INTERACTION BETWEEN NONACHLAZINE AND NORADRENALIN ON A MODEL PHOSPHOLIPID MEMBRANE

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The binding of nonachlazine (NON), imipramine (IMI), and noradrenalin (NA) with model phospholipid membrane vesicles (liposomes) was investigated. Binding was determined by the quenching effect of these substances on a 3-methoxybenzanthrone (MBA) fluorescent probe present in the membrane. The method of calculating the parameters of binding of the substances with the membrane based on a change in intensity of fluorescence of MBA was developed. The binding constants of NON, IMI, and NA, with the membrane were $(4.3 \pm 10.3) \cdot 10^3\ M^{-1}$, $(2.7 \pm 0.2) \cdot 10^3\ M^{-1}$, and $(0.7 \pm 0.15) \cdot 10^3\ M^{-1}$ respectively. NON and IMI were shown to be able to compete with NA for the binding sites. These competitive interactions can be regarded as the probable mechanism of the blocking of reverse NA transport through the axonal and vesicular membranes characteristic of NON and IMI.

KEY WORDS: noradrenalin; imipramine; nonachlazine; liposomes; fluorescence.

The writers showed previously that nonachlazine (NON) reduces the reverse transport of noradrenalin (NA) in rat heart muscle. Electron-microscopic and fluorescence-histochemical observations have led to the hypothesis that the blocking of mediator uptake takes place at the level of its passage through the axonal and vesicular membranes. However it is not yet clear how NON can block the passage of NA through the membrane.

It was therefore decided to investigate the possibility of direct interaction between these substances in a membrane. The well-known blocker of mediator uptake, imipramine (IMI), which also blocks at the level of the axonal membrane [1], was used for comparison.

EXPERIMENTAL METHOD

Phospholipids were isolated from hens' eggs [6] and artificial phospholipid membrane vesicle-liposomes were obtained [5]. Interaction between drugs on the model phospholipid membrane was studied by the fluorescence probe method [3]. Fluorescence spectra were recorded on the instrument described previously [4] with a correction for the spectral sensitivity of the apparatus. The total light energy of the fluoresence spectrum in the region 470-600 nm was measured on the ÉF-3 Ma fluorometer during excitation by light 436 nm. The properties of the 3-methoxybenzanthrone (MBA) fluorescent probe were described previously [2]. The parameters of binding of the drugs with the membranes were determined from their quenching effect on fluorescence of the MBA probe in the membrane. It was assumed that quenching obeys the Stern–Volmer equation [8]:

$$K_q r \cdot \frac{b}{N} = \left(\frac{I_0}{I} - 1\right) = i,$$

where I_0 and I represent the intensity of fluorescence in the absence and presence of the quenching agent respectively; r the concentration of quencher bound with the membrane; N the concentration of binding sites; b the number of binding sites of the quenching agent (in moles) per liter volume of membrane accessible to the

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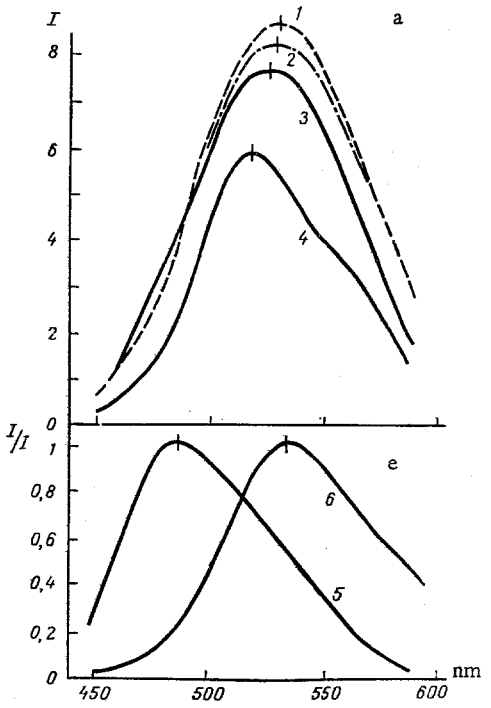

Fig. 1. Fluorescence spectra of MBA in suspension of liposomes (a) and in organic solvents (b): 1) MBA (5 μM) in suspension of lysosomes (0.4 mg lipid/ml); 2) the same in presence of 1.5 mM NA; 3) the same in presence of 0.5 mM NON; 4) the same in presence of 0.5 mM IMI. Solvent: 0.01 M Tris–HCl, pH 7.5; 5) MBA (5 μM) in heptane solution; 6) the same in ethanol solution.

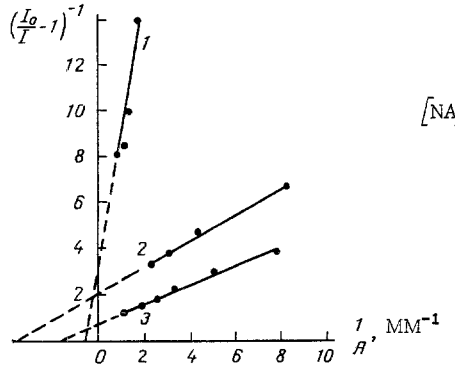

Fig. 2

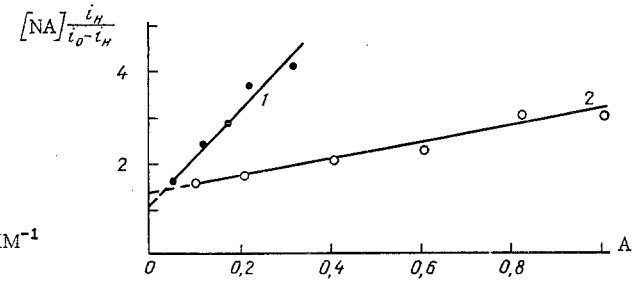

Fig. 3

Fig. 2. Determination of binding constants of NA (1), NON (2), and IMI (3) with liposomes (0.4 mg/ml in 0.01 M Tris–HCl solution, pH 7.5). Explanation in text.

Fig. 3. Competition of NON (1) and IMI (2) with NA for binding sites with membrane of liposomes. I_0 and I) Intensity of fluorescence of MBA in absence and presence of added substances respectively; $i_0 = [(I_0/I) - 1]$ in absence, and $i_n = [(I_0/I) - 1]$ in presence of 1.7 mM NA; [NP]concentration of noradrenalin; A) concentration of NON (1) or IMI (2).

quencher; K_q the quenching constant. In that case, quenching is described by equations (1-3):

$$r + C = A \quad (1); \qquad \frac{r}{C(N-r)} = K_b \quad (2);$$

$$K_q \cdot r \cdot \frac{b}{N} = i, \quad (3)$$

where A is the concentration of the quenching agent, C the concentration of quenching agent not bound with the membrane, and K_b the binding constant of the quenching agent with the membrane. The value of K_b was determined by the method of double reciprocal coordinates [2]. The value of N was measured by carrying out determinations at two different concentrations of membrane (N_1 and N_2) and an equal concentration of quenching agent (A). In that case, according to (1) and (2):

$$N_1 K_b (A - r_1) = r_1 (K_b A + 1) - K_b r_1^2, \quad (4)$$

$$N_1 q K_b (A - r_2) = r_1 (K_b A + 1) - K_b r_2^2, \quad (5)$$

where $q = N_2/N_1$. According to (3): $r_1/r_2 = i_1/i_2 \cdot 1/q$. (6).

Substitution of (6) in (4) and (5) gives an equation for determining r_2:

$$K_b + \frac{1}{A - r_2} = \frac{i_1}{i_2} K_b + \frac{1}{A - r_2 \frac{i_1}{i_2} \cdot \frac{1}{q}}, \quad (7)$$

and substitution of r_2 in (2) gives the value of N_2.

EXPERIMENTAL RESULTS AND DISCUSSION

NON and IMI lowered the intensity of fluorescence of the probe and shifted the maximum of fluorescence of MBA in the lysosomes (Fig. 1). The effect of NA was much weaker. The position of the maximum of fluorescence of MBA in the liposomes (530 nm) is evidence that the probe was located in a polar environment close to the membrane surface. This follows from comparison of the fluorescence spectra of MBA in solvents with different polarities. The maximum of fluorescence of MBA in the liposomes was similar to that in the polar alcohols and it differed appreciably from the fluorescence spectrum in nonpolar heptane. Since NON and IMI quench the fluorescence of MBA, which was located in the surface layer of the membrane, presumably the substances were bound to the membrane and also were located in its surface layer.

By using the quenching data it was possible to determine the binding constants (K_b) of the preparations with liposomes (Fig. 2). Since a shift of the maximum of fluorescence took place under the influence of the drugs, the total light energy of the fluorescence spectrum of MBA was subsequently recorded. Approximate values of K_b were determined (intercepts on the horizontal axis), into which corrections were introduced [2]. The values of K_b thus obtained were as follows: $(4.3 \pm 0.3) \cdot 10^3$ M^{-1} for NON, $(2.7 \pm 0.2) \cdot 10^3$ M^{-1} for IMI, and $(0.7 \pm 0.15) \cdot 10^3$ M^{-1} for NA.

To determine the number of binding sites for NON in the membrane, quenching of the fluorescence of the probe was measured in a concentration of NON of 0.12 mM and a concentration of liposomes of 0.2 and 0.4 mg/ml, giving values of $i_1 = 0.13$ and $i_2 = 0.1$. Substitution of these values in equations (1-7) showed that for two molecules of phospholipids there was one NON binding site.

Both IMI and NON, on the one hand, and NA on the other hand, could thus bind with the membrane of the liposomes, but NON and IMI had much greater affinity for the membrane than NA. It could accordingly be postulated that the two substances could compete with NA for the binding sites with the membrane. To test this hypothesis, NON and IMI were added to liposomes in the absence and in the presence of 1.7 mM NA. When plotted in Hunter and Downce coordinates [7], the results fell on straight lines having a certain slope, and intercepting a segment on the vertical axis equal to the reciprocal of K_q for NA. All this, according to the method of Hunter and Downce [7], is evidence in support of competition between NON and IMI, on the one hand, and NA on the other hand for the same binding sites on the membrane. NON and IMI thus therefore effectively prevent NA from binding with the membrane. If such competition between NA and the other substances studied in fact exist not only in the model membrane, but also in axonal and granular membranes, the relationships described above can be regarded as a possible mechanism of the blocking of reverse NA transport, a characteristic feature of the substances studied.

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EFFECT OF HEPARIN ON LIPID PEROXIDATION REACTION OF ERYTHROCYTES AND THEIR RESISTANCE

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In a physiological concentration (10 units/ml) heparin activates the ascorbate-dependent lipid peroxidation of lipids and reduces their resistance in citrate–phosphate buffer, pH 3.0. In a concentration of 100 units/ml heparin does not affect thermal (62°C) oxidation of methyloleate; this is evidence that heparin is not a direct-action prooxidant.

KEY WORDS: heparin; erythrocytes; peroxidation of lipids.

Heparin has been shown to participate in the humoral regulation of metabolism as a universal inhibitor of many enzymic processes [6, 12, 17]. The biological effect of heparin is due to its polyanionic structure and its marked ability to form complexes. When adsorbed on cell membranes heparin essentially changes their physicochemical properties, and thereby regulates the permeability of the membranes, their charge, and the cell metabolism [13, 14, 16]. According to some workers, heparin increases the osmotic and reduces the acid resistance of erythrocytes [7]; other workers conclude that it destabilizes cell membranes, evidently by activating phospholipase A_1 [20]. This enzyme removes the free fatty acids from structural phospholipids, and in conjunction with its actions on permeability and the charge, this may lead to activation of lipid peroxidation processes (LPP). LPP are known to play an important role in the regulation of the physicochemical properties of membranes under normal and pathological conditions [3, 4, 8]. A stationary level of LPP in the body is essential for its normal function and it is controlled by a whole series of factors [5], including hormones [19]. According to the present writers, LPP can be controlled by heparin, which activates the lipolipid system [18], on the one hand, and blocks glutathione reductase activity on the other hand [1].

The object of the present investigation was to study the possible effect of heparin on LPP of erythrocytes and their stability and also the anti- and prooxidant properties of heparin.

EXPERIMENTAL METHOD

Blood was taken from fasting donors into sodium oxalate and washed twice with physiological saline. Heparin (Richter) was first purified with ether. The erythrocytes were incubated for 10 min at 37°C with 10

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