POLYPHOSPHOINOSITIDES IN NORMAL AND NEOPLASTIC RODENT ASTROCYTES

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Summary - Polyphosphoinositides were identified in dispersed cell cultures of normal newborn hamster astrocytes and of a chemically transformed adult rat astrocytoma (C6) and are therefore presumed to be constituents of immature astrocytes in brain. Small amounts were also detected in astrocytomas grown as subcutaneous tumors. These lipids were metabolically highly active, accounting for a substantial fraction of $^{32}P_i$ incorporated into phospholipids. Astrocytes may thus contain a small pool of polyphosphoinositides metabolically distinct from that in myelin.

Polyphosphoinositides are located primarily in the central nervous system where they are concentrated principally in myelin (1-7). However, these lipids are also present in structures of nervous tissue other than myelin (4,7,8). One indication of such an extra-myelin localization of polyphosphoinositides is that a fraction of triphosphoinositide, associated with brain regions rich in gray matter, becomes labeled more rapidly than total brain triphosphoinositide following intraperitoneal injection of $^{32}P_i$ into rats (4,9).

Of the neuroglial cell types present in brain, oligodendrocytes are assumed to contain polyphosphoinositides, because of their involvement in the elaboration of myelin (10). A logical question is whether an extra-myelin pool of these lipids is present in astrocytes, which are derived developmentally from the same precursor cell type, spongioblasts, as are oligodendrocytes (11).

In the present communication we describe studies on the presence and metabolism of polyphosphoinositides in normal and neoplastic rodent astrocytes grown in dispersed cell cultures (12-15) and as subcutaneous tumors in newborn hamsters (12-15).

METHODS

Three types of astrocytic cultures were utilized: primary normal newborn hamster brain astrocytes (12); subcultures of these astrocytes both before and after spontaneous neoplastic transformation, which occurred between the 30th and 40th passage in vitro and was detected by the ability of the cells to produce tumors following subcutaneous inoculation into newborn hamsters; and an adult rat brain astrocytoma cell line (line C6), chemically transformed by N-nitrosomethylurea (16). The derivation of these cell lines, their histological identification as astrocytes, and techniques for preparation, maintenance and subcultivation have previously been described (12,16).

Just prior to use of the cells in biochemical studies, the nutrient medium was decanted and cells in culture flasks were washed with Krebs-Ringer bicarbonate buffer, pH 7.4, containing glucose (1 mg/ml). Dispersions from whole newborn hamster brains were prepared by repeatedly drawing up and expelling pooled whole brains, suspended in the buffer, through a widemouthed pipet.

Two incubation procedures were followed, in both of which the cells were incubated in fresh Krebs-Ringer bicarbonate-glucose buffer together with $^{32}P_i$ (1 μCi/ml) for 60 min at 37°. In one procedure, cells were scraped from the cell culture flasks, incubated on a Dubnoff metabolic shaker and the incubation terminated by addition of 5.7 vol. of $CHCl_3$-CH_3OH (1/1). In the other procedure, incubations were performed directly in the flasks. The radioactive medium was then poured off, the cells were cooled to 0° and rapidly scraped and suspended in a minimal quantity of buffer and 5.7 vol. of solvent added. Portions of the suspensions were sonicated and used for protein analysis (17).

Four types of astrocytomas grown as subcutaneous tumors were also analyzed for polyphosphoinositides. These tumors were produced by subcutaneous inoculation into newborn hamsters of 1-3 x 10^6 cells from the following cultured astrocytoma lines: N-nitrosomethylurea-transformed adult rat (C6 and C2A)

(16); SV 40-transformed newborn hamster (line N1) (13,14); SV 40-transformed fetal hamster (line THA15) (13,14); and spontaneously transformed newborn hamster (52nd subculture). For chemical analyses, the subcutaneous astrocytomas were dissected out as rapidly as possible and frozen in liquid nitrogen or on dry ice.

Polyphosphoinositides were extracted, separated by thin-layer chromatography and located and assayed for radioactivity as described elsewhere (9). The levels of di- and triphosphoinositide were measured (7) in samples from parallel incubations without ^{32}P. Inorganic ^{32}P was obtained from International Chemical and Nuclear Corp., Waltham, Mass. as HCl-free solution.

RESULTS AND DISCUSSION

Concentration of polyphosphoinositides in cultured astrocytes and astrocytomas Polyphosphoinositides were present in both normal newborn hamster astrocytes and chemically transformed adult rat astrocytes grown in cell culture (Table I) and comprised from 0.1-0.3% of total phospholipid. The levels of triphosphoinositide were similar to one another and to that in newborn hamster brain suspension. The concentrations of diphosphoinositide were more variable and somewhat lower than were those of triphosphoinositide. The values obtained for polyphosphoinositides should be considered minimal since partial breakdown of these lipids may have occurred either during collection and incubation of the cultured cells or within a few minutes post mortem in the case of the tumors (18).

Substantially lower quantities of polyphosphoinositides were present in the subcutaneous astrocytomas as compared to the cultured cells. The highest amounts were found in astrocytomas produced from line C6 and were (nmole/100 mg protein): TPI, 2.81 and DPI, 0.68. Other subcutaneous astrocytomas (produced from lines C2A, N1 or THA15) contained less than 3 nmoles of polyphosphoinositides/100 mg protein. Neither phospholipid could be detected in astrocytomas derived from the spontaneously transformed astrocyte cell line (52nd subculture) nor, indeed, in these cells in culture (Table I).

TABLE I

CONCENTRATIONS AND SPECIFIC ACTIVITIES OF POLYPHOSPHOINOSITIDES OF BRAIN CELL PREPARATIONS

Preparation	Diphospho-inositide		Triphospho-inositide	
	nmole / 100 mg protein	cpm[1] / μmole P	nmole / 100 mg protein	cpm[1] / μmole P
Newborn hamster brain astrocytes:				
Primary culture	-[2]	-[2]	11.3	105,800
12th subculture	7.1	18,700	13.3	113,200
26th subculture	-	33,300[3]	-	301,000[3]
43rd subculture	7.3	5,400	11.2	18,100
52nd subculture	0	-	0	-
Adult rat brain astrocytoma cells (C6)	6.0	82,500	19.1	317,000
Newborn hamster brain dispersion	4.1	53,500	15.4	90,200

[1]Values normalized to a specific activity of ^{32}P in the incubation medium, equal to 1 x 10^6 cpm/μmole.
[2]Although the area to which diphosphoinositide migrated on thin-layer plates contained about 10% as many counts as the triphosphoinositide area, P levels were too low for accurate measurement.
[3]Values were calculated, assuming the same levels of di- and triphosphoinositide as those obtained from the 12th and 43rd subcultures.

Identification of triphosphoinositide in normal and neoplastic astrocytes

To obtain more definite evidence for the presence of triphosphoinositide in two of the cell lines (i.e., C6 and the 12th subculture of the normal hamster astrocytes), polyphosphoinositides obtained from cells labeled with ^{32}P were separated by thin-layer chromatography. Presumptive identification of triphosphoinositide was provided by radioautography of the plate which revealed a discrete band at the same position to which authentic triphosphoinositide migrated (Fig. 1). Appropriate areas of silica gel were eluted with three

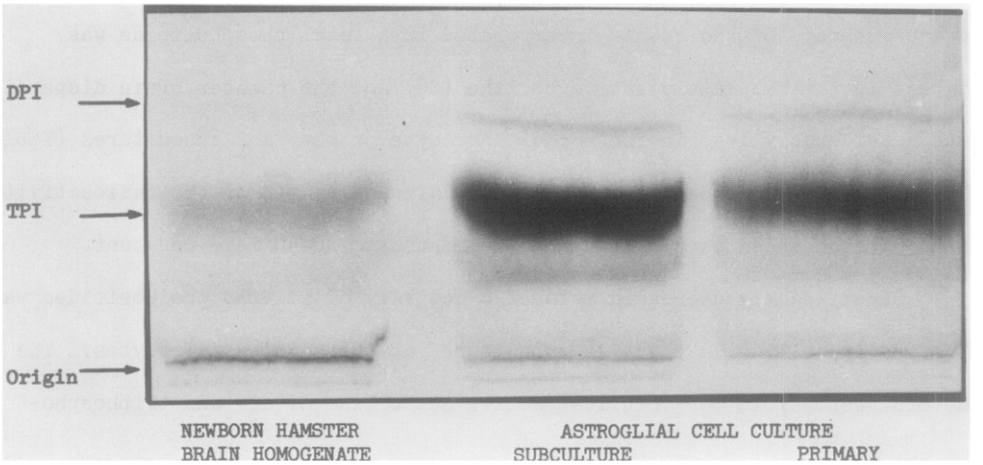

Fig. 1. Radioautograph of a thin-layer chromatographic separation of polyphosphoinositides. Two successive solvent systems were used: 1) $CHCl_3$-CH_3OH-4.3 N NH_4OH (90/65/20); 2) n-propanol - 4.3 N NH_4OH, containing 10^{-2}M cyclohexanediaminetetraacetate (65/35) (7).

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5 ml portions of n-propanol-4.3N NH_4OH (40/60). The solution was centrifuged to remove silica gel and dried in vacuo. The residue was subjected to mild ethanolic alkaline hydrolysis (19) in the presence of highly purified carrier triphosphoinositide from beef brain and the hydrolysis products separated by chromatography and electrophoresis (20). In both cases, the bulk of the recovered counts was in glycerylphosphorylinositoldiphosphate, the major product produced on degradation of triphosphoinositide by this procedure, with lesser amounts in inositol triphosphate, glycerylphosphorylinositolmonophosphate, inositol diphosphate, P_i (trace) and at the origin.

Metabolism of polyphosphoinositides in cultured astrocytes Polyphosphoinositides in cultures of line C6, as well as in primary cultures and early (12th and 26th) subcultures of the normal hamster astrocytes, incorporated substantial quantities of isotope. Thus triphosphoinositide in these cells achieved specific activities which were several times higher than those of other phospholipids (Eichberg, Hauser and Shein, unpublished experiments) and comparable to or higher than the specific activity of the lipid in newborn hamster brain dispersions (Table I). The total radioactivity found in triphosphoinositide

as a percentage of the counts incorporated into total phospholipids was highest in the neoplastic astrocyte line (C6) and the hamster brain dispersion with considerably less in the normal astrocyte primary and subcultures (Table II) Diphosphoinositide labeling accounted for only 4-11% of the radioactivity in polyphosphoinositides, although it amounted to 20-40 mole per cent.

Significantly decreased metabolic activity of polyphosphoinositides was observed in cells from the 43rd subculture of the hamster astrocytes. The factors responsible for this less active metabolism of di- and triphospho-

TABLE II

INCORPORATION OF ^{32}P INTO TOTAL PHOSPHOLIPIDS AND TRIPHOSPHOINOSITIDE OF BRAIN CELL PREPARATIONS

Preparation	Total phospho-lipids (A)	Triphospho-inositide (B)	$\frac{B}{A}$ x 100
	cpm/50 mg protein		%
Newborn hamster brain astrocytes:			
Primary culture 1	31,400	2,600	8.3
2	64,900	4,800	7.4
12th subculture	69,900	3,200	4.6
26th subculture	96,100	7,700	8.0
43rd subculture	21,900	250	1.1
Adult rat brain astrocytoma cells (C6)	53,600	10,000	18.6
Newborn hamster brain dispersion 1	18,600	2,900	15.6
2	33,200	6,500	19.6

Each value for total phospholipid labeling represents the average from duplicate incubations except for the 43rd astrocyte subculture where triplicate incubations were performed. Triphosphoinositide values were obtained from pooled, neutral solvent extracted tissue residues. For further details see the text.

Values are normalized to 5 x 10^6 cpm of ^{32}P in the incubation medium.

inositide in cells at this culture level are unknown. These cells were morphologically less well-differentiated than cells from earlier subcultures and had undergone neoplastic transformation in vitro. It is noteworthy that the rat astrocytoma cell line, C6, although neoplastic, exhibited stable, well-differentiated astrocytic morphology upon repeated subculturing in vitro and also displayed high metabolic activity of polyphosphoinositides.

The findings that polyphosphoinositides are constituents of normal newborn hamster astrocytes in dispersed cell culture, as well as of an adult rat astrocytoma cell line, strongly suggest that these lipids are components of immature astrocytes in brain. Such a fraction might constitute a metabolic pool of these substances distinct from the bulk of polyphosphoinositides located in myelin. The high metabolic activity of these lipids in cultured astrocytes incubated under arbitrary conditions indicates that they may play a significant role in the still unknown function of these cells in the central nervous system.

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