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The effect of sodium carbenoxolone on the permeability of phosphatidylcholine and phosphatidylcholine: cholesterol liposomes

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Sodium carbenoxolone has been widely used in the treatment of gastric ulcer since the first successful trials of Doll and colleagues [1, 2]. Its specific mode of action however, remains unknown although many of its pharmacological and biochemical properties have been described [3–6].

Among the interesting properties of sodium carbenoxolone which improves the defence mechanism of the stomach is its ability to stimulate the amount of gastric mucus secreted. Johnston *et al.* [7] have shown that the incorporation of many hexose sugars into the glycoprotein fraction of the gastric mucosa of man, ferret and the rat is increased with carbenoxolone treatment. Lipkin [8] has also shown that carbenoxolone treatment in mice decreased the turnover rate of gastric epithelial cells giving rise to a more stable cell population.

Gastric mucosal cell membranes, like all other mammalian membranes contain phospholipids. Spenney *et al.* [9] have characterised canine gastric mucosal membranes and shown phosphatidylcholine (lecithin) to be the most abundant phospholipid, followed by phosphatidylethanolamine. Two membrane fractions were isolated by these workers, one with a density of 1.04 and one with a density of 1.10. The former had a phospholipid:cholesterol ratio of 1.30 (molar ratio), and the latter 0.89.

Bangham *et al.* [10] first showed that when aqueous dispersions of pure phospholipids were sonicated they formed enclosed vesicles surrounded by a lipid bilayer (liposomes). Although these artificial bilayers possess a higher resistivity and lower ion permeability than natural membranes, (see Robinson [11]), cellular membranes might be likened to these lipid bilayers with their properties modified by the presence of protein. Phospholipid liposomes with incorporated markers have been widely used for investigating the membrane effects of many series of compounds. Papahadjopoulos [12] and Singer [13] showed that local anaesthetics can reduce the cation permeability of model phospholipid bilayer membranes and Heap *et al.* [14] showed some interesting correlations between different steroid classes (corticosteroids, androgens and oestrogens) and their effects on the permeability of phosphatidylcholine liposomes.

The present work describes the effect of a wide concentration range of the anti-ulcerogenic drug, sodium carbenoxolone, on the permeability of phosphatidylcholine and phosphatidylcholine:cholesterol (1:1 molar ratio) liposomes to sodium.

Liposomes were prepared using egg lecithin (Grade 1, B.D.H., England) and cholesterol (Biochemical standard, B.D.H., England). Both were stored as 30 mM solutions in chloroform at −20°. Purity of the egg lecithin was checked before each experiment using t.l.c. Silica gel G plates were run in chloroform–methanol–water–ammonia (75:30:4:0.5), sprayed with 50% sulphuric acid and developed by heating at 110° for 15 min [15]. The egg lecithin used always ran as one spot (R_f value 0.61). There were no traces of lysolecithin or free fatty acids.

For the preparation of lecithin liposomes, 50 μmoles were taken to dryness in a 25-ml round-bottomed flask. For lecithin–cholesterol liposomes, 50 μmoles of each were taken to dryness from the chloroform solutions. The dried films were then shaken with 0.9 ml 160 mM ^{22}NaCl (25 μCi/ml) and 0.1 ml 160 mM Tris–HCl (pH 7.4) for 5 min. The flask was flushed exhaustively with nitrogen before shaking to prevent oxidation of the lipids. The suspension was then sonicated for 60 min in a Pulsation 50 bath (Kerry Ultrasonics Ltd.). The temperature of the water in the bath was kept below 28° and the flask flushed with nitrogen every 10 min. T.l.c. of the lipids after sonication showed no presence of oxidation products. After sonication the lipid was allowed to equilibrate at room temperature (under nitrogen) for 24 hr before use. Untrapped ^{22}Na was removed by column chromatography. The 1-ml aliquot of liposomes was passed over 3 g of hydrated Sephadex G-50 prepared in non-radioactive 144 mM NaCl–16 mM Tris–HCl (buffered saline, pH 7.4). Liposomes were eluted in the 12th–15th 1-ml fraction from the column. These fractions were bulked and diluted to 25 ml with buffered saline.

Series of dialysis bags (8/32 visking tubing), previously washed, tied at one end and left soaking overnight in buffered saline, were then prepared containing 1 ml of the liposome suspension. Further aliquots were taken for estimation of the total liposome–^{22}Na content by γ-radioactive measurement. Liposome containing dialysis bags were then placed in 10-ml screw-top test tubes containing 8 ml of buffered saline or various concentrations of sodium carbenoxolone in buffered saline. Tubes were then slowly rotated at 37° (10 rev/min). At intervals the tube contents (diffusate) were taken for γ-counting and calculation of sodium leakage from the liposomes.

Figure 1 shows the leakage rates of sodium from lecithin and lecithin:cholesterol liposomes in the presence of buffered saline, (control rates). It can be seen that the leakage of sodium from lecithin liposomes was linear over 6 hr with approximately 1% of the total entrapped sodium leaking out per hr. Further experiments showed that this linear rate continued for at least 24 hr. Leakage rates from lecithin–cholesterol liposomes were significantly reduced (Fig. 1), as found by Johnson [16].

Table 1 shows the effect of sodium carbenoxolone (10^{-3}–10^{-6} M) on sodium leakage from liposomes over a 6-hr period. Other experiments using non-radioactive liposomes in the dialysis bags and sodium [^{14}C]carbenoxolone (10^{-3}–10^{-6} M) in the test tubes established that equilibration of carbenoxolone with the contents of the dialysis bag was achieved after 3 hr. The results in Table 1 show that over the range 10^{-3}–10^{-6} M sodium carbenoxolone had a biphasic effect on the sodium permeability of both lecithin and lecithin cholesterol liposomes. A concentration of 10^{-3} M caused complete lysis of lecithin liposomes within 2 hr. Lecithin cholesterol liposomes, however, were more resistant and complete lysis was not apparent until 6 hr incubation. Lower concentrations of sodium carbenoxolone (10^{-4} and 10^{-5} M) also increased sodium leakage from both types of liposomes but not to the same extent as 10^{-3} M. Again the lecithin cholesterol liposomes were more resistant. After 6 hr, 82.1 and 9.5 per cent of the total entrapped sodium had leaked from lecithin liposomes in the presence of 10^{-4} M and 10^{-5} M sodium carbenoxolone, respectively. The control value at this time was 6.97 per cent. Values for lecithin cholesterol liposomes were 7.7 and 6.3 per cent with controls leaking 2.74 per cent.

Sodium carbenoxolone at 10^{-6} M showed a different effect. The leakage of sodium from lecithin liposomes was lower than the controls at 2, 4 and 6 hr, the greatest stabili-

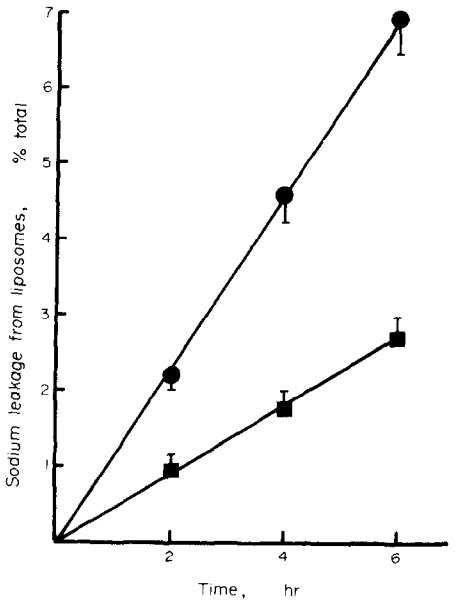

Fig. 1. Leakage of ^{22}Na from lecithin liposomes (●——●) and lecithin cholesterol (1:1 molar ratio) liposomes (■ - - ■). Sodium leakage is expressed as a percentage of total entrapped liposomal sodium. Results are the mean ±S.E.M. of four experiments.

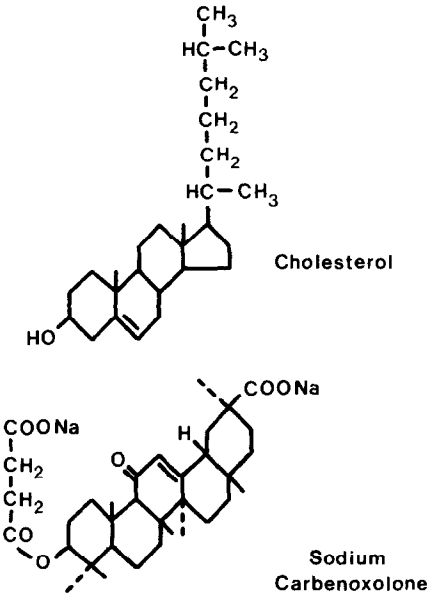

Fig. 2. The structure of cholesterol and sodium carbenoxolone.

sation being at 4 hr (67 per cent of control values). This stabilising effect was even more apparent with the lecithin–cholesterol liposomes. Sodium leakage was 47, 58 and 58 per cent of control values at 2, 4 and 6 hr, respectively. Lower concentrations of sodium carbenoxolone (10^{-7} and 10^{-8} M) were shown to have no effect on the leakage of sodium from either type of liposome.

These results show that sodium carbenoxolone is a very "membrane active" compound and at concentrations at which it is likely to exist next to gastric mucosal cells after oral administration (usual dose 100–300 mg twice daily) may cause a significant decrease in plasma membrane permeability. An increase in the stability of weakened gastric mucosal cells in ulcerogenic patients by sodium carbenoxolone could explain the decreased cell turnover noted by Lipkin [8] and may explain the increase in glycoprotein synthesis which occurs after carbenoxolone treatment.

It is worth noting that liposomes prepared with a 1:1 molar ratio of lecithin and cholesterol, which is very similar to the lipid composition of gastric mucosal cell plasma membranes, showed the maximal stabilising properties of carbenoxolone. Cholesterol and sodium carbenoxolone are somewhat similar in structure (Fig. 2) and both can cause a decrease in permeability of lecithin liposomes. It is suggested that insertion or uptake of the highly lipophilic sodium carbenoxolone molecules into phospholipid bilayers is altered by the presence of cholesterol in such a way that the labilising or detergent-like effect of carbenoxolone is reduced. The values for 10^{-4} M carbenoxolone in Table 1 best illustrate this point. After 6 hr, 82.1 per cent of total entrapped sodium was released from lecithin liposomes but only 7.7 per cent from lecithin cholesterol liposomes. Enhancement of the stabilizing properties of carbenoxolone at 10^{-6} M on lecithin cholesterol

Table 1. The effect of sodium carbenoxolone (10^{-3}–10^{-6} M) on the sodium leakage from (a) lecithin and (b) lecithin–cholesterol liposomes over a 6-hr period

Time (h)	Control sodium leakage*	Sodium carbenoxolone concn (M)			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}
(a) Lecithin liposomes					
2	100 (2.19)	3870 ± 126 (84.7)†	498 ± 61 (10.9)†	129 ± 17 (2.8)‡	68 ± 8 (1.5)†
4	100 (4.62)	1880 ± 99 (86.9)†	1510 ± 36 (69.8)†	118 ± 13 (5.5)†	67 ± 12 (3.1)†
6	100 (6.97)	1250 ± 68 (87.1)†	1178 ± 87 (82.1)†	136 ± 9 (9.5)†	84 ± 9 (5.8)†
(b) Lecithin cholesterol liposomes					
2	100 (1.01)	6369 ± 284 (64.3)†	109 ± 4 (1.1)‡	109 ± 8 (1.1)^{NS}	47 ± 7 (0.48)†
4	100 (1.78)	4422 ± 214 (78.7)†	213 ± 24 (3.8)†	149 ± 10 (2.7)†	58 ± 9 (1.0)†
6	100 (2.74)	3086 ± 119 (84.5)†	282 ± 19 (7.7)†	231 ± 17 (6.3)†	58 ± 11 (1.6)†

* Results are expressed as a percentage of control leakage (control = 100). Figures in parentheses represent leakage as a percentage of total liposomal entrapped sodium; i.e. control values are as shown in Fig. 1. Complete equilibration of labelled sodium between the 1-ml liposome content of the dialysis bag and the 8-ml content of the tube would therefore be equivalent to 88.8% (8/9) leakage of total liposomal entrapped sodium. Levels of significance are shown (Student's *t*-test) comparing these "percentage of total" values with controls. † $P < 0.01$, ‡ $P < 0.05$, NS, no significant difference.

Results are the mean ±S.E.M. of four experiments.

liposomes was also significant; 1.6 per cent of entrapped sodium was released after 6 hr from lecithin–cholesterol liposomes compared to 5.8 per cent from lecithin liposomes.

The molecular composition of biological membranes and the concentration of carbenoxolone are therefore both important in determining the effect of the drug on membrane permeability. Studies to determine the actual membrane concentration of carbenoxolone in different liposomal systems may give more information on its molecular interactions in lipid membranes.

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p-Chlorophenylalanine-induced enhancement of the effects of morphine on the adrenal medulla

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The actions of morphine on the adrenal medulla can be explained in large part by its combined direct and centrally mediated stimulation of the sympatho–adrenal axis [1–4]. Thus, acute administration of morphine results in adrenal catecholamine depletion and trans-synaptic induction of the catecholamine biosynthetic enzymes, tyrosine hydroxylase and dopamine β-hydroxylase, as well as increased formation of new storage vesicles [1,2]. Upon chronic administration, however, the ability of morphine to deplete catecholamines disappears and levels increase to supranormal values; this recovery is related directly to the stimulation-induced increases in catecholamine biosynthetic enzymes and storage vesicles [1]. However, there is evidence that other factors may operate in limiting both the degree to which morphine can deplete adrenal catecholamines and the degree to which enzyme induction can occur. Morphine even in large doses appears to be incapable of evoking the full degree of stimulation of which the sympatho–adrenal axis is capable, and no additional induction of adrenal tyrosine hydroxylase is evident at doses of morphine exceeding 40 mg/kg [1,2]. Mueller *et al.* [5] and Breese *et al.* [6] have shown that depletion of central serotonin with *p*-chlorophenylalanine (PCPA) can enhance the sympatho–adrenal effects of other stimulatory agents, such as insulin or amphetamine. Since chronic morphine enhances serotonin turnover [7,8], it is possible that sympatho–adrenal stimulation by morphine is limited in part by enhancement of serotonergic negative input in the brain-stem [9]. In the present study, the action of PCPA on chronic morphine-induced stimulation of the adrenal medulla has been examined.

Male Sprague–Dawley rats (Zivic–Miller) weighing 200–250 g were given morphine HCl subcutaneously twice daily as follows: 10 mg/kg for 2 days, followed by 40 mg/kg for 2 days, followed by 100 mg/kg thereafter. Controls received saline on the same schedule. After 1 week at the highest dose, saline- or morphine-treated rats received saline or PCPA methyl ester HCl (150 mg/kg, i.p.) once daily for 2 days and were killed 24 hr after the second PCPA injection; animals continued to receive morphine or saline concurrently with PCPA or saline and thus were killed 12 hr after the last injection of morphine.

Adrenals from the rats in the four groups (control, PCPA, morphine, PCPA plus morphine) were excised and each pair was homogenized in 2 ml of 0.15 M KCl. Aliquots (0.1 ml) were deproteinized with 1.9 ml of 3.5% perchloric acid, centrifuged at 26,000 *g* for 10 min, and the supernatant was analyzed for catecholamines by the trihydroxyindole method using an autoanalyzer [10]. Duplicate 0.2 ml aliquots of the homogenate were used for analysis of dopamine β-hydroxylase activity by the periodate oxidation method [11], using 10 μM[^{3}H]-tyramine as substrate and *p*-hydroxymercuribenzoate (optimal concentration, 0.5 mM) to inactivate endogenous inhibitors. The