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ABSENCE OF DIGLYCERIDE KINASE ACTIVITY IN THE PHOTORECEPTOR CELLS OF DROSOPHILA MUTANTS

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Diglyceride kinase (DG) activity was found to be almost absent in the photoreceptor cells of Drosophila vision mutants, norpA (no receptor potential A). Other enzymes related to the phosphatidic acid (PA) metabolism, such as monoacyl-glycerophosphate acyltransferase and PA phosphatase, were normal in the mutant. The deficiency in PA content in the retinular cells of norpA, demonstrated by ^{32}P incorporation in vivo is, therefore, due to abnormal reduction in DG kinase activity. The DG kinase was found to be localized in the retinular cell by assaying its activity in the mutants rdgA (receptor cells degenerated) and sine oculis (compound eyes absent).

Drosophila has been fully utilized for analyzing photoreceptor mechanisms by genetically dissecting each process involved (1). There is a mutant called norpA (no-receptor potential A), which has been shown to generate abnormally small receptor potentials, although rhodopsin is present in the eyes (2). The amount of rhabdomere specific proteins in the mutant retinular cells are reduced (3) and the mutant rhabdomere contain few intramembranous particles (4).

In our previous paper (5), we analyzed phospholipids of the mutant head homogenate, and demonstrated that ^{32}P incorporation into phosphatidic acid (PA) is drastically reduced in the mutant. The reduction in PA content was also shown by chemical analyses. It was further found that PA is localized almost exclusively in the retinular

Abbreviations: DG, diglyceride; EGTA, ethylene glycol bis (β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid; GP, glycerophosphate; GPAT, glycerophosphate acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Pi, inorganic phosphate; PS,phosphatidylserine; TLC,thin layer chromatograph

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cells by examining rdgA (receptor degeneration A) and sine oculis (without eyes) mutants.

Major purpose of this paper is to analyze the mutant effect on PA metabolism in vivo and to investigate the enzymatic basis of the deficit. PA content in the mutant eyes is examined by feeding ^{32}P-inorganic phosphate and it was found that PA synthesis in vivo, indeed, is very low. We further demonstrate that, among the enzymes involved in PA metabolisms, diglyceride (DG) kinase activity is almost absent in the mutant heads.

Materials and Methods

Materials : As a normal strain, an inbred Drosophila melanogaster strain of Canton-S was used, from which all the mutants used were derived. To examine the defect of norpA mutants, two alleles, norpA^{JM11} (JM11) and norpA^{EE5} (EE5) were used as in the previous experiment (4). A mutant, rdgA^{KO14} (KO14; receptor degeneration A gene) and sine oculis (without compound eyes) were also used to test whether DG kinase is localized only in the retinular cell.

Chemicals : 1-^{14}C oleoyl-CoA (specific activity; 59.5 mCi/mmol), 1-^{14}C palmitoyl-CoA (specific activity; 56.9 mCi/mmol), γ-^{32}P ATP (specific activity; 2,600 Ci/mmol) and ^{32}P-inorganic phosphate (specific activity; 50 Ci/mmol) were purchased from New England Nuclear Co. DG and PA were purchased from Serdary Research Laboratories, Inc. Monoacyl-glycerophosphate was kindly supplied by Prof. Y. Nozawa, Gifu University. Other chemicals used are of reagent grade.

Labeling in vivo of phospholipids : About 100 µCi of ^{32}P labeled inorganic phosphate was added into 2 ml of 1% sucrose solution. A filter paper with a diameter of 30 mm was placed at the bottom of a vial on which 200 µl of the radioactive solution were dripped. About 100 young flies, from six to twelve hours after eclosion were first starved for 30 minutes, and then transfered into the vial with radioactive phosphate. Flies were kept there for 24 hours. Labeled flies were collected and frozen in the deep-freezer (-80°C) until the extraction of phospholipids. The amount of phosphate ingested was monitored by counting the radioactivity of the whole fly head.

Assay of DG kinase activity : The method used here was similar to that described by Lapetina and Hawthorn for rat cerebral cortex (6). Flies 4 to 7 days after eclosion were etherized lightly, frozen and kept in a deep-freezer (-80°C) until the enzyme assay. For the experiments, they were manually decapitated on a dry ice plate and submitted to homogenization. Details of the homogenization procedure were described previously (5). The enzyme activity was determined by measuring incorporation of ^{32}P from γ-^{32}P ATP into PA. The standard reaction medium contained, in a final volume of 200 µl, 100 mM KCl, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM KF, 1 mM EGTA, 2 mM DG, 1 mM γ-^{32}P ATP, 20 mM phosphate buffer (pH 6.8) and head homogenate (0.17 mg protein). Immediately before incubations, DG was sonicated for 5 min using a Microprobe (Artek, Dismembrane sonicator). DG suspension was prepared

freshly for each experiment. Deoxycholate (2 mM) was added to the DG suspensions in some experiments. The reaction mixture was incubated for 5 min at 24°C, and the reaction was stopped by adding 0.5 ml of 12% ice cold perchloric acid. Lipids were extracted and separated as described previously (5).

Assay of monoacyl-glycerophosphate acyltransferase : The method was similar to that of Imai et al. (7). The incubation mixture contained, in a final volume of 0.2 ml, 100 mM Tris-HCl buffer (pH 7.4), 0.1 mM monoacyl glycerophosphate (GP), 1-^{14}C oleoyl-CoA (0.06 µCi/4mmol; final concentration, 20 µM). The reaction was initiated by the addition of 0.17 mg protein of fly head homogenate, which was prepared by homogenizing 20 heads in Tris-HCl buffer solution. After 2-minute incubation at 24°C, the reaction was terminated by adding 4 ml of chloroform/methanol/1N HCl (200:100:0.75) mixture. Lipids were separated by thin layer chromatography (TLC), the corresponding area of PA was scraped into vial and the radioactivity was counted.

Assay of PA phosphatase : PA phosphatase activity was assayed by measuring the amount of orthophosphate liberated, according to the method of McCaman et al. (8) with a slight modification. A hundred heads were homogenized in 0.2 ml of Tris-HCl buffer (pH 7.4). Incubation was started by adding 100 µl of homogenate (0.4 mg protein) to 100 µl of PA containing Tris buffer solution (final concentration of PA was 4 mM). After 30 min incubation at 24°C, the reaction was stopped by adding 10 µl of 10% ice cold albumin solution, and 0.2 ml of 10% ice cold trichloroacetic acid (9). The mixture was centrifuged and aliquot (0.3 ml) of the supernatant was taken for the inorganic phosphate assay. The amount of inorganic phosphate released was corrected for the amount liberated from the enzyme preparation in the absence of substrate. In the absence of fly homogenate, no inorganic phosphate was liberated from PA. Protein concentration was determined by the method of Lowry et. al. (10).

Results

Deficiency in PA synthesis of norpA retinular cells :

In our previous paper (5), we demonstrated that PA content in the retinular cell of norpA mutants was markedly reduced. In order to confirm the PA deficit under more physiological conditions, in vivo, we fed flies with labeled inorganic phosphate. The two dimensional TLC autoradiographs of the labeled phospholipids extracted from heads of normal and norpA flies are given in Fig. 1. Among the total phospholipids, the percent radioactivity of PA was found to be 0.53% in normal flies (n=14) and 0.12% (n=12) in norpA^{EE5}. Therefore, the mutant was also shown in vivo to have defect in ^{32}P incorporation into PA.

Enzymatic activity for PA metabolism :

Since PA is synthesized either from DG by phosphorylation with DG kinase or from monoacyl-glycerophosphate by acylation with

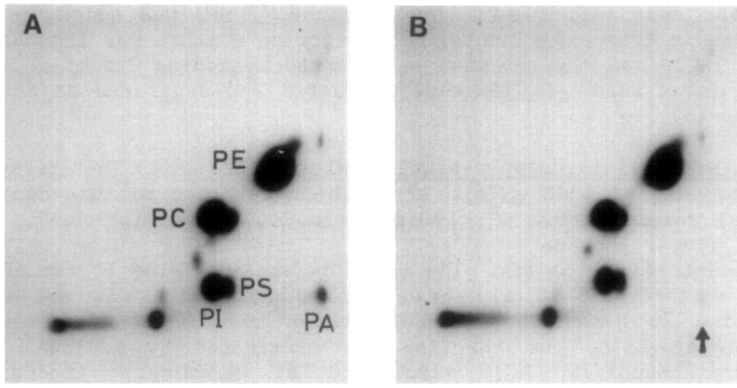

Fig. 1 Autoradiographs of two dimensional TLC pattern of in vivo labeled phospholipids. Radioactive phospholipids were extracted from (a) normal and (b) norpA^{EE5} heads. All phospholipids were labeled in vivo by feeding ^{32}Pi. Details are explained in the Materials and Methods. Labeled phospholipids were extracted from fly heads with acidic chloroform-methanol solution and developed in the first dimension with chlorofrom/methanol 28% NH_4OH (65:35:5, v/v) and in the second dimension with chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v). Arrows indicate the spot corresponding to PA. As for abbreviations, see foot note.

monoacyl-GPAT, we measured the activity of these enzymes by using head homogenates. As is shown in Table 1, DG kinase activity of the two alleles of norpA mutant was reduced markedly to 59% and 18% of that in the normal flies. The difference between the two alleles is in parallel with the severity of the mutant syndrome. On the other hand, no change was found in the activity of monoacyl-GPAT in the mutants.

Table I
Comparison of enzymatic activity related to PA metabolism between normal and mutant strains

	normal	norpA^{JM11}	norpA^{EE5}
DG Kinase [a]	9.7 ± 3.4 (12)	5.7 ± 2.2 (6)	1.7 ± 0.5 (7)
PA Phosphatase [b]	0.10 ± 0.01 (4)	0.09 ± 0.01 (4)	0.10 ± 0.02 (5)
Monoacyl-GPAT [c]	5.6 ± 0.8 (6)	5.5 ± 0.2 (4)	5.3 ± 0.7 (4)

The assay conditions are described in the Methods. Values are the means ± S.D. Number of experiments is give in the parenthesis.
a) Enzymatic assay was performed under optimal condition deduced from the data in Fig. 2. The activity is expressed as nmol/mg protein/5 min.
b) The activity is expressed as nmol/mg protein/30 min. Assay condition is the same as was used for brain tissues (8). Optimal conditions for this enzymes of Drosophila is under study.
c) The activity is expressed as nmol/mg protein/min. The assay conditions originally devised for platelet (7) were used throughout the experiments.

Oleoyl-^{14}C-CoA and palmitoyl-^{14}C-CoA were used in this assay, but both gave identical results. PA phosphatase activity was also compared. No abnormality was found in the mutants. Therefore, we conclude that the deficiency in PA of norpA is caused by the reduction in the DG kinase activity.

Characterization of Drosophila DG kinase :

Experiments were carried out to test whether DG kinase activity of the mutant is abnormal under any assay conditions. In these experiment, incubation time, concentration of DG, ATP and Mg^{2+} were changed. Results for normal and norpA head homogenates are summarized in Fig. 2.

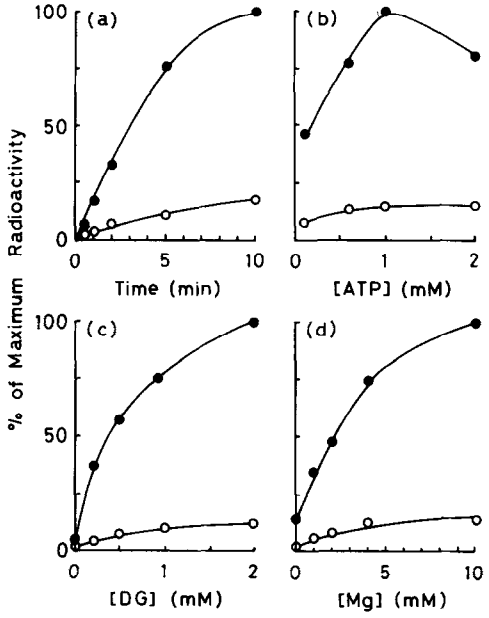

Fig. 2 Characteristics of DG kinase activity in crude fly head homogenates. Results are presented as percentage of the maximum activity of normal preparations. Data expressed by (●) are for normal and (○) are for mutant, norpA^{EE5}. Note that DG kinase activity in the mutant is extremely low under any assay conditions. (a) Time course of DG kinase reaction. Effects of (b)ATP, (c) DG and (d) Mg^{2+} on DG kinase activity. The DG kinase activity thus measured was not sensitive to the Ca^{2+} concentration. After the reaction was terminated, lipids were extracted with acidic chloroform-methanol solution and developed by one dimensional TLC with the mixture of chloroform/acetone/methanol/acetic acid/water (40:17:15:12:8, v/v). Labeled PA was scraped and counted. The results presented are obtained using the same DG suspensions. Addition of deoxycholate in the reaction medium (final concentration, 2 mM) activated DG kinase about 2 folds, but the activity ratio between normal and mutant was not changed. The data are mean values for three different preparations, each with duplicate experiments.

The DG kinase activity of norpA was extremely low under any assay conditions.

Discussion

In our previous paper (5), we showed with phosphorylation experiments and with chemical analyses by using head homogenates that PA in the photoreceptor membrane of norpA is drastically reduced. This conclusion is confirmed in this paper by analyzing labeled phospholipids after feeding flies with ^{32}P inorganic phosphate. PA content in the retinular cell of norpA was found to be only 20% of that in the normal flies.

We further demonstrated that the mutants have markedly reduced DG kinase activity, while other enzymes related to PA metabolisms, such as monoacyl-GPAT and PA phosphatase activities are normal in the mutants. Therefore, the reduction in PA content in the mutant is the result of reduced DG phosphorylation.

In order to ask whether DG kinase is also localized in the retinular cells, the enzyme activity was assayed for rdgA (retinular cells degenerated) and sine oculis (compound eyes absent). The activities of the kinase in the two mutants' head homogenates were about 0.3 nmol/mg protein/5 min; the value being only 3% of normal flies (9.7 nmol/mg protein/5 min). This indicates that DG kinase must be localized in the compound eyes, especially in the retinular cells which show severe degeneration in the rdgA mutants. These results are consistent with our previous observation that PA is highly localized in the Drosophila retinular cells. These findings suggest a possibility that PA and/or DG kinase must play an important role in the visual transduction processes in Drosophila.

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