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THE ASYMMETRIC DISTRIBUTION OF PHOSPHATIDYLCHOLINE IN RAT BRAIN SYNAPTIC PLASMA MEMBRANES

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Abstract—The distribution of phosphatidylcholine between inner and outer monolayers of rat brain synaptic plasma membrane was investigated by means of a phosphatidylcholine specific exchange protein. About 70% of the total membranal phosphatidylcholine was in the outer leaflet, 33% of which was exposed and readily exchanged in intact synaptosomes while the remainder was exchangeable following osmotic shock. Permeabilization of the synaptic plasma membranes by overnight incubation in buffer or by saponin (<0.08%) exposed an additional 30% of phosphatidylcholine to exchange, presumably from the inner cytoplasmic leaflet. Phosphatidylcholine is therefore asymmetrically distributed in the synaptosomal plasma membrane, as it is in other plasma membranes.

The two sides of biological membranes are structurally and functionally asymmetric. While the asymmetry of protein and carbohydrate is absolute, the same lipid components are present on both halves of the membrane bilayer, albeit in different proportions, thus presenting an asymmetry of distribution. For most plasma membranes, the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS) are predominantly located at the cytoplasmic side while choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM) are mostly in the external half of the membrane (for reviews, see Bishop and Bell, 1988; Rothman and Lenard, 1977; Zachowski and Devaux, 1990; Zwaal, 1978; Bretcher, 1972; Gordesky and Marineti, 1973; Op den Kamp, 1979).

Lipid asymmetry may result from the sidedness of the respective biosynthetic and catabolic enzymes, as has been found in bacteria, or may result after synthesis, during transport of the lipid to the cell surface (Zachowski and Devaux, 1990). Lipid asymmetry is maintained throughout the cell life in spite of transmembrane movements which tend to randomize the lipid distribution. One such movement is the simple diffusion of lipid molecules from one to the other side of the bilayer, or "flip-flop". As measured in red cells, the translocation half time for PC (~13 h), is greater than for PE and PS (Frank *et al.*, 1985; Lubin *et al.*, 1989). Another randomizing movement is facilitated diffusion (Bishop and Bell, 1988), by means of transport protein(s) called "flippase(s)". The preferential location of aminophospholipids at the cytoplasmic face of the membrane is maintained by two processes: an active, ATP-dependent transfer of PE and PS, mediated by a specific aminophospholipid translocase (Zachowski and Devaux, 1990) and the interaction of the aminophospholipids with the membrane skeletal proteins (Frank *et al.*, 1985).

Though information on phospholipid asymmetry in a large number of membranes has been obtained, the brain synaptosomes have been little investigated. Two previous studies on synaptic plasma membranes dealt with the distribution of aminophospholipids only, and the reported results were contradictory (Smith and Loh, 1976; Fontaine *et al.*, 1980). This is the first report on the asymmetric distribution of PC in brain synaptic plasma membranes, which we have investigated using a PC specific exchange protein.

EXPERIMENTAL PROCEDURES

Synaptosomes and synaptic plasma membranes

Male Sprague–Dawley rats were decapitated and the cerebral cortex rapidly dissected over ice. Synaptosomes were

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prepared essentially according to the method of Booth and Clark (1978) with all procedures carried out at 4°C. Cortex (2–2.5 g) was homogenized in 10 ml of isolation medium (0.32 M sucrose, 10 mM EDTA, 10 mM Tris–HCl pH 7.4) by hand in a Dounce homogenizer and then diluted to 20 ml with the same medium and centrifuged at 1100 *g* for 5 min. The supernatants were centrifuged at 15,000 *g* for 10 min and the resulting pellet was resuspended by gentle homogenization in 1.2 ml of isolation medium. After dilution to 7.2 ml (with 15% w/w Ficoll in 0.32 M sucrose, 50 μM EDTA, 10 mM Tris–HCl pH 7.4), the suspension was placed at the bottom of a centrifuge tube. Three ml of 7.5% (w/w) Ficoll in the same sucrose–EDTA–Tris medium was layered on top, followed by 3 ml of isolation medium. The gradient was centrifuged in a swinging bucket rotor at 99,000 *g* for 30 min. Synaptosomes were collected from the interface of 7.5 and 15% Ficoll, diluted with isolation medium and sedimented at 78.000 *g* for 30 min. The synaptosomal preparation was fixed in 1.5% paraformaldehyde and 1.5% glutaraldehyde and post-fixed in 2% osmium tetroxide for electron micrographic examination. It appeared as a population of closed, rounded membranes which enclosed mitochondria and synaptic vesicles.

Synaptic plasma membranes (SPM) were isolated from the above synaptosomal preparations following osmotic shock and fractionation in a sucrose gradient (Cotman and Matthews, 1971). Synaptosomes suspended in 1 ml of isolation medium were osmotically lysed by dilution with a 20-fold vol of cold 6 mM Tris–HCl (pH 8.2), and left overnight at 4°C with continuous shaking. The lysed synaptosomes were centrifuged at 54,000 *g* for 15 min and the pellet suspended in 0.32 M sucrose (pH 7.4), applied on top of a gradient formed by layering 38, 35, 32.5 and 25% (w/w) sucrose (pH 7.4). Following centrifugation at 99,000 *g* for 30 min SPM were collected from the 25%–32.5% sucrose interface, diluted with isolation medium and sedimented at 78,000 *g* for 30 min. The PC content of the synaptic plasma membranes was calculated from the total phospholipid in the SPM and the phospholipid distribution in SPM, determined in repeated thin layer chromatographic separations (Shina *et al.*, 1990) i.e. PC, 41.9±0.5%; PE, 35.8±4; PS, 12.8±0.6; SM, 5.6±0.2 and phosphatidylinositol, 3.7±0.4 (mean±SE, $n = 11$).

Synaptosomes prepared from 2–2.5 g cortex were suspended in 5 ml of a buffer containing 0.25 M sucrose and 1 mM EDTA in 50 mM Tris–HCl pH 7.4 (SET buffer). The suspensions were adjusted to a constant phospholipid concentration by means of absorbance readings. In order to do this we prepared a range of dilutions from a synaptosomal suspension. One aliquot of each dilution was used to determine absorbance at $O.D._{450nm}$, and another aliquot to extract the total lipids and estimate lipid phosphate as described under "PC exchange experiments". By relating the $O.D._{450nm}$ values to the phospholipid contents we constructed a standard curve. Each synaptosomal preparation was thus adjusted to contain 1.5–2.55 μmol PL/ml. Similarly, by adjusting the $O.D._{450nm}$ of the SPM suspensions, we made them up to 0.3–0.5 μmol PL/ml.

Small unilamellar vesicles (SUV)

For the PC-exchange reaction, synaptosomes or SPM were incubated with SUV containing [^{14}C]PC (L-α-l-palmitoyl-2-[l-^{14}C]oleoyl-sn-glycero-3-phosphocholine; New England Nuclear, 52.6 mCi/mmol) and triolein ([2-^{3}H]glycerol trioleate, Amersham 26.8 Ci/mmol), which served as a non-exchangeable marker for the extent of vesicles sticking to the membranes. The SUV had the following mol % composition: 70 PC (non-labeled plus labeled) 25 PE, 5 cardiolipin and 0.1 butylated hydroxytoluene, all from Sigma. The lipid mixture was dried under a N_2 stream, suspended in SET–BSA (5 mg BSA/ml) buffer and sonicated in a W-385 sonicator (Heat-Systems-Ultrasonics) using 3 cycles of 5 min each, over ice. The SUV used for PC exchange with intact synaptosomes contained 5 μmol PL/ml and for exchange with SPM, 1 μmol PL/ml, both supplemented with 0.5 μCi of [^{14}C]PC. The 5-fold difference in SUV PL content corresponds to the 5-fold difference in the PL content of the synaptosome suspensions, vs SPM suspensions.

PC exchange protein (PCEP)

PCEP, isolated from beef liver, catalyzes the transfer of PC (Wirtz *et al.*, 1976) and was purified as described by Kamp *et al.* (1977) as modified by Westerman *et al.* (1983). Greater than 700-fold purification (approx. 20% pure) was achieved by carrying the procedure through the CM-cellulose step. At this stage of purification no other phospholipid exchange proteins and no phospholipase activities are present in the preparation. The exchange protein was stored in 50% glycerol at −20°C. Before use, the PCEP solution was dialyzed for 24 h against SET buffer, with four changes at 4°C. One unit of PCEP transfers 1 nmol of PC/h at 37°C (Zilversmit, 1984; Crain, 1990).

PC exchange experiments

One ml aliquots of either synaptosomal or SPM suspensions were incubated at 37°C with 1 ml of SUV containing 4000 U of PCEP and adjusted to a final volume of 5 ml with SET–BSA. The exchange was terminated by adding 10 ml of SET buffer at 4°C and the synaptosomes or SPM were then immediately sedimented at 15,000 *g* for 15 min or 100,000 *g* for 30 min, respectively, followed by two washings with SET buffer. The amount of radio-labeled PC was always determined in SPM which were either directly subjected to PC exchange or derived from intact synaptosomes which were subjected to PC exchange prior to isolation of SPM.

Total lipids were extracted from SPM by methanol: chloroform mixtures (Marinetti *et al.*, 1959) and the extract washed with $CaCl_2$ (Folch *et al.*, 1957). An aliquot of the extract was taken for determination of total lipid phosphorus (Bartlett, 1959) and another, dissolved in 10 ml of Quicksafe A (Zuisser Analytic, Frankfurt) was counted in a liquid scintillation counter (Beckmann LS 1801). The ^{14}C counts, corrected for ^{3}H (non-specific sticking of SUV), indicated the amount of PC in the membrane which was exchanged for PC from the vesicles. The percent of total SPM–PC in the exchangeable pool, assumed for intact synaptosomes to be PC in the outer monolayer, was calculated as described previously (Steck *et al.*, 1976). This calculation was based upon the total PC in the SPM (see synaptosomes and synaptic plasma membranes section) and the total mass (60% of PC in SUV) and specific activity of the exchangeable PC in the SUV, determined by complete exchange with multilammelar vesicles (Crain, 1982; Zilversmit and Hughes, 1976), given that PCEP catalyzes a 1 for 1 exchange (Zilversmit and Hughes, 1976) of SUV–PC for SPM–PC. Exchange in the absence of the PCEP was negligible, the values being between 0–0.5%.

Saponin treatment and assays

SPM preparations were supplemented with saponin (Merck, Darmstadt) and incubated for 3 h with SUV and PCEP to achieve PC exchange as described above. Samples were incubated in parallel with saponin but without SUV and PCEP. The pellets obtained from saponin-treated SPM were washed twice with SET buffer and homogeneously dispersed in a sonicating bath before dividing into aliquots for determinations of protein (Markwell *et al.*, 1978), total lipid phosphorus (Bartlett, 1959) and Na^+–K^+–ATPase (EC 3.6.1.4) (Bonting *et al.*, 1961).

Iodinated human growth hormone (I^{125} hGH) was a gift from Mrs Aviva Zilberberg (Dept. of Pediatric Endocrinology, Beilinson Hospital, Petah Tikva). Pituitary hGH purchased from KABI VITRUM, Stockholm, Sweden, was iodinated by the lactoperoxidase-catalyzed reaction essentially according to Thorell and Johansson (1971). The monomeric form of Mr 22,000 was obtained by gel filtration and used immediately.

RESULTS

PC exchange in intact synaptosomes

Studies of sidedness employ the general strategy of using a non-penetrant probe, first on the intact membrane in which only the externally exposed components are available to the probe, and then on the permeabilized membrane in which the probe may reach components from both sides. Using the PC specific exchange protein we first established the amounts of PC which are available for exchange on the surface of intact synaptosomes.

The time-course presented in Fig. 1 shows that the PC exchange levels off between 2 and 3 h, reaching a maximal mean value $33 \pm 2\%$ ($n = 12$) of total PC in the SPM. The leakage of LDH (EC 1.1.1.27) into the medium (see legend to Fig. 1) was constantly small, reflecting synaptosomal intactness for the entire 3 h incubation period. The effect of varying the amount of PCEP was tested at the 2 h incubation point (Fig. 1). Using 2000 U of PCEP resulted in $27.7 \pm 1\%$ ($n = 6$) of PC exchanged while 4000 U of PCEP gave $32.8 \pm 0.8\%$ ($n = 4$). This relatively small difference indicated a near-saturation condition even at 2000 U of PCEP. For the curve in Fig. 1 and all other exchange experiments, 4000 U PCEP were used.

PC exchange in synaptic plasma membranes

The PC exchange in SPM (Fig. 2) levels off between 2–3 h of incubation, at a higher value than that obtained with intact synaptosomes. The mean exchange value after 3 h of incubation was $71 \pm 2\%$ ($n = 10$) of the total PC in the SPM. In the time-course experiment illustrated in Fig. 2, the maximal exchange reached was slightly lower than this mean, but still significantly higher than that in synaptosomes

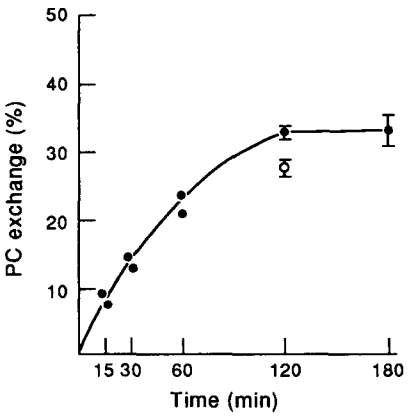

Fig. 1. Time course of PC exchange in intact synaptosomes. A synaptosomal suspension in SET–BSA buffer containing 1.5–2.5 μmol PL/ml was mixed with an equal vol of SUV containing 5 μmol of PL/ml and 0.5 μCi of ^{14}C–PC. The mixture was supplemented with PCEP and incubations carried out at 37°C for the times indicated. Leakage of lactate dehydrogenase into the medium, tested as an indicator of synaptosomal integrity, was as follows: 4.9 ± 0.4, $n = 4$ at 0 time, 6.5 ± 0.8, $n = 20$ at 15 min, 10.5 ± 1, $n = 4$ at 30 min and 8.5% at 180 min. Following PC exchange the synaptosomes were sedimented, washed, osmotically lysed and SPM separated on a sucrose gradient. ●, exchange with 4000 U PCEP/ml; ○, exchange with 2000 U PCEP/ml. All points are single values except for: means ± SE at: 2000 U, 120 min, $n = 4$; 4000 U, 120 min, $n = 6$ and 4000 U, 180 min, $n = 12$.

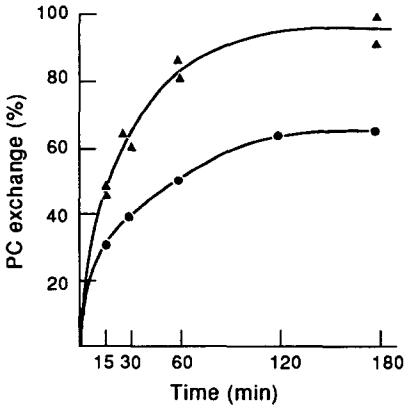

Fig. 2. Time course of PC exchange in synaptic plasma membranes. A synaptic plasma membrane suspension in SET–BSA buffer containing 0.3–0.5 μmol PL/ml was mixed with an equal volume of SUV containing 1 μmol of PL/ml and 0.5 μCi of ^{14}C–PC. The mixture was supplemented with 4000 U of PCEP/ml and incubated at 37°C for the times indicated. ●, SPM used for exchange immediately after preparation. Values shown are means (SE values are smaller than the symbols), $n = 4$. ▲, SPM stored overnight at 4°C in SET buffer before exchange. Each point represents a single experiment.

(Fig. 1). In addition to reaching a higher maximal value, the time-course of PC exchange in SPM differs from that obtained with intact synaptosomes by showing more rapid exchange (Figs 1 and 2). For example 30% PC exchange is achieved after 2 h incubation of the synaptosomes, but in only 15 min incubation of the SPM, suggesting a much greater availability of PC in the latter preparation.

In view of the tendency of biological membrane preparations to reseal following osmotic lysis (Bodemann and Passow, 1972; Low *et al.*, 1973; Bloj and Zilversmit, 1976) it was expected that the SPM preparation would not allow free access of PCEP to the cytoplasmic side of the membrane. Both the abundance of closed structures in the SPM preparations (Fig. 3) and the incomplete PC exchange obtained seem to support the conclusion that the inner membrane leaflet was not exposed to the exchange protein.

In order to further prove that SPM were not permeable to PCEP, we performed an experiment using a protein of similar size (Mr 22,000) viz. hGH. An SPM preparation was divided into two equal samples, pelleted and a volume of SET buffer equal to that of the pellet, containing [I^{125}]hGH, was added. One of the samples was, in addition, supplemented with saponin to a final concentration of 10%. Both samples were incubated for 3 h at 37°C with shaking. At the end of the incubation aliquots were removed, the remainder was pelleted and aliquots were again removed from the clear supernatants. The radioactivity in the aliquots taken before sedimentation and from the supernatants are given in Table 1.

Table 1. Exclusion of [I^{125}]hGH from SPM

	cpm/0.04 ml	
	A	B
Before centrifugation	16,900	15,500
Supernatant	25.000	14,000

SPM suspended in SET buffer were supplemented with [I^{125}]hGH. Sample B contained, in addition, 10% saponin. After 3 h at 37°C, aliquots of 0.04 ml were removed before centrifugation and from the supernatants after centrifugation and the radioactivity determined.

As expected, for the sample in which the SPM were permeabilized by saponin, the distribution of [I^{125}]hGH was even, resulting in similar cpm values in the aliquot containing SPM (before centrifugation) and in that removed from the supernatant. In

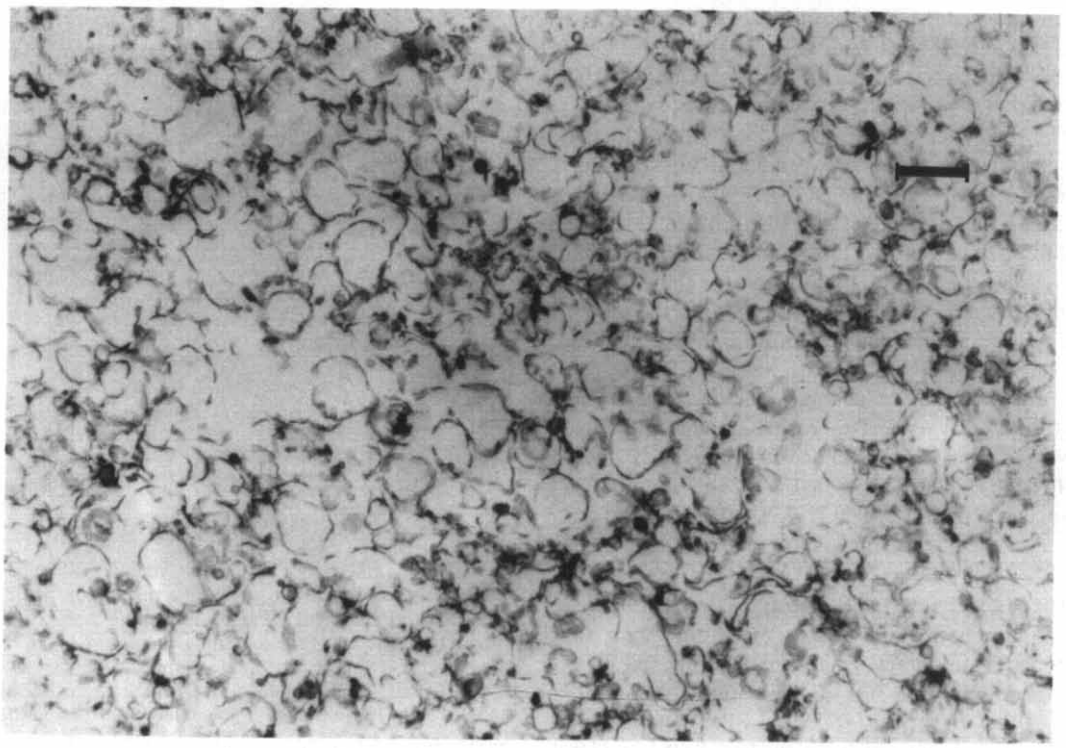

Fig. 3. Electron micrograph of SPM. SPM pellets were fixed in 1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 and postfixed in 2% osmium tetroxide. 1 cm bar = 4 μm.

contrast, the supernatant of the sample without saponin was twice as concentrated in [I^{125}]hGH than that of the sample with saponin, indicating that the protein was excluded from the SPM. The radioactivity in this supernatant being less than double that of the total aliquot suggests that some dilution by the liquid trapped within the initial SPM pellet occurred.

An attempt to permeabilize the SPM by three cycles of freeze–thaw failed to promote full exchange of PC (85%, two experiments). In contrast, an almost complete exchange of PC, viz. 94%, was achieved when SPM preparations were left overnight at +4°C in SET buffer (Fig. 2).

Effects of saponin

Saponin has been extensively used for permeabilization of biological membranes (Wassler *et al.*, 1987; Tohmatsu *et al.*, 1989; Burgess *et al.*, 1983). We attempted therefore to facilitate the access of PCEP to the membrane interior by treatment of the SPM with increasing concentrations of saponin. It was necessary, however, to avoid saponin concentrations which induce solubilization of membrane components, indicating membrane disorganization. In the experiments illustrated in Fig. 4, we estimated protein and phospholipid contents of SPM after exposure to saponin. We also assayed the activity of Na^+–K^+–ATPase, an integral membrane protein functioning across the hydrophobic core of the membrane (Karlish *et al.*, 1977). The results presented in Fig. 4 show that, when treated with up to about 0.08% saponin, the SPM do not lose any lipid, protein or Na^+–K^+–ATPase activity. At higher saponin concentrations there was some membrane solubilization and loss of Na^+–K^+–ATPase activity. At 1% saponin, the membrane lost only 10% of the protein but about 40% of the phospholipid and most of its Na^+–K^+–ATPase activity (Fig. 4).

In view of these results we chose to treat SPM with concentrations of saponin not higher than 0.08%. As seen in Fig. 5, increasing concentrations of saponin promoted the exchange of PC until at 0.08% all of the PC became exchangeable.

DISCUSSION

The specific PC exchange protein from beef liver catalyzes a mol/mol exchange of exposed PC in membranes for PC in an external source, such as SUV (Wirtz *et al.*, 1976; Kamp *et al.*, 1977; Zilversmit, 1984; Crain, 1990). Using PCEP and intact synaptosomes we obtained data indicating that a pool of PC, comprising 33% of the total membrane PC, is exposed on the membrane surface and is readily available for exchange. A higher proportion of membranal PC, viz. about 70% of the total, is exchanged when synaptic plasma membranes, instead of intact synaptosomes, are used for exchange. We consider this 70% to represent the total of externally located PC, viz. both the pool which is readily exposed (33%) and an additional pool which, although at the membrane surface, becomes available to exchange only after the synaptosomes are osmotically shocked. The question may be asked whether the additional PC exchanged in SPM represents PC located at the cytoplasmic side

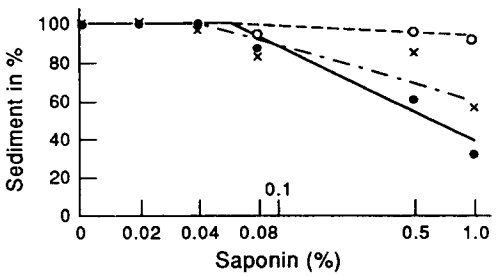

Fig. 4. Solubilization of protein, phospholipid and Na^+–K^+–ATPase from SPM by saponin. SPM were supplemented with saponin and incubated for 3 h as in Fig. 2. Following sedimentation and two washes with SET buffer, the pellet was homogeneously dispersed by sonication and aliquots used for protein, lipid phosphate and Na^+–K^+–ATPase determinations. ×, phospholipid; ○, protein; ●, Na^+–K^+–ATPase.

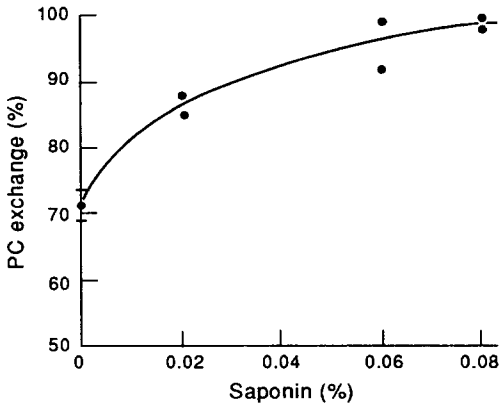

Fig. 5. Exchange of PC in saponin-treated SPM. SPM suspensions were supplemented with increasing concentrations of saponin and then subjected to PC exchange as described in Fig. 2. Incubations were for 3 h. Each point represents a single experiment except for mean ± SE, $n = 10$ for zero saponin.

of the membrane. A number of findings, however, argue against this possibility. There is a known tendency of membranes to form vesicles following lysis, thus regaining impermeability to proteins if not necessarily to small solutes (Bodemann and Passow, 1972; Low *et al.*, 1973; Bloj and Zilversmit, 1976). Indeed, electron micrographs of SPM preparations show mainly closed structures (Fig. 3). Further proof for the impermeability of SPM to a protein of the same size as the PCEP was provided by showing that [I^{125}]hGH (MR 22,000) is excluded from SPM preparations.

Bloj and Zilversmit (1976) found that PC in the outer layer of resealed red cell ghosts reacts readily with exchange protein, though in intact red cells it does not. PC exchange in the latter condition required larger amounts of PCEP (Van Meer *et al.*, 1980). This resembles our data which indicate a better exposure of PC in SPM than in intact synaptosomes. For the preparation of SPM, the synaptosomes have to be osmotically shocked in conditions which favor the detachment of loosely bound proteins, i.e. low ionic strengths, mildly alkaline pH and presence of EDTA (Steck, 1974). This may result in a more loosely packed bilayer structure (Van Meer *et al.*, 1980) promoting additional exposure of PC in the outer half of the membrane, thus increasing the total to about 70%. The finding of 70% PC in the external leaflet of the SPM is close to the proportion of PC in the outer leaflet of other biological membranes (Bretcher, 1972; Gordesky and Marinetti, 1973; Op den Kamp, 1979). Freysz *et al.* (1982) found, e.g., that about 75–80% of the PC is in the outer leaflet of brain microsomal vesicles.

The cytoplasmic side of the SPM could be made accessible to exchange by overnight incubation or by permeabilization of the membrane with saponin. Saponins are naturally occurring glycosides with lytic activity, due to their property of forming complexes with cholesterol, thus leading to membrane permeabilization (Wassler *et al.*, 1987). It has been shown that the size of the molecules crossing the permeabilized membrane are a function of saponin concentration. In order to permeabilize platelets or hepatocytes to Ca^{2+}, amounts of saponin well under 0.01% are sufficient (Tohmatsu *et al.*, 1989; Burgess *et al.*, 1983). At 0.01% saponin, the LDH contents of hepatocytes is completely released indicating that proteins may cross the membrane (Wassler *et al.*, 1987). Permeabilization of membrane to proteins is not associated with membrane disorganization at saponin concentrations as high as 0.05%. Thus, 0.05% saponin did not release an integral protein marker enzyme from hepatocytes (Wassler *et al.*, 1987). At 0.05% saponin, an integral membrane protein could be digested with proteases from both sides of the membrane, while its buried part remained intact, showing that the hydrophobic core of the membrane was not disorganized (Rotier *et al.*, 1984).

In agreement with the above studies, our data show that saponin, at concentrations below 0.8%, does permeabilize the membrane without perturbation of the membrane structure. This is indicated by the full retention of the membranal protein, lipid and Na^+–K^+–ATPase activity. Since this enzyme is an integral protein spanning the membrane (Karlish *et al.*, 1977) its unimpaired activity reflects a normal membranal environment. At the time, however, the membrane becomes permeable to PCEP, with resulting exchange of PC from both sides of the membrane, thus reaching 100%. Permeabilization and full PC exchange occurred also following overnight storage of the SPM preparations.

In summary, our data indicate that in the SPM, as in most other membranes investigated, PC is asymmetrically distributed. On the outer surface about 70% of the PC is exposed, out of which 33% is more readily available for exchange than the remainder. About 30% of the PC is on the cytoplasmic side of the membrane and not exchanged unless the membrane is permeabilized.

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