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Nonpolar lipid and phospholipid methylation during development of

Dictyostelium discoideum

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The life-cycle of the cellular slime mold Dictyostelium discoideum is characterized by development from a vegetative stage of solitary amoebae via cell aggregation and a ‘slug’ stage to a fruiting body. It has been suggested that phospholipid methylation is involved in CAMP-induced CAMP-synthesis, which is the mechanism underlying cell aggregation. Therefore, we have examined changes in lipid methylation during development. Individual amoebae incorporated the methyl group of [methyl-3H]methionine both in phospholipid and certain nonpolar lipid components. Nonpolar methyl acceptors, characterized by thin-layer chromatography, were probably Δ22-stigmasten-3β-ol, Δ22-stigmasten-3β-ol fatty acid ester and ubiquinone. The activities of nonpolar and phospholipid methyltransferases, as assayed in a homogenate with S-adenosyl[methyl-3H]methionine as a methyl donor, showed a strong decline from the vegetative stage to the slug stage, which was not followed by a subsequent rise during the final stage. When the activities of both enzymes were measured in intact cells during development, the decline of the amount of enzyme was masked by a 60-fold increase of the specific activity of intracellular S-adenosyl[methyl-3H]methionine during cell differentiation. We conclude that the activities of phospholipid N-methyltransferase and adenylate cyclase (or the number of cyclic AMP receptors on the cell surface) are not regulated in parallel during development.

Introduction

Individual amoebae of the cellular slime mold Dictyostelium discoideum live in the soil and feed on bacteria. Exhaustion of the food supply induces cell aggregation which is mediated by positive chemotaxis to cyclic adenosine 3’,5’-monophosphate (cAMP) [1]. Pulses of cAMP are secreted by the aggregation center, whereas the amoebae detect the signal by means of cell-surface receptors [2-5]. After cell aggregation, the aggregate becomes a pseudoplasmodium or ‘slug’, moving over the substrate and finally differentiating into a fruiting body, which consists of a stalk with spores embedded in a slime droplet at its top.

In the presence of [methyl-3H]methionine or S-adenosyl[methyl-3H]methionine, intact cells or homogenates of D. discoideum incorporate the methyl-3H group into mono- and dimethylphosphatidylethanolamine (PME, PMME) and phosphatidylcholine (PC) [6,7]. Phosphatidylcholine is mainly synthesized from CDP-choline and diacylglycerol, but it can also be formed from phosphatidylethanolamine (PE) by three successive methylations [8]. The methyl donor is S-adenosylmethionine (AdoMet), which is synthesized from methionine and ATP. Methylation gives rise to the formation of a methylated lipid and S-adenosyl-homocysteine as end products. As described for higher organisms [9], D. discoideum seems to
possess two different phospholipid methyltransferases. The first enzyme shows a low $K_m$ for AdoMet (6 $\mu$M) and it converts PE into PME. The second enzyme has a rather low affinity for AdoMet (60 $\mu$M) and it produces PC from PME via PMME [7]. Slime mold phospholipid methyltransferases have been reported to be activated by cyclic guanosine 3',5'-monophosphate (cGMP) [6] and free calcium via the formation of a calcium-calmodulin complex [7]. From observations on the transient increase of phospholipid methylation after stimulation of aggregative cells with cAMP, it has been suggested that phospholipid methylation in *D. discoideum* could be related to CAMP-induced cAMP-secretion or cell differentiation [6]. Differentiation-induced changes in phospholipid methylation have actually been observed in human B cells [10], hamster sperm [11], ganglionic neurons [12] and chicken lens fiber cells [13].

Here, we report about changes in phospholipid methylation during development of *D. discoideum* from the vegetative stage to the fruiting body. Methyl groups were not only found to be incorporated into phospholipids, but also into certain nonpolar lipid components. An attempt has been made to characterize the methylated compounds.

**Materials and Methods**

**Materials.** [methyl-3H]Methionine (74 Ci/mmol) was from Amersham International, Buckinghamshire, U.K. S-Adenosyl[methyl-3H]-methionine (78 Ci/mmol) was a product of New England Nuclear, Boston, U.S.A. Adenosine and S-adenosylmethionine were obtained from Boehringer Mannheim, F.R.G. S-adenosylhomocysteine was a product of Serva, Heidelberg, F.R.G. 10-Camphorsulphonic acid was from Merck, Darmstadt, F.R.G.

**Organisms.** Dictyostelium discoideum NC-4(H) was used for all experiments. Cells were grown in association with *Escherichia coli* 281 on a solid medium (3.3 g peptone, 3.3 g glucose, 4.5 g KH$_2$PO$_4$, 1.5 g Na$_2$HPO$_4$·2H$_2$O and 15 g agar per liter) and were harvested in phosphate buffer (10 mM Na$_2$HPO$_4$/KH$_2$PO$_4$, pH 6.5). They were freed from bacteria by repeated centrifugation (3 times at 150 × g for 3 min). Amoebae were starved in two different ways, depending on the type of experiment:

(a) in suspension (10$^7$ cells/ml in phosphate buffer as above, for 4$\frac{1}{2}$ h on a gyratory shaker at 150 rpm and 20–22°C);

(b) on non-nutrient agar plates (10$^8$ cells/Petri dish of 10 cm diameter) for different periods of time at 20°C. Non-nutrient agar consisted of 4.5 g KH$_2$PO$_4$, 1.5 g Na$_2$HPO$_4$·2H$_2$O and 15 g agar per liter.

**Incubation systems and lipid extraction.** After starvation, cells were collected by centrifugation and resuspended in phosphate buffer (see above) at a density of 10$^8$/ml. [methyl-3H]Methionine was added (5 or 10 $\mu$Ci/ml) and the reaction was stopped after different intervals of time by the addition of 0.1 vol. 70% HClO$_4$. In experiments with cell homogenates, the amoebae were disrupted by short sonication in ice after resuspending in Tris-chloride buffer (50 mM, pH depending on the type of experiment, 1.5 mM MgCl$_2$ added). Sonication was performed with a Branson Sonifier with microtip at position 4 (3 times for 5 s at 0–4°C). Homogenates were incubated with S-adenosyl[methyl-3H]methionine (1 $\mu$Ci/10$^7$ cells) and the reaction was stopped after 2 min by the addition of 0.1 vol. 70% HClO$_4$.

Acid-insoluble material from homogenates or intact cells was precipitated by centrifugation (4 min, 8000 × g) and the pellet (corresponding to 10$^7$ cells) was washed with 1 ml 6% HClO$_4$. After removal of the supernatant, lipids were extracted with 1 ml of chloroform/methanol/2 M HCl (6:3:1, v/v). After at least 30 min of shaking on an Eppendorf mixer, the organic phase was washed three times with 0.5 ml of 0.05 M KCl in 50% methanol. Between washes, phase separation was rapidly obtained by centrifugation (2 min, 8000 × g). After washing, the aqueous phase was carefully removed and the organic phase was evaporated under a stream of nitrogen. This extraction method is a modification of the procedure described in Refs. [9,14 and 15].

**Thin-layer chromatography of lipids** The residue which remained after evaporation was dissolved in 100 $\mu$l of chloroform. This solution was spotted on silica gel plates which had been activated by heating for at least 1 h at 110°C. The test-tubes were subsequently washed with 50 $\mu$l of chloroform and
this solution was also applied to the thin-layer plates. Different mobile phases were used for development:

(A) propionic acid/1-propanol/chloroform/water (2 : 2 : 1 : 1) in paper-lined tanks [16];
(B) chloroform/methanol/water/25% ammonia 120 (75 : 6 : 2) in paper-lined tanks [17];
(C) ligroin/diethyl ether/acetic acid (50 : 50 : 1) in normal tanks (modified after ref. [18]);
(D) benzene/methanol (95: 5) in normal tanks [19];
(E) chloroform in normal tanks [20].

After development, plates were dried at room temperature. Lipid spots were visualized by exposure of the plate to iodine vapour, and were identified by comparison of their \( R_F \) values to those of external standards. Spots were scraped from the thin-layer plate and eluted with 1 ml of methanol by at least 15 min of vigorous shaking on a gyratory shaker at 250 'pm. After elution, radioactivity was determined by liquid scintillation counting with automatic quench correction.

Determination of the specific activity of intracellular \( S \)-adenosyl[methyl-\( ^3\)H]methionine. Cells of different developmental stages were resuspended in phosphate buffer (see above, density log/ml) and were incubated with \( S \)-adenosyl[methyl-\( ^3\)H]methionine (10 \( \mu \)Ci/ml) for 60 min (gyratory shaker at 150 rpm, 20–22°C). After this period, incubation was ended by addition of 0.1 vol. of 70% HClO\(_4\). Protein was sedimented by centrifugation, and the supernatant was neutralized to pH4.5 by addition of a solution of 50% KHCO\(_3\), (w/v). KClO\(_4\) was sedimented by centrifugation and the supernatant was passed through 0.45 \( \mu \)M Millipore filters before application to a Lichrosorb 10 RP 18 HPLC column. The column was eluted with 0.2 M sodium acetate, 0.025 M camphorsulphonic acid, 15% methanol (pH 4.6) [21]. The size of the intracellular AdoMet pool was determined from the integrated area of the AdoMet peak as measured by the UV detector, in comparison with standard samples. The amount of radioactivity in the AdoMet peak was determined by liquid scintillation counting in 0.5 ml fractions of the eluate.

**Results**

As reported previously [6,7], aggregative cells of \( D. \) discoideum incorporate the methyl group of \( [methyl-^3\)H]methionine into phospholipid. As shown in Fig. 1A, B, separation of the methylated compounds by thin-layer chromatography shows radioactivity to be associated with PME, PMME, PC and lysophosphatidylcholine (LPC), PC being the major radioactive phospholipid.

When homogenates of aggregative cells are incubated with \( S \)-adenosyl[methyl-\( ^3\)H]methionine, PME, PMME and PC become labeled. Under these conditions, PME is the major radioactive component (Fig. 1C).

In intact cells, 25–50% of amount of label elutes at the solvent front in the same position as the nonpolar lipid fraction (Fig. 1A, B). Since nonpolar lipid methylation has not been described previously in \( D. \) discoideum, we wanted to know whether the radioactive peak at the solvent front was due to enzymatic methylation or to an artifact.

Incorporation of the methyl group into nonpolar lipid increases with time during a 50 min period of incubation (Fig. 2A). When the cells are put in a boiling water bath for 2 min before subsequent incubation with label at 20°C, labeling of the neutral lipid fraction is completely blocked. Addition of 0.1% methanol to the incubation medium has no effect on nonpolar lipid methylation. When the incubation temperature is lowered from 20 to 2°C, the reaction rate is decreased to 7% of that observed at 20°C (Fig. 2A).

In intact cells, the amount of methyl-\( ^3 \)H groups into phospholipid increases linearly with time during a 50 min period of incubation at 20°C (Fig. 2B). Lowering the incubation temperature to 2°C causes a 14-fold decrease of the reaction rate. Addition of 0.1% methanol to the incubation medium induces an initial increase of phospholipid methylation, but after 10 min, the velocity of methylation has returned to that of the control suspension. 2 min at 100°C before incubation does not totally block the incorporation of methyl groups into phospholipid in the cells, but it causes a slowing down of about 65% (Fig. 2B).

The pH-optimum of nonpolar lipid methylation in a cell homogenate is narrow and situated at pH 8 (Fig. 3A). Phospholipid methylation shows a
different dependency on pH, an optimum being observed at pH 9 (Fig. 3B, C).

When the nonpolar lipid fraction is separated into different lipid classes by thin-layer chromatography, radioactivity co-elutes in all mobile phases, mainly with cholesterol and ubiquinone (Fig. 4A–C). Besides the separations shown in the figure, we also used acetone as a mobile phase and methanol/water (90:10 v/v) as the eluent in a reversed-phase separation on silanized silica gel (values not shown).

In a cell homogenate, both phospholipid methylation and nonpolar lipid methylation show a dose-dependent inhibition by S-adenosylhomocysteine (Fig. 5). Nonpolar lipid methylation is affected with an apparent $K_i$ of 8.7 μM, whereas the $K_i$ for phospholipid is 21 μM.

When intact cells of different developmental stages are incubated with [methyl-$^3$H]methionine, the incorporation of methyl groups into phospholipid decreasing during development from the vegetative stage to the formation of the 'slug' (Fig. 6A). During the slug stage, however, phospholipid radioactivity shows a strong increase. After complete differentiation to the fruiting body, the amount of label in phospholipid decreases again to a low value (Fig. 6A).

A similar labeling pattern during development is observed for those nonpolar lipids which co-clute with sterol and sterol esters (Fig. 6B). Radioactivity in the position of ubiquinone does not decline during the first 12 h of starvation, but its develop-

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Fig. 1. Separation of methylated lipids of aggregative cells of *D. discoideum* by thin-layer chromatography on silica gel 60 (Merck). (A) Mobile phase 1-propanol/propylic acid/chloroform/water (2:2:1:1, v/v); reaction products in intact cells after labeling with [methyl-$^3$H]methionine. (B) Mobile phase chloroform/methanol/water/25% ammonia (120:75:6:2, v/v); reaction products in intact cells after labeling with [methyl-$^3$H]methionine. (C) Mobile phase 1-propanol/propylic acid/chloroform/water (2:2:1:1, v/v); reaction products in homogenates after labeling with S-adenosyl[methyl-$^3$H]methionine. Abbreviations indicate the position of external standards: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylerine; PME, phosphatidylmonomethylthanolamine; PMME, phosphatidyltrimethylthanolamine; FFA, free fatty acids; PE, phosphatidylethanolamine; PA, phosphatidic acid; NL, nonpolar lipid.
Fig. 2. Incorporation of methyl groups from [methyl-\(^3\)H]methionine into (A) Nonpolar lipids (B) phospholipids of D. discoideum. Aggregative cells were incubated with [methyl-\(^3\)H]methionine (10 \(\mu\)Ci/10\(^6\) amoebae) in phosphate buffer. The incubation was started at zero time and was terminated after the indicated intervals by addition of 0.1 vol. 70% HClO\(_4\). Extraction of lipids, separation on silica gel with solvent A and measurement of radioactivity in the different lipid fractions were performed as described in Materials and Methods. The following experimental conditions were used: ○, intact cells at 20°C; □, intact cells at 20°C with 0.1% methanol in the medium; ■, intact cells at 2°C; △, boiled cells subsequently incubated with label at 20°C.

Fig. 3. Dependency of lipid methylation on pH. Homogenates of aggregative cells of D. discoideum were incubated with S-adenosyl[methyl-\(^3\)H]methionine (1 \(\mu\)Ci/10\(^6\) cells) in 50 mM Tris-chloride buffer of different pH during 2 min at 20°C. After incubation, the reaction was stopped by addition of 0.1 vol. 70% HClO\(_4\). Lipids were extracted and separated on silica gel with solvent A as described in Materials and Methods. (A) Incorporation of radioactivity in nonpolar lipid, (B) in PME, and (C) in PC and PMME. Each experimental point is a mean of duplicate observations.

The enzyme pattern is similar to that of phospholipid and sterol in that it shows an increase after 14 h (Fig. 6C).

When homogenates are incubated with S-adenosyl[methyl-\(^3\)H]methionine, the activity of phosphatidylethanolamine \(N\)-methyltransferase is found to show a strong decline during early development (Fig. 7A). From 12 to 20 h of starvation, the amount of enzyme remains more or less constant, but it shows a further decline during fruiting body formation. Nonpolar lipid methyltransferase, as measured in a homogenate, also shows a decline after the vegetative stage (Fig. 7B). Hence, the decrease of activity is already very strong during the first 4 h of starvation and is followed by a less rapid fall during development of the slug stage. After the onset of slug migration, the activity of the nonpolar lipid methyltransferase remains constant during the remaining 10 h of observation (Fig. 7B). Only after complete differentiation to
Fig. 4. Separation of methylated lipids in aggregative cells of D. discoideum by thin-layer chromatography on silica gel 60 (Merck). (A) Mobile phase ligroin/diethyl ether/acetic acid (50:50:1, v/v); reaction products in intact cells after labeling with $[\text{methyl}-^3\text{H}]$methionine. (B) Mobile phase benzene/methanol (95:5); reaction products in intact cells after labeling with $[\text{methyl}-^3\text{H}]$methionine. (C) Mobile phase chloroform; reaction products in intact cells after labeling with $[\text{methyl}-^3\text{H}]$methionine. Abbreviations indicate the position of external standards: PL, phospholipids; MAG, monooacylglycerol; DAG, diacylglycerol; CH, cholesterol; FFA, free fatty acids; UQ, ubiquinone; MFA, fatty acid methyl esters; TAG, triacylglycerol; YBP, yellow-brown pigment; NN, unknown compound (see Discussion).

Fig. 5. Inhibition of lipid methylation by S-adenosylhomocysteine (AdoHcy). Homogenates of aggregative cells were incubated for 2 min with S-adenosyl[ methyl-$^3$H]methionine (1 $\mu$Ci/10$^7$ cells) in 50 mM Tris-chloride buffer (pH 8) in the presence of different amounts of S-adenosylhomocysteine. After incubation, the reaction was stopped by the addition of 0.1 vol. 70% HClO$_4$. Lipids were extracted and separated on silica gel with solvent A as described in Materials and Methods.

the fruiting body, does nonpolar lipid methyltransferase activity decrease to a very low value (Fig. 7B).

An examination of the specific activity of intracellular S-adenosyl[methyl-$^3$H]methionine during cell differentiation showed this parameter to increase from 1950 dpm/nmol in the vegetative stage via 21500 dpm/nmol after cell aggregation (8 $\frac{1}{2}$ h of development) to 127 000 dpm/nmol at culmination (23 h of development).
Discussion

Incorporation of radioactivity from \([\text{methyl}^-3\text{H}]\text{methionine}\) into the nonpolar lipid fraction of aggregative amoebae seems to be due to enzymatic transmethylation and not to be an artifact, for:
(a) the reaction is apparently enzyme-catalyzed. Product concentration increases with time, but not in cells which have been placed in a boiling water bath for 2 min before incubation (Fig. 2A). The process shows a strong dependency on the incubation temperature, lowering the temperature from 20 to 2°C giving rise to a 14-fold decrease in rate (Fig. 2A);
(b) the reaction is a transmethylation and not an esterification of the substrate with free radioactive methanol. The addition of a large amount of unlabeled methanol to the incubation medium has no effect on the incorporation of radioactivity into nonpolar lipid (Fig. 2A);
(c) the reaction occurs also in homogenates of aggregative cells when these are incubated with S-adenosyl[\text{methyl}^-3\text{H}]\text{methionine}\) (Fig. 1C) and it can be blocked by the addition of a sufficient amount of S-adenosyl homocysteine (Fig. 5);
(d) the reaction shows a sharp pH-optimum (Fig. 3A), which is different from that of phospholipid methylation (Fig. 3B–C).

On the basis of this evidence, we assume the radioactivity at the solvent front (Fig. 1A, B) to represent transmethylation of certain nonpolar lipid components. Since methylation of nonpolar lipids has not been described previously in \(D.\ discoideum\), we have attempted to characterize the methylated compounds.

Fig. 6. Methylation of lipids in intact cells of \(D.\ discoideum\) during development. After different periods of starvation on non-nutrient agar, amoebae were harvested and incubated for 60 min with \(L-[\text{methyl}^-3\text{H}]\text{methionine}\) (10 μCi/10⁶ cells) in phosphate buffer as described in Materials and Methods. The incubation was started at the times indicated in the figure, and it was terminated by addition of 0.1 vol. 70% HClO₄. Lipids were extracted and separated by thin-layer chromatography with mobile phase C as described in Materials and Methods. The incubation was started at the times indicated in the figure, and it was terminated by addition of 0.1 vol. 70% HClO₄. Lipids were extracted and separated by thin-layer chromatography with mobile phase C as described in Materials and Methods. (A) Radioactivity in total phospholipid. (B) Radioactivity in sterols and sterol esters. (C) Radioactivity in ubiquinone. FB indicates complete development to fruiting bodies (more than 48 h of starvation). Each experimental point is a mean ± S.D. of three independent observations.
Incorporation of radioactivity from \([\text{methyl-}^3\text{H}]\text{methionine}\) into non-polar lipids can theoretically be due to the following mechanisms (Fig. 8):

1. Addition of methyl groups to double bonds in the acyl chain, leading to the formation of a methylene fatty acid as an intermediate and a branched fatty acid as the end product. This process causes the synthesis of 10-methylstearic acid in bacteria [18]. The bacterial enzyme is membrane bound and it does not use free oleic acid as a substrate, but it catalyzes a reaction between \(S\)-adenosylmethionine and olefinic acid chains in phospholipid molecules [18];

2. Addition of methyl groups to double bonds in the acyl chain, leading to the formation of a cyclopropane fatty acid as the end product. This process has been well-characterized in bacteria [22,23]. Since acyl groups of phospholipids may be liberated by the action of phospholipases, which are very active in \(D.\) discoideum [24], mechanisms 1 and 2 may cause the accumulation of methylated fatty acids;

3. Methylation of carboxyl groups of fatty acids, leading to the formation of fatty acid methyl esters. This mechanism has been characterized in bacteria [25] and rat lung [26], but the presence of natural fatty acid methyl esters has been demonstrated in several other tissues of vertebrates [27–29]. Mammalian cells also contain an enzyme capable of hydrolysis of fatty acid methyl esters. The activity is membrane bound and localized in the microsomal fraction [30,31];

4. Esterification of free fatty acids with free methanol. Methanol can be produced from \(S\)-adenosylmethionine by the concerted action of protein \(O\)-methyltransferase and protein methyl-esterase. Porcine pancreas is known to contain an enzyme which catalyzes the esterification of alcohols with free fatty acids [32];

\(\mu\)Ci/10^7 cells in Tris-chloride buffer (50 mM, 1.5 mM MgCl₂, pH 8). The reaction was stopped by the addition of 0.1 vol. 70% HClO₄. Lipids were extracted and separated by thin-layer chromatography with mobile phase A, as described in Materials and Methods. (A) Total amount of radioactivity in PME, PMME, PE and LPC. (B) Total amount of radioactivity in nonpolar lipid. Each experimental point is a mean ± S.D. of four independent observations. FB indicates complete development to fruiting bodies (more than 48 h of starvation).
(5) formation of S-methyl-N-oleoylmercaptotoethylamide from oleoyl coenzyme A and S-adenosylmethionine. This process has been well-characterized in rat lung [19]. The methylating enzyme is localized mainly in the microsomal fraction and is probably identical with thiol-S-methyltransferase. It has been detected not only in lung, but also in several other tissues of mammals. The product migrates in acidic solvents near free fatty acid during thin-layer chromatography [19]; (6) formation of methoxy groups in quinones and a two-carbon side-chain in sterols from S-adenosylmethionine. Ubiquinone contains two methoxy groups, which are derived from S-adenosylmethionine [33,34]. It is the major methyl-accepting nonpolar lipid in basophilic leukemia cells, retina and parotid gland [20]. The methylating enzyme is localized mainly in mitochondria [20,34]. The major sterol of D. discoideum is Δ22-stigmasterol [35,36], which the amoebae synthesize from acetate via mevalonic acid [37]. Two carbon atoms of stigmasterol are derived from methyl groups of methionine [38].

When the nonpolar lipids of D. discoideum are separated by thin-layer chromatography, two major radioactive peaks are observed which co-elute with authentic ubiquinone and cholesterol in all mobile phases (Fig. 4). Radioactivity does not appear in free fatty acid (Fig. 4A), which indicates that mechanisms 1 and 2 described above are not of major importance. Although D. discoideum lipids contain significant amounts of 17- and 19-carbon cyclopropane fatty acids, the amoebae do not synthesize these acyl chains themselves, but they are derived from lipids of ingested bacteria [39]. There is hardly any incorporation of radioactivity into
fatty acid methyl esters (Figs. 4A-C), so that mechanisms 3 and 4 cannot be very active. Mechanism 4 is very unlikely as an explanation for our data, since addition of unlabeled methanol to the incubation medium of intact cells does not cause any decline of nonpolar lipid radioactivity (Fig. 2A). Mechanism 5 apparently does not occur in slime molds, since, in acidic solvents, no radioactivity co-elutes with free fatty acid (Fig. 4A). Although one of the radioactive peaks co-elutes with cholesterol, the methyl acceptor cannot be cholesterol itself, since the cholesterol molecule does not contain any of the methyl groups which are derived from S-adenosylmethionine. The major sterol of *D. discoideum*, stigmatenol [35,36], however, is known to possess a methionine-derived two-carbon side-chain [38] and to co-elute with cholesterol during thin-layer chromatography [40].

The methylated nonpolar lipid components were scraped from the TLC plate, eluted from the silica gel with methanol and reapplied on a thin-layer plate with authentic ubiquinone and stigmatenol. In all mentioned mobile phases, the eluted compounds were recovered in the same positions as the authentic standards.

For these reasons we conclude that the major nonpolar lipids which are methylated in aggregative amoebae are stigmatenol and ubiquinone. The presence of the latter compound has also been demonstrated in slime molds [40]. In some solvents, a third radioactive peak is observed, which is separated from sterol and which shows more hydrophobic behavior (Fig. 4B, C). This peak might represent esters between stigmatenol and fatty acids which are common in *D. discoideum* [40].

Attempts to identify the esters by elution, saponification and chromatography of the saponification products, however, were not successful. The amount of radioactivity incorporated in the peak is usually very low and, after saponification, little radioactivity is recovered.

When the intact cells are labeled with [*methyl-3H]*methionine after different periods of food depletion, the incorporation of methyl groups into polar and nonpolar lipid shows a strong increase after 14 h of starvation (Fig. 6A–C). The amount of labeling of lipids from [*methyl-3H]*methionine in intact cells is dependent on a number of factors, including the rate of methionine uptake, the rate of synthesis and breakdown of S-adenosylmethionine and the activity of lipid methyltransferases. Therefore, we also attempted a direct measurement of lipid methyltransferase activity by incubation of a cell homogenate with S-adenosyl[*methyl-3H]*methionine. Under these conditions, incorporation of methyl groups into phospholipid is linear for several minutes. When homogenates produced from cells in different developmental stages are incubated with S-adenosyl[*methyl-3H]*methionine, the activities of phospholipid methyltransferases and nonpolar lipid methyltransferases show a dramatic decline from the vegetative stage to the onset of slug migration, which is not followed by any increase during subsequent development (Fig. 7A, B).

The discrepancy between the results obtained with homogenates and intact cells can be explained by the assumption that a dramatic increase in the specific activity of S-adenosylmethionine occurs during development. Such an increase could be due to a starvation-induced decline in the size of the intracellular pool of methionine. For this reason, we examined changes in the specific radioactivity of intracellular S-adenosyl[*methyl-3H]*methionine. The specific activity of intracellular S-adenosyl[*methyl-3H]*methionine was found to increase 60–70-fold during cell differentiation from the vegetative stage to the fruiting body. This change appears to be the cause of the strong increase of [*methyl-3H]*incorporation into all lipid classes as observed in intact cells after 14 h of starvation (Fig. 6).

It has been suggested that phospholipid methylation is involved in receptor-adenylate cyclase coupling [16,41] and in the initial morphologic and biochemical responses of white blood cells to chemotactants [42–44]. In *D. discoideum*, cAMP-mediated chemotaxis and cAMP-mediated cAMP secretion take place from 4 to 12 h and probably also from 20 to 28 h after food depletion, during aggregation and culmination phases of development [45]. Both cAMP-binding to cell surface receptors and the activity of cAMP-phosphodiesterase show maxima from 8 to 12 and 20 to 28 h [45], whereas adenylate cyclase activity peaks at 8 h [46]. Such a pattern of developmental regulation, however, could not be observed for the activity of phosphatidylethanolamine-N-methyl-
transferase (Fig. 7A). The changes in enzyme activity during development suggest an involvement in the synthesis of new membranes for cell growth or in the mechanism of phagocytosis rather than a role in the process of cAMP-mediated chemotaxis.

The strong decline of the activity of phosphatidylethanolamine-N-methyltransferase (Fig. 7A) coincides with a 4-fold increase of general phospholipid synthesis [47], whereas the relative proportions of the major phospholipid classes remain relatively constant [48]. Therefore, *D. discoideum* seems to be no exception to the general rule that the CDPcholine and transmethylation pathways of phosphatidylcholine synthesis are regulated in coordination, an increase in the former resulting in a decrease in the latter and vice versa [8].

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**References**