Characterization of gastric-mucosal membranes

Distribution of lipid- and protein-associated amino groups across pig gastric microsomes

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Two NH_2 -reactive probes (2,4,6-trinitrobenzenesulphonic acid and 1-fluoro-2,4-dinitrobenzene) were used to study the vectorial orientation of the membrane-associated free NH_2 groups across pig gastric microsomal vesicles. Unlike 1-fluoro-2,4-dinitrobenzene, 2,4,6-trinitrobenzenesulphonic acid is ordinarily an impermeant probe that becomes permeant in the presence of K^+ and valinomycin. Although 2,4,6-trinitrobenzenesulphonic acid alone reacts with about 28% of the total microsomal phosphatidylethanolamine, 2,4,6-trinitrobenzenesulphonic acid in the presence of valinomycin plus K^+ or 1-fluoro-2,4-dinitrobenzene alone reacted with 75% of the phosphatidylethanolamine. Under similar conditions the free NH_2 groups associated with the microsomal proteins also exhibited an asymmetric labelling pattern, the intra- and extra-vesicular orientation being 74 and 26% respectively.

The NH_2 -containing phospholipids, such as phosphatidylethanolamine and phosphatidylserine, have recently been reported to be distributed asymmetrically across different biological membranes like erythrocytes (Gordesky *et al.*, 1975; Marinetti & Love, 1976), mitochondrial inner and outer membranes (Marinetti *et al.*, 1976; Nilsson & Dallner, 1977; Krebs *et al.*, 1979), microsomes (Nilsson & Dallner, 1977; Higgins & Dawson, 1977; Sundler *et al.*, 1977), synaptosomes (Fontaine *et al.*, 1980) and Golgi membranes (Nilsson & Dallner, 1977; Sundler *et al.*, 1977). Asymmetric distribution of protein-bound NH_2 groups across erythrocyte membranes has also been suggested (Marinetti & Love, 1976). Such studies necessitate prior knowledge of the degree of purity and tightness and sidedness of the membrane vesicles to get meaningful data. The purified gastric microsomes are well-suited for such studies since these membranes have previously (Forte & Lee, 1977; Sachs *et al.*, 1977; Lee *et al.*, 1979) been characterized as nearly homogeneous tightly-sealed vesicles. Thus these vesicles are highly enriched in K^+ -stimulated ATPase, which is stimulated several-fold in the presence of valinomycin (Forte & Lee, 1977; Sachs *et al.*, 1977; Lee *et al.*, 1979). The K^+ ionophore also induces a rapid accumulation of 9-aminoacridine (Lee *et al.*, 1979) by the microsomes in the presence of K^+ , Mg^{2+} and ATP. The

studies suggested that most of the K^+ -stimulated ATP hydrolytic sites of these tight microsomal vesicles are exposed to the vesicle exterior.

The present paper deals with the vectorial distribution of the phosphatidylethanolamine and protein-bound NH_2 groups across the purified microsomes from the pig fundic mucosa, using the impermeant probe 2,4,6-trinitrobenzenesulphonic acid, which becomes permeant in the presence of K^+ plus valinomycin (Gordesky *et al.*, 1975) and the penetrating probe 1-fluoro-2,4-dinitrobenzene (Gordesky *et al.*, 1975). Our data demonstrate that under optimal conditions about 75% of the total gastric microsomal phosphatidylethanolamine reacts with either 2,4,6-trinitrobenzenesulphonic acid or 1-fluoro-2,4-dinitrobenzene, the intra- and extra-vesicular orientation being 58% and 17% respectively. Since 25% of the total phosphatidylethanolamine does not react with the probes except in detergent-treated microsomes it is not possible to predict their orientation. The microsomal protein-bound NH_2 groups also exhibit an asymmetric distribution, the intra- and extra-vesicular orientation being 74% and 26% respectively. The quantitative values for the membrane-bound NH_2 groups across the gastric microsomal vesicles reported in the present paper have been calculated after taking into account the proportion of leaky vesicles in the microsomal population and hence are somewhat different from those previously reported (Ray *et al.*, 1980; Sen *et al.*, 1980).

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Methods and materials

Fresh pig stomachs were purchased from the local slaughterhouse. The gastric microsomal membranes were harvested by a procedure described previously (Ray, 1978). The proteins were assayed by the procedure of Lowry *et al.* (1951).

The ATPase was assayed as previously described (Ray, 1978). Briefly, the incubation mixture contained, in a total volume of 1 ml, 50 μ mol of Pipes (piperazine-1,4-diethanesulphonic acid) buffer (pH 6.8), 1 μ mol of $MgCl_2$, 2 μ mol of Tris/ATP, 150 μ mol of KCl, 20 μ g of membrane protein in the presence and absence of 10 μ M-valinomycin. After 6 min incubation at 37°C the reactions were stopped by 1 ml of 12% trichloroacetic acid. The P_i was assayed by the procedure of Sanui (1974). The valinomycin-stimulated ATPase activity was calculated as the rate of P_i production in presence of valinomycin minus the rate in the absence of the ionophore.

Vesicular accumulation of H^+ was measured at room temperature by the method of Lee *et al.* (1979) using the change in fluorescence intensity (quenching) of 9-aminoacridine, which is proportional to the amount of dye taken up by the microsomes.

Labelling of the microsomal phosphatidylethanolamine with 2,4,6-trinitrobenzenesulphonic acid (Pierce Chemical Co.) or 1-fluoro-2,4-dinitrobenzene (Eastman Kodak) was done by incubating the microsomes (1 mg) with different concentrations of freshly prepared 2,4,6-trinitrobenzenesulphonic acid (1–5 mM) or 1-fluoro-2,4-dinitrobenzene (1–2 mM) in 250 mM-sucrose/5 mM-Pipes/Tris buffer (pH 6.0–9.0) for 15–90 min at room temperature. To make 2,4,6-trinitrobenzenesulphonic acid permeable to the microsomal vesicles 150 mM- K^+ and 10 μ M-valinomycin was included in the assay medium. The reactions were stopped by dilution with about 10 vol. of ice-cold sucrose/Pipes/Tris buffer of appropriate pH and centrifuged for 60 min at 150 000 g. The lipids were extracted from the pellet by the method of Bligh & Dyer (1959). The pellet after lipid extraction was used for quantification of 2,4,6-trinitrobenzenesulphonic acid bound to protein. The lipid extract was concentrated under N_2 and the trinitrobenzyl phosphatidylethanolamine and dinitrobenzyl phosphatidylethanolamine were separated from free phosphatidylethanolamine by t.l.c. (0.25 mm plate thickness; Merck Silica gel) with chloroform/methanol/water (65:25:4, by vol.) as solvent system. The derivatives could be located from the yellow colour and free phosphatidylethanolamine was detected with the ninhydrin spray reagent. The bands of phosphatidylethanolamine and its derivatives were scraped off, extracted twice with 3 ml of chloroform/methanol (1:1, v/v) and assayed for lipid phosphorus by the

Bartlett (1959) procedure. The trinitrobenzyl phosphatidylethanolamine and dinitrobenzyl phosphatidylethanolamine were also quantified by measuring the light absorption at 337 and 345 nm respectively.

The delipidated membranes described above were dissolved in 3 ml of 1% sodium dodecyl sulphate and the absorbance was read at 337 nm for trinitrobenzyl-protein and 345 nm for dinitrobenzyl-protein (Marinetti & Love, 1976). The absorbance values were converted into nmoles from a standard curve drawn by plotting the absorbance of different concentrations of trinitrobenzylvaline against the concentration of valine.

Results and discussion

The microsomes used in the present study were highly enriched in valinomycin-stimulated ATPase. The K^+ -stimulated ATPase activities measured as the rate of P_i production at 37°C, before and after addition of 10 μ M-valinomycin, were 4.8–6.5 and 25–30.5 μ mol/h per mg of protein respectively. Fluorescence quenching of 9-aminoacridine induced by valinomycin under the conditions described above using 200 μ g of microsomes was 70–80%. These data suggest that the microsomes used in the present study were predominantly (about 85%) in the form of tightly sealed vesicles and had most of the ATP hydrolytic sites facing the extravesicular space.

Pig gastric microsomes contain the highest amount of phosphatidylethanolamine (96.1 nmol/mg) representing 40.4% of the total phospholipids and a trace amount (0.7%) of phosphatidylserine (Sen & Ray, 1979a).

Fig. 1 shows the effects of different concentrations of 2,4,6-trinitrobenzenesulphonic acid on the labelling of gastric microsomal phosphatidylethanol-

Fig. 1. Effects of different concentrations of 2,4,6-trinitrobenzenesulphonic acid on the labelling of phosphatidylethanolamine (PE) associated with pig gastric microsomes

Data are from three separate experiments $\pm$ S.E.M.

amine in the absence of valinomycin plus K^+ . Optimum labelling was observed with 2 mM-2,4,6-trinitrobenzenesulphonic acid.

The kinetics of labelling of microsomal phosphatidylethanolamine by 2,4,6-trinitrobenzenesulphonic acid is shown in Fig. 2. The labelling was complete within 15 min in 2 mM-2,4,6-trinitrobenzenesulphonic acid compared with 30 min with a lower plateau in 1 mM-2,4,6-trinitrobenzenesulphonic acid, suggesting that the optimum concentration of 2,4,6-trinitrobenzenesulphonic acid for the labelling of microsomal phosphatidylethanolamine would be around 2 mM. The slight increase in labelling with 5 mM-2,4,6-trinitrobenzenesulphonic acid (Fig. 1) may be due to partial penetration of the probe at high concentrations.

The data in Table 1 demonstrate the effect of pH on the labelling of microsomal phosphatidylethanolamine by 1-fluoro-2,4-dinitrobenzene and 2,4,6-trinitrobenzenesulphonic acid (with and with-

Fig. 2. Kinetics of reaction of 2,4,6-trinitrobenzenesulphonic acid with gastric microsomal phosphatidylethanolamine

Data are from three separate experiments, ○, 1 mM-2,4,6-trinitrobenzenesulphonic acid; ▲, 2 mM-2,4,6-trinitrobenzenesulphonic acid.

out valinomycin plus K^+). Maximum labelling of phosphatidylethanolamine was observed at pH 8.0. 2,4,6-Trinitrobenzenesulphonic acid, which is ordinarily a non-penetrating probe, can be made penetrating in the presence of valinomycin plus K^+ , thus exhibiting an extent of phosphatidylethanolamine labelling similar to the penetrating probe 1-fluoro-2,4-dinitrobenzene (Table 1). The data show that at pH 8.0 inclusion of valinomycin plus K^+ elevates the labelling by 2,4,6-trinitrobenzenesulphonic acid from 28% of the total microsomal phosphatidylethanolamine to 75%. Considering about 15% of the vesicles were leaky, hence non-responsive to valinomycin plus K^+ , the corrected values for extra- and intra-vesicular phosphatidylethanolamine would be 16 and 56 nmol/mg of protein respectively.

2,4,6-Trinitrobenzenesulphonic acid also reacts with the free NH_2 groups of the membrane protein, approaching a plateau around 2 mM (Fig. 3a). There was more trinitrobenzyl-protein in the presence than in the absence of valinomycin plus K^+ (Fig. 3b). From the extent of interaction of 2,4,6-trinitrobenzenesulphonic acid with the protein NH_2 groups in the presence and the absence of valinomycin plus K^+ (Fig. 3b), and taking into account that 15% of the vesicles were leaky, it is calculated that the protein-bound free NH_2 groups exposed to the vesicle exterior are 11.8 nmol/mg of protein, the remaining 45 nmol being within the vesicles. The NH_2 groups exposed to the vesicle exterior have recently been demonstrated to play critical roles in ATPase function (Ray *et al.*, 1980).

It should be noted that about 25% of the phosphatidylethanolamine did not react with any of the probes (Table 1), although both probes reacted with over 95% of the phosphatidylethanolamine when the microsomes were solubilized with Triton X-100 (Table 2). Similar failure of a significant portion (about 40%) of the total phosphatidylethanolamine being labelled by permeant probes has been reported for erythrocyte ghosts (Bonsall

Table 1. Effects of different pH values on the labelling of gastric microsomal phosphatidylethanolamine by 2,4,6-trinitrobenzenesulphonic acid or 1-fluoro-2,4-dinitrobenzene

Labelling with 2,4,6-trinitrobenzenesulphonic acid or 1-fluoro-2,4-dinitrobenzene was done at a concentration of 2 mM. K^+ (150 mM) plus 10 μ M-valinomycin (Val- K^+) was used when indicated. Details are given in the Methods and materials section. Data are means $\pm$ S.E.M. ($n = 4$).

pH	Trinitrobenzyl phosphatidylethanolamine		Dinitrobenzyl phosphatidylethanolamine	
	(nmol/mg of protein)	Proportion of total phosphatidylethanolamine (%)	(nmol/mg of protein)	Proportion of total phosphatidylethanolamine (%)
7.0 (without Val- K^+)	17.7 $\pm$ 1.9	18.4	56.5 $\pm$ 3.0	58.8
7.0 (with Val- K^+)	50.0 $\pm$ 3.2	52.0	58.3 $\pm$ 4.5	60.7
8.0 (without Val- K^+)	27.0 $\pm$ 2.8	28.1	76.0 $\pm$ 3.0	73.2
8.0 (with Val- K^+)	72.0 $\pm$ 5.0	75.0	75.5 $\pm$ 3.5	78.6
8.5 (with Val- K^+)	72.5 $\pm$ 6.0	75.5	75.8 $\pm$ 4.5	78.9

Table 2. Labelling of microsomal phosphatidylethanolamine with 2,4,6-trinitrobenzenesulphonic acid in the presence or absence of Triton X-100

The labelling was carried out at pH 8.0. Details are given in the Methods and materials section. Data are means $\pm$ S.E.M. ($n = 3$).

Conditions	Trinitrobenzyl phosphatidylethanolamine (nmol/mg of protein)	Proportion of total phosphatidylethanolamine labelled (%)
2,4,6-trinitrobenzenesulphonic acid (with Val-K ⁺)	70.0 $\pm$ 3.5	72
2,4,6-trinitrobenzenesulphonic acid (with 0.1% Triton)	78 $\pm$ 3.2	81.0
2,4,6-trinitrobenzenesulphonic acid (with 1.0% Triton)	92.1 $\pm$ 2.0	96.0

Fig. 3. Labelling of the protein-bound NH₂ groups by 2,4,6-trinitrobenzenesulphonic acid

(a) shows the effects of different concentrations of 2,4,6-trinitrobenzenesulphonic acid in the presence of 150 mM-K⁺ and 10 μ M-valinomycin; (b) shows the effects of 2 mM-2,4,6-trinitrobenzenesulphonic acid in the presence and absence of valinomycin plus K⁺. Abbreviation used: Val, valinomycin plus K⁺. Results are means $\pm$ S.E.M. ($n = 3$).

& Hunt, 1971; Gordesky & Marinetti, 1973) and mitochondrial particles (Marinetti *et al.*, 1976; Krebs *et al.*, 1979). This non-availability of a significant portion of the phosphatidylethanolamine of biological membranes to various NH₂ reactive chemical probes appear to be due to some intrinsic molecular architecture of the biomembranes. Studies with the erythrocyte membranes (Marinetti & Love, 1974) demonstrated that a significant portion (about 37%) of the phospholipids was found to be in close proximity to the neighbouring proteins so that they could react with the NH₂-cross-linking agent difluorodinitrobenzene. Such approaches using difluorodinitrobenzene may be useful for further characterization of the small amounts of microsomal phospholipids (about 4%) demonstrated to be essential for the functional stability of the gastric K⁺-stimulated ATPase (Sen & Ray, 1979b, 1980).

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