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COMPARTMENTATION OF MEMBRANE PHOSPHATIDYLETHANOLAMINE FORMED BY BASE-EXCHANGE REACTION IN RAT BRAIN MICROSOMES

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The compartmentation of membrane phosphatidylethanolamine (PE) formed by base-exchange reaction in rat brain microsomal vesicles has been investigated. After labelling membrane PE by base-exchange in vitro, microsomal vesicles were treated with trinitrobenzenesulfonic acid (TNBS). The amount of membrane PE reacting with TNBS depends on the duration and the temperature of the reaction as well as on the TNBS concentration. It was found that almost all of the labelled PE molecules, but only about 24% of membrane PE, were accessible to TNBS, under very mild reaction conditions. It is concluded that PE labelled by base-exchange is completely localized in the cytoplasmic leaflet of microsomal vesicles.

Introduction

An asymmetrical distribution of lipids has been reported to occur in various membranes [1–11]. It may arise from a different localization of lipid-metabolizing enzymes and/or from a redistribution of lipids taking place after their synthesis.

Phosphatidylethanolamine (PE) can be synthesized in brain microsomes through at least two pathways: (a) net synthesis [12,13], and (b) base-exchange reaction [14–16]. The sidedness of ethanolamine, choline and serine base-exchange enzymes has recently been examined in rat brain microsomes by Buchanan and Kanfer [17], who reported that ethanolamine base-exchange is localized on the inner surface of microsomal vesicles. However, no attempt has been made to determine whether the PE newly-synthesized by base-exchange is asymmetrically distributed in the membrane.

In this work, the localization of the PE formed by base-exchange reaction in rat brain microsomal membranes has been investigated by means of trinitrobenzenesulfonic acid (TNBS), which has been reported to be, under certain conditions, a non-penetrating probe [1].

Materials and Methods

Materials. [1-^{3}H]Ethanolamine hydrochloride (specific radioactivity, 19.5 Ci/mmol) and [2-^{14}C]ethanolamine hydrochloride (spec. act. 60 Ci/mol) were purchased from Amersham, International, U.K. Hepes was produced by Fluka (Buchs, Switzerland). 2,4,6-Trinitrobenzenesulfonic acid was obtained from Pierce Eurochemie (Rotterdam, The Netherlands). Other reagents were purchased from the common commercial sources and solvents were freshly distilled before use.

Abbreviations: PE, phosphatidylethanolamine; TNBS, trinitrobenzenesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TNPh-PE, trinitrophenylphosphatidylethanolamine.

Base-exchange reaction. Wistar male rats (150–200 g body wt.) were fasted 12 h before killing. Brain microsomes were prepared and their purity was assessed as previously described [14]. The base-exchange reaction was performed in stoppered test tubes containing, in a final volume of 0.2 ml, the following incubation mixture: 1 μCi [1-^{3}H]ethanolamine (spec. act., 19.5 Ci/mmol), 40 mM Hepes (pH 8.1) and 2.5 mM $CaCl_2$. The reaction was started by adding 0.25–0.30 mg microsomal protein and was continued for 20 min at 37°C, unless otherwise specified. The reaction was stopped adding 0.01 ml of 25 mM $CdCl_2$.

Reaction with TNBS. Immediately after stopping the base-exchange reaction, 4 ml of a buffered TNBS solution (40 mM Hepes (pH 8.1), 25 mM KCl, 0.2 M sucrose and various concentrations of TNBS) were added to each tube. The reaction with TNBS, performed for various times at carefully controlled temperatures, was stopped by adding 1 ml of saturated glycine solution.

Extraction and analysis of lipids. The extraction of lipids was performed as described by Folch et al. [18]. The final chloroform phase was dried under vacuum and lipids chromatographed on silica gel G plates (0.3 mm thickness). To separate unreacted PE and trinitrophenylphosphatidylethanolamine (TNPh-PE) a two-dimensional TLC was performed using the following developing mixtures: (a) chloroform/methanol/1.6 M NH_4OH (70:30:5, v/v); and (b) chloroform/methanol/acetic acid/acetone/water (70:15:15:30:7.5, v/v). The spots were visualized by exposure to I_2 vapours and identified using pure reference standards. After I_2 sublimation, the spots corresponding to PE and TNPh-PE were scraped from the plate. Very small amounts of trinitrophenylethanolamine remaining in the chloroform phase were well separated from the lipid by the described chromatographic procedure.

The unreacted lipid was eluted three times with 3 ml chloroform/methanol/water/acetic acid (50:39:10:1, v/v), each time. The eluate was then divided into two parts: one was used to determine phosphorus content, whereas the other was counted for radioactivity. TNPh-PE was eluted three times with 3 ml of methanol/chloroform (2:1, v/v), each time. TNPh-PE was determined spectrophotometrically at 340 nm. The same sample was counted for radioactivity. Alternatively, TNPh-PE mass was determined by measuring the phosphorus content.

Reaction of liposomes with TNBS. In some instances, labelled microsomal lipids were extracted before reaction with TNBS. Liposomes were subsequently prepared by sonicating the dried lipid extract with 2 ml 40 mM HEPES (pH 8.1)/25 mM KCl/0.2 M sucrose. The subsequent reaction with 0.6 mM TNBS, the extraction and the analyses were performed as described elsewhere in this paper.

In vivo lipid labelling. PE of rat brain microsomes was labelled in vivo by injecting intracranially 40 μCi [1-^{3}H]ethanolamine (5 μl, spec. act., 19.5 Ci/mmol). Animals were killed 24 h after the injection, and brain microsomes (about 6 mg protein) were prepared as described above. The base-exchange reaction was performed under the usual conditions for 30 min with 200 nCi [2-^{14}C]ethanolamine (spec. act., 60 Ci/mol).

Analyses. PE and TNPh-PE were determined as P_i after digestion with 70% (w/v) perchloric acid [19]. The radioactivity was measured by scintillation as described [20]. Protein was evaluated following the method of Lowry et al. [21].

Results

General

Under our experimental conditions the PE of rat brain microsomal membranes reacted with TNBS to form TNPh-PE. The reaction depended on various factors, such as time, temperature and TNBS concentration. In the experiments described below, the reaction of radioactive PE formed in vitro by base-exchange and the reaction of the bulk of membrane PE with the probe were evaluated. In parallel experiments, total lipid radioactivity (PE + TNPh-PE) was shown to remain constant during the reaction with TNBS.

Time-dependence of TNPh-PE formation

Fig. 1 shows the time-dependence of the reaction of membrane PE with TNBS at three concentrations of TNBS.

About 95% of the radioactive membrane PE was transformed into TNPh-PE in 4 h at any TNBS concentration, although the formation of

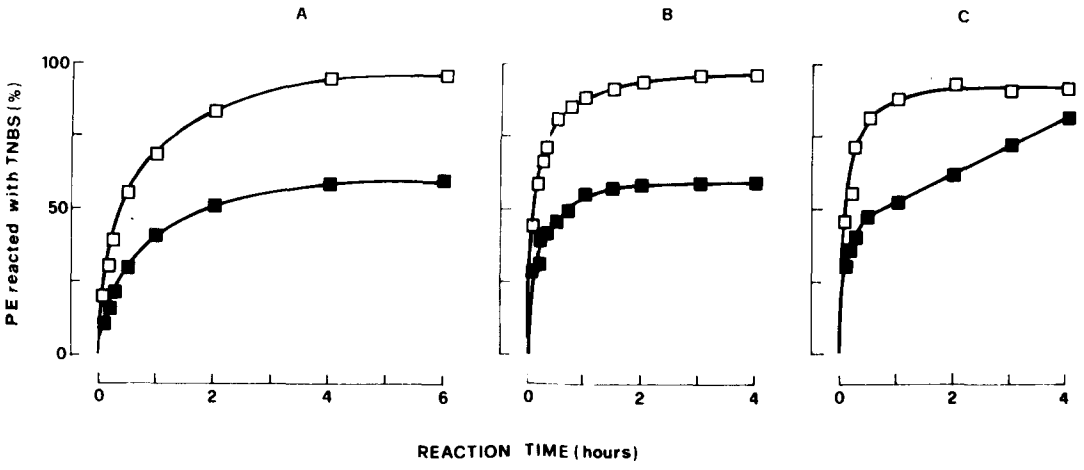

Fig. 1. Time-dependence of TNPh-PE formation. After the base-exchange reaction (cf. Materials and Methods) microsomal vesicles were incubated for the indicated time intervals at 23°C with (A) 0.6 mM, (B) 1 mM and (C) 2 mM TNBS. Results are expressed as percent of total PE radioactivity (□———□) or nmol (■———■) transformed into TNPh-PE. Reacted plus unreacted PE was 92.5 ± 5 nmol and 155 ± 10 nCi in each sample.

radioactive TNPh-PE became faster upon increasing the concentration of the probe. With 0.6 mM and 1 mM TNBS, however, the mass of reacted PE never exceeded 55–60% of the total. Only with 2 mM TNBS did this value reach 80%. As a consequence, the specific radioactivity of TNPh-PE remained fairly constant in the course of the experiments performed with 0.6 mM and 1 mM TNBS, but decreased upon increasing the duration of the reaction when 2 mM TNBS was used. Clearly, the PE formed in vitro by base-exchange reacted with the probe more quickly and more completely than did the bulk of PE present in microsomal vesicles.

Temperature-dependence of TNPh-PE formation

Fig. 2 shows the temperature-dependence of the reaction of membrane PE with TNBS.

About 95% radioactive PE reacted with any concentration of TNBS, provided that the temperature was maintained above 20°C. At 4°C, 81% of labelled PE reacted with 0.6 mM TNBS, this percentage increasing to 92% if TNBS was 2.4 mM.

However, the temperature-dependence of the reaction of the bulk of membrane PE was different. Indeed, only 60% of the total PE mass reacted with 1.2 mM and 2.4 mM TNBS, provided that the temperature was kept below 20°C. At higher temperatures, more than 90% of PE reacted with 2.4 mM TNBS and about 70–75% with 1.2 mM TNBS. Only 24% of membrane PE reacted with 0.6 mM TNBS at 4°C. This percentage increased to 60–65% upon increasing the temperature. As a consequence, the specific radioactivity of TNPh-PE decreased upon increasing the temperature of the reaction (Fig. 2). The specific radioactivity of TNPh-PE formed below 20°C with 1.2 mM and 2.4 mM TNBS was about 3.0–3.2 Ci/mol and decreased to a limit value of about 2 Ci/mol upon increasing the temperature of the reaction. If the microsomal vesicles reacted with 0.6 mM TNBS, the temperature-dependence of the specific radioactivity of TNPh-PE was strikingly different. At low temperatures (4–10°C), the specific radioactivity of TNPh-PE was 6 Ci/mol and decreased to a minimum of 3.0–3.5 Ci/mol upon increasing the reaction temperature.

From the experiments on the temperature-dependence of TNPh-PE formation, we may assume that the radioactive PE formed by the base-exchange reaction does not easily mix with the bulk of membrane PE, as proved by the variation in the specific radioactivity of TNPh-PE, at low temperature and at low concentration of TNBS (Fig. 2).

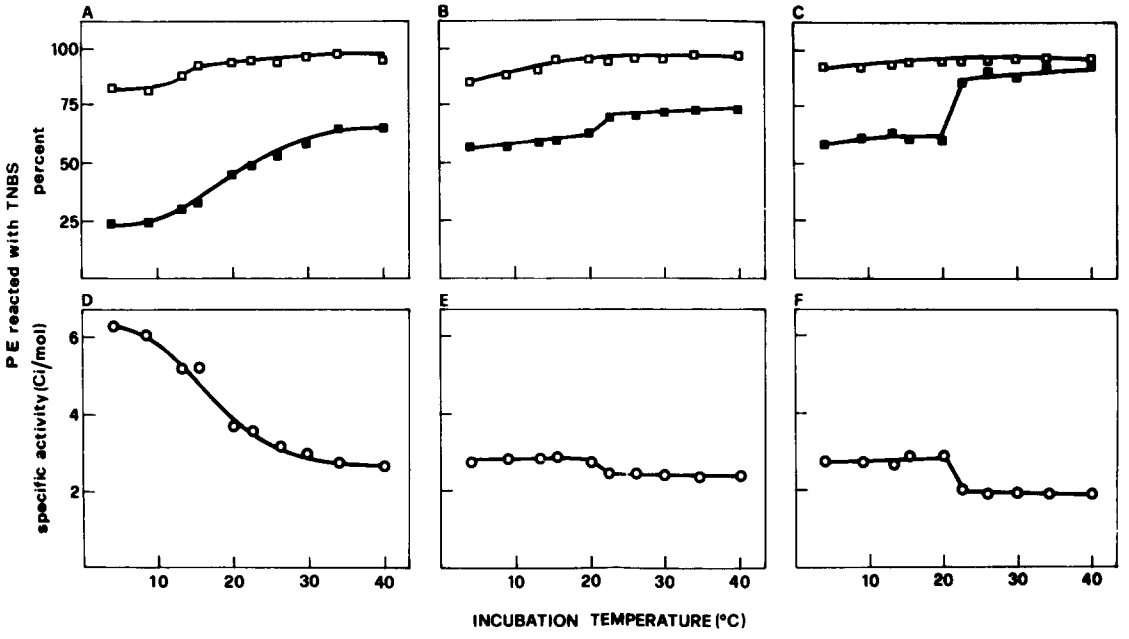

Fig. 2. Temperature-dependence of TNPh-PE formation. After the base-exchange reaction (cf. Materials and Methods), microsomal vesicles were incubated for 3 h with TNBS at the indicated temperatures (°C). Results are expressed as percent of total PE radioactivity (□———□) or nmol (■———■) transformed into TNPh-PE. The specific radioactivity (Ci/mol) of TNPh-PE (○———○) is also shown (lower row). (A) and (D) 0.6 mM TNBS; (B) and (E) 1.2 mM TNBS; (C) and (F) 2.4 mM TNBS. Reacted plus unreacted PE was 92 ± 5 nmol and 155 ± 10 nCi in each sample.

TNBS concentration-dependence of TNPh-PE formation

The dependence of the formation of TNPh-PE on the concentration of TNBS is shown in Fig. 3.

It is evident that the reaction of TNBS with membrane PE preferred the radioactive lipid formed by base-exchange. 1 mM TNBS represents a somewhat critical concentration. Indeed, at this concentration almost all labelled PE reacted with the probe, whereas only 60% of total membrane PE was present as TNPh-PE. At higher TNBS concentrations, the radioactivity of TNPh-PE did not increase, unlike its mass. Moreover, the shape of the curve representing the formation of TNPh-PE vs. TNBS concentration changed at about 1 mM TNBS, as expected if TNBS disrupted microsomal membranes at concentrations higher than 1 mM [1].

TNPh-PE formation from doubly-labelled PE

Microsomal membranes were labelled injecting [^{3}H]ethanolamine intracerebrally and then by incubating them in vitro with [^{14}C]ethanolamine (for base-exchange Materials and Methods). After the reaction with TNBS (0.6 mM; 23°C; 4 h), 97% of

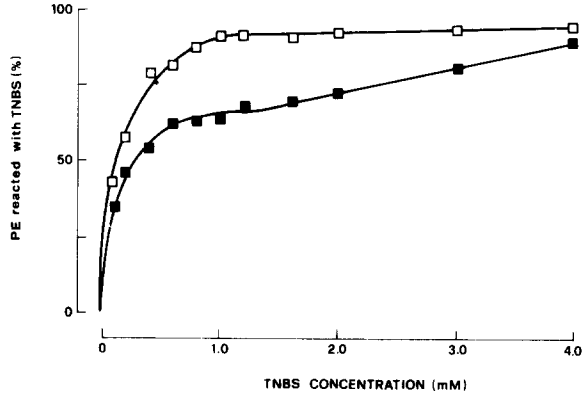

Fig. 3. Dependence of TNPh-PE formation on TNBS concentration. After base-exchange reaction (cf. Materials and Methods) microsomal vesicles were incubated for 3 h at 23°C with the indicated concentrations of TNBS. Results are expressed as percent of total PE radioactivity (□–□–□) or nmol (■–■–■) transformed into TNPh-PE. Reacted plus unreacted PE was 90 ± 6 nmol and 160 ± 10 nCi in each sample.

^{14}C-labelled PE was recovered as TNPh-PE, whereas only 62% of ^{3}H-labelled PE was transformed into TNPh-PE under the same conditions. The specific radioactivity of [^{3}H]PE and [^{3}H]TNPh-PE was the same (0.11 Ci/mol) after 4 h, whereas the specific radioactivity of [^{14}C]PE was 0.006 Ci/mol and that of [^{14}C]TNPh-PE was 0.13 Ci/mol. This finding means that PE synthesized in vivo had mixed with membrane PE and reacted with the probe only partially, contrary to the case of PE synthesized in vitro by base-exchange.

Treatment of membranes with disrupting agents

The reported experiments indicate that two or more pools of PE, reacting differently with the probe, are present at membrane level. If this assumption is true, membrane disgregating agents should influence the reaction with TNBS. The results obtained from treating microsomal membranes with disrupting agents after base-exchange and before the reaction with TNBS are reported in Table I.

TABLE I

REACTION OF MICROSOMAL PE WITH TNBS AFTER THE TREATMENT WITH MEMBRANE-DISRUPTING AGENTS

After the base-exchange reaction, microsomal membranes were treated as indicated below, then reacted with 0.6 mM TNBS at 23°C for 3 h. Data represent the mean of four experiments ± S.E. Total membrane PE was 280 ± 10 nmol/mg protein. Each sample contained 0.3 mg microsomal protein.

Treatment	TNPh-PE (nCi/mg protein)	TNPh-PE (Ci/mol)	PE (nCi/mg protein)
None	597 ± 20	3.67 ± 0.23	32.8 ± 3.2
Deoxy-cholate [a]	294 ± 25	2.71 ± 0.21	313.0 ± 18.0
Sonication [b]	557 ± 54	2.61 ± 0.25	62.8 ± 14.9
Sonication + deoxy-cholate [c]	268 ± 29	2.57 ± 0.20	287.1 ± 19.0
Liposomes [d]	264 ± 49	2.11 ± 0.06	227.3 ± 24.0

[a] Before adding TNBS, 0.5% (final concentration) of deoxycholate was added to the microsomes.

[b] Before adding TNBS, microsomes were sonicated four times (15 s, each time) with an MSE sonicating apparatus (100 W).

[c] Sonication (as in footnote b) was done in the presence of deoxycholate (as in a).

[d] After base-exchange, and before the reaction with TNBS, lipids were extracted and liposomes prepared as described under Materials and Methods.

The specific radioactivity of TNPh-PE in non-treated microsomes (controls) was 3.67 Ci/mol and decreased following the sonication of the membranes and/or treatment with 0.5% deoxycholate. The specific radioactivity decreased even more if lipids were extracted from the membranes and used to prepare liposomes before the reaction with the probe (cf. Materials and Methods). A variation in the specific radioactivity of TNPh-PE is to be expected if disrupting agents produce a mixing of newly-synthesized, labelled PE with non-labelled PE present in the membranes; it is worth noticing that the theoretical specific radioactivity expected after a complete mixing of membrane PE would be 2.2 Ci/mol, this value being very close to that obtained after the reaction of TNBS with liposomes (2.1 Ci/mol). The results reported in Table I would therefore suggest the presence of more than one pool of membrane PE.

Discussion

The experiments reported in this work permit the identification of several PE pools in rat brain microsomal membranes. These pools can be distinguished following their reactivity with TNBS.

Two main pools of membrane PE can be demonstrated after reaction of microsomal vesicles with TNBS under various conditions: (a) one pool comprising about 60% of membrane PE and promptly reacting with the probe; and (b) one pool, comprising about 40% of membrane PE and reacting with the probe only at the highest concentrations and temperatures tested in this work. Probably, another pool, comprising about 24% of membrane PE and reacting with 0.6 mM TNBS at low temperatures, can be identified (Fig. 2). However, at higher temperatures and TNBS concentrations, this pool would not be distinguished from pool (a). Finally, a small amount of membrane PE never reacts with the probe, this result being in accordance with previously reported data [1,4,22–24].

One of the major problems is represented by the localization in the membrane structure of the pools demonstrated by the reaction with TNBS.

The experiments reported in this work evidence the presence of several pools of membrane PE, but cannot definitely prove that the easily reacting pools are localized on the ouside surface of the vesicles, and vice-versa. Almost complete reaction of microsomal PE with TNBS was achieved only when the concentration of the probe was 2.4 mM, whereas with 0.6 mM TNBS the TNPh-PE formed did not exceed 24% below 10°C and 60% at a higher temperature. However, in all cases, about 90% of the radioactive PE formed by base-exchange in vitro reacted with TNBS. Since almost all radioactive PE reacts with TNBS between 4 and 10°C, when only 24% of the total PE have reacted with the probe, it may be assumed that under this conditions nearly all the PE facing the outer surface was accessible to TNBS. The increase in TNPh-PE formation up to 60% at 20°C may be due to the permeation of the probe through the microsomal membranes, which should be rate-limiting under the conditions used. The use of a higher concentration of TNBS, such as 2.4 mM, may disrupt the microsomal vesicles, allowing almost complete reaction of the PE with TNBS, thus confirming previously reported data [1].

Nevertheless, whatever the mass distribution of PE among the two leaflets, the PE molecules newly synthesized by base-exchange are localized in the outer leaflet of the microsomal vesicles, corresponding to the cytoplasmic leaflet.

Buchanan and Kanfer [17] reported that the enzyme catalyzing ethanolamine base-exchange is localized on the luminal surface of rat brain microsomes. On the other hand, the same authors [17] studied the localization of enzyme molecules and did not consider the product of the reaction. Therefore, it would seem that PE is synthesized by an enzyme present on the inner surface of microsomal vesicles being immediately transferred to the outer surface. However, inactivation of enzymes by mild proteolytic digestion could be a rather ambigous test for assessing enzyme topology [2].

The results shown in Table I indicate that the PE formed by base-exchange does not mix with the other pools of membrane PE, unless disrupting agents are used. Disrupting agents can indeed lower the specific activity of TNPh-PE (Table I), although different treatments can differently affect the reactivity of labelled PE with the probe (Table I), probably as a result of different ways of modifying the structure of the membrane. Therefore, although the effects of the agents tested in this work are not similar, a common result is the lowering of the specific activity of TNPh-PE; this would further support the idea that an alteration of membrane structure, produces a mixing of the different PE pools.

From the data reported in this work it can be concluded that the labelled PE formed by base-exchange does not mix with the bulk of membrane PE. However, 24 h after the intracerebral injection of radioactive ethanolamine (cf. Results) the distribution of PE radioactivity paralleled the distribution of PE mass. Therefore, it would be interesting to know whether the PE formed by base-exchange could, in some instances, move to the pool slowly reacting with TNBS. The experiments reported here have been performed with radioactive PE synthesized in vitro and it cannot be excluded that in vivo a translocation of PE formed by base-exchange occurs. Indeed, the geometrical properties of microsomal vesicles are hardly similar to those of the endoplasmic reticulum in the intact cell; moreover, the interaction among membranes and soluble cytoplasmic compounds could be relevant in this connection.

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