concentrations of inositol have been reported for giant neurons of Deiters' nucleus (Sherman et al., 1977) and the molecular layer of cerebellar flocculus (Godfrey et al., 1982). In contrast, based on experiments with dissected layers of cerebellar cortex (Stewart et al., 1969), whole sections of cerebral cortex (Godfrey, 1989), and neuroblastoma and glioma cells (Glanville et al., 1989), it has been concluded that concentrations of inositol in glia may exceed those in

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; phloretin, 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone; phlorizin, phloretin 2′-β-D-glucoside dihydrate.

neurons. Thus, inositol stores in neurons could potentially be more susceptible than those in glia to depletion by agents such as Li^+. An additional consideration is that both inositol uptake (Inoue et al., 1996; Patishi et al., 1996) and phosphoinositide turnover (Hwang et al., 1990) are reported to be highly variable in different regions of the brain. Hence, inositol concentration and metabolism may be similarly regionally diverse.

To address a lack of information regarding inositol concentrations in CNS neurons and the mechanisms whereby those concentrations are attained, inositol homeostasis has been investigated in NT2-N neurons. NT2-N cells are derived from the human teratocarcinoma cell line NT2, which is induced to irreversibly differentiate to a postmitotic neuronal phenotype by means of retinoic acid treatment (Andrews, 1984; Andrews et al., 1984; Pleasure et al., 1992). NT2-N cells have a highly polarized and distinctly neuronal morphology, characterized by a single axon and multiple dendrites. In addition, NT2-N neurons express many CNS neuron-specific proteins such as neurofilament subunits (Lee and Andrews, 1986), neural cell adhesion molecules and neuronal microtubule-associated proteins (Pleasure et al., 1992), NMDA receptors (Younkin et al., 1993), choline acetyltransferase (Zeller and Strauss, 1995), and tyrosine hydroxylase (Iacovitti and Stull, 1997). These cells also express functional phosphoinositide signaling proteins such as M_3 and M_5 muscarinic receptors, G_q, and phospholipase C-β (Wolf et al., 1995; Squires et al., 1996). NT2-N neurons are thus nearly identical to primary neurons in culture by numerous morphological, immunocytochemical, biochemical, and electrophysiological criteria. The present results indicate that after differentiation of NT2 stem cells to NT2-N neurons, the concentration of inositol increases ~10-fold, and that this increase can be accounted for by increased uptake and decreased efflux of inositol. A preliminary account of part of this study has been reported previously (Novak et al., 1998).

MATERIALS AND METHODS

Materials

myo-[2-^{3}H]Inositol (80 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.). D-[6-^{3}H]Glucose 6-phosphate (60 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). 3-*O*-[^{3}H]Methyl-D-glucose (60 Ci/mmol), 3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone (phloretin), phloretin 2′-β-D-glucoside dihydrate (phlorizin), bis(trimethylsilyl)trifluoroacetamide, all *trans*-retinoic acid, uridine, 5-fluoro-2′-deoxyuridine, cytosine β-D-arabinofuranoside, NAD, *myo*- and *scyllo*-inositol, and *myo*-inositol monophosphate phosphohydrolase (inositol monophosphatase; EC 3.1.3.25) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Powdered Dulbecco's modified Eagle's medium (DMEM), 0.25% (wt/vol) trypsin, 10× trypsin-EDTA [0.5% (wt/vol) trypsin, 5.3 m*M* EDTA], 1× Versene (0.2 g/L of EDTA in phosphate-buffered saline), 50× penicillin/streptomycin (5,000 U/ml of penicillin G sodium and 5,000 μg/ml of streptomycin sulfate), and Hanks' balanced salt solution were from GIBCO (Grand Island, NY, U.S.A.). Fetal calf serum was obtained from BioWhittaker (Walkersville, MD, U.S.A.). Matrigel basement membrane matrix and poly-D-lysine hydrobromide were from Collaborative Biomedical Products (Bedford, MA, U.S.A.). Tissue culture supplies were obtained from Corning Glass Works (Corning, NY, U.S.A.) and Sarstedt (Newton, NC, U.S.A.). Coomassie Plus and bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL, U.S.A.). UniverSol-ES liquid scintillation cocktail was obtained from ICN (Costa Mesa, CA, U.S.A.). Dowex-1 resin (100–200 mesh; X8 formate) was from Bio-Rad Laboratories (Hercules, CA, U.S.A.). High-performance TLC plates (0.20 mm) were obtained from Merck (Darmstadt, Germany). En^{3}Hance surface autoradiography enhancer was from Du Pont/New England Nuclear (Boston, MA, U.S.A.). One-month-old male Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN, U.S.A.). Rat cerebellar granule cells, L929 mouse fibroblast cells, and 1321N1 human astrocytoma cells were gifts from Dr. M. E. Gnegy (University of Michigan), Dr. M. D. Uhler (University of Michigan), and Dr. R. K. Palmer (University of North Carolina), respectively.

Cell culture

NT2 stem cells (passages 50–80) were grown in 75-cm^2 tissue culture flasks containing DMEM supplemented with 10% (vol/vol) fetal calf serum and (during retinoic acid treatment) 1× penicillin/streptomycin. NT2 cells were differentiated into the neuronal phenotype (NT2-N) as previously described (Pleasure et al., 1992). In brief, NT2 cells were treated with 10 μ*M* retinoic acid twice per week for 5 weeks. After retinoic acid treatment, cells were rinsed with 1× Versene, detached from culture flasks with 0.25% (wt/vol) trypsin, and replated into 225-cm^2 flasks (replate I). Two days later, cells were rinsed with Hanks' balanced salt solution and treated with 1× trypsin-EDTA. Flasks were then struck repeatedly to remove NT2-N cells, which were replated into 6-cm dishes previously coated with 10 μg/ml poly-D-lysine and 1:36 Matrigel in DMEM (replate II). NT2-N cells were seeded at a density of 2.5–5.0 × 10^6 cells/dish. For the first 3 weeks after replate II, NT2-N cells were maintained in DMEM supplemented with 10% (vol/vol) fetal calf serum, 1× penicillin/streptomycin, 10 μ*M* 5-fluoro-2′-deoxyuridine, 10 μ*M* uridine, and 1 μ*M* cytosine β-D-arabinofuranoside. After 3 weeks, NT2-N cells were maintained in conditioned medium obtained from replate I. NT2-N cells were used for experiments between 4 and 5 weeks after replate II. NT2 cells used for inositol efflux experiments were frequently plated on dishes previously coated with poly-D-lysine and Matrigel to improve adherence to the substratum; this treatment had no observable effect on inositol transport characteristics. Both NT2 and NT2-N cells were grown at 37°C in a humidified atmosphere of 90% air and 10% CO_2.

Rat cerebellar granule cells were prepared essentially as described by del Río et al. (1998). These cells and SH-SY5Y human neuroblastoma, 1321N1 human astrocytoma, and L929 mouse fibroblast cells were maintained in DMEM supplemented with 10% (vol/vol) fetal calf serum as described above. Inositol-free SH-SY5Y cells were grown as described above, except that cells were maintained in inositol-free DMEM supplemented with dialyzed fetal calf serum.

Inositol mass

Cells were washed with a modified Puck's D_1 solution (Honegger and Richelson, 1976), detached from culture flasks or dishes by either scraping or incubation with D_1 containing 1× trypsin-EDTA, and centrifuged at 1,000 *g* for 1 min.

Similar values for inositol content were obtained with each procedure. Rats were killed by decapitation. Cell or rat tissue was lysed in 1 ml of water and ultrasonicated with a Sonic Dismembrator 60 (Fisher Scientific, Hampton, NH, U.S.A.). Proteins were determined and 0.25–1.0 mg of lysate protein was extracted with 1.5 ml of $CHCl_3/CH_3OH$ (1:2) and 1 ml of $CHCl_3$. *scyllo*-Inositol (500 nmol) was added to some extracts as an internal standard. Extracts were centrifuged at 2,400 *g* for 5 min. The upper aqueous phase was blown free of CH_3OH under N_2, stored at −80°C for 1 h, and lyophilized overnight. Samples were resuspended in 50 μl of pyridine and 50 μl of bis(trimethylsilyl)trifluoroacetamide and heated at 70°C for 2 h. Samples were stored in a desiccator overnight and centrifuged at 2,400 *g* for 5 min. Inositol mass was determined with an HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) fitted with an SP 2250 capillary column (Supelco, Bellefonte, PA, U.S.A.). Samples (2 μl) were injected with the injector temperature at 220°C and the oven temperature at 130°C. After 6 min, the oven temperature was ramped at 5°C/min to 155°C. The temperature was held for 5 min and ramped at 25°C/min to 240°C. The temperature was again held for 5 min and then returned to the initial temperature. The detector temperature was 350°C. An HP 3396A integrator (Hewlett-Packard) was used to calibrate signal intensity for *myo*- and *scyllo*-inositol, which eluted as single peaks with retention times of 11.8 and 11.2 min, respectively.

Intracellular water space

Intracellular water spaces were measured essentially as previously described by Kletzien et al. (1975). NT2 and NT2-N cells, attached to 6-cm dishes, were washed with 5 × 2 ml of buffer A without glucose (142 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, and 30 m*M* HEPES, pH 7.4) and incubated with various concentrations of 3-*O*-[^{3}H]methyl-D-glucose at 37°C for 30 min. Cells were then washed with 5 × 2 ml of ice-cold buffer A without glucose containing 0.1 m*M* phloretin and lysed with 2 × 1 ml of ice-cold 6% (wt/vol) trichloroacetic acid. Lysates were transferred to test tubes on ice and centrifuged at 2,400 *g* for 5 min at 4°C. Intracellular 3-*O*-[^{3}H]methyl-D-glucose in 1 ml of supernatant was determined by liquid scintillation spectroscopy after the addition of 10 ml of UniverSol scintillation fluid.

Inositol uptake

A modification of the method of Batty et al. (1993) was used to determine inositol uptake. NT2 and NT2-N cells, attached to 6-cm dishes, were washed with 4 × 2 ml of buffer A (142 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, 30 m*M* HEPES, and 5.6 m*M* D-glucose, pH 7.4) and allowed to equilibrate in 2.85 ml of the same buffer at 37°C for 30 min. Reactions were initiated after the addition of 150 μl of various concentrations of [^{3}H]inositol (5 μCi/μmol) and allowed to proceed at either 0 or 37°C for 1 h. To terminate the reactions, the buffer was rapidly aspirated and the cells were washed with 5 × 2 ml of ice-cold buffer A containing 1 m*M* inositol and lysed with 2 × 1 ml of ice-cold 6% (wt/vol) trichloroacetic acid. Lysates were transferred to test tubes on ice and centrifuged at 2,400 *g* for 5 min at 4°C. Radioactivity in the supernatant was determined as previously described. The pellet was dissolved in a solution of 5% (wt/vol) sodium dodecyl sulfate and 0.1 *M* NaOH and analyzed for protein. In experiments to study the effect of hypertonicity, cells were maintained for 24 h in either isotonic (350 mOsm) medium or medium made hypertonic (500 mOsm) by the addition of NaCl. When the Na^+ dependence of inositol uptake was examined, cells were washed and incubated in either buffer A or Na^+-free buffer A (which contained 142 m*M* choline chloride instead of NaCl and 3.6 m*M* $KHCO_3$ instead of $NaHCO_3$).

In some experiments, the uptake of inositol into NT2-N cells was monitored in prolonged incubations. Under these conditions, NT2-N cells, attached to 6-cm dishes, were incubated at 37°C with conditioned medium containing 10 μCi/ml *myo*-[2-^{3}H]inositol for up to 96 h. Reactions were terminated and radioactivity associated with supernatant (cytosol) fraction was determined as described above. To determine the incorporation of [^{3}H]inositol into phospholipids, 1 ml of water, 1 ml of $CHCl_3$, and 1.5 ml of $CHCl_3/CH_3OH$ (1:2) were added to the tissue pellets, and lipids were extracted and analyzed for ^{3}H-labeled phosphoinositides as described by Fisher et al. (1984). The remaining pellet was dissolved in a solution of 5% (wt/vol) sodium dodecyl sulfate and 0.1 *M* NaOH and analyzed for protein content.

Inositol efflux

NT2 and NT2-N cells, attached to 6-cm dishes, were incubated at 37°C for 1 h in medium containing 20 μCi/ml *myo*-[2-^{3}H]inositol (short-term labeling) or 18 h in medium containing 10 μCi/ml *myo*-[2-^{3}H]inositol (long-term labeling). After preincubation, the cells were washed with 3 × 2 ml of buffer A and incubated in 4 ml of buffer A at 37°C. Aliquots of extracellular medium (50 μl) were periodically removed and centrifuged at 7,800 *g* for 5 min to remove cell debris and radioactivity was determined. To terminate the reactions, the medium was rapidly aspirated and the cells washed with 3 × 2 ml of ice-cold buffer A. Cells were lysed with 2 × 1 ml of ice-cold 6% (wt/vol) trichloroacetic acid and water-soluble radioactivity determined as previously described. Total water-soluble radioactivity present initially in the cells was calculated as the sum of that recovered in the extracellular medium and that remaining in the lysate at the end of the assay. Efflux at any given time was then expressed as the ratio of radioactivity present in extracellular medium to total [^{3}H]inositol.

Inositol synthase

L-*myo*-Inositol-1-phosphate synthase (inositol synthase; EC 5.5.1.4) activity was measured by a modification of the procedure of Culbertson et al. (1976). Rats were killed by decapitation and brain or testis suspended in ice-cold homogenization buffer [50 m*M* Tris-HCl and 0.02% (wt/vol) NaN_3, pH 7.4] at a final concentration of 0.5 g/ml. Tissue was homogenized in glass homogenizers at 4°C. One 75-cm^2 flask of NT2 cells and eight to ten 6-cm dishes of NT2-N cells (~1 mg of protein) were incubated with D_1 containing 1× trypsin-EDTA. Cells were removed, resuspended in 0.1 ml of homogenization buffer, and triturated. Both rat homogenates and cell tissue lysates were transferred to microfuge tubes and centrifuged at 19,500 *g* for 15 min at 4°C. The supernatants were then removed and proteins determined. To initiate the reaction, 10 μl of supernatant (~100–200 μg of protein) was added to 10 μl of incubation buffer (28 m*M* NH_4Cl, 5.4 m*M* $MgCl_2$, 150 m*M* Tris acetate, pH 7.4, 1.6 m*M* NAD, 4 m*M* D-glucose 6-phosphate, and 1 mCi/ml D-[6-^{3}H]glucose 6-phosphate), and the reaction was allowed to proceed at 37°C for 1 h. Reactions were terminated by placing reaction tubes in a boiling water bath for 5 min. Control samples were boiled for 5 min before the addition of incubation buffer. Inositol monophosphatase (10 μl of 0.25 U/ml; 1 U produces 1.0 μmol/min of phosphate at pH 7.4 and 37°C) was added and each sample incubated at 37°C for 1 h to convert inositol 1-phosphate to inositol. The sample volume was increased to 0.5 ml by the addition of

water, and 0.5 ml of Dowex-1 resin was added to bind anionic compounds. The resin was washed and pelleted by centrifugation and the supernatant, which contained inositol, was taken to dryness either by lyophilization or vacuum centrifugation. The supernatants were then resuspended in 40 μl of unlabeled inositol (5 mg/ml) and 20 μl was spotted onto preheated TLC plates. The plates were run twice for 6–12 h in a tank equilibrated with 120 ml of propanol/pyridine/water (3:1:1, by volume), sprayed with En^{3}Hance, and exposed to x-ray film for 2–4 days. The band comigrating with an authentic [^{3}H]inositol standard was scraped and radioactivity was determined.

Data analysis

Results shown represent mean ± SEM values for the number (n) of independent experiments performed. Duplicate or triplicate values were obtained for each parameter measured. Student's two-tailed *t* tests were used to evaluate the statistical differences between the means of paired or unpaired sets of data.

RESULTS

Teratocarcinoma stem cells (NT2) differentiate into CNS neurons (NT2-N) after retinoic acid treatment

Previous reports (Andrews, 1984; Andrews et al., 1984) have described the retinoic acid-mediated differentiation of NT2 teratocarcinoma cells to postmitotic NT2-N neurons. Before differentiation, NT2 stem cells showed the rapid proliferation, lack of contact inhibition, and amorphous phenotype characteristic of cancer cells (Fig. 1A). After 5 weeks of treatment with 10 μM retinoic acid, however, the cells ceased to divide and began to aggregate. After selective replating and 1 week of treatment with mitotic inhibitors, the cell population was enriched for NT2-N neurons, which propagated neurites as residual NT2 cells died (Fig. 1B). Three weeks later, cultures of NT2-N neurons were obtained, organized into an interconnected network of prominent ganglia that elaborated processes resembling axons and dendrites (Fig. 1C). These cultures have previously been shown to be >95% pure by immunocytochemical criteria (Lee and Andrews, 1986; Pleasure et al., 1992).

Neuronal differentiation of NT2 cells results in an increased intracellular concentration of inositol

To assess the effect of neuronal differentiation on inositol homeostasis, inositol concentrations within NT2 and NT2-N cells were compared. NT2-N neurons contained approximately eight times the mass of inositol present in NT2 stem cells (195 vs. 24 nmol/mg of protein; Table 1). As both cell types were maintained in the presence of similar concentrations of extracellular inositol (~100 μM), these data indicate that neuronal differentiation is accompanied by a substantial accumulation of inositol. By comparison, the concentration of inositol in primary cultures of rat cerebellar granule cells was ~20% of that in NT2-N neurons. SH-SY5Y human neuroblastoma cells, a frequently used model of PNS neurons, contained approximately one-half the concentration of inositol present in NT2-N cells. As observed previously for SK-N-SH neuroblastoma cells (Stubbs and Agranoff, 1993), the concentration of inositol in SH-SY5Y cells could be further reduced to 16 nmol/mg of protein by growth in inositol-free medium for 1–4 weeks. Inositol concentrations in 1321N1 human astrocytoma cells and L929 mouse fibroblast cells were also lower than those in NT2-N cells. The concentrations of

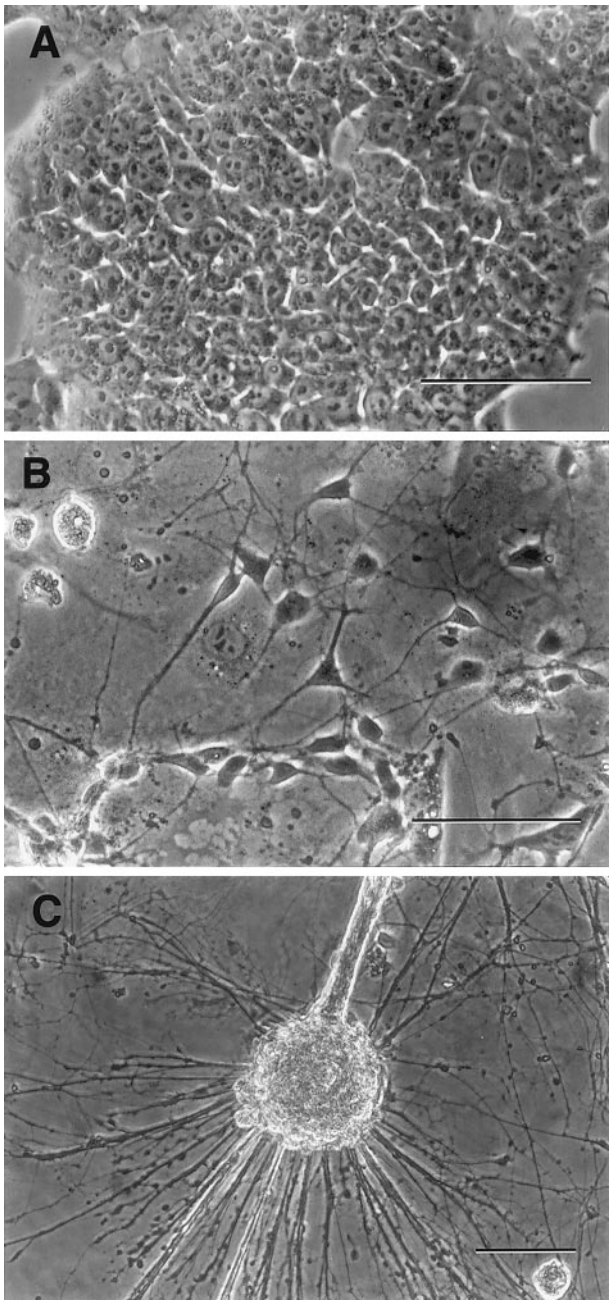

FIG. 1. Phase-contrast photomicrographs illustrating the morphological changes that occur during retinoic acid-mediated differentiation of NT2 cells and subsequent treatment of NT2-N cells with mitotic inhibitors. **A:** Untreated NT2 teratocarcinoma stem cells. **B:** Individual NT2-N neurons, 7 days after replate II as described in Materials and Methods. **C:** NT2-N ganglia, 28 days after replate II. Bar = 0.1 mm.

TABLE 1. *Concentrations of inositol in cultured cells and rat tissues*

Cell/tissue	Inositol (nmol/mg of protein)
NT2 stem cells	24.5 ± 1.9 (10)
NT2-N neurons	195.2 ± 22.6 (8)
Rat cerebellar granule cells	41.1 ± 9.8 (3)
SH-SY5Y human neuroblastoma cells	111.7 ± 12.1 (5)
SH-SY5Y human neuroblastoma cells (IF)	16.1 ± 1.6 (5)
1321N1 human astrocytoma cells	76.2 ± 8.1 (3)
L929 mouse fibroblasts	37.0 ± 7.3 (3)
Cerebral cortical gray matter	60.9 ± 5.5 (3)
Cerebral cortical white matter	79.5 ± 0.6 (3)
Testis	21.2 ± 1.1 (3)
Liver	4.6 ± 0.7 (3)

Cell or tissue lysates were prepared as described in Materials and Methods and inositol mass was determined by gas chromatography. SH-SY5Y cells (IF) were maintained in inositol-free medium for 4 weeks. Results shown are mean ± SEM values for the number of separate experiments indicated in parentheses.

inositol in gray and white matter of rat brain were approximately one-third those in NT2-N neurons, whereas those of rat testis and liver were very low (<11 and 2%, respectively, of that in NT2-N neurons).

To obtain a more physiologically relevant measurement of intracellular concentrations of inositol, the intracellular water spaces in NT2 and NT2-N cells were determined. Cells were incubated in the presence of increasing extracellular concentrations of 3-*O*-[^{3}H]-methyl-D-glucose and intracellular concentrations monitored at equilibrium. A plot of these two parameters yields a line whose slope is the volume of intracellular water with respect to protein (data not shown). Determination of water space by this method requires that 3-*O*-methyl-D-glucose not be metabolized or actively transported, and these assumptions were validated by the linearity of the plot and its extrapolation through the origin (Kletzien et al., 1975). The water spaces inside NT2 and NT2-N cells were 15.1 ± 0.1 and 11.2 ± 0.1 μl/mg of protein, respectively (n = 3). Based on these data, the intracellular concentrations of inositol in NT2 stem cells and NT2-N neurons were calculated to be 1.6 and 17.4 m*M*, respectively.

Neuronal differentiation of NT2 cells is accompanied by an increased uptake of inositol

Of the possible mechanisms underlying the high concentrations of inositol in NT2-N neurons, inositol uptake is the most thoroughly characterized (Yorek et al., 1986; Fruen and Lester, 1991; Wiesinger, 1991; Batty et al., 1993). Uptake of [^{3}H]inositol into NT2 and NT2-N cells was determined during short-term (1 h) incubations (Fig. 2). Under these conditions, uptake was linear with time from 5 min onward at concentrations of inositol up to 500 μ*M* (data not shown). Dose–response curves and the associated Eadie–Hofstee plot (Fig. 2A) indicated a maximal velocity of inositol uptake (V_{max}) of 1.6 nmol/mg of protein/h into NT2 cells, which increased to 3.7 nmol/mg of protein/h after neuronal differentiation. The EC_{50} values for inositol uptake were 70 and 36 μ*M* for NT2 and NT2-N cells, respectively. Because of the limited availability of NT2-N cells, changes in inositol uptake after differentiation were routinely examined at two selected concentrations of inositol, i.e., 50 μ*M*, a value close to the EC_{50}, and 250 μ*M*, a value that approximates the V_{max} (Fig. 2B). At both concentrations of inositol, uptake into NT2-N cells was approximately double that observed for NT2 cells ($p < 0.001$).

In most cells, high-affinity inositol uptake is mediated by an Na^+/*myo*-inositol transporter. Batty et al. (1993) and others have described this transporter on the basis of its Na^+ dependence and competitive inhibition by phlorizin. In addition, transcription of the Na^+/*myo*-inositol transporter is up-regulated during conditions of chronic hypertonicity (Nakanishi et al., 1989; Isaacks et al., 1994). The uptake of [^{3}H]inositol into NT2 and NT2-N

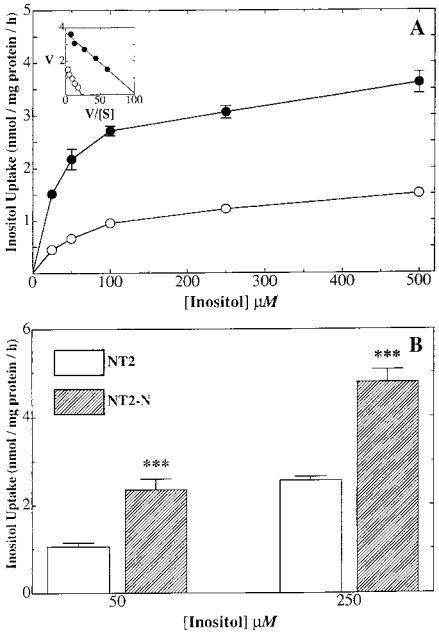

FIG. 2. Uptake of *myo*-inositol into NT2 and NT2-N cells. **A:** NT2 (○) or NT2-N (●) cells, adherent to 6-cm dishes, were washed four times with buffer and incubated with [^{3}H]inositol (5 μCi/μmol) at the concentrations indicated at either 0 or 37°C for 1 h. Cells were then washed five times with ice-cold buffer containing 1 m*M* inositol and lysed with trichloroacetic acid. Lysates were centrifuged and radioactivity present in supernatants determined. Results shown are the differences between mean ± SEM values of triplicate samples at 0 and 37°C for NT2 or NT2-N cells assayed on the same day in a single experiment. Uptake at 0°C was typically 5–10% of uptake at 37°C. Where error bars are not shown, they fell within the symbol. SEM was calculated as the square root of $[(SEM_1)^2 + (SEM_2)^2]$, where SEM_1 and SEM_2 are the standard errors of the mean for inositol uptake at 0 and 37°C. **Inset:** Eadie–Hofstee plot of dose–response data. **B:** NT2 or NT2-N cells were incubated with 50 or 250 μ*M* [^{3}H]inositol as described in A. Results shown are mean ± SEM values for four to eight separate experiments. ***Different from NT2 cells, $p < 0.001$.

TABLE 2. *Characterization of inositol uptake in NT2 and NT2-N cells*

Characteristic	Inositol (μM)	Phlorizin (mM)	% of control uptake NT2	% of control uptake NT2-N
Na^+ dependence	50	—	95.2 ± 1.0	97.4 ± 0.9[a]
	250	—	91.7 ± 1.2	93.6 ± 1.9[a]
Phlorizin inhibition	50	0.2	47.5 ± 4.3	52.3 ± 5.3[a]
	50	1.0	20.6 ± 0.8	21.3 ± 0.4[a]
Hypertonic stimulation	250	—	279.9 ± 39.9	226.0 ± 23.3[a]

NT2 or NT2-N cells, adherent to 6-cm dishes, were incubated with [^{3}H]inositol for 1 h, then lysed with trichloroacetic acid and centrifuged. Radioactivity in the lysate supernatant was determined as described in Materials and Methods. For Na^+-dependence assays, cells were incubated in either buffer A or Na^+-free buffer A (which contained choline chloride instead of NaCl). For hypertonic stimulation assays, cells were maintained for 24 h in either isotonic (350 mOsm) medium or medium made hypertonic (500 mOsm) by the addition of NaCl. Results shown are mean ± SEM values for three separate experiments. Control values for inositol uptake in NT2 cells were 2.2 ± 0.4 and 3.2 ± 0.5 nmol/mg of protein/h (n = 4–5) at inositol concentrations of 50 and 250 μM, respectively. The corresponding values for NT2-N cells were 3.8 ± 0.5 and 5.4 ± 0.3 nmol/mg of protein/h (n = 4–6).

[a] Not different from NT2 cells, $p > 0.05$.

cells was almost completely (>90%) Na^+-dependent at 50 and 250 μM concentrations of inositol in both cell types (Table 2). The inclusion of phlorizin caused a dose-dependent inhibition of uptake in both NT2 and NT2-N cells consistent with competitive antagonism of the transporter. Furthermore, maintenance of both NT2 and NT2-N cells in hypertonic medium (500 mOsm) for 24 h stimulated inositol uptake to a similar degree (two- to threefold). These data imply that an Na^+/*myo*-inositol transporter is involved in high-affinity uptake in both cell types, and that the characteristics of inositol uptake are not affected by neuronal differentiation. However, the two cell types differed with respect to changes in inositol mass after hypertonic exposure. Although hypertonicity resulted in an increase in inositol concentration in NT2 cells consistent with the increase in uptake (282 ± 40% of control, n = 3), little or no increase in inositol concentration was observed in NT2-N neurons (120 ± 8% of control, n = 4; different from NT2 cells, $p < 0.01$).

The concentration of inositol present in NT2-N neurons (17.4 mM) is 150–200-fold higher than that of the extracellular medium (~100 μM). To determine how readily the intracellular pool of inositol in NT2-N cells could exchange with extracellular inositol, the kinetics of [^{3}H]inositol uptake into the neurons were monitored in prolonged incubations (1–96 h; Fig. 3). These long-term incubation assays were feasible in NT2-N neurons because of the postmitotic state of the cells. The uptake of label into both the water-soluble and the lipid fractions of the neurons was curvilinear and approached a plateau by 96 h (Fig. 3A and B, respectively), although the appearance of radioactivity in the water-soluble fraction preceded the incorporation of label into the lipid fraction. By 96 h, the cells had attained a specific activity of ~300 dpm/pmol inositol, a value similar to the specific activity of inositol calculated to be present in the extracellular medium. Equilibrium labeling of the inositol lipid pool was achieved by ~48 h, at which time ~1% of the water-soluble radioactivity could be recovered in the

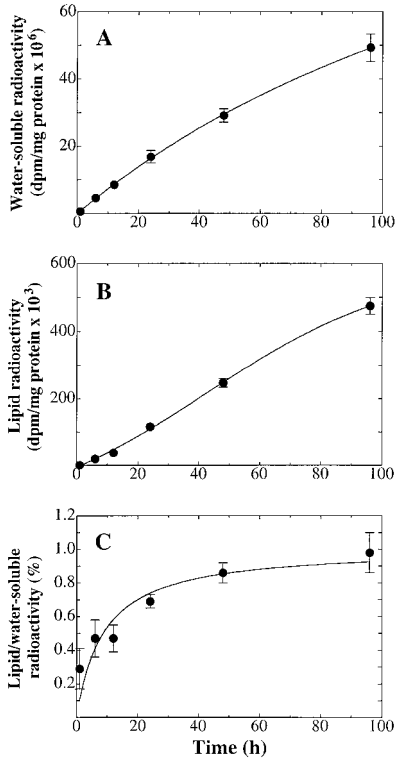

FIG. 3. Long-term labeling of NT2-N cells with [^{3}H]inositol. NT2-N cells, adherent to 6-cm dishes, were incubated at 37°C with medium containing 10 μCi/ml [^{3}H]inositol for 1–96 h. Cells were then washed five times with ice-cold buffer containing 1 mM inositol and lysed with trichloroacetic acid. Lysates were centrifuged and radioactivity present in supernatants determined. Lipids were extracted from pellets as described in Materials and Methods. **A:** Water-soluble radioactivity. Values were determined by quantification of radioactivity in lysate supernatants. **B:** Radioactivity in phospholipids. Values were determined by quantification of radioactivity in organic fractions. **C:** Ratio of radioactivity present in phospholipid relative to that present in water-soluble fraction. Results shown are mean ± SEM values for three separate experiments. Where error bars are not shown, they fell within the symbol.

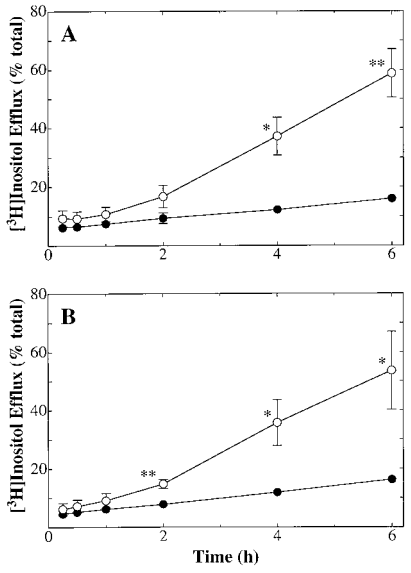

FIG. 4. Efflux of *myo*-inositol from NT2 and NT2-N cells. **A:** Short-term prelabeling of cells with [^{3}H]inositol. NT2 (○) or NT2-N (●) cells, adherent to 6-cm dishes, were incubated with medium containing 20 μCi/ml [^{3}H]inositol for 1 h. Cells were washed three times with buffer and incubated at 37°C. Aliquots of extracellular medium were periodically removed and radioactivity was determined. Cells were then washed three times with ice-cold buffer and lysed with trichloroacetic acid. Lysates were centrifuged and radioactivity present in supernatants was determined. **B:** Long-term prelabeling of cells with [^{3}H]inositol. NT2 or NT2-N cells were incubated with medium containing 10 μCi/ml [^{3}H]inositol for 18 h. Efflux of inositol was then determined as described in A. Data are expressed as [^{3}H]inositol recovered in the extracellular medium relative to [^{3}H]inositol present initially in the cells. Results shown are mean ± SEM values for three separate experiments. Where error bars are not shown, they fell within the symbol. *Different from NT2-N cells, $p < 0.05$. **Different from NT2-N cells, $p < 0.01$.

phosphoinositide fraction (Fig. 3C). More than 95% of the radioactivity found in lipid after 24 h was in the form of phosphatidylinositol, with the remainder associated with the polyphosphoinositides (data not shown).

Neuronal differentiation of NT2 cells is associated with a decreased efflux of inositol

Changes in inositol efflux from NT2 and NT2-N cells were also evaluated as a potential mechanism whereby changes in inositol concentration could be effected. After prelabeling cells for 1 h with [^{3}H]inositol, the efflux of label was monitored for 6 h. After an initial lag period of ~30 min, efflux of label from NT2 stem cells proceeded at an approximately linear rate of 9.0 ± 1.8%/h (n = 3), whereas efflux from NT2-N neurons was significantly lower (1.7 ± 0.1%/h, n = 3; different from NT2 cells, $p = 0.01$; Fig. 4A). The kinetics of [^{3}H]inositol efflux from NT2 and NT2-N cells prelabeled with [^{3}H]inositol for 18 h were comparable to those obtained for the shorter prelabeling period and in NT2 cells proceeded at four to five times the rate of that observed for NT2-N neurons (Fig. 4B). More than 95% of NT2 and NT2-N cells excluded the vital dye trypan blue throughout the course of the efflux period, a result that indicates that a reduction in cell viability did not account for the differences in the release of [^{3}H]inositol between the two cell types.

Neither NT2 nor NT2-N cells exhibit measurable inositol synthase activity

An additional potential mechanism to explain the observed increases in inositol concentration after neuronal differentiation is an increased de novo synthesis of inositol mediated by L-*myo*-inositol-1-phosphate synthase (inositol synthase, EC 5.5.1.4). This enzyme, which catalyzes the cyclization of D-glucose 6-phosphate to L-inositol 1-phosphate, has been found in the seminiferous tubules of testis (Eisenberg, 1967) and in the capillary endothelium of brain (Wong et al., 1987). Cell lysates and rat tissue homogenates were incubated with [^{3}H]glucose 6-phosphate, and [^{3}H]inositol 1-phosphate thus produced was converted to [^{3}H]inositol, which was then separated by TLC. As anticipated, inositol synthase activity was readily measured in rat testis, whereas the activity in brain (0.12 ± 0.06 nmol/mg of protein/h, n = 3) was <8% of that present in testis (1.57 ± 0.35 nmol/mg of protein/h, n = 5). No production of [^{3}H]-inositol was detected when equivalent amounts of either NT2 or NT2-N lysates were assayed under the same conditions. These data suggest that de novo synthesis of inositol plays little or no role in the increased intracellular inositol concentration in NT2-N neurons.

DISCUSSION

myo-Inositol is required for phosphoinositide synthesis and for osmotic regulation within the CNS. Consequently, its cellular concentrations and proper disposition within the CNS can be critical to neurological and behavioral function. It is well established that concentrations of inositol in brain are much higher than those in plasma (5–50 m*M* vs. 0.05–0.1 m*M*, respectively; Palmano et al., 1977; Sherman et al., 1977; Stokes et al., 1983; Pouwels and Frahm, 1998) as well as in other organs, but the role that neurons play in the maintenance of these substantial inositol stores has yet to be defined. This report is the first, to our knowledge, in which the concentration of inositol in a pure population of cultured CNS neurons has been directly measured. It has been suggested that inositol is primarily (Glanville et al., 1989) or even exclusively (Brand et al., 1993) to be found in glia. The present results indicate, however, that neurons, as has been reported for glia (Isaacks et al., 1994), are able to maintain high intracellular concentrations of inositol, a conclusion in agreement with earlier observations of Sherman et al. (1977). The concentrations of inositol in NT2-N neurons (195 nmol/mg of protein) exceeded those found in astrocytoma cells (76 nmol/mg of protein), rat brain gray and white matter (~70 nmol/mg of protein), and human brain (60 nmol/mg of protein; Stokes et al., 1983). However, the concentration of inositol present in NT2-N neurons (195 nmol/mg of protein; 17.4 m*M*) is consistent with the

value reported for a neuron-rich tissue, the Purkinje cell layer of cat cerebellar flocculus (~20 m*M;* Godfrey et al., 1982). Nonetheless, it remains possible that the concentration of inositol may differ considerably among distinct populations of neurons. For example, the concentration of inositol in rat cerebellar granule cells (~40 nmol/mg of protein) was <25% of that in NT2-N neurons.

The high concentration of inositol in NT2-N neurons may, in part, be accounted for by an increased rate of inositol uptake. Stimulation of inositol uptake after differentiation is not limited to cells of neuronal lineage and has, for example, been reported for HL60 promyeloid cells (Baxter et al., 1991). If the elevations in inositol concentration in NT2-N cells that result from differentiation of NT2 cells were solely dependent on the two- to threefold increase in inositol uptake, an intracellular concentration of ~20 m*M* could hypothetically be attained within 48 h. Direct determination of [^{3}H]inositol uptake indicated that the activity of the transporter in NT2-N neurons permitted the equilibration of extracellular inositol with the intracellular pool within ~96 h. Despite the differences in the capacity of inositol uptake in the precursor stem cells and differentiated neurons, the characteristics of uptake (EC_{50}, Na^+ dependence, phlorizin inhibition, and response to hypertonic stimulation) were essentially the same and comparable to those observed for other cell lines (Strange et al., 1991; Wiesinger, 1991; Batty et al., 1993; Isaacks et al., 1994). Whereas chronic hypertonicity evoked a stimulation of inositol uptake in both NT2 and NT2-N cells, there was no commensurate increase in the concentration of inositol in NT2-N cells. These data suggest that the concentration of inositol in NT2-N neurons is maximal under isotonic conditions, and that any increases that may occur after hypertonic exposure are not sustained. One implication of this result is that CNS neurons may have a limited capacity to accumulate inositol during hypernatremic stress and that the use of inositol for osmotic regulation may thus be primarily characteristic of glia. Glia have previously been reported to exhibit substantial increases in inositol concentration in response to hypertonicity (Strange et al., 1991; Isaacks et al., 1994).

Neuronal differentiation of NT2 cells was also accompanied by a decreased efflux of inositol. Inositol efflux from both NT2 and NT2-N cells occurred over a relatively prolonged time course (hours) and was unaffected by Cl^- channel inhibitors such as niflumic acid and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (data not shown). In contrast, inositol efflux from C6 glioma cells (Jackson and Strange, 1993) and rat astrocyte cultures (González et al., 1995) occurs within minutes and is mediated by a nonselective Cl^- channel. A low rate of efflux associated with NT2-N neurons, observed under conditions of both acute and prolonged prelabeling with [^{3}H]inositol, may contribute to the high concentrations of inositol present in these cells.

A third potential mechanism whereby inositol could accumulate in NT2-N cells is from de novo synthesis via cyclization of glucose 6-phosphate (Eisenberg, 1967). Whereas inositol synthase activity could be measured in both rat brain and testis, no activity was detected in either NT2 or NT2-N cell lysates when assayed under the same conditions. The absence of synthase activity from NT2-N cells and its presence in brain is consistent with previous immunocytochemical data localizing the enzyme primarily to the vasculature (Wong et al., 1987). The activity of inositol synthase in brain was <8% of that in testis, a value consistent with the ~5% ratios previously observed for rat (Eisenberg, 1967) and bovine (Mauck et al., 1980) tissues. Despite the relatively high activity of inositol synthase in testis, the low concentration of inositol in this tissue (21 nmol/mg of protein) suggests that de novo synthesis may not contribute significantly to the maintenance of cellular inositol.

In summary, differentiated NT2-N human neurons maintain concentrations of inositol 10 times higher than their undifferentiated precursor cells. Enhancement of inositol uptake and reduction of inositol efflux after differentiation may account for this difference. Although previous studies with neural tissues have led to the suggestion that the inositol content of neurons is much less than that of glia, the present study reports the high concentration of inositol in a differentiated human neuronal cell line. There may be a wide variation in the constitutive inositol content among neural cell types, and the present study cautions against generalization in this regard.

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