Metabolism of [18F]fluorodiacylglycerol in Rat Hippocampal Neurons in Vitro

III. 3. Metabolism of [^{18}F]fluorodiacylglycerol in Rat Hippocampal Neurons *in Vitro*

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Introduction

Positron emission tomography (PET) has been contributed for the clinical diagnosis of various diseases, especially brain disorders by measuring cerebral blood flow, oxygen consumption and metabolism. Recent developments in positron-emitting radio labeled ligands have made it to measure the binding capacity of many kinds of neurotransmitter receptors in humans. However, it is difficult to measure exactly the neuronal activities in the central nervous system (CNS) at present. In order to solve this problem, Imahori *et al.* have focused on the diacylglycerol as a second messenger in intracellular signal transduction system and designed carbon-11 labeled 1,2-*O*-diacylglycerol ([^{11}C]DAG) as an excellent tracer to enter the receptor-mediated Phosphoinositide (PI) turnover[1]. These attempts are of interest in relation to the viewpoint for imaging second messenger systems in human brains.

We recently succeeded to synthesize the DAG analogues with fluorine-18 (*rac*-1,2-[^{18}F]FDAG). We also observed that these compounds can produce unique patterns in PI turnover, as compared with [^{11}C]DAG. The administrated *rac*-1,2-[^{18}F]FDAG in rats was mainly metabolized into phosphatidyl-ethanolamine (PE) and phosphatidylcholine (PC), which are potent substrates for phospholipase D (PLD)[2]. These results indicate that *rac*-1,2-[^{18}F]FDAG can be a novel tracer to evaluate receptor-mediated neuronal activities linked with PLD.

In order to examine exactly possible role of novel tracer with fluorine-18, we carried out in vitro study using hippocampal neurons.

Methods

Isolation and culture of rat hippocampal neurons

Rat embryonic hippocampal neurons were obtained from pregnant Sprague-Dawley rats with 18 days gestation. Brains were removed quickly from the embryos. Typically, hippocampi were dissected from the brains, and placed in 5.0 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.125% trypsin/ 0.01% DNase for 10 min at 37°C. After adding 1 ml of fetal calf serum (FCS) to inhibit the activity of trypsin, cells

were dissociated by trituration with 1 ml and 200 μl polypropylene pipet tips. The preparation was centrifuged at 800 *g* for 5 min and then the pellet was gently re-suspended in Neurobasal/B27 (25μM 2-mercaptoethanol) medium. The cells were seeded on a 25 cm^2 flask coated with poly-D-lysine at concentration of 3.2×10^5 cells /cm^2.

Radio synthesis of rac-1,2-[^{18}F]FDAG

rac-1,2-[^{18}F]FDAG was synthesized from no-carrier-added [^{18}F]fluoride[3] and solubilized to Ca^{2+}- and Mg^{2+}-free PBS with negative charged liposome[4].

Metabolism of rac-1,2-[^{18}F]FDAG on rat hippocampal neurons

Rat hippocampal neurons cultured for 7-10 days were used. After washing twice cell surface with 2 ml of PBS, *rac*-1,2-[^{18}F]FDAG preparation in this study (0.5 mCi /2 ml /flask) was applied to the neurons with or without indicated drugs, and the cells were incubated up to 60 min. The reaction was terminated by removing the solution and adding 2 ml of ice-cold PBS to each flask.

Lipid extraction

After stopping the reaction, cell surface was washed twice by adding 2 ml of ice-cold PBS. The cells were detached by adding 0.125% trypsin, replaced to 15 ml tube, centrifuged at 800 *g* for 5 min. After removing supernatant, the cells were further washed by adding 1 ml of PBS and repeated passings through a 1 ml blue pipet tip. After centrifugation of the cell suspension at 1200 *g* for 5 min, 400 μl of chloroform/methanol (2/1 v/v) and 20μl of water were added into the pellet; the mixture was sonicated at 30°C for 5 min and centrifuged at 1200 *g* after adding 200 μl of methanol. The supernatant was transferred to other tube, and 100 μl of chloroform/methanol (1/2 v/v) and 5 μl of 5% KCl solution were added into the pellet by the same procedure as described above. Then the supernatants were collected.

Metabolites analysis

Radioactivities in the extract was measured with a well-type gamma counter and the radioactive metabolites in the extract were separated by thin layer chromatography (TLC) using a silica gel plate and a solvent system (chloroform:acetone: methanol:acetic acid:water, 45:15:13:12:8 v/v). The radioactivity in each metabolite was measured using image analyzer BAS5000.

Results and Discussion

Lipidic fractions were extracted from neurons exposed to *rac*-1,2-[^{18}F]FDAG up to 60min. *rac*-1,2-[^{18}F]FDAG metabolites produced by phospholipid turnover such as [^{18}F]PE,

[^{18}F]PC and [^{18}F]phosphoinositides ([^{18}F]PIs) were detected by TLC analysis (Fig. 1(A)). By measuring these activities, the present study was showed that [^{18}F]PE and [^{18}F]PC were the main metabolites (37% and 24% in whole phospholipid, respectively)(Fig. 1(B)). These findings suggest that *rac*-1,2-[^{18}F]FDAG was trapped on cell surfaces of neurons and incorporated into the phospholipid turnover associated with the intracellular signal transduction, particularly PLD.

rac-1,2-[^{18}F]FDAG metabolism into phospholipids without a drug stimulus reached a steady state at 30 min. On the other hand, the stimulus with carbachol, agonist of muscarinic cholinergic receptor (mAChR) conjugated with PLC, caused a time-dependent increase of the *rac*-1,2-[^{18}F]FDAG incorporation at least up to 60 min (Fig. 2(A)). As shown in Fig. 2(B), the increase of whole phospholipid was mainly due to the incorporation of *rac*-1,2-[^{18}F]FDAG into [^{18}F]PE.

There was no correlation between metabolism of *rac*-1,2-[^{18}F]FDAG and carbachol concentration (>10μM). The metabolic ratio into phospholipids was significantly larger than the control (about 150% of control). Among them, [^{18}F]PIs and [^{18}F]PE was increased as compared with the control (150% and 170% of control respectively), whereas [^{18}F]PC did not show significant difference (Fig. 3). These responses were inhibited by pre-incubation of atropine, mAChR antagonist (Fig. 4). Since PIs and PE are substrates for PLC and PLD, respectively, it is conceivable that *rac*-1,2-[^{18}F]FDAG is incorporated into the phospholipid turnover associated with neuronal signal transduction. No difference was observed in metabolism into [^{18}F]PC between carbachol-treated and the control neurons. The results suggest that the conversion of *rac*-1,2-[^{18}F]FDAG into [^{18}F]PC is caused by re-construction of cellular membrane.

When neurons were treated with phorbol 12-myristate 13-acetate (PMA), PKC-dependent PLD activator, the *rac*-1,2-[^{18}F]FDAG metabolic ratio into [^{18}F]PIs had a low value as compared with carbachol-treated neurons (about 120% of control)(Fig. 5). It is well known that PMA doesn't activate PLC directly. Therefore, our findings suggest that PLD activation caused the increase of *rac*-1,2-[^{18}F]FDAG metabolic ratio into [^{18}F]PE because that *rac*-1,2-[^{18}F]FDAG could not be incorporated into the PI turnover. It also suggests that the increase of [^{18}F]PE in carbachol-treated neurons is not due to the PLC activation but due to the PLD activation.

Both stimulus studies with carbachol and PMA showed 5-fold higher metabolic ratio into [^{18}F]PE as compared with into [^{18}F]PIs. Taken together, these results suggest that *rac*-1,2-[^{18}F]FDAG is incorporated into the PE-PLD turnover mainly and metabolized into [^{18}F]PE finally.

In conclusion, these results demonstrate the activation of *rac*-1,2-[^{18}F]FDAG metabolism mediated by PLD in rat hippocampal neurons. Therefore, we suggest that *rac*-1,2-[^{18}F]FDAG may be useful as a novel tracer for imaging signal transductions in brain

neurons.

References

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3) Furumoto S., Iwata R., Ido T., *J. Labelled Compd. Radiopharm.* (in press).
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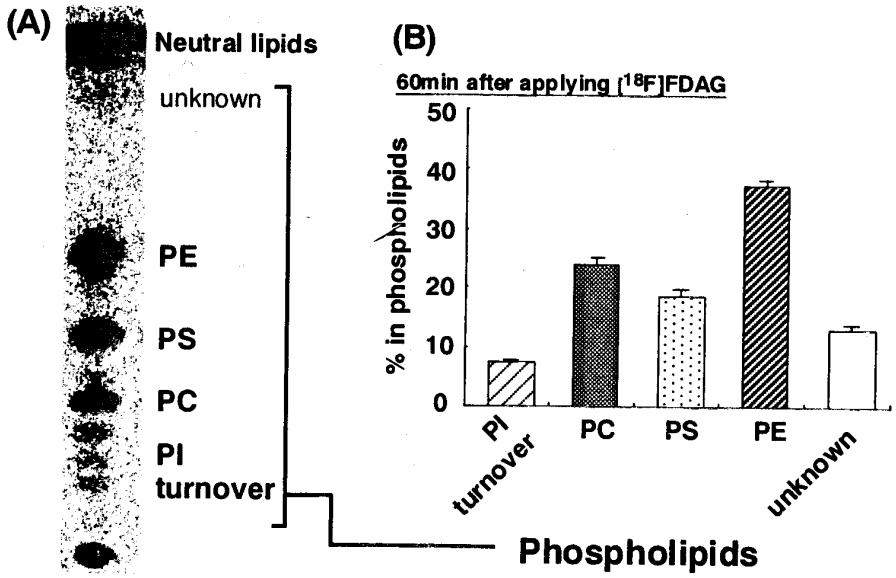

Fig. 1. Chromatographic analysis of phospholipids in rat hippocampal neurons using [^{18}F]FDAG.
(A) radio-TLC profile of rat hippocampal neurons after 60min labeling. Chloroform/ Acetone/ MeOH/ AcOH/ Water = 45/ 15/ 13/ 12/ 8. (B) Phospholipids ratio in rat hippocampal neurons after 60min labeling. Each column represents the mean ± S.E.M. (n=15).

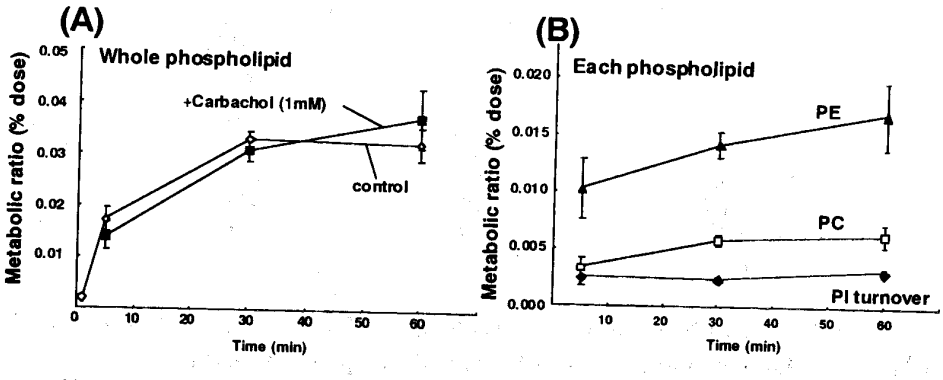

Fig. 2. Time course of [^{18}F]FDAG metabolic ratio into phospholipids of neurons exposed to carbachol (1mM) and [^{18}F]FDAG
Each point represents the mean ± S.E.M.(n=3-15).

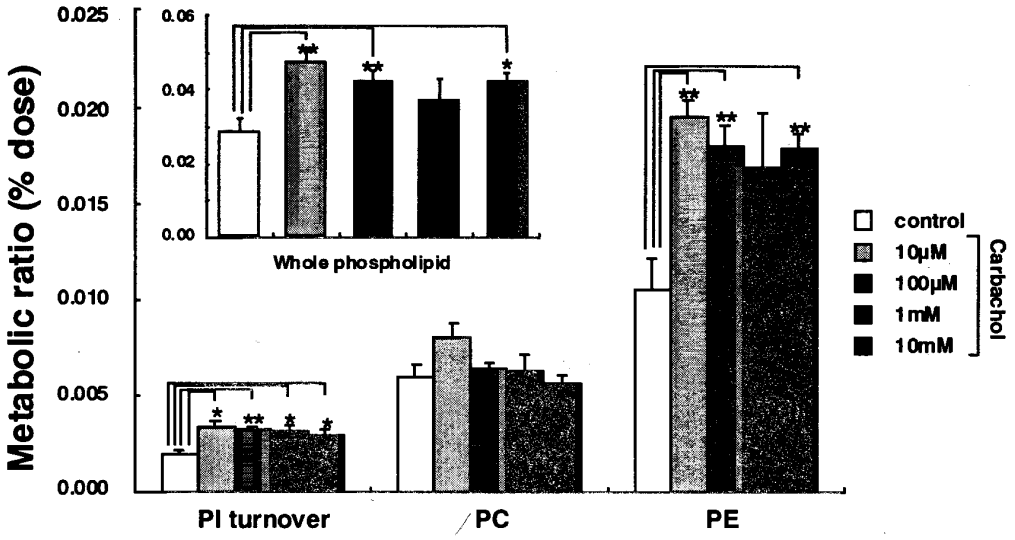

Fig. 3. [^{18}F]FDAG metabolic ratio into phospholipids of neurons exposed to carbachol and [^{18}F]FDAG for 60min

Each column represents the mean ± S.E.M.(n=3-14). **:P<0.01, *:P<0.05: significantly different from the control by Student's *t* -test.

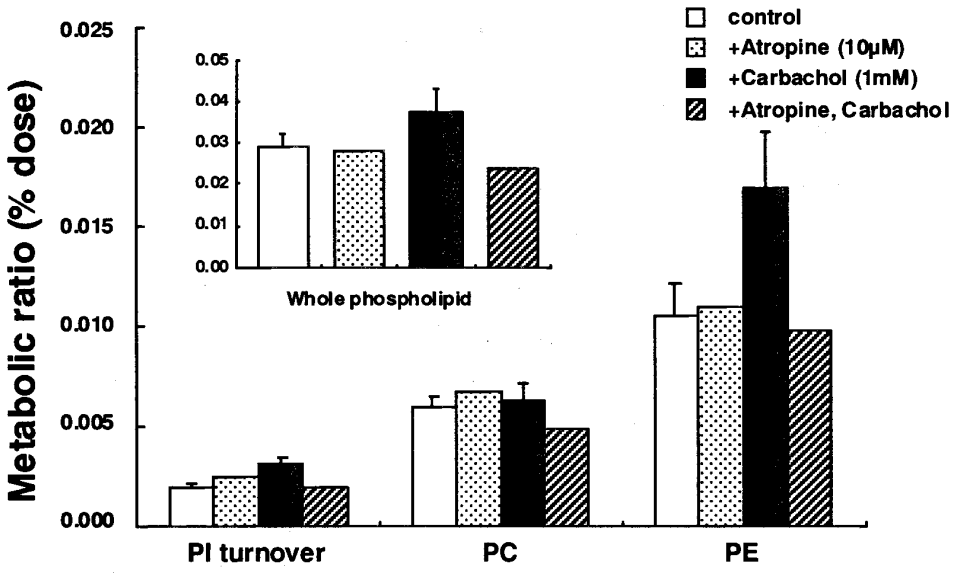

Fig. 4. [^{18}F]FDAG metabolic ratio into phospholipids of neurons exposed to carbachol and [^{18}F]FDAG for 60min after pre-incubated with atropine for 10min

Open- and closed-column represents the mean ± S.E.M. (n=3-15).

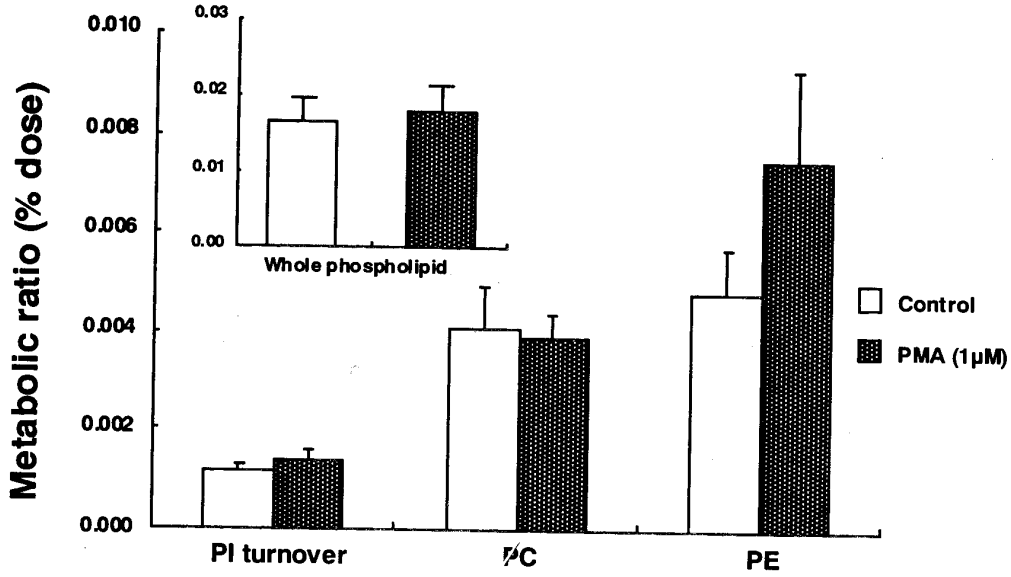

Fig. 5. [^{18}F]FDAG metabolic ratio into phospholipids of neurons exposed to PMA and [^{18}F]FDAG for 60min

Each column represents the mean ± S.E.M.(n=3-5).