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Advances in Brief

Rapid Incorporation of Carbon-11-labeled Diacylglycerol as a Probe of Signal Transduction in Glioma[1]

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Abstract

We have synthesized and characterized a positron-emitting carbon-11-labeled 1,2-diacylglycerol to study phosphoinositide turnover in tumor cells. Rapid incorporation of the 1,2-diacylglycerol was observed in the C6 glioma cell line. The incorporated lipid fraction consisted chiefly of phosphoinositide pool and another phospholipid pool in the proliferative state. When the state was inhibited by (−)-3D-3-deoxy-3-fluoro-*myo*-inositol, incorporation into the phosphoinositide pool decreased selectively. This suggested that phosphoinositide turnover is the leading regulator of tumor proliferation potential. On the basis of the concept of carbon-11-labeled 1,2-diacylglycerol as a specific probe for visualizing the tumor signal transduction *in vivo*, we obtained proliferating images of implanted C6 glioma cells in the rat brain by autoradiography and visualized the proliferation signal in human glioma by positron emission tomography.

Introduction

Major advances in cell biology have been made in methods for analyses of a series of signal transduction systems linked to cell surface receptors (1, 2). The role of phosphoinositide-mediated signaling and related events remains one of the most intensively investigated areas in tumor growth (3–5). This signaling system involves the PLC[3]-dependent hydrolysis of membrane phosphoinositides to form inositol polyphosphate and 1,2-DAG. In the brain, which is characterized by highly efficient synaptic neurotransmission, the DAG level usually remains low to prepare for signaling when needed (6). In contrast, the DAG level is high in proliferating cells, suggesting continuous release of proliferation signals (7–9). It was reported that treatment of several cell lines with growth factors resulted in a biphasic increase in DAG level, suggesting that an initiation trigger and a long-term enhancer coexist in the DAG-associated proliferation signals (10). An important key to interpret the proliferation signal can be found if the DAG metabolism, which regulates the DAG mass level, is studied in different signaling pathways in both the central nervous system and proliferating cells. However, no assessments of exogenously added DAG metabolism have yet been attempted. Recently, we synthesized carbon-11-labeled 1,2-DAG (1, 2-[^{11}C]DAG) and studied its metabolic pathway in the central nervous system (11). In this previous study, i.v. administered 1,2-[^{11}C]DAG was converted only into phosphoinositides along the PI-recycling process and did not appear in other phospholipids, such as PE and PC. In this paper, we describe specific metabolism of 1,2-[^{11}C]DAG in a tumor cell line and discuss the balance between the phosphoinositide pool and other phospholipid pools in the proliferating state, which represents tumor proliferation potential. Upon such interpretation, we demonstrate the first informative case of human glioma by using PET.

Materials and Methods

Cell Cultures and Growth Inhibition Study. C6 glioma cells obtained from American Type Culture Collection (Rockville, MD) were used between the 38th (no. 8573) and 58th passages. The culture medium was L-15 Medium (modified) supplemented with 10% FBS, without antibiotics. C6 glioma cells were plated at 1.82×10^6 cells/well in 7-cm^2 × 6-well culture plates in 2 ml of the culture medium, including each bioactive compound, for 48 h at 37°C under 5% CO_2 in air. Bioactive compounds were dipalmitoyl PA (0.5 and 1 mM), 3-dFI (0.5 and 1 mM), or phorbol 12,13-dibutyrate (50 and 100 nM). After incubation, the action of drugs was stopped by placing the flasks on ice and removing the medium. Cells were easily scraped off with a Pasteur pipette with 0.05% trypsin and 0.5 mM EDTA and counted with the use of a cell counter.

Carbon-11-labeled 1-Butyryl-2-Stearoyl-*rac*-Glycerol. The synthesis of *rac*-1,2-[^{11}C]DAG (l-[l-^{11}C]butyryl-2-stearoyl-*rac*-glycerol) was described in detail in our previous report (12). The half-life of ^{11}C is 20.38 min. [^{11}C]Ethylketene, which was synthesized by using a cyclotron, was trapped in pyridine solution containing 1 μmol of 2-stearoylglycerol. The acylation reaction was performed by using [^{11}C]ethylketene under a no-carrier-added condition at room temperature and within 5 min. Carbon-11-labeled 1-butyryl-2-stearoyl-*rac*-glycerol was synthesized by a simple method and had a high specific activity (5 Ci/μmol, at the end of synthesis).

Chromatographic Analysis of Phosphoinositide Intermediates in C6 Glioma Cells. C6 glioma cells were cultured up to a concentration of 1×10^8 cells/ml in 75-cm^2 Falcon flasks with 10 ml of L-15/FBS. The medium was replaced with 2 ml of Ca^{2+}, Mg^{2+}-free-Hanks solution containing 3.5 mCi of 1,2-[^{11}C]DAG (specific activity; 5 Ci/μmol). After 5- and 20-min incubations were terminated, cells were washed three times with 2 ml of PBS on ice. They were centrifuged at 300 × *g* for 5 min at 4°C, and the supernatant was discarded. The cell pellets were homogenized in 0.5 ml of double-distilled water by sonication for 3 min, followed by addition of 0.5 ml of the solvent (chloroform:methanol; 3:2, v/v) and centrifuged at 3000 rpm (1660 × *g*; Kubota KR/702) at 4°C for 3 min. The extracted lipid samples were spotted onto TLC plates (length, 75 mm; Silica Gel 60), which were then developed in one-dimension in a solvent system consisting of the basic polar solvents—chloroform:methanol:ammonium hydroxide:water, 20:15:3:2 (v/v) as a mobile phase and Silica Gel 60 (activated) as a stationary phase for analysis of ^{11}C-labeled phosphoinositides (PI, PIP, and PIP_2) and ^{11}C-labeled phospholipids (PE and PC). The developed TLC plates were put in contact with the imaging plate of the FCR system for 1 h, and then digitized images were obtained. The positions of the various radioactive phospholipids were visualized by the FCR system and identified by comparison with the authentic compounds. The PA, PI, PIP, PIP_2, and other phospholipids (PE and PC) and corresponding authentic compounds were visualized with common lipid-locating agents such as I_2 or molybdenum blue spray for phosphate on the same TLC plates.

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[3] The abbreviations used are: PLC, phospholipase C; DAG, diacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-monophosphate; PIP_2, phosphatidylinositol-4,5,-bisphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; FCR, Fuji computed radiography system; FBS, fetal bovine serum; PET, positron emission tomography; PDBu, phorbol 12,13-dibutyrate; 3-dFI, (−)-3D-3-deoxy-3-fluoro-*myo*-inositol.

Phospholipid Analysis of C6 Glioma Cell Line by Using Bioactive Compounds. C6 glioma cells were cultured up to a concentration of 1×10^8 cells/ml in 75-cm^2 Falcon flasks with 10 ml of L-15 with FBS. After the cells were cultured without FBS for 24 h, they were incubated with PA (1 mM), 3-dFI (1 mM), or phorbol 12,13-dibutyrate (100 nM) for 30 min. Then the medium was replaced with 2 ml of Ca^{2+}, Mg^{2+}-free-Hanks solution containing 3.5 mCi of 1,2-[^{11}C]DAG (specific activity; 5 Ci/μmol). After 20-min incubations were terminated, cells were washed three times with 2 ml of PBS on ice. The TLC analyses were done as described above.

Autoradiography of Implanted C6 Glioma. Wistar rats (weighing 80–130 g) were implanted with the cell pellets of C6 glioma suspended in 10 μl of L-15. After 21 days, tracer was administered through the tail vein under transiently anesthetized conditions induced by halothane (1–1.5%) in room air. An i.v. bolus injection of 1,2-[^{11}C]DAG, dissolved in 0.4 ml saline with 0.1% BSA and 0.5% DMSO, was given, and 30 min later, the rats were killed by decapitation. The brain involved with tumor was rapidly removed and frozen in powdered dry ice. Autoradiographs were reconstructed from adjacent brain sections (40-μm thick) with the FCR system.

Technical Procedure on PET Study and Human Subject. Human application of the DAG-PET system and quality control of 1,2-[^{11}C]DAG (l-[l-^{11}C]butyryl-2-palmitoyl-*rac*-glycerol) followed the Guidelines for the committee established by the PET Committee in Nishijin Hospital (Kyoto, Japan) in October 1993. Carbon-11-labeled DAG was solubilized by using human serum albumin (5% human serum albumin solution). After i.v. injection of [^{11}C]DAG (20 mCi/50 kg), dynamic scans were obtained by using a positron camera (HEADTOME III; Shimadzu Co., Kyoto, Japan). The spatial resolution was 8.6 mm in full width at one-half of the maximum in plane resolution, whereas the average axial resolution was 13.6 mm. A sequential scanning was performed 11 times at 2–4-min intervals, and the total examination took 42 min. The patient was a 73-year-old woman who suffered from brain tumor in the left basal ganglia. The histological diagnosis of the tumor was astrocytoma grade III, certified by stereotactic biopsy.

Results

As shown in Fig. 1, the positions of the various phospholipid radioactivities were visualized by the FCR system and were identified by comparison with authentic standards in the developing system. In proliferating cells (C6 glioma), 1,2-[^{11}C]DAG was metabolized into the polyphosphoinositide and into the PE-PC pool within 5 min when the cells were incubated in FBS-free medium for 24 h (Fig. 1*A*). Compared with a radio-TLC profile of 5-min labeling (Fig. 1*A*), a

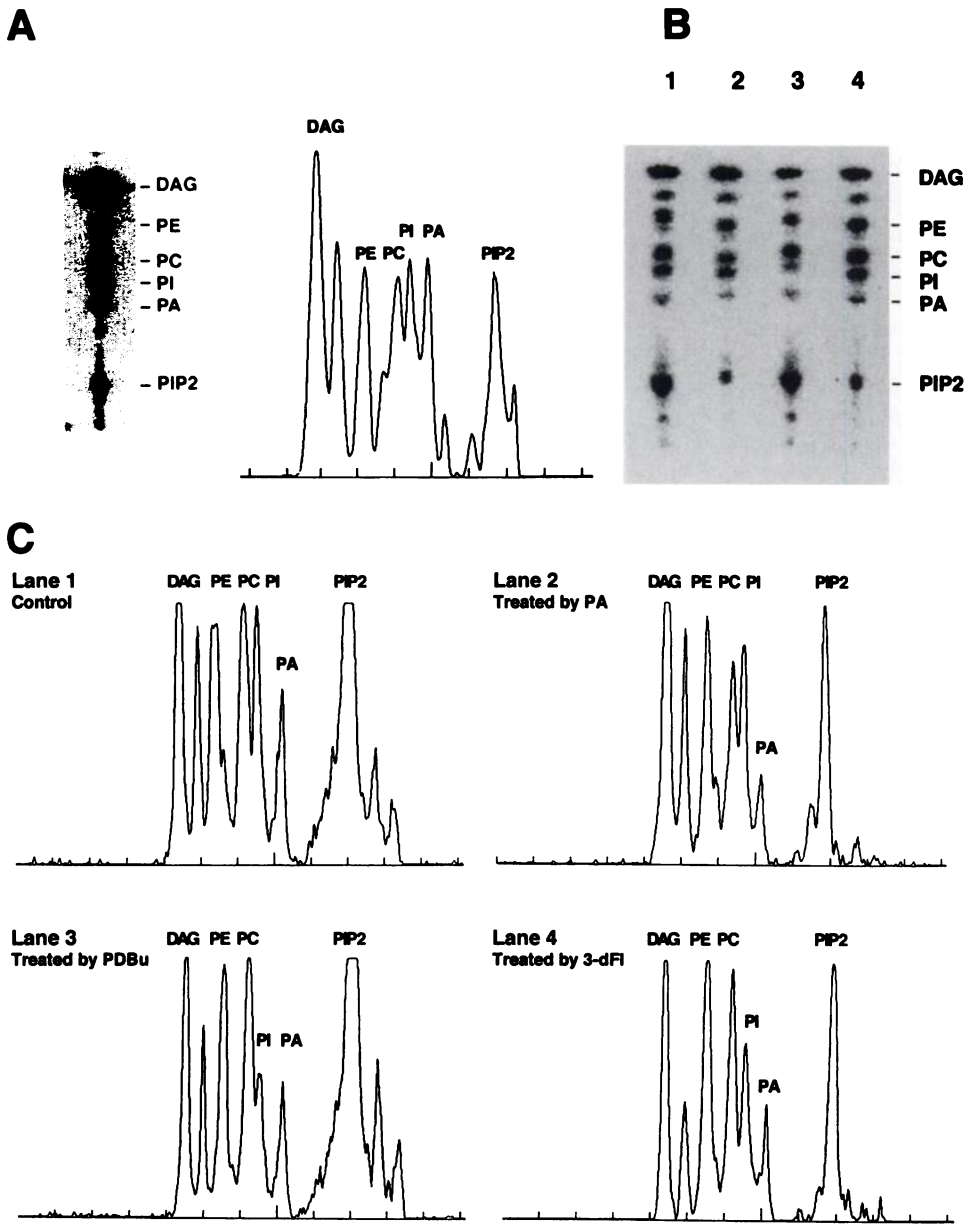

Fig. 1. Chromatographic analysis of phosphoinositide intermediates in C6 glioma cells using bioactive compounds. *A*, radio-TLC profile of C6 glioma after 5-min labeling. *B*, radio-TLC of C6 glioma after 20-min labeling. *Lane 1*, control C6 glioma cells. *Lane 2*, cells treated by dipalmitoyl phosphatidic acid (1.0 mM). *Lane 3*, cells treated with phorbol 12,13-dibutyrate (100 nM). *Lane 4*, cells treated with (−)-3D-3-deoxy-3-fluoro-*myo*-inositol (1.0 mM). *C*, radio-TLC profiles of C6 glioma after 20-min labeling. Each profile corresponds to each lane (*B*). Each range of profile was settled within the appropriate limits of PE, PC, PI, and PA fractions. These results have been confirmed by another three experiments.

Table 1 *Effects on PI-recycling process by bioactive compounds*

Effects on PI-recycling process by dipalmitoyl PA, PDBu, and 3-dFI. Each fraction area after 20-min labeling was measured based on the radio-TLC profiles. The fraction area was calculated by FWHM × height (pixels). The DAG fraction was used as the reference because it was thought to be in equilibrium to the concentration of medium ^{11}C-labeled DAG. We calculated the percentage of fraction by using the formula: (each fraction area after 20-min labeling)/(DAG fraction area after 20-min labeling) × 100 (%). The numbers are averages of three determinations in three experiments. Data, average ± SD.

	% of fractions of ^{11}C-labeled phosphoinositide intermediates					
	DAG	PE	PC	PI	PA	PIP_2
Control	100%	104 ± 9	116 ± 6	78 ± 11	54 ± 4	182 ± 18
PA (1.0 mM)	100%	76 ± 8	66 ± 7	67 ± 7	23 ± 7	82 ± 15
PDBu (100 nM)	100%	105 ± 9	131 ± 10	61 ± 7	48 ± 4	274 ± 26
3-dFI (1.0 mM)	100%	119 ± 9	113 ± 8	81 ± 5	32 ± 3	119 ± 13

Table 2 *Inhibition effects on cell growth*

Inhibition effects on cell growth by dipalmitoyl PA, 3-dFI, and PDBu. C6 glioma cells were plated at 1.82 × 10^6 cells/well in 7 cm^2 × 6-well culture plates in L-15 and 10% FBS with each bioactive compound for 48 h at 37°C under 5% CO_2 in air. The numbers are averages of six to nine determinations in two experiments. Data, average ± SD.

	Cell number after 48-h incubation
Cell growth ratio	1.82 × 10^6 cell/well in 7 cm^2
Control	4.0 ± 0.6
PA (0.5 mM)	3.6 ± 0.5
PA (1.0 mM)	3.7 ± 0.5
3-dFI (0.5 mM)	4.2 ± 0.6
3-dFI (1.0 mM)	2.9 ± 0.4[a]
PDBu (50 nM)	3.8 ± 0.6
PDBu (100 nM)	3.0 ± 0.5[a]

[a] Significance by $P < 0.01$, Student's t test.

radio-TLC profile of 20-min labeling showed an increase in PIP_2 fraction in reference to the PA and PI fractions (Fig. 1, *B* and *C*, *Lane 1*). When PA was added, the specific activity decreased in the phosphoinositide pool (Fig. 1, *B* and *C*; *Lane 2*) compared with *Lane 1* (control), probably because of a dilution effect of PA in the PI-recycling pathway. When PDBu was added, the PI-PIP_2 pathway showed a reduction in PI but an increase in PIP_2 (Fig. 1, *B* and *C*; *Lane 3*). Thus, in the radio-TLC analysis, the changes in PI differed from those in PIP_2. The increase in PIP_2 agrees with the previous report that phorbol ester promotes the synthesis of PIP and PIP_2 (13). These quantitative values are summarized in Table 1.

3-dFI is known to suppress cell proliferation (14). As shown in Table 2, inhibition experiments have revealed significant suppression of the proliferation of C6 glioma cells. As shown in Fig. 1, *B* and *C* (*Lane 4*), a treatment with 3-dFI resulted in a decrease in PIP_2. According to the radio-TLC (Fig. 1, *Lane 4*; Table 1), 3-dFI probably inhibited the formation of PIP_2 in the process of phosphorylation of PI 4-kinase, PI 3-kinase (5), and PIP kinase. This result agreed with the other experiences in which PI and PIP kinase activities were linked with proliferation and malignancy (15, 16).

We then studied animal models of brain tumor, produced by transplanting cultured C6 glioma cells into the subcortical region of the rat brain. Fig. 2*A* shows a histological section, stained with hematoxylin & eosin, obtained 21 days after implantation. The adjacent autoradiograph, obtained by 1,2-[^{11}C]DAG, showed a more intense accumulation of radioactivity in the tumor-affected area when compared with the normal brain (tumor:normal brain ratio of radioactivity; T:N ratio = 2.7-3.2).

Tumor Imaging by PET Using ^{11}C-labeled DAG. Fig. 3 shows a PET study using l-[l-^{11}C]butyryl-2-palmitoyl-*rac*-glycerol in a patient with astrocytoma grade III. The dynamic PET images demonstrate a gradual incorporation profile in the lesion. The activity then remained at a constant level. In equilibrium period (32–40 min after i.v. injection; legion profiles, 25, 28, and 31 in Fig. 3*B*), mean value of the tumor lesion was 3734 cps (% SD, 13%) and that of contralateral normal area was 2422 cps (% SD, 16%). We can see a more extensive area incorporating 1,2-[^{11}C]DAG than that of magnetic resonance image with gadolinium enhancement (Fig. 3*A*). The PET data revealed that 1,2-[^{11}C]DAG was actively incorporated to the malignant tumor cells, showing high radioactivity and tumor:normal ratio (T:N ratio = 1.54).

In two early scans taken at 6 min (profiles 1 and 4), radioactivity in the blood stream was predominant, especially in the right transverse sinus. Thereafter, radioactivity in the brain parenchyma and glioma increased gradually and reached equilibrium. The tumor in the left basal ganglia demonstrated high DAG uptake; furthermore, it was thought that the tumor suppressed uptake in the whole left hemisphere by inadequate neural signal transduction. In contrast, adequate synaptic transmission was suggested especially in the right prefrontal cortex (profiles 22–31).

Discussion

In previous studies of PI turnover, its metabolism was measured by prelabeling methods using ^{32}P or by quantification of metabolic products after [^{3}H]inositol treatment. With these techniques, however, it was difficult to obtain *in vivo* images of PI turnover because of poor incorporation of the labeling compounds into the PI-recycling pathway. We reported previously that 1,2-[^{11}C]DAG can easily permeate through common biological membranes and is rapidly incorporated in cell membranes (11). In neural cells, incorporation of 1,2-[^{11}C]DAG

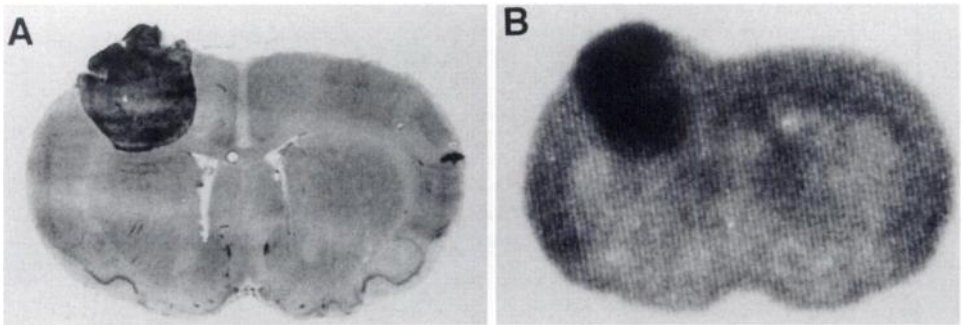

Fig. 2. Autoradiography of implanted C6 glioma. *A*, histological section of a rat brain (Wistar, male) stained with hematoxylin-eosin, obtained after 21 days after implantation of C6 glioma cells. *B*, the adjacent autoradiograph obtained by 1,2-[^{11}C]DAG. These experiments were performed three times each on two rats.

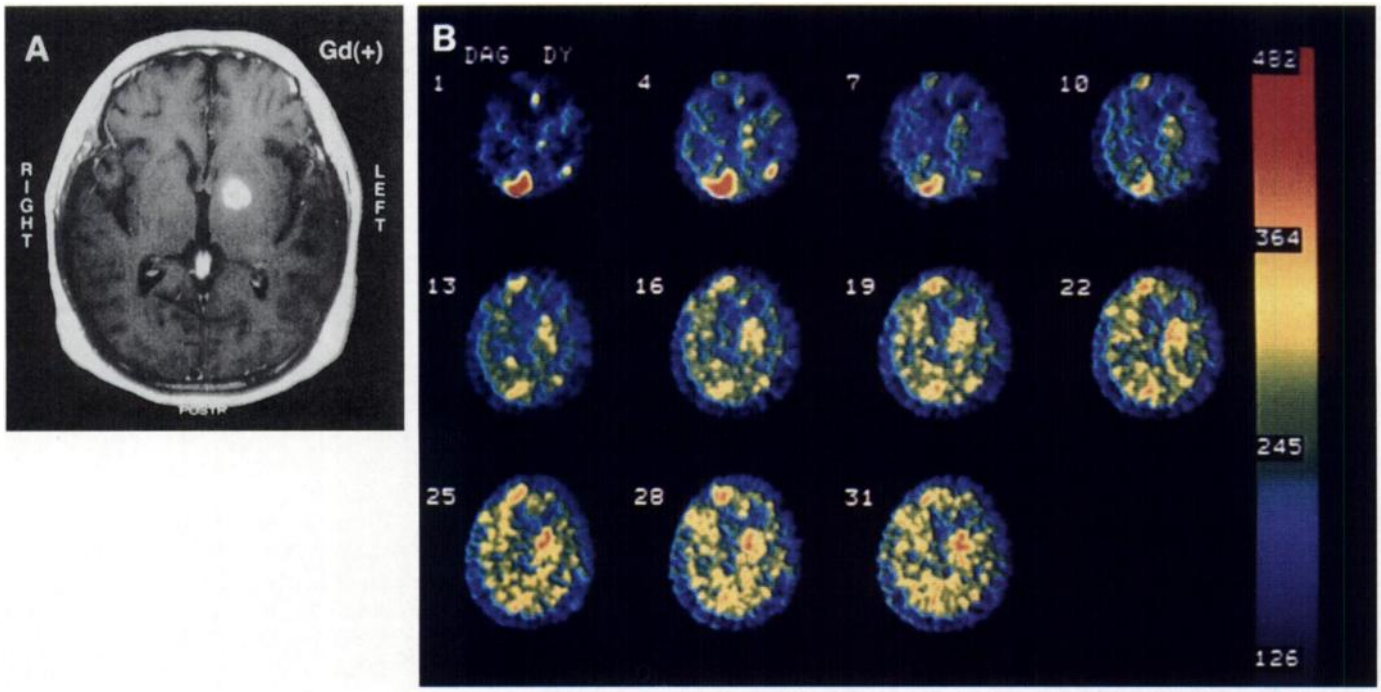

Fig. 3. PET using ^{11}C-labeled DAG. *A*, magnetic resonance image with gadolinium (*Gd*) enhancement. The brain tumor was located in the left basal ganglia. *B*, PET study using l-[l-^{11}C]butyryl-2-palmitoyl-*rac*-glycerol in a patient with astrocytoma grade III. A sequential scanning was performed 11 times at 2–4-min intervals, and the total examination took 42 min.

was weighted into PI turnover and did not appear in other phospholipids such as PE and PC (Fig. 4). This is supported by the finding that intrinsic *sn*-1,2-DAG appears transiently after degradation by PLC and disappears rapidly within a few seconds because of its conversion within the PI-recycling process (6). For this reason, it is simple to evaluate the potential of signal transduction in the central nervous system (11).

In the present study using C6 glioma cells, the radioactive fractions of PE and PC were seen with PI turnover intermediates (Fig. 1). This should reflect the specificity of tumor proliferation. It was reported that degradation components from PC *per se* may be second messengers in the proliferating cells (10) and have been shown to contain high 1,2-DAG concentrations in transformed cell lines (7–9). In proliferating cells, accumulation of 1,2-[^{11}C]DAG increased with preservation of a balance in both metabolic pools (phosphoinositide and PE-PC fractions). The incorporation of radioactivity into the PE-PC fraction may be assumed to develop in compensation for an increase in native 1,2-DAG concentration (Fig. 4).

Autoradiographic studies indicated that the incorporation of 1,2-[^{11}C]DAG was higher in the lesions that developed as a result of implantation of C6 glioma cells than it was in normal brain (Fig. 2*B*). To further understand the metabolism of exogenously added DAG in tumors, we must pay attention to the results of treatments using PDBu and 3-dFI (Fig. 1, *B* and *C*). After actions with both compounds, the

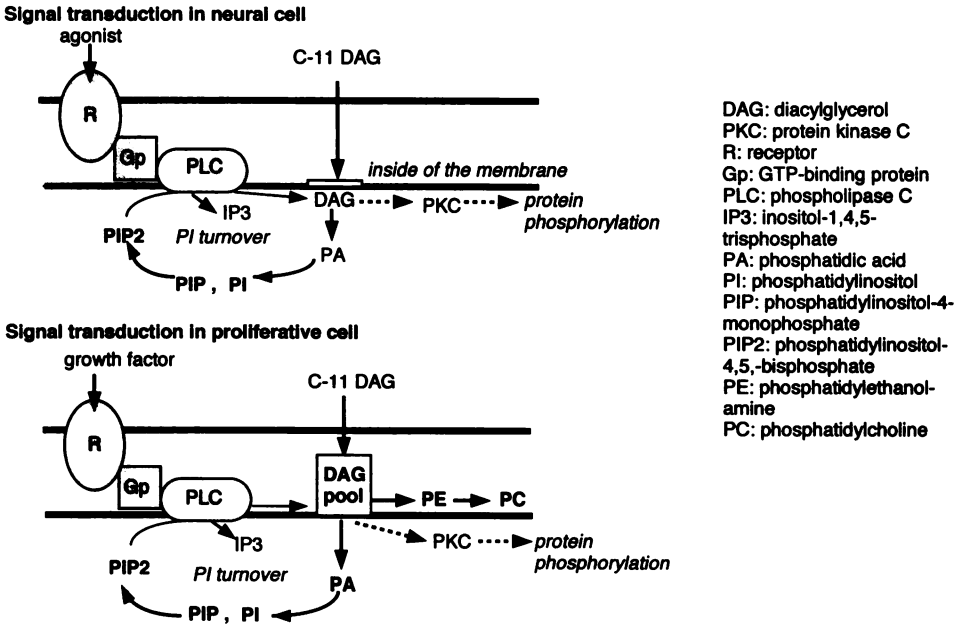

Fig. 4. Schematic PI-recycling process according to the studies of exogenous added 1,2-[^{11}C]DAG. *IP3*, inositol-1,4,5-trisphosphate; *R*, receptor; *Gp*, GTP-binding protein; *PKC*, protein kinase C; *PIP2*, phosphatidylinositol-4,5-bisphosphate.

DAG: diacylglycerol
PKC: protein kinase C
R: receptor
Gp: GTP-binding protein
PLC: phospholipase C
IP3: inositol-1,4,5-trisphosphate
PA: phosphatidic acid
PI: phosphatidylinositol
PIP: phosphatidylinositol-4-monophosphate
PIP2: phosphatidylinositol-4,5,-bisphosphate
PE: phosphatidylethanolamine
PC: phosphatidylcholine

radioactive densities of PE and PC showed little variation in reference to the membrane-bound fraction of 1,2-[^{11}C]DAG. This membrane-bound state of 1,2-[^{11}C]DAG has been verified in our experiments in erythrocytes. This binding was intercalation into the cell membrane. On the other hand, the radioactive density of PI and PIP_2 showed large variations (Table 1). Phorbol ester is known to suppress phosphoinositide hydrolysis (17). A reduction in the PI pool was noted in Fig. 1, *B* and *C* (*Lane 3*) with increasing in the PIP_2, suggesting that PDBu suppresses PI-dependent PLC activity with activating PI kinase and PIP kinase. These changes may result in decreasing mass of native DAG formation and an increased consumption of PA and PI. Although the involvement of diverse intracellular signaling systems was suggested in the growth of C6 glioma, the predominance of PI turnover seems likely. When the proliferative state was inhibited by 3-dFI (Table 2), the phosphoinositide pool decreased selectively (Table 1). This suggested that PI turnover is the leading regulator of tumor proliferation potential, and variation in the PE-PC fraction is secondary. High concentrations of the native 1,2-DAG in transformed cells should provide an opportunity for mixing into the structural phospholipid (Fig. 4). The radioactive PE and PC pool seemed to be less varied (Table 1) because of the large native pooling mass. Thus, the large incorporation into the PE and PC pools may also reflect the proliferation potential.

In conclusion, 1,2-[^{11}C]DAG served as a probe for the rapid metabolism that occurs in PI turnover. The general elevation in 1,2-[^{11}C]DAG incorporation made it possible to visualize the status of *in vivo* tumor signal transduction for the proliferation potential using PET. We demonstrated the first informative case of glioma. The tumor image obtained by DAG-PET can be of great value for the diagnosis of brain tumors.

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