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Factors affecting the reaggregation of rat brain microsomes solubilized with octyl glucoside and their relationship with the base-exchange activity of reaggregates

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Rat brain microsomal membranes disaggregated by exposure to octyl glucoside were recovered by centrifugation after dialytic removal of the detergent. The composition of the dialysis medium (divalent cations, pH) was important to this effect; indeed, the reaggregation process which occurred during the dialytic step required the presence of either Ca^{2+} or Mg^{2+} and a slightly acidic pH. The lipid protein/ratio and choline and ethanolamine base-exchange of recovered particles depended on the conditions of dialysis although their lipid composition did not. The lipid composition of membranes was also varied by adding PE or PC to octyl glucoside-microsome suspensions. This treatment produced reaggregates possessing a low content of cholesterol and varying PC/PE ratios. Both choline and ethanolamine base-exchange activities were related to this parameter.

Introduction

It has been reported [1] that rat brain microsomes solubilized with octyl glucoside recover their ability to synthesize PE by base-exchange after the dialytic removal of the detergent in the presence of suitable concentrations of Ca^{2+} in the dialysis medium.

The PE formed by base-exchange in membranes reaggregated after exposure to octyl glucoside is compartmented [1], similar to the PE formed by base-exchange in native membranes [2]. This indicates that the reassembly process taking place upon detergent removal implies an ordered reaggregation of membrane components and a probable recovery of sidedness.

Octyl glucoside is a non-ionic detergent which has been used to reconstitute protein into phospholipid vesicles, its dialytic removal being followed by the formation of heterogeneous proteolipid aggregates [3–6]. Moreover, in order to study phospholipid protein interactions in model membranes, an octyl glucoside dilution procedure for achieving variable phospholipid/protein ratios has been described [7].

It is generally agreed that the phospholipid composition of membranes is important for their biological activity. Furthermore, cholesterol plays several roles in membrane functioning, influencing the permeability of membranes to cations and

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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interacting with proteolipid and membrane protein [8]. Therefore, the possibility of varying the lipid composition of brain microsomes exposing them to octyl glucoside and removing the detergent in various experimental conditions has been investigated and exploited to investigate the effect of modifications of membrane lipid on membrane-bound enzymes, such as choline and ethanolamine base-exchange.

Materials and Methods

Materials. [1-^{3}H]Ethanolamine hydrochloride (specific radioactivity of 30000 Ci/mol) and [*methyl*-^{3}H]choline (spec. act. 78000 Ci/mol) were purchased from Amersham International (Amersham, U.K.). Hepes buffer and octyl-β-D-glucopyranoside were produced by Boehringer Biochemia (Mannheim, F.R.G.). Non-polar polystyrene absorbent (Bio-Beads SM-2) and Bio-Sil were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Other reagents were obtained from Carlo Erba (Milano, Italy).

Assay of the base-exchange reaction. Sprague-Dawley male rats (150–200 g body wt.) were used to prepare brain microsomes, as described previously [9]. The base-exchange reaction was assayed incubating, in a final volume of 0.15 ml the following mixtures: (a) 0.1 mM [1-^{3}H]ethanolamine (spec. act. 66.6 Ci/mol), 40 mM Hepes (pH 8.0) and 2.5 mM $CaCl_2$ [10], or (b) 0.05 mM [*methyl*-^{3}H]choline (spec. act. 200 Ci/mol), 40 mM Hepes (pH 8.0) and 4.0 mM $CaCl_2$ [11]. The reaction was started by addition of 0.25–0.30 mg of microsomal protein, continued for 20 min at 37° C and stopped with 2 ml of chloroform/methanol (2 : 1, by vol.).

Treatment of membranes with octyl glucoside and dialytic procedures. Octyl glucoside (80 mM, 0.5 ml) was added to 0.5 ml of microsomal suspension (3 mg protein). The mixture was allowed to equilibrate for 15 min at room temperature, poured into a 1-cm-wide dialysis bag and dialyzed at 4° C in the presence of Bio-Beads in the external medium, to increase the velocity and the effectiveness of detergent removal [1,12].

Different dialysis media were used in this work, namely: (1) 0.32 M sucrose, 2 mM 2,4-dithioerythritol and various concentrations of $CaCl_2$ or $MgCl_2$ (adjusted to pH 6.0) and (b) 0.32 M sucrose, 2 mM 2,4-dithioerythritol, 10 mM Hepes (pH 8.0) and suitable concentrations of $CaCl_2$. The dialysis medium (150 ml) and the Bio-Beads (0.5 g) were replaced after 2 h and dialysis continued up to 16 h. Dialyzed membranes were subsequently centrifuged, without any further dilution, for 60 min at $140000 \times g$ in a SW 50 Beckman Rotor. The pellet was finally resuspended in 0.4 ml of 0.32 M sucrose + 2 mM 2,4-dithioerythritol.

Addition of exogenous phospholipid to octyl glucoside-treated microsomal membranes. Exogenous phospholipids were extracted from rat liver [13] and separated on a Bio-Sil (100–200 mesh) column as described [14]. Aliquots of PC or PE (0.5 to 3.5 μmol) were transferred to test tubes and solvent evaporated. Solubilized membranes (1 ml, 2 mg microsomal protein, 40 mM octyl glucoside) were then added to the same tubes and lipid solubilized by vortexing to clarification (about 5–6 min). Solubilization was checked by measuring turbidity at 550 nm. The mixture was then dialyzed as described above, against a medium containing 0.32 M sucrose, 2 mM 2,4-dithioerythritol, 2.5 mM $CaCl_2$ and adjusted to pH 6. The content of the dialysis bag was layered on 0.5 M sucrose and centrifuged for 60 min at $140000 \times g$ with a SW-50 Beckman rotor. The pellet, containing the reaggregated vesicles, was harvested, resuspended in 0.4 ml of 0.32 M sucrose + 2 mM 2,4-dithioerythritol and used for further analyses, whereas the supernatant and the floating excess lipid were discarded.

Analyses. Lipids were extracted and analyzed following described procedures [1,13]. Phospholipid was assayed as inorganic P_i after digestion with perchloric acid [15]. Cholesterol was purified and determined as described [16]. Protein was assessed following the method of Lowry et al. [17] and radioactivity measured as described [18].

Results

Effects of dialysis conditions on some properties of reaggregated particles

The microsomal material which had been solubilized by octyl glucoside could be recovered by centrifugation after dialytic removal of the

detergent [1]. The composition of the dialysis medium (presence of cations and pH) was important to this effect. The recovery of microsomal protein by centrifugation was almost complete after dialysis at pH 6 in the presence of 5.0 mM of either Ca^{2+} or Mg^{2+}. An increase of pH from 6.0 to 8.0 and/or a decrease of ion concentration decreased protein recovery. As shown by electron microscopy (Fig. 1), particles recovered after dialysis and centrifugation were similar to native microsomes.

The phospholipid/protein ratio of reaggregated particles also depended on pH and ion concentration (Table I). At pH 6.0 and with 2.5 mM of either Ca^{2+} or Mg^{2+}, the ratio was similar to that of native membranes. However, it decreased with decreasing cation concentrations or shifting pH from 6.0 to 8.0. Although the phospholipid/protein ratio depended on the conditions of dialysis, the lipid composition of reaggregates did not and was similar to that of native microsomes (about 30% PE, 45% PC, 15% phosphatidylserine and 10% phosphatidylinositol + sphingomyelin) [10].

Although Ca^{2+} and Mg^{2+} were similar as to their ability to affect protein recovery and phospholipid/protein ratio, their action on base-exchange was different. The addition of Mg^{2+} to dialysis medium (pH 6.0) inhibited choline and ethanolamine (only at low concentrations) base-exchange activities (Table I), which, however, were both stimulated by Ca^{2+}. At pH 8.0 no increase of base-exchange could be observed increasing the concentration of Ca^{2+} although, in the same conditions, the phospholipid/protein ratio and protein recovery increased. This apparent inconsistency might, however, be explained considering that the base-exchange activity was calculated on total reaggregated protein basis and therefore any effects due to specific reaggregation of some proteins cannot be ruled out, especially considering that the reaggregation at pH 8.0 was less complete than at pH 6.0.

It should be noticed that using reaggregation conditions (2.5 mM Ca^{2+}, pH 6.0) which pro-

TABLE I

THE EFFECT OF DIFFERENT DIALYSIS CONDITIONS ON SOME PROPERTIES OF REAGGREGATED PARTICLES

Conditions of dialysis: A, Ca^{2+}, pH 6.0; B, Ca^{2+}, pH 8.0; C, Mg^{2+}, pH 6.0. Phospholipid is expressed as nmol/mg protein ± S.E. (four determinations); native (non-dialyzed) microsomes contained 715 ± 8 nmol phospholipid/mg protein (10 determinations). The base-exchange activity is expressed as pmol/mg protein per h ± S.E. (four determinations, at least).

Concn. (mM)	Dialysis conditions	Phospho-lipid	Base-exchange activity	
			choline	ethanolamine
0	A	285 ± 30	136 ± 9	375 ± 15
	B	124 ± 8	95 ± 5	248 ± 12
	C	275 ± 25	136 ± 8	375 ± 15
0.5	A	550 ± 25	151 ± 12	430 ± 35
	B	135 ± 15	76 ± 3	213 ± 6
	C	470 ± 28	109 ± 10	309 ± 14
1.0	A	610 ± 45	164 ± 11	576 ± 32
	B	146 ± 35	71 ± 2	199 ± 3
	C	550 ± 25	84 ± 9	300 ± 13
2.5	A	720 ± 48	174 ± 15	795 ± 50
	B	186 ± 22	65 ± 2	198 ± 3
	C	695 ± 42	76 ± 5	450 ± 47
5.0	A	830 ± 55	184 ± 17	1011 ± 57
	B	220 ± 20	64 ± 1	187 ± 3
	C	810 ± 45	73 ± 5	625 ± 51

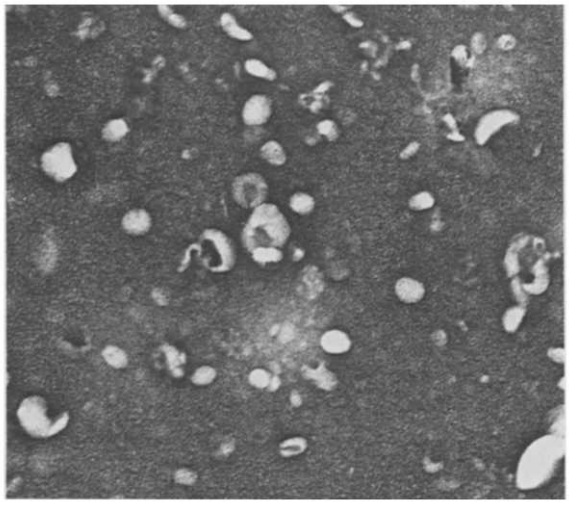

Fig. 1. Electron microscopic appearance of reaggregated vesicles. Microsomes exposed to octyl glucoside and dialyzed as described under Materials and Methods. The dialysis medium contained 2.5 mM Ca^{2+}, at pH 6.0. The negative-staining was performed as described in Ref. 22.

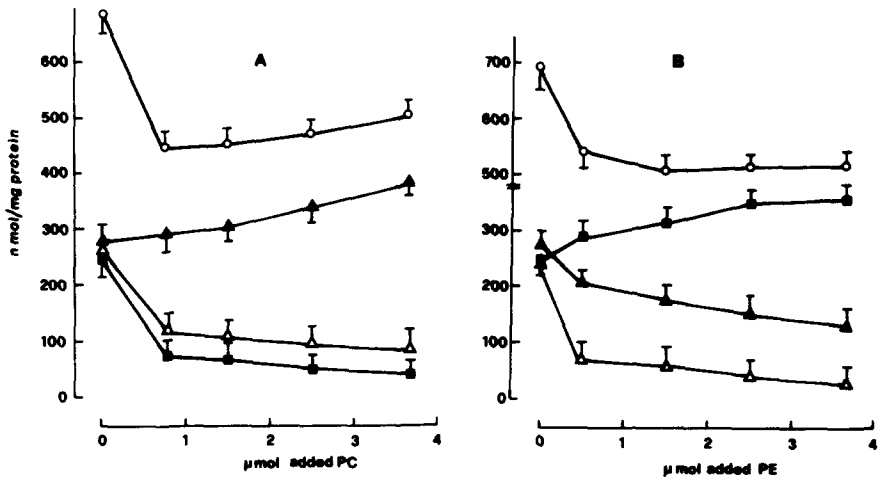

Fig. 2. The effect of the addition of PC (A) or of PE (B) on the composition of reaggregated membranes. The indicated amounts of PC (A) or PE (B) were added to the detergent/microsome mixtures. After dialysis (pH 6.0, 2.5 mM $CaCl_2$) membranes were pelleted at 140000 × g × 60 min. Values are the mean of five experiments ± S.E. ○——○, phospholipid; △——△, cholesterol; ■——■, PE; ▲——▲, PC.

duced particles having phospholipid/protein ratios similar to those of native membranes, the recovered base-exchange activity was about 85% of that of microsomes not exposed to detergent, but dialyzed in the same conditions [1]; however, the activities reported in this work may appear low due to very low precursor concentrations used (very far from saturating) [10].

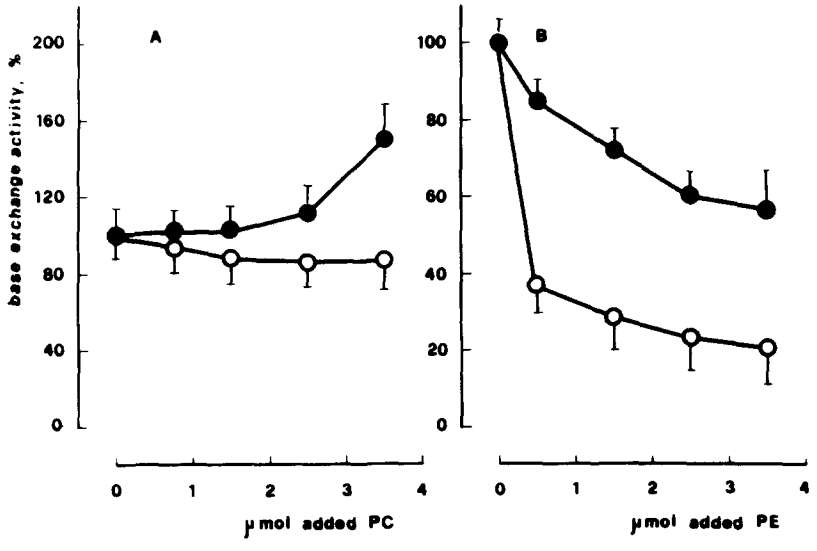

Fig. 3. The effect of the addition of PC (A) or of PE (B) to the detergent-membrane mixtures on the choline and ethanolamine base-exchange reactions of reaggregates. The indicated amounts of PC (A) or of PE (B) were added to the detergent-microsome mixtures. After dialysis (pH 6.0, 2.5 mM $CaCl_2$) membranes were pelleted at 140000 × g × 60 min. ●——●, ethanolamine base-exchange (795 ± 50 pmol/mg protein per h, in reaggregates to which no phospholipid had been added, corresponding to 100% in the figure); ○——○, choline base-exchange (174 ± 15 pmol/mg protein per h, in microsomes to which no phospholipid had been added, corresponding to 100% in the figure). The reported values are the mean of five experiments ± S.E.

Treatment of detergent-membrane mixtures with exogenous phospholipid

The addition (before the dialysis step) of exogenous phospholipid to membranes solubilized with octyl glucoside modified strongly the lipid composition and the base-exchange activity of the vesicles recovered after dialysis and centrifugation.

The treatment of the membranes with PC produced a decrease of PE, of cholesterol and of the phospholipid/protein ratio (Fig. 2A). The lipid composition of the membranes was also influenced by the addition of PE to detergent-membrane mixtures. In this case, a decrease of the phospholipid/protein ratio, a very large decrease of cholesterol, an increase of PE and a decrease of PC were observed (Fig. 2B). Therefore, PE affected the lipid composition of the reaggregates increasing itself and decreasing all other examined lipid, whereas the addition of PC decreased all lipid except itself which increased.

The modification of the lipid composition of reaggregates, produced by the addition of either PE or PE to membrane-detergent mixtures, affected the base-echange reactions (Fig. 3). Indeed, the addition of PC stimulated ethanolamine base-exchange although it did not influence choline base-exchange (Fig. 3A), whereas the addition of PE inhibited both choline and ethanolamine base-exchange activities (Fig. 3B).

Discussion

Reaggregates with a wide range of phospholipid/protein ratios can be obtained varying the concentrations of either Ca^{2+} or Mg^{2+} in the dialysis medium (Table I), which emphasizes the role of divalent cations in the formation of proteolipids during the removal of octyl glucoside from mixed micelles. From this point of view, our results agree with previously reported data on the reassembly of *Mycoplasma* membranes [19], which also requires an acidic pH and the presence of Mg^{2+} in the dialysis medium.

Suzuki and Kanfer [20] have recently purified ethanolamine base-exchange and have shown that this enzymic activity is stimulated by the addition of exogenous phospholipids. This finding agrees with our results which demonstrate that an increase of phospholipid/protein ratio, obtained by modifying the concentration of Ca^{2+} in the dialysis medium, stimulates choline and ethanolamine base-exchange (Table I). However, if reaggregation is performed in the presence of Mg^{2+} the dependence of base-exchange activity on the phospholipid/protein ratio is less clear, possibly because of the inhibitory action of this ion on base-exchange [9]. Therefore, base-exchange may be considered as a marker of the reaggregation state of the membrane provided that reaggregation is performed in suitable conditions.

The composition of reaggregates and their phospholipid/protein ratio is influenced by the addition of exogenous PC or PE to octyl glucoside/membrane mixtures (Fig. 2). Indeed, different populations of micelles (particularly phospholipid/ octyl glucoside/ cholesterol micelles containing small amounts of protein) would form in the presence of detergent [7]. The decrease of the concentration of octyl glucoside occurring during the dialysis would remove the detergent from micelles leading to the formation of low-density aggregates which cannot be pelleted by centrifugation ($140\,000 \times g \times 60$ min). On the other hand, micelles containing a relatively high amount of protein can produce recoverable particles having a low content of cholesterol. The addition of PC produces aggregates very poor in PE and vice-versa, probably because of a redistribution of endogenous phospholipids between low-density and high-density micelles.

The adidtion of PE or PC produces reaggregates whose composition is different from that of those obtained varying the concentration of cations in the dialysis medium. Indeed, in the latter case, the lipid composition of reaggregates is similar to that of native membranes, whereas in the former it is amply altered (Fig. 2). This may explain why ethanolamine base-exchange is positively correlated to the phospholipid/protein ratio when the ratio is modified only by detergent and dialysis (Table I) but does not depend on this parameter when it is varied adding PC or PE to detergent/membrane mixtures (Fig. 3). The base-exchange reaction is sensitive to the PC/PE ratio of reaggregates. Indeed, when it is low, the base-exchange activity is also low (Figs. 2 and 3); however, it increases when the ratio approaches or

exceeds the one present in native membranes. It is therefore possible that a certain amount of PC in the enzymic environment is necessary for the base-exchange activity. The hypothesis that the activity of base-exchange in native membranes might be regulated by its lipid environment is indeed very attractive. The fact that in some instances (Table I, Fig. 3) choline and ethanolamine base-exchange activities are differently affected by some parameters, may be due to the presence in rat brain microsomes of different systems for the base-exchange of choline or ethanolamine [21].

In conclusion, this work indicates the possibility of obtaining a wide range of reaggregates treating rat brain microsomal membranes with octyl glucoside and removing it by dialysis under different conditions.

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