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HYPOGLYCEMIC DRUGS INCREASE LIVER PLASMA MEMBRANE MICROVISCOSITY IN VITRO

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Summary

The effect of hypoglycemic drugs chlorpropamide and phenformin has been tested on isolated liver plasma membranes as to their microviscosity parameters. Results reported suggest that both drugs are able to increase in vitro plasma membrane microviscosity in a dose-dependent way.

Hypoglycemic agents, belonging to both sulfonylureas and biguanides, exert their action on biological membranes either at the intracellular level or on the plasma membrane itself [1]. In addition, it has recently been suggested that biguanide binding makes plasma membranes more rigid, decreasing their fluidity [2]. In previous research we reported that chlorpropamide (1-[*p*-chlorophenylsulfonyl]-3-propylurea) and phenformin (N^1-β-phenethylformamidinyl iminourea), a sulfonylurea and a biguanide, respectively, act on isolated liver plasma membrane, modulating the activity of membrane-bound enzymes such as (Na^+-K^+)-ATPase and cyclic AMP-phosphodiesterase [3]. This body of evidence prompted us to investigate as to the role of plasma membrane fluidity in the mechanism of action of both chlorpropamide and phenformin on liver, and results reported herein indicate that an in vitro treatment of isolated liver plasma membranes with both drugs significantly increases their microviscosity.

Liver plasma membranes were prepared, as already described [4, 5], from male Sprague Dawley rats, 'Nos' strain, 150 g average body weight, supplied by Nossan Ltd., Correzzana, Italy. Animals were fed ad libitum using a standard diet according to Miller and Phillips [6] with addition of inorganic salts and vitamins [7] and had free access to water.

The change of cholesterol to phospholipid (C/PL) molar ratio was ob-

tained as recently suggested by Shinitzky [8]. Briefly, one volume of liver membrane suspension (1 mg protein/ml) was incubated 12 h at 37°C with two volumes of a heat-inactivated 10% human serum in 0.05 M Tris-HCl (pH 7.5)/0.1 M NaCl containing 100 000 U/l sodium penicillin G [9]; the cholesterol depleting medium contained 0.25 mg/ml additional egg lecithin, about 0.35 μmol phospholipid/mg membrane protein, employing L-β,γ-dipalmitoyl-α-lecithin from Fluka A.G., Buchs, Switzerland. The cholesterol enriching medium contained 0.13 mg/ml additional cholesterol; chromatographically pure cholesterol was obtained from Merck, Darmstadt, F.R.G. After incubation, plasma membranes were extensively washed with the Tris-buffered saline and finally resuspended at the original protein concentration. Cholesterol was determined as total cholesterol [10]: phospholipids were determined according to Bartlett [11].

For fluorescence polarization measurements plasma membranes were labelled with 1,6-diphenyl-1,3,5-hexatriene in the following way: $2 \cdot 10^{-3}$ M diphenylhexatriene in tetrahydrofuran was diluted 1:1000 just before use with Tris-buffered saline, the membrane suspension was then mixed in a 1:1 ratio with diluted diphenylhexatriene at a final protein concentration of about 50 μg/ml. The mixture was usually incubated 15 min at 37°C in the absence or presence of the two hypoglycemic drugs at the concentration indicated; no significant change in the pattern of results could be observed when, after 15 min of diphenylhexatriene labelling, the incubation of membranes continued for an equal time in the presence of the drugs under investigation at any concentration tested. Fluorescence polarization measurements were carried out with an Aminco-Bowman spectrophotofluorometer equipped with two Glan prism polarizers; excitation was set at 366 nm and emission at 430 nm, with two additional 2 nm slits on both light paths. The time of exposure of the sample to the excitation light did not exceed 10 s to reduce diphenylhexatriene photoisomerization [12]. The temperature of the sample was checked with an accuracy of ± 0.1°C using a thermistor thermometer. The degree of fluorescence polarization, P, which was not dependent on sample dilution, was calculated from the formula

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 1}$$

being $I_{\parallel}$ and $I_{\perp}$ the fluorescence intensities recorded with the analyzing polarizer oriented respectively parallel and perpendicular to the direction of the polarized excitation beam; correction for light-scattering from the sample as well as for a grating correction factor [13] were introduced.

Microviscosity was obtained employing the Perrin equation [12]

$$\frac{r_o}{r} = 1 + C(r) \frac{T\tau}{\eta}$$

where the fluorescence anisotropy, r, can be obtained as follows:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 2}$$

being $r_o = 0.362$ for diphenylhexatriene, $C(r)$ a structural parameter of the fluorophor related to each r, $\bar{\eta}$ the microviscosity, T the absolute temperature and τ the excited state lifetime [12]. The excited state lifetime, τ, was calculated from a plot of the total fluorescence intensity $F = I_{\parallel} + 2I_{\perp}$ against temperature with a τ_o value of 11.4 ns for diphenylhexatriene [12], being 8.9 ± 0.1 ns at 37°C as assessed by single photon counting technique [14].

Proteins were estimated according to the method of Lowry et al. [15] using bovine serum albumin as a standard.

Both chlorpropamide and phenformin increase liver plasma membrane microviscosity (Table I) in a dose-dependent way, chlorpropamide being definitely more effective at low concentration. The temperature dependence of microviscosity of liver membranes, treated or not treated with both hypoglycemic agents, has been determined in the temperature range 6—41°C (Fig. 1). The plot of log $\bar{\eta}$ vs. the reciprocal of the absolute temperature ($1/T$) gives a straight line from which the apparent flow activation energy ($\triangle$E) can be calculated [16]. The observed value of $\triangle$E for control membranes was 9.0 kcal·mol^{-1}, which is in reasonable agreement with values reported from mammalian membranes [16]; both phenformin and chlorpropamide treatment significantly reduced the $\triangle$E value to 8.1 and 5.7 kcal·mol^{-1} respectively. The decrease of the flow activation energy of liver plasma membranes treated with both hypoglycemic agents is suggestive of a more ordered conformation, as indicated by the increase of microviscosity parameters (Table I).

The microviscosity of isolated liver plasma membranes was modulated in vitro by altering their cholesterol content with a simple technique established for erythrocytes, which proved to be rather satisfactory also for liver membranes [8, 9]. Liver membranes show, as expected [16], increased or decreased microviscosity following the increase or decrease of cholesterol/phospholipid (C/PL) molar ratio; in addition control serum treated membranes show increased C/PL ratio owing to the higher cholesterol content of

TABLE I

DOSE-RESPONSE STUDY OF THE EFFECT OF CHLORPROPAMIDE AND PHENFORMIN ON MICROVISCOSITY PARAMETERS OF ISOLATED LIVER PLASMA MEMBRANES

Plasma membranes were incubated 15 min at 37°C in the absence of the two drugs, at the concentration indicated, before the measurement; diphenylhexatriene labelling of plasma membranes took place at the same time. Results reported as microviscosity $\bar{\eta}$ (poise at 37°C) ± S.D. as a mean of three experiments carried out on different membrane preparations; the average degree of fluorescence polarization, P, is reported between brackets. *$p < 0.05$ (at least), as estimated by Student's t-test with respect to controls.

Drug concentration	Chlorpropamide		Phenformin	
(M)	$\bar{\eta}$	P	$\bar{\eta}$	P
0	2.69 ± 0.13	(0.261)	—	—
$1 \cdot 10^{-5}$	2.91 ± 0.12	(0.262)	2.73 ± 0.10	(0.258)
$1 \cdot 10^{-4}$	3.09 ± 0.17*	(0.269)	2.77 ± 0.11	(0.262)
$1 \cdot 10^{-3}$	3.44 ± 0.27*	(0.282)	3.32 ± 0.19*	(0.274)
$1 \cdot 10^{-2}$	4.70 ± 0.33*	(0.314)	3.90 ± 0.22*	(0.286)

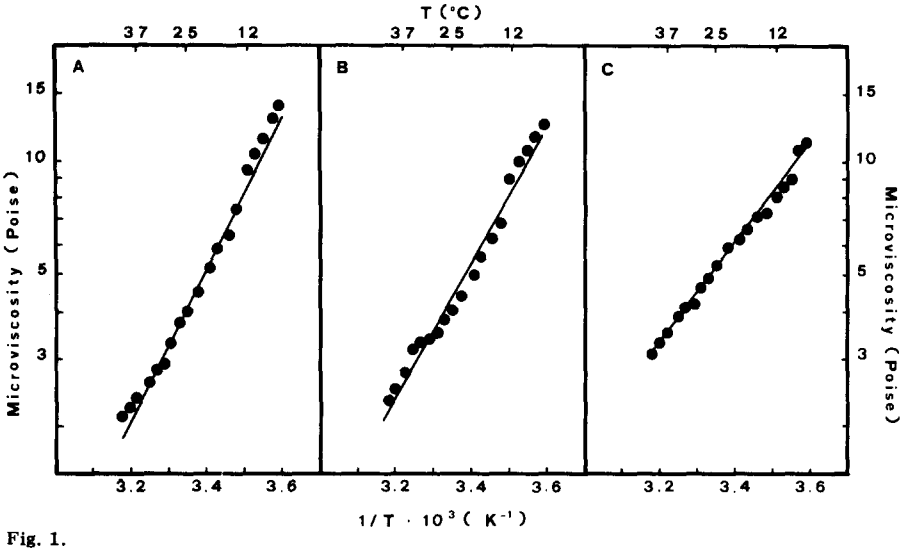

Fig. 1.

Temperature dependence of microviscosity ($\bar{\eta}$) of liver plasma membranes untreated (A, $r = 0.98$), treated with $1 \cdot 10^{-3}$ M phenformin (B, $r = 0.97$) or chlorpropamide (C, $r = 0.99$) as reported in Table I. The concentration giving half-maximal effect has been selected for both drugs (Table I; see also Ref. 3). Each point represents the average of three separate experiments carried out on different plasma membrane preparations. Straight lines have been drawn by regression analysis, the slope being significantly different ($p < 0.05$ for phenformin; $p < 0.001$ for chlorpropamide) with respect to control experiments as assessed by analysis of variance of regression lines.

TABLE II

EFFECT OF CHLORPROPAMIDE AND PHENFORMIN ON MICROVISCOSITY PARAMETERS OF LIVER PLASMA MEMBRANES WITH DIFFERENT CHOLESTEROL/PHOSPHOLIPID (C/PL) MOLAR RATIO

For each group of experiments, four different plasma membrane preparations, untreated or treated as reported in Table I being $1 \cdot 10^{-3}$ M the concentration of both drugs, were employed. The degree of fluorescence polarization P, microviscosity $\bar{\eta}$ (poise at 37°C) and C/PL ratios are reported as means ± S.D. of four experiments. [1] $p < 0.05$ at least, as estimated by Student's t-test with respect to controls.

	P	$\bar{\eta}$	C/PL (molar ratio)
Untreated membranes			0.56 ± 0.06
Control	0.249 ± 0.008	2.41 ± 0.19	
Chlorpropamide	0.272 ± 0.004[1]	3.02 ± 0.11[1]	
Phenformin	0.272 ± 0.007[1]	3.03 ± 0.19[1]	
Control serum treated membranes			0.73 ± 0.14
Control	0.255 ± 0.007	2.53 ± 0.25	
Chlorpropamide	0.292 ± 0.014[1]	3.67 ± 0.33[1]	
Phenformin	0.283 ± 0.008[1]	3.54 ± 0.30[1]	
Cholesterol enriched membranes			1.38 ± 0.20
Control	0.269 ± 0.006	2.96 ± 0.14	
Chlorpropamide	0.275 ± 0.014	3.12 ± 0.40	
Phenformin	0.275 ± 0.007	3.15 ± 0.21	
Cholesterol depleted membranes			0.52 ± 0.18
Control	0.244 ± 0.004	2.35 ± 0.12	
Chlorpropamide	0.253 ± 0.006[1]	2.64 ± 0.18[1]	
Phenformin	0.258 ± 0.009[1]	2.88 ± 0.25[1]	

human with respect to rat serum (Table II) [9, 17]. Untreated as well as control serum treated or cholesterol depleted membranes are significantly sensitive to both hypoglycemic agents as to an increase of microviscosity; whereas plasma membranes made more rigid by an artificial increase of C/PL ratio are not significantly responsive to both drugs (Table II).

Our results clearly indicate that chlorpropamide and phenformin, in a concentration range which can be attained in therapeutic treatment (see Ref. 3), decrease membrane fluidity as from their effect on lipid microviscosity parameters. These data, which are consistent with previous observations related to membrane-bound enzymes [3] again stress that the plasma membrane is perhaps a major target for the action of both drugs; in particular, it now becomes evident that there is a role for membrane fluidity in the mechanism of action of these hypoglycemic agents, as suggested by Schäfer [2] for biguanides. In this connection it has been shown very recently that sulfonylureas increase the number of insulin receptors in liver membranes after an in vivo treatment [18]; such a phenomenon could be, at least partially, explained on the basis of increased exposure of receptors sites which should follow the increase of membrane microviscosity elicited by the binding of drugs to the plasma membrane [9, 19].

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