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Increased Expression of $G\alpha_{q/11}$ and of Phospholipase-Cβ1/4 in Differentiated Human NT2-N Neurons: Enhancement of Phosphoinositide Hydrolysis

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Abstract: The CNS is enriched in phosphoinositide-specific phospholipase C (PLC) and in the G proteins linked to its activation. Although the regional distributions of these signaling components within the brain have been determined, neither their cell type-specific localizations (i.e., neuronal versus glial) nor the functional significance of their high expression has been definitively established. In this study, we have examined the expression of phosphoinositide signaling proteins in human NT2-N cells, a well characterized model system for CNS neurons. Retinoic acid-mediated differentiation of NT2 precursor cells to the neuronal phenotype resulted in five- to 15-fold increases in the expression of PLC-β1, PLC-β4, and $G\alpha_{q/11}$ (the prime G protein activator of these isozymes). In contrast, the expression of PLC-β3 and PLC-γ1 was markedly reduced following neuronal differentiation. Similar alterations in cell morphology and in the expression of PLC-β1, PLC-β3, and $G\alpha_{q/11}$ expression were observed when NT2 cells were differentiated with berberine, a compound structurally unrelated to retinoic acid. NT2-N neurons exhibited a significantly higher rate of phosphoinositide hydrolysis than NT2 precursor cells in response to direct activation of either G proteins or PLC. These results indicate that neuronal differentiation of NT2 cells is associated with dramatic changes in the expression of proteins of the phosphoinositide signaling system and that, accordingly, differentiated NT2-N neurons possess an increased ability to hydrolyze inositol lipids. **Key Words:** G proteins, $G_{q/11}$, G_o—Retinoic acid—Phospholipase C isozymes—Berberine—Inositol phosphates.
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Receptor-stimulated phosphoinositide hydrolysis, with the attendant formation of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol, represents a major signal transduction pathway in the CNS (for review, see Fisher et al., 1992). Agonist occupancy of phosphoinositide-linked receptors results primarily in the activation of heterotrimeric guanine nucleotide binding proteins (G proteins), chief of which is $G_{q/11}$ (Simon et al., 1991; Jope et al., 1994). $G\alpha_{q/11}$ is expressed at relatively high concentrations in highly specialized regions of the CNS, such as frontal cortex, olfactory bulb, caudate-putamen, hippocampal pyramidal cells, and cerebellar Purkinje cells (Mailleux et al., 1992; Milligan, 1993; Friberg et al., 1998). $G\alpha_{q/11}$ is linked preferentially to the activation of the β1 and β4 isozymes of phosphoinositide-specific phospholipase C (PLC) (Taylor et al., 1991; Jiang et al., 1994; Lee et al., 1994), both of which colocalize with $G\alpha_{q/11}$ (Ross et al., 1989; Roustan et al., 1995). High levels of PLC-β1 expression have been linked to neuronal differentiation and synaptic plasticity in the postnatal rat cortex (Hannan et al., 1998). In contrast, PLC-β3, which is preferentially activated by $G\beta\gamma$ subunits, is expressed only minimally in brain (Tanaka and Kondo, 1994; Watanabe et al., 1998) at a level estimated to be 1% of that of PLC-β1 (Jhon et al., 1994).

Despite the known regional distribution and enrichment of $G\alpha_{q/11}$ and PLC isozymes in the brain, a number of issues remain to be addressed. One is whether the expression of these phosphoinositide signaling proteins is homogeneous within a region of the brain or, alternatively, whether these components are enriched in specific neuronal and/or glial cell populations. Although previous studies, based on immunohistochemical criteria, have indicated that the expression of phosphoinositide signaling proteins may be cell type-specific, some of these reports are inconsistent (e.g., for $G\alpha_{q/11}$ localization to glia versus neurons, see Mailleux et al., 1992 versus Friberg et al., 1998, respectively) or incomplete (e.g., for

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Abbreviations used: db-cAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dibutyryl cyclic AMP); G protein, heterotrimeric guanine nucleotide binding protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate) tetralithium salt; PAGE, polyacrylamide gel electrophoresis; PBS-Tw, phosphate-buffered saline with 0.1% (vol/vol) Tween 20; PLC, phosphoinositide-specific phospholipase C; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

localization of the PLC-β family but not individual isozymes, see Mizuguchi et al., 1991). A separate issue is whether the relative enrichment of $G\alpha_{q/11}$ and PLC-β in neural tissues results in correspondingly higher rates of phosphoinositide turnover. Such a relationship might indicate that the concentrations of $G\alpha_{q/11}$ and/or PLC-β are rate-limiting for inositol lipid hydrolysis.

NT2-N neuronal cells, which are obtained by retinoic acid treatment of NT2 human teratocarcinoma precursors and comprise a relatively pure population of postmitotic CNS neurons (Andrews, 1984; Pleasure et al., 1992), offer an ideal cell culture model with which to address these issues. NT2-N neurons aggregate into highly polarized ganglia with morphologically identifiable axons and dendrites; they express numerous CNS neuron-specific proteins, such as neurofilaments (Lee and Andrews, 1986), neural cell adhesion molecules (Pleasure et al., 1992), NMDA receptors (Younkin et al., 1993), and tyrosine hydroxylase (Iacovitti and Stull, 1997), and they form functional synapses (Sheridan and Maltese, 1998; Hartley et al., 1999). These cells do not, however, express glial or PNS neuronal markers (Lee and Andrews, 1986; Pleasure et al., 1992). We have recently shown that differentiation of NT2 cells to the neuronal phenotype is associated with an ~10-fold increase in *myo*-inositol content, a result that is consistent with the known high concentration of this polyol in brain (Novak et al., 1999*b*). However, phosphoinositide signaling has not previously been systematically investigated in NT2-N neurons. We now report that differentiation of NT2 stem cells to the neuronal phenotype results in dramatic changes in the expression of phosphoinositide signaling proteins. Specifically, the concentrations of $G\alpha_{q/11}$, PLC-β1, and PLC-β4 are markedly increased (five- to 15-fold), whereas that of PLC-β3 is greatly reduced. Furthermore, the enhanced expression of $G\alpha_{q/11}$, PLC-β1, and PLC-β4 observed for NT2-N neurons is accompanied by substantial increases in the rate of phosphoinositide hydrolysis in response to direct activation of either G proteins or PLC. A preliminary account of part of this study has been reported previously (Novak et al., 1999*a*).

MATERIALS AND METHODS

Materials

myo-[^{3}H]Inositol (80 Ci/mmol), enhanced chemiluminescence reagents, high-range molecular weight rainbow markers, and peroxidase-conjugated sheep anti-mouse IgG were obtained from Amersham (Arlington Heights, IL, U.S.A.). Monoclonal antibody to PLC-γ1 was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Peroxidase-conjugated goat anti-rabbit IgG and polyclonal antibodies to PLC-β1, PLC-β2, PLC-β3, PLC-β4, $G\alpha_{q/11}$, $G\alpha_i$, $G\alpha_o$, and $G\alpha_s$ were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Immobilon-P polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, U.S.A.). All *trans*-retinoic acid, berberine chloride, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate [dibutyryl cyclic AMP (db-cAMP)], and digitonin were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Ionomycin and guanosine 5′-*O*-(3-thiotriphosphate) tetralithium salt (GTPγS) were from Calbiochem (La Jolla, CA, U.S.A.). Cell culture reagents and supplies were obtained as described previously (Novak et al., 1999*b*) except fetal calf serum, which was supplied by Summit Biotechnology (Fort Collins, CO, U.S.A.). Bicinchoninic acid protein assay reagents were obtained from Pierce (Rockford, IL, U.S.A.). UniverSol-ES liquid scintillation cocktail was from ICN (Costa Mesa, CA, U.S.A.). Dowex AG 1-X8 resin (100–200 mesh; formate) was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). C6 glioma cells were a gift from Dr. William Mancini (University of Michigan), originally obtained from the American Type Culture Collection. Forebrain samples from 50-day-old male Sprague–Dawley rats (220–250 g) were a gift from Dr. Kirk Frey (University of Michigan).

Cell culture

NT2 stem cells were cultured and induced to differentiate to NT2-N neurons by means of treatment with retinoic acid (5 weeks) and mitotic inhibitors (3 weeks) as previously described (Novak et al., 1999*b*). In some experiments, partial differentiation of NT2 cells was achieved by maintenance (72 h) in medium supplemented with either 10 μM retinoic acid, 10 μg/ml berberine, or 100 μM db-cAMP followed by maintenance (72 h) in medium alone. Human SH-SY5Y neuroblastoma and rat C6 glioma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum at 37°C and passaged once each week by incubation with buffer containing 0.05% (wt/vol) trypsin and 0.53 mM EDTA.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

NT2 or NT2-N cells, adherent to 6-cm dishes, were rinsed with ~3 ml of ice-cold buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 30 mM HEPES, and 5.6 mM D-glucose, pH 7.4) and incubated on ice with 300–500 μl of lysis buffer [50 mM NaCl, 20 mM HEPES, 1 mM EGTA, 1% (vol/vol) Triton X-100, 30 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4 (Wahl et al., 1988)]. Cell lysates were scraped into microcentrifuge tubes on ice and centrifuged at 7,800 *g* for 2 min to remove debris. Supernatants (25 μg) were boiled in sample buffer [200 mM Tris (pH 6.7), 5% (wt/vol) SDS, 20% (vol/vol) glycerol, 1 M β-mercaptoethanol, and 0.1% (wt/vol) bromphenol blue] for 5 min and electrophoresed through 10% polyacrylamide gels (Laemmli, 1970). Proteins were then transferred to PVDF membranes for immunoblot analysis.

Immunoblot analysis

Membranes were treated with phosphate-buffered saline (138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.2 mM KH_2PO_4, pH 7.4) supplemented with 0.1% (vol/vol) Tween 20 (PBS-Tw), 1% (wt/vol) bovine serum albumin, and 0.01% (wt/vol) NaN_3 for 1 h to block nonspecific binding sites. Primary antibodies were diluted in blocking solution (0.1–0.5 μg/ml final concentration) and incubated with membranes for 1 h, which were then washed three times with PBS-Tw to remove excess antibody. Secondary antibodies were diluted in PBS-Tw (1:10,000) and incubated with membranes for 1 h, which were again washed three times with PBS-Tw. Immunoreactive proteins were detected by enhanced chemiluminescence. In some experiments, membranes were subsequently incubated in stripping solution [0.1 M Tris (pH 8.0), 2% (wt/vol) SDS, and 100 mM β-mercaptoethanol] for 30 min at 52°C,

washed twice with PBS-Tw, and reprobed with a different primary antibody as described above. Quantitative analysis of autoluminograms was performed by computer-assisted imaging densitometry (MCID; Imaging Research, St. Catharines, Ontario, Canada).

Measurement of phosphoinositide hydrolysis

Intact cells. NT2 stem cells and NT2-N neurons were prelabeled for 48–72 h at 37°C in medium supplemented with 10–20 μCi/ml [^{3}H]inositol, by which time labeling of inositol lipids had achieved isotopic equilibrium (Novak et al., 1999*b*). Cells adherent to 6-cm dishes were rinsed with 2 ml of buffer A and incubated for 15 min at 37°C in 1.5 ml of the same buffer that contained 30 m*M* LiCl with or without 20 m*M* NaF or 1 μ*M* ionomycin. Reactions were terminated by the rapid aspiration of buffer and the addition of 2 × 1 ml of 6% (wt/vol) ice-cold trichloroacetic acid. Cell lysates were scraped into centrifuge tubes on ice and centrifuged at 2,400 *g* for 5 min at 4°C. The supernatants were washed with 5 × 2 ml of water-saturated diethyl ether and neutralized with $NaHCO_3$. Radioactivity associated with ^{3}H-inositol phosphates in the supernatants was quantified as previously described (Fisher et al., 1984). The cell pellets were analyzed to determine the incorporation of [^{3}H]inositol into phosphoinositides as described (Novak et al., 1999*b*). Production of inositol phosphates was then normalized as the ratio of ^{3}H-inositol phosphates to total radioactivity (i.e., that present in inositol phosphates and inositol phospholipids).

Permeabilized cells. NT2 and NT2-N cells were prelabeled as above, rinsed with 2 ml of KGEH buffer (139 m*M* potassium glutamate, 10 m*M* EGTA, 30 m*M* HEPES, 10 m*M* LiCl, 4 m*M* $MgCl_2$, and 2 m*M* ATP, pH 7.4), and incubated for 5 min at 37°C in 2 ml of the same buffer supplemented with 20 μ*M* digitonin. Permeabilized cells were then rinsed with 2 ml of KGEH buffer and incubated for 60 min in 1.5 ml of the same buffer that contained various concentrations of Ca^{2+} with or without 50 μ*M* GTPγS. Required free Ca^{2+} concentrations were achieved by the addition of various amounts of $CaCl_2$ to the KGEH buffer that contained 10 m*M* EGTA as previously described (Fisher et al., 1989). Reactions were terminated by the removal of the buffer to centrifuge tubes on ice and the addition of 2 × 0.5 ml of 6% (wt/vol) ice-cold trichloroacetic acid to the permeabilized cells. Cell lysates were scraped into the same centrifuge tubes and centrifuged at 2,400 *g* for 5 min at 4°C. Release of ^{3}H-inositol phosphates was then quantified as described above.

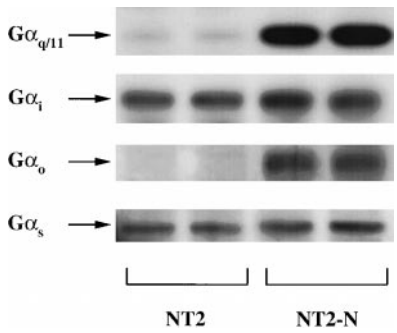

FIG. 1. G protein expression in NT2 precursors and NT2-N neurons. Cell lysates were centrifuged, and duplicate aliquots of supernatant (25 μg) were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for G$\alpha_{q/11}$, Gα_i, Gα_o, or Gα_s as described in Materials and Methods. Blots are representative of 11, five, four, and six independent experiments, respectively.

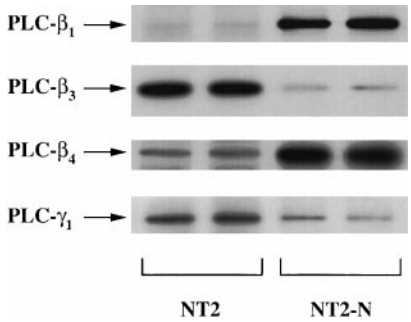

FIG. 2. PLC expression in NT2 precursors and NT2-N neurons. Cell lysates were centrifuged, and duplicate aliquots of supernatant (25 μg) were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for PLC-β1, PLC-β3, PLC-β4, or PLC-γ1 as described in Materials and Methods. Blots are representative of 11, 12, three, and four independent experiments, respectively.

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

Neuronal differentiation of NT2 cells results in selective changes in the expression of G proteins and PLC isozymes

To assess the effect of neuronal differentiation on signal transduction proteins, the expression of various G proteins in NT2 stem cells and 9-week-old, retinoic acid-induced NT2-N cells was determined by immunoblot (Fig. 1). The immunoreactivity of G$\alpha_{q/11}$, which is the prime activator of PLC-β1 and PLC-β4 (Taylor et al., 1991; Jiang et al., 1994), was markedly increased in NT2-N neurons (1,470 ± 320% of that in NT2 cells, n = 11, $p < 0.0001$). The expression of the neuron-enriched G protein Gα_o (Strittmatter et al., 1990) was also increased following the appearance of the neuronal phenotype (2,860 ± 460% of that of NT2 cells, n = 4, $p < 0.05$). In contrast, only small increases in the expression of Gα_i and Gα_s were observed following differentiation (180 ± 10% and 280 ± 50% of that present in NT2 cells, respectively, n = 5–6, $p < 0.05$). The expression of PLC isozymes following neuronal differentiation was also measured (Fig. 2). Immunoreactivity associated with PLC-β1 in NT2-N neurons increased to 1,260 ± 360% of that present in NT2 precursors (n = 11, $p < 0.001$). PLC-β4 expression in NT2-N neurons was also elevated with respect to that in NT2 cells (560 ± 170%, n = 3, $p < 0.05$). These two PLC isozymes are also enriched in specific regions of the CNS (Watanabe et al., 1998). In contrast, the immunoreactivity of

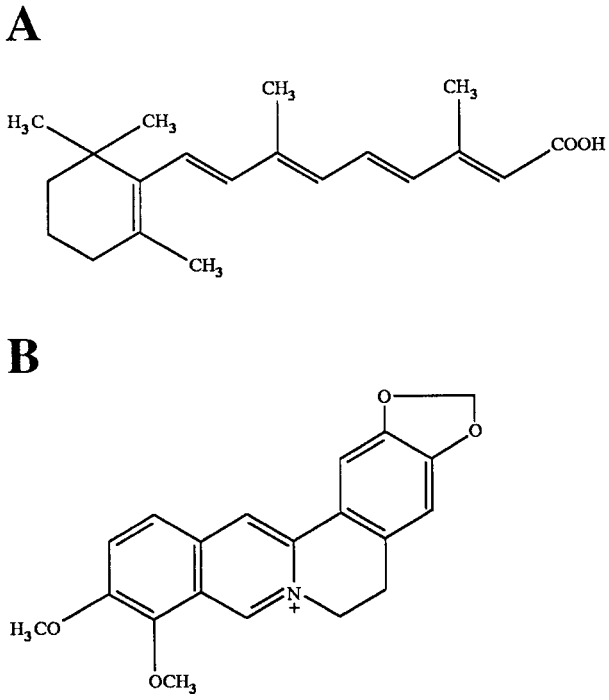

FIG. 3. Chemical structures of all *trans*-retinoic acid (**A**) and berberine (**B**).

PLC-β3 was relatively low in NT2-N neurons (18 ± 4% of that in NT2 cells, n = 12, $p < 0.001$), as was that of PLC-γ1 (36 ± 6%, n = 4, $p < 0.05$). PLC-β2 immunoreactivity was not detected in either NT2 or NT2-N cells (data not shown).

Both retinoic acid and berberine treatment of NT2 cells results in a neuronal phenotype and altered expression of phosphoinositide signaling proteins

Although the acquisition of neuronal morphological characteristics following retinoic acid treatment of NT2 cells is well established (Andrews, 1984; Pleasure et al., 1992), we nonetheless wished to determine whether the observed changes in the expression of phosphoinositide signaling proteins were specific to the use of retinoic acid alone. To evaluate this possibility, differentiation of NT2 cells to the neuronal phenotype was promoted by means of treatment with either 10 μM all *trans*-retinoic acid or 10 μg/ml berberine (Fig. 3), a structurally unrelated alkaloid (Chang, 1991). In addition, some cells were treated with 100 μM db-cAMP, which has been reported to induce differentiation of these cells to a nonneuronal phenotype (Andrews et al., 1986). For these experiments, incubation with differentiating agents was limited to 72 h, because prolonged treatment with berberine reduced cell viability. Before differentiation, NT2 precursors appeared amorphous and proliferated rapidly (Fig. 4A). NT2 cells incubated with either retinoic acid or berberine, however, ceased to divide and demonstrated a dramatically altered morphology, such as cell polarization and the elaboration of neurites (Fig. 4B and C, respectively). These properties were also observed for fully differentiated NT2-N neurons (Fig. 4E). In contrast, cells incubated with db-cAMP did not acquire this neuronal morphology, but became larger and epithelioid (Fig. 4D).

Neuronal alterations in morphology were accompanied by specific changes in the expression of G proteins and PLC isozymes (Figs. 5 and 6). The expression of $G\alpha_{q/11}$, which was strongly up-regulated following complete (9-week) differentiation to NT2-N neurons (see Fig. 1), rose to ~300% of that present in NT2 cells following a 72-h treatment with either retinoic acid or berberine. In contrast, differentiation of NT2 stem cells to an epithelioid phenotype had no effect on the $G\alpha_{q/11}$ immunoreactivity (Fig. 5, top). Similarly, expression of PLC-β1 increased significantly following either retinoic acid or berberine treatment, but was unchanged by exposure of NT2 cells to db-cAMP (Fig. 6, top). $G\alpha_i$, whose concentration in NT2-N neurons was only slightly higher than that in NT2 stem cells, remained relatively constant with all three treatments (Fig. 5, bottom). Conversely, the immunoreactivity of PLC-β3, which declines following 9 weeks of neuronal differentiation (see Fig. 2), also decreased by 60–80% during a 72-h exposure to either retinoic acid or berberine, whereas PLC-β3 expression was unchanged following treatment with db-cAMP (Fig. 6, bottom).

Expression of G proteins and PLC isozymes in NT2-N neurons approximates that observed for brain

It is not yet established how individual cell populations within the brain contribute to the overall expression of G proteins and PLC isozymes. To address this question, concentrations of G proteins and PLC isozymes in NT2-N neurons were compared with those in rat C6 glioma cells (a glial cell model), human SH-SY5Y neuroblastoma cells (a PNS neuronal cell model), and rat forebrain (Figs. 7 and 8). The immunoreactivity of $G\alpha_{q/11}$ in NT2-N neurons was comparable to that observed for forebrain, whereas $G\alpha_{q/11}$ expression was significantly lower in C6 glioma and SH-SY5Y neuroblastoma cells (Fig. 7, top). In addition, $G\alpha_o$, which was undetectable in either C6 or SH-SY5Y cells, was readily detectable in NT2-N neurons and prominent in whole brain (Fig. 7, bottom). These data imply that the $G\alpha_{q/11}$ and $G\alpha_o$ in brain are present at higher concentrations in neurons than in glia, and that these same G proteins are expressed more robustly in neurons of the CNS (NT2-N) than in those of the PNS (SH-SY5Y). The lack of detectable $G\alpha_o$ in C6 glioma cells may indicate that this G protein is specific to neurons in the brain, as has been suggested (Gierschik et al., 1986); alternatively, the expression of $G\alpha_o$ may vary among glial cell models of different lineages. Although PLC-β1 was enriched in NT2-N neurons with respect to SH-SY5Y cells, the highest concentration of this isozyme was observed for C6 cells and forebrain, a result that suggests both glial and neuronal contributions to the PLC-β1 content of brain (Fig. 8, top). As previously described, the expres-

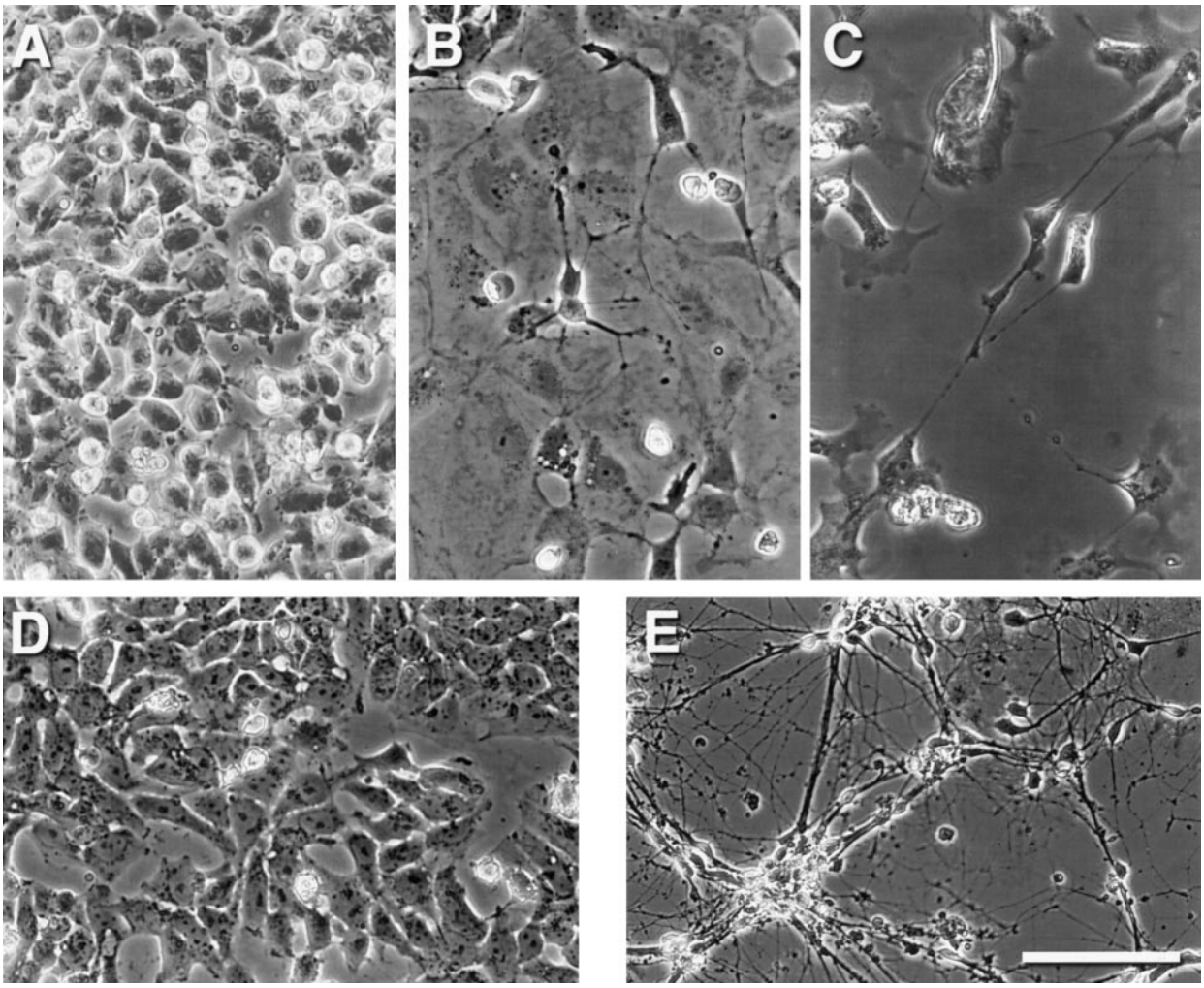

FIG. 4. Morphological evidence of neuronal differentiation of NT2 cells following treatment with either retinoic acid or berberine. Phase-contrast photomicrographs show the following: untreated NT2 precursors (**A**); NT2 cells incubated with either 10 μM retinoic acid (**B**), 10 μg/ml berberine (**C**), or 100 μM db-cAMP (**D**) for 72 h as described in Materials and Methods; and 9-week-old, retinoic acid-induced NT2-N neurons (**E**). Bar = 160 μm.

sion of PLC-β3 was low in NT2-N neurons (see Fig. 2), as is observed for whole brain (Watanabe et al., 1998), whereas relatively high expression of this isozyme was observed for C6 and SH-SY5Y cells (Fig. 8, bottom). The high expression of PLC-β3 in C6 cells is at odds with the relative lack of this isozyme in rat forebrain, a brain region in which glia far outnumber neurons. One explanation for this discrepancy might be that the glial population represented by C6 cells is comparatively rare or, alternatively, that these cells are reflective of glia in hindbrain, a region in which PLC-β3 expression is relatively higher than in forebrain (Tanaka and Kondo, 1994; Watanabe et al., 1998).

Increased expression of G$\alpha_{q/11}$, PLC-β1, and PLC-β4 is associated with increased rates of phosphoinositide hydrolysis

To determine whether the increased expression of G$\alpha_{q/11}$, PLC-β1, and PLC-β4 was accompanied by an increased rate of inositol lipid hydrolysis, ^{3}H-inositol phosphate production was determined for NT2 stem cells and NT2-N neurons following either direct G protein or PLC activation (Fig. 9). Compared to NT2 precursors, NT2-N neurons exhibited an equivalent incorporation of [^{3}H]inositol into lipid with respect to protein (data not shown). In addition, radioactivity was recovered primarily in phosphatidylinositol (96%), with the remainder of the radioactivity present in phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in both cell types. Activation of phosphoinositide hydrolysis at the level of G$\alpha_{q/11}$ following incubation with 20 mM NaF (Fisher et al., 1993) resulted in a fivefold higher release of inositol phosphates in NT2-N neurons compared with NT2 stem cells (Fig. 9A). Similarly, direct stimulation of PLC by treatment with the Ca^{2+} ionophore ionomycin (Fisher et al., 1989) produced a fourfold greater increase in inositol phosphate generation in

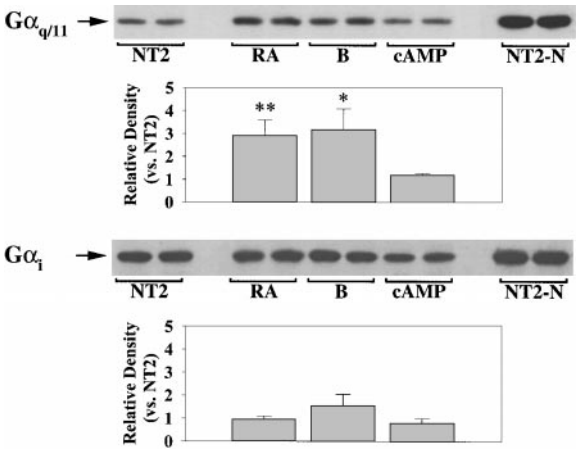

FIG. 5. G protein expression in retinoic acid-, berberine-, or db-cAMP-treated NT2 cells. NT2 precursors were incubated with either 10 μM retinoic acid (RA), 10 μg/ml berberine (B), or 100 μM db-cAMP (cAMP) as described, and duplicate aliquots of cell lysate supernatant (25 μg) were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for $G\alpha_{q/11}$ (**top**) or $G\alpha_i$ (**bottom**). NT2 and NT2-N cell lysates were included for comparison. Densitometric analyses of G protein expression relative to NT2 cells are presented as means ± SEM for three to five independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, different from NT2 cells.

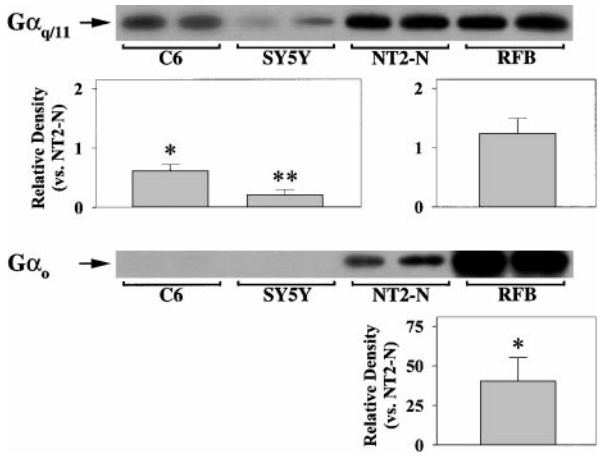

FIG. 7. G protein expression in C6 glioma cells, SH-SY5Y neuroblastoma cells, NT2-N neurons, or rat forebrain. Cell lysates from either C6, SH-SY5Y (SY5Y), or NT2-N cells or tissue lysate from rat forebrain (RFB) were centrifuged, and duplicate aliquots of supernatant (25 μg) were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for $G\alpha_{q/11}$ (**top**) or $G\alpha_o$ (**bottom**). Densitometric analyses of G protein expression relative to NT2-N cells are presented as means ± SEM for four independent experiments, with the exception of $G\alpha_o$ expression in C6 and SH-SY5Y cells, in which no immunoreactive bands were detected. $^*p < 0.05$, $^{**}p < 0.01$, different from NT2-N neurons.

NT2-N neurons than that observed for NT2 precursors. Inositol phosphate production in NT2 and NT2-N cells was also examined with a permeabilized cell paradigm (Fig. 9B). Incubation with 1–10 μM free Ca^{2+} to stimulate PLC directly did not result in significant stimulation of inositol phosphate release from either NT2 or NT2-N cells. In contrast, the addition of GTPγS to permeabilized NT2-N neurons resulted in an approximately fourfold greater increase in inositol phosphate

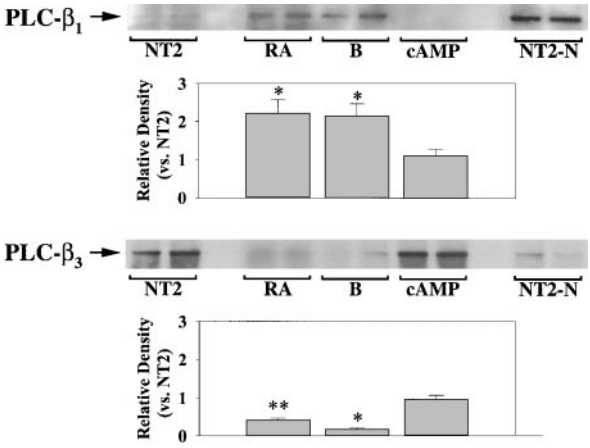

FIG. 6. PLC isozyme expression in retinoic acid-, berberine-, or db-cAMP-treated NT2 cells. NT2 precursors were incubated with either 10 μM retinoic acid (RA), 10 μg/ml berberine (B), or 100 μM db-cAMP (cAMP) as described, and duplicate aliquots of cell lysate supernatant (25 μg) were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for PLC-β1 (**top**) or PLC-β3 (**bottom**). NT2 and NT2-N cell lysates were included for comparison. Densitometric analyses of PLC isozyme expression relative to NT2 cells are presented as means ± SEM for four to seven independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, different from NT2 cells.

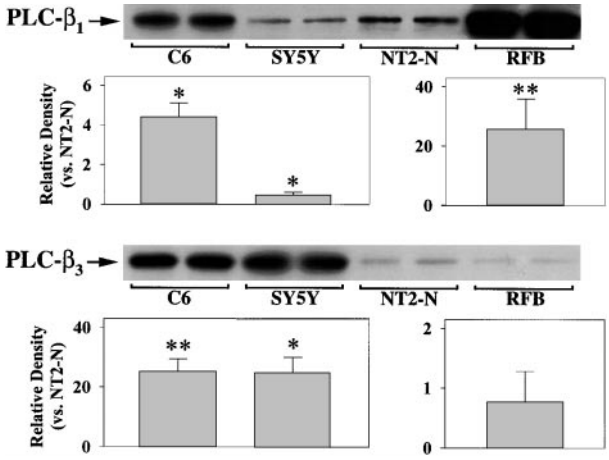

FIG. 8. PLC isozyme expression in C6 glioma cells, SH-SY5Y neuroblastoma cells, NT2-N neurons, or rat forebrain. Cell lysates from either C6, SH-SY5Y (SY5Y), or NT2-N cells or tissue lysate from rat forebrain (RFB) were centrifuged, and duplicate aliquots of supernatant (25 μg) were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted for PLC-β1 (**top**) or PLC-β3 (**bottom**). Densitometric analyses of PLC isozyme expression relative to NT2-N cells are presented as means ± SEM for three to five independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, different from NT2-N neurons.

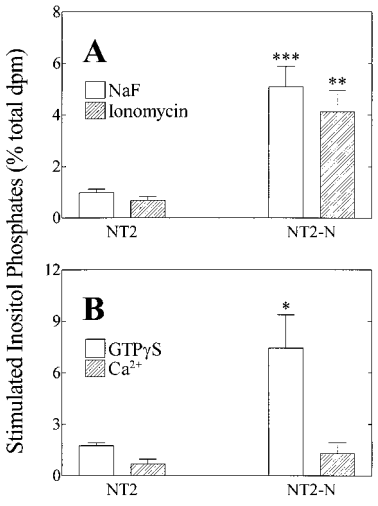

FIG. 9. Inositol phosphate production by NT2 precursors and NT2-N neurons. Cells were prelabeled with 10–20 μCi/ml [^{3}H]-inositol for 48–72 h. Intact cells (**A**) were incubated in the presence or absence of either 20 m*M* NaF or 1 μ*M* ionomycin at 37°C for 15 min. Permeabilized cells (**B**) were incubated in the presence or absence of either 50 μ*M* GTPγS or 1 μ*M* Ca^{2+} at 37°C for 60 min. Inositol phosphates were quantified as described in Materials and Methods and expressed as the difference between the means of experimental and control replicates. Data shown are means ± SEM for three to six independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, different from NT2 cells.

release than that observed for NT2 cells. Consequently, direct activation of G proteins in both intact and permeabilized cells robustly stimulated inositol phosphate production in NT2-N neurons compared to NT2 stem cells.

DISCUSSION

In this report, we demonstrate that the differentiation of human NT2 cells to the neuronal phenotype is accompanied by neuron-specific increases in the expression of $G\alpha_{q/11}$ and of PLC-β1/4. The expression of $G\alpha_{q/11}$ increased ~15-fold following CNS neuronal differentiation to a concentration similar to that found in rat brain (~0.1% of membrane protein; Milligan, 1993). Comparison of G protein expression in NT2-N cells relative to other neural tissues suggests that $G\alpha_{q/11}$ in brain may be more highly expressed in neurons than in glia, particularly in neurons of the CNS rather than in those of the PNS. PLC-β1 and PLC-β4, each of which is activated by and colocalized with $G\alpha_{q/11}$ (Taylor et al., 1991; Jiang et al., 1994; Jope et al., 1994), were also enriched upon differentiation to NT2-N neurons, although the high expression of PLC-β1 in C6 cells implies both neuronal and glial contributions to the content of this isozyme in brain. As PLC-β1 and PLC-β4 have been localized to different regions of the CNS (Watanabe et al., 1998), the presence of both isozymes at relatively high concentrations in the NT2-N preparation suggests that it may constitute a mixed cell population. Alternatively, the NT2-N neuron may represent a fetal stage of CNS neuronal development, as previously indicated (Pleasure et al., 1992), in which the cell has yet to commit to the expression of a specific PLC-β isozyme.

The observed increases in $G\alpha_{q/11}$, PLC-β1, and PLC-β4 expression with neuronal differentiation of NT2 cells were specific for these isoforms, because the expression of both PLC-β3 and PLC-γ1 decreased with differentiation. The latter data are consistent with the low concentration of these isozymes reported for brain (Tanaka and Kondo, 1994; Watanabe et al., 1998) and the probable astroglial localization of PLC-γ1 (Choi et al., 1989). It is noteworthy that the expression of $G\alpha_i$, which can activate PLC-β3 via the release of $G\beta\gamma$ subunits, was minimally influenced by neuronal differentiation (see Fig. 1). This observation, along with the pronounced reduction in the expression of PLC-β3 during differentiation, suggests that in NT2-N neurons, the predominant mechanism for activation of PLC-β is via $G\alpha_{q/11}$. The robust and specific increases in PLC-β1 and PLC-β4 expression that were observed in the present study following neuronal differentiation of NT2 cells are in marked contrast to the results obtained in a previous report in which no increased expression of PLC-β1 was detected (Wolf et al., 1995). Although an explanation for this discrepancy is not immediately apparent, it should be noted that we observed similar changes in $G\alpha_{q/11}$, PLC-β1, and PLC-β3 expression regardless of whether neuronal differentiation of NT2 precursors was induced by retinoic acid or by berberine, a structurally distinct differentiating agent. In contrast, when NT2 cells were treated with db-cAMP, they acquired an epithelioid phenotype and showed no change in phosphoinositide signaling protein expression. Collectively, these results indicate that (a) the appearance of the neuronal phenotype is accompanied by marked changes in the expression of phosphoinositide signaling proteins and (b) the biochemical changes observed appear to be independent of the agent used to induce differentiation.

The expression of $G\alpha_o$, a signal transduction protein not directly involved in phosphoinositide hydrolysis, was also altered in a manner consistent with neuronal differentiation, because its concentration in NT2-N neurons was ~30 times higher than in NT2 stem cells. $G\alpha_o$ has been estimated to comprise 1–2% of the total membrane protein of brain (Sternweis and Robishaw, 1984; Gierschik et al., 1986) and 10% of the membrane protein of brain growth cones (Strittmatter et al., 1990). In addition, increased expression of $G\alpha_o$ parallels neurite proliferation during neuronal differentiation of PC12 cells (Zubiaur and Neer, 1993; Strittmatter et al., 1994). Although the $G\alpha_o$ content of NT2-N neurons was much less than that of whole brain, possibly because NT2-N cells represent an immature neuronal phenotype and require the presence of glia for further maturation (Hartley et al., 1999), it is noteworthy that no $G\alpha_o$ was detected in either C6 glioma or SH-SY5Y neuroblastoma cells, a result that further strengthens the association of this G protein with CNS neuronal differentiation.

Although the concentrations of phosphoinositide signaling proteins such as $G\alpha_{q/11}$ and PLC-β1/4 are reportedly high in the CNS (Milligan, 1993; Watanabe et al., 1998), the relationship between this enhanced protein expression and the rate of phosphoinositide turnover in discrete cell populations remains to be determined. In the present study, we observed that direct activation of $G\alpha_{q/11}$ in either intact or permeabilized NT2-N neurons resulted in a significantly greater stimulation of phosphoinositide hydrolysis than in NT2 precursors. Similarly, direct stimulation of PLC with the Ca^{2+} ionophore ionomycin also resulted in a greater increase in inositol lipid turnover in intact NT2-N neurons than in NT2 stem cells. These findings are consistent with the possibility that the increased expression of $G\alpha_{q/11}$ and/or PLC-β1/4 results in a greater ability of neuronal cells to hydrolyze inositol lipids. A corollary of this conclusion would be that the concentrations of $G\alpha_{q/11}$ and/or PLC-β1/4 are rate-limiting for inositol lipid hydrolysis. In the absence of G protein stimulation, the basal release of inositol phosphates was higher from NT2-N neurons than from NT2 stem cells (data not shown), a finding that suggests that PLC activity may limit the rate of phosphoinositide hydrolysis. This situation would be analogous to that observed for the cyclic AMP signaling system, in which the activity of the effector enzyme adenylyl cyclase, rather than the availability of G protein, is rate-limiting for second messenger production (Alousi et al., 1991; Post et al., 1995). In the present study, the increased ability of neurons to hydrolyze inositol lipids does not appear to be due to differences in the concentrations of inositol lipid substrates initially present, because lipids in both cell types were labeled with [^{3}H]inositol to an equivalent extent. However, the possibility of compartmentation of phosphoinositide pools within NT2 or NT2-N cells cannot be excluded.

We conclude that the neuronal differentiation of NT2 cells is accompanied by neuron-specific changes in the expression of phosphoinositide signaling components as well as stimulated hydrolysis of inositol lipids. The NT2-N cell system may thus serve as a useful and representative model with which to further investigate phosphoinositide signaling in human CNS neurons.

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