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DRUG-INDUCED SURFACE POTENTIAL CHANGES OF LIPID VESICLES AND THE ROLE OF CALCIUM*

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Abstract—The effects of four different drugs with local anesthetic properties were investigated on the surface potential of phospholipid vesicles composed of electrostatically neutral lipids (phosphatidylcholine), negatively charged lipids (phosphatidylserine) and a mixture of acidic and neutral lipids (soy bean lipids). Propranolol, tetracaine, lidocaine and procaine decrease the negative surface potential of phosphatidylserine and soy bean liposomes and increase that of phosphatidylcholine liposomes. The drugs interact with the liposomes in such a way that the protonated amine groups point towards the polar head groups of the phospholipids and the rest of the molecule is probably incorporated into the hydrophobic core of the lipid bilayer. The same sequence in drug activity normally measured in biological tissues (propranolol > tetracaine > lidocaine > procaine) is found for the surface potential change of the phospholipids. Calcium prevents the binding of the drugs to phosphatidylserine, especially the binding of lidocaine and procaine. Because of its high affinity for negative surface charges, Ca^{2+} chelates with phosphatidylserine and blocks the incorporation of the drug molecule. Vice versa, when incorporated into the liposomal bilayer, the drug blocks the interaction of calcium. These antagonistic effects are only observed in liposomes made from acidic phospholipids and not in those made from pure electrostatically neutral lipids like phosphatidylcholine.

Calcium and cardiodepressant drugs, like some local anesthetics and β-blocking agents, are known to be antagonists in heart muscle. Negative inotropic drug action is overcome by increased calcium. The molecular mechanism is still a matter of debate. The hypothesis of competitive antagonism is strongly supported by postulating a common binding site for both calcium and drugs with local anesthetic properties. Different suggestions have been made to explain the antagonistic effect of Ca^{2+} and local anesthetics in nerves. While several authors propose an antagonistic behavior [1–4], Narahashi *et al.* [5] recently discussed two independent actions of Ca^{2+} and local anesthetics in squid axon membranes (for review see also Ref. 6). Calcium in nerves plays a different physiological role than in heart muscle, where it is the fundamental ion of excitation–contraction coupling. As pointed out by Langer [7, 8] the force of contraction in the heart is strongly dependent on the physical state of the glycocalyx, a thin surface coat, surrounding the heart cell. This glycocalyx, attached to the lipid bilayer of the membrane, consists mainly of glycolipids and glycoproteins, which serve as binding sites for Ca^{2+} with their sialic acid residues. Calcium is first bound to these sites before it crosses the membrane via the slow channel or via a carrier system to trigger intracellular Ca^{2+} release from the sarcoplasmic reticulum. Besides glycolipids and glycoproteins the heart cell membrane contains a variety of phospholipids, among which negatively charged lipids like cardiolipin, phosphatidylinositol and phosphatidylserine may also serve as binding sites for bivalent ions. Such binding has been extensively studied in artificial membrane systems like phospholipid bilayers and liposomes [9–19]. Bivalent ion binding is antagonized by cationic amine anesthetics [20–26].

We recently reported that tertiary amine local anesthetics and a β-blocker with local anesthetic activity when interacting with liposomes strongly change the surface potential of such particles at acidic, neutral and alkaline pH [27]. When composed of electrostatically neutral lipids (phosphatidylcholine) the surface potential of the liposomes is increased (made more positive) by these drugs and when the liposomes contain negatively charged lipids the negative surface potential is decreased. The results clearly demonstrate that positively charged amine drugs interact with the membrane surface of phospholipid vesicles. Since ion binding also occurs at the membrane surface, local anesthetics and Ca^{2+} should show antagonistic behavior, if competing for the same binding site. In order to determine the antagonistic properties of some drugs with local anesthetic activity, the surface charge of differently charged liposomes was measured with the newly developed method of laser Doppler electrophoresis.

MATERIALS AND METHODS

Highly purified egg yolk phosphatidylcholine was kindly donated by L. Michaelis (Department of Biochemistry, University of Hull, U.K.). Phosphatidyl-*l*-serine from bovine brain was purchased from Koch-Light Laboratories. Phosphatidylserine but not phosphatidylcholine was slightly contaminated with calcium. In the suspensions used for the electrophoresis experiments, a Ca^{2+} concn of about 15 μM has been detected by atomic absorption spectrophotometry. The precise determination of the Ca^{2+} was difficult because of the unknown

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percentage of ionized calcium strongly complexed with phosphatidylserine. The buffer solution did not contain any detectable amount of calcium.

The liposomes were prepared by evaporating the organic solvent under vacuum and by adding the appropriate buffer solution (1 mM KCl, 1 mM glycine, pH 7) to the N_2-dried phospholipids. The suspensions were hand shaken for 5 min before sonication in a probe-type sonicator (Branson Sonifier B-15, 3 mm tip dia.) at a nominal frequency of 20 kHz. The samples were sonicated for different periods of time, depending on the concn of calcium in the buffer solutions. One hour was needed for the phospholipid suspension containing 1 mM $CaCl_2$ whereas for EDTA-treated samples sonication for 3 min was sufficient to get a transparent suspension. After a final adjustment of pH with KOH, the samples were directly used for the measurements of electrophoretic mobilities. The size of the liposomes was determined by dynamic laser light scattering techniques confirming earlier results [27]. An average particle radius (a) of 1700 ± 600 Å has been calculated. A certain percentage of multilamellar liposomes cannot be excluded in these preparations. This is of minor importance for studies of drug effects, as in electrophoresis the charge density at the surface of spherical particles is related to the electrophoretic mobility, which is essentially independent of size, as pointed out by Wiersema *et al.* [28].

The lipid concn was 0.33 mg/ml in all experiments. The mobility of liposomes was determined by electrophoretic laser light scattering (LAZYPHER, BTG) using a 15 mW He–Ne laser and the standard electrophoretic chamber with a small capillary of 0.5 mm inner dia. and 20 mm length. The applied electric field was adjusted to 50 V/cm. The scatttered and frequency shifted laser light was detected in the heterodyne mode by a photomultiplier and the intensity fluctuations analyzed by a spectrum analyzer (Intertechnique). According to the relation $2\pi f_D = \vec{\mathbf{K}}\mu\vec{\mathbf{E}}$, the Doppler frequency (f_D) is directly proportional to the particle mobility (μ) ($\vec{\mathbf{K}}$ is the scattering vector and $\vec{\mathbf{E}}$ the applied electric field). From the mobility values it is possible to calculate the surface potential of the liposomes and their effective surface charge density applying the Gouy–Chapman theory. For conversion of the mobility to the zeta potential a value of $f(\kappa a) = 1.32$ for Henry's function has been introduced into the equations. This value is based on an average particle radius of 1700 Å and a buffer molarity of 2 mM. Experiments were performed at room temp (21°). All further experimental and theoretical details are described in the literature [27, 29, 30].

The following drugs were used: (±)-propranolol hydrochloride (Ayerst), tetracaine hydrochloride (Hoechst), lidocaine hydrochloride (Asta) and procaine hydrochloride (Hoechst).

RESULTS

In a first set of experiments liposomes from phosphatidylcholine were prepared in 1 mM KCl, 1 mM glycine and for comparison in 1 mM $CaCl_2$, 1 mM glycine. Then the samples were titrated with the different drugs and the electrophoretic mobility of the liposomes measured as a function of drug concn. The results are summarized in Fig. 1. The electrophoretic mobility, and also the zeta potential (initially zero because of the electrostatic neutrality of the phosphatidylcholine at pH 7), are increased with increasing drug concn. The drugs adsorb to the liposomes, adding positive charges (from the protonated secondary or tertiary amine group) to its surface. The strongest effects are observed with propranolol, moderate effects with tetracaine and only very small effects at high concns of lidocaine and procaine. Calcium has only a very small influence on the drug effect.

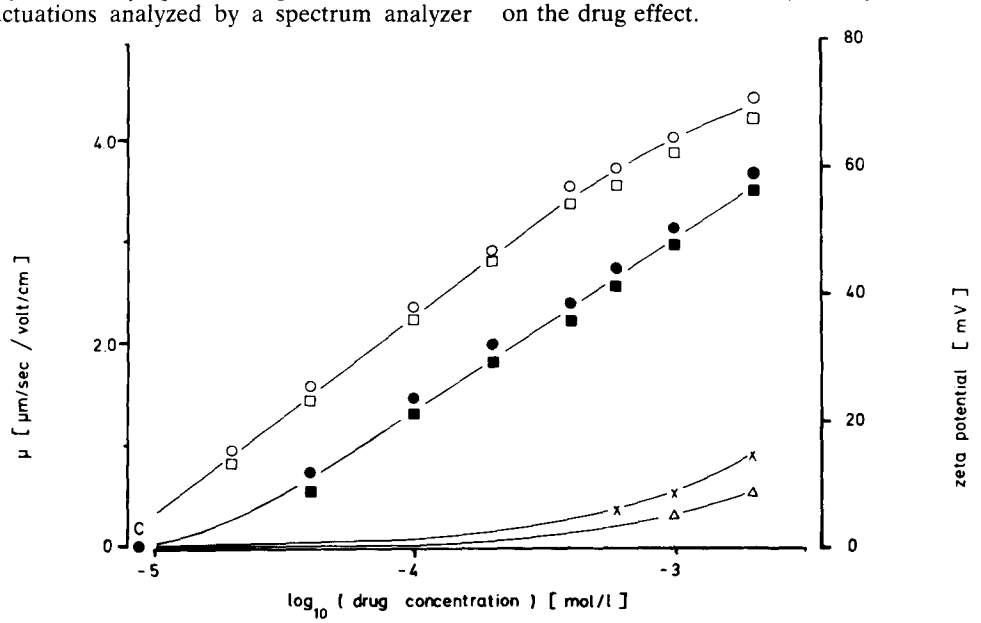

Fig. 1. Electrophoretic mobility (left ordinate) and zeta potential (right ordinate) of liposomes made from phosphatidylcholine as a function of drug concn: (○) propranolol, (●) tetracaine, (x) lidocaine, (△) procaine. Buffer medium: 1 mM KCl, 1 mM glycine, pH 7. □ and ■ indicate experiments with propranolol and tetracaine in a buffer medium where 1 mM KCl was replaced by 1 mM $CaCl_2$. Lipid concn = 0.33 mg/ml. C = control.

In a second set of experiments liposomes from phosphatidylserine, a phospholipid with a net negative charge at pH 7, were prepared in buffer solutions composed of 1 mM KCl, 1 mM glycine and different concns of EDTA or $CaCl_2$ (Fig. 2a–d). Addition of EDTA increases the mobility, i.e. the surface potential, indicating a complexation of divalent ions from the lipid surface. Ca^{2+} on the other hand strongly decreases the mobility of phosphatidylserine liposomes. Propranolol when added to the liposomes decreases the negative surface charges; consequently the electrophoretic mobility decreases. The effects are most pronounced in buffer solutions containing EDTA and are very weak at high calcium concns (Fig. 2a).

Similar results are obtained with tetracaine (Fig. 2b), although compared to propranolol the effects are smaller. When the phosphatidylserine liposomes are prepared in pure KCl, glycine (1 mM each) in the absence of EDTA and $CaCl_2$, small concns of propranolol or tetracaine cause an increase in the electrophoretic mobility, which is probably due to a displacement of divalent ions from the liposome surface. This effect is not observed at higher Ca^{2+} concns.

Lidocaine and procaine (Fig. 2c and d) do not change the mobility of calcium-treated phosphatidylserine liposomes. In pure 1 mM KCl, 1 mM glycine the mobility is slightly less. Clear effects are observed in the presence of EDTA. Lidocaine and

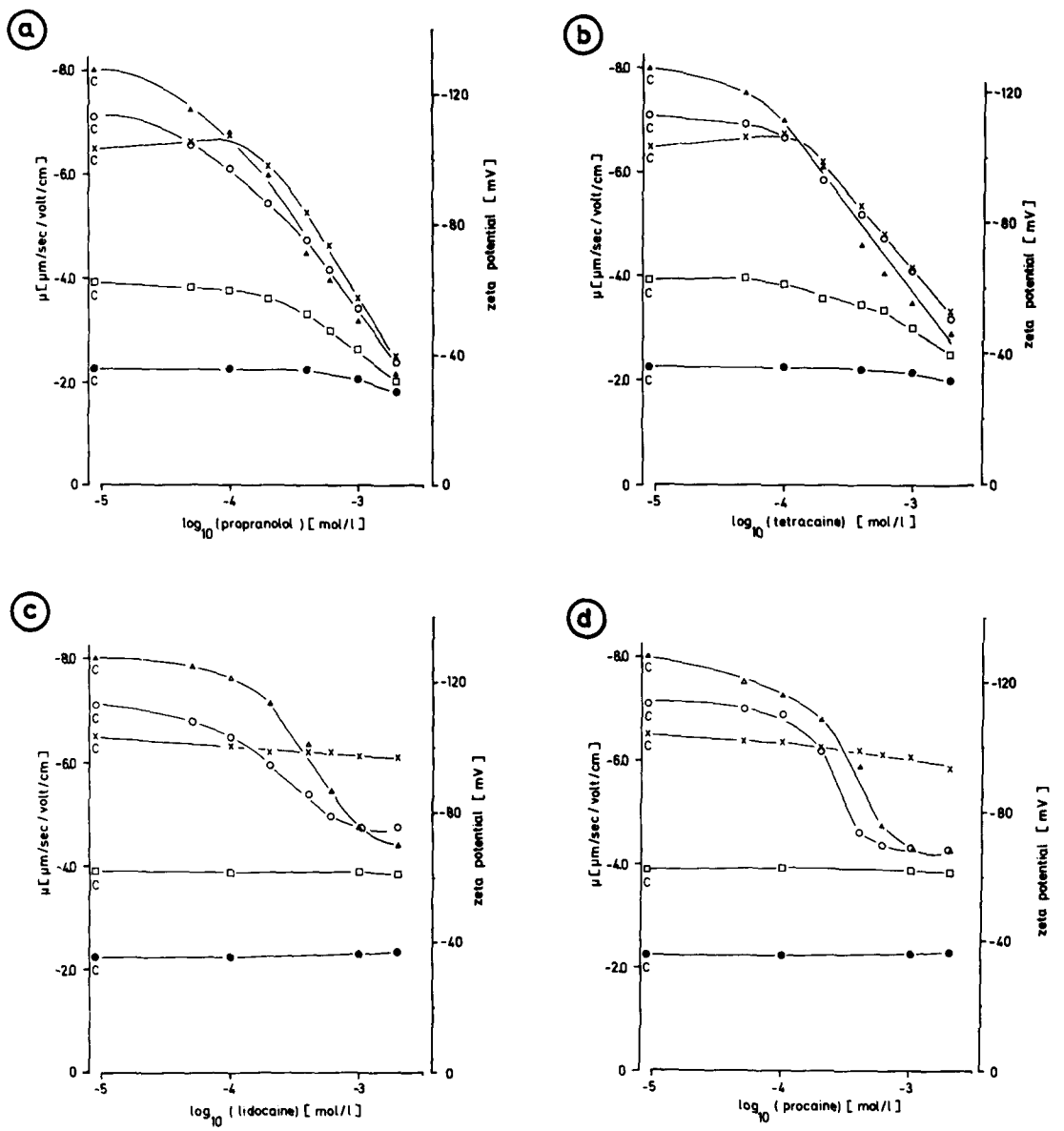

Fig. 2a–d. Electrophoretic mobility (left ordinate) and zeta potential (right ordinate) of liposomes made from phosphatidylserine as a function of drug concn: (a) propranolol, (b) tetracaine, (c) lidocaine, (d) procaine. The buffer medium contained, in addition to 1 mM KCl, 1 mM glycine, pH 7: (△) 0.3 mM EDTA, (○) 0.2 mM EDTA, (□) 0.1 mM $CaCl_2$, (●) 1 mM $CaCl_2$. x indicates a control curve in 1 mM KCl, 1 mM glycine alone. Lipid concn = 0.33 mg/ml. C = control.

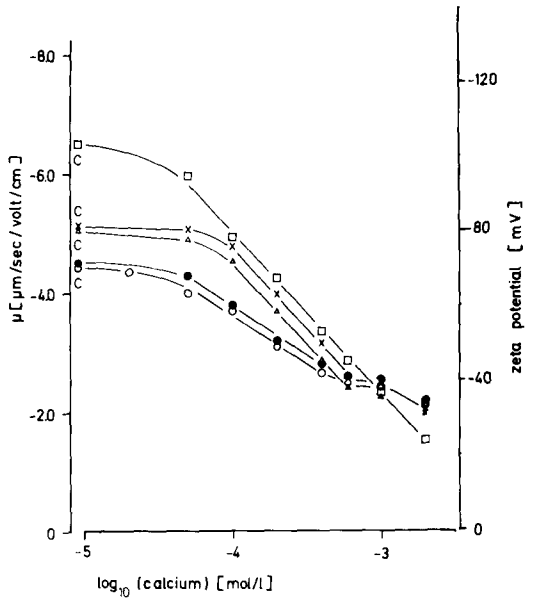

Fig. 3. Electrophoretic mobility (left ordinate) and zeta potential (right ordinate) of liposomes made from phosphatidylserine and prepared in the presence of different drugs as a function of calcium concn. The buffer medium contained, in addition to 1 mM KCl, 1 mM glycine, pH 7: (○) 0.4 mM propranolol, (●) 0.4 mM tetracaine, (x) 1 mM lidocaine, (△) 1 mM procaine, (□) untreated liposomes in 1 mM KCl, 1 mM glycine, pH 7. Lipid concn = 0.33 mg/ml. C = control.

procaine now decrease the electrophoretic mobility by compensating negative surface charges. Saturation is reached at about 1 mM drug concn.

In a third set of experiments liposomes from phosphatidylserine were prepared and sonicated in buffer solutions (1 mM KCl, 1 mM glycine) already containing different concns of the drugs. The change in the electrophoretic mobility is then measured as a function of calcium concn. The results are plotted in Fig. 3. For comparison, the effect of Ca^{2+} on untreated liposomes is also shown. Calcium decreases the electrophoretic mobility, and hence the surface potential, of all the liposomes. The effect is strongest in untreated liposomes, moderate in liposomes containing lidocaine and procaine and weakest in liposomes containing propranolol and tetracaine.

When the experiments are performed with liposomes prepared from a phospholipid mixture (electrostatically neutral and negatively charged phospholipids), similar results are obtained to those with liposomes from phosphatidylserine alone. Calcium inhibits the effects of tetracaine and propranolol (Fig. 4) and, vice versa, the drugs when incorporated into the liposomes inhibit the effect of Ca^{2+} (Fig. 5).

DISCUSSION

From the mobility values of phosphatidylserine liposomes in 1 mM KCl, 1 mM glycine, 0.3 mM EDTA under control conditions an effective surface charge density (σ) of 1 e^-/505 Å^2 is calculated. This value is too low in comparison to σ = 1 e^-/60 Å^2 for a phospholipid with one net negative charge; this value is supposed to be the surface area of one phosphatidylcholine molecule [31, 32]. Several reasons may account for the discrepancy. (1) A small amount of divalent ions is still bound to the phospholipid and is not complexed by EDTA. (2) EDTA itself exerts an effect on the surface charge via complexed Ca^{2+}. (3) The zeta potential corresponds to the potential at the plane of shear and not necessarily to the potential at the surface of the vesicle. The plane of shear, however, is located a few Ångstroms away from the lipid surface, where the electrostatic potential has already dropped significantly. This normally leads to an underestimation of charge density. (4) The Gouy–Chapman theory does not hold for high surface potentials. This is the central issue of the current discussion which is also stressed in the paper of O'Brien and White [33] who recalculated the forces acting on a particle in electrolyte solution under electrophoretic conditions. According to their calculations the relation between the zeta potential and mobility is linear only for low zeta potentials ($\zeta < 50$ mV). For high zeta potentials μ reaches a maximum value and then decreases for $\zeta > 125$ mV and $\kappa a > 3$ in the μ (ζ, κa) diagram. These findings are interpreted the following way: electrophoretic retarding forces resulting from the counter ion cloud around the particle increase at a faster rate with ζ than does the driving force. Consequently the measured mobility values for high zeta potentials (as is the case for pure phosphatidylserine liposomes) are less than expected and cannot be taken for an exact calculation of charge densities.

The scope of this paper is not to prove the validity of Gouy–Chapman theory (this will be the topic of a separate paper) but to show in comparative measurements [34] the different drug actions in the presence of calcium at distinct concns.

The results demonstrate the influence of the four drugs on the surface potential of electrostatically neutral and negatively charged liposomes. Drug interaction with liposomes was tested by measuring liposome mobility at different ionic concns. Results should be different for drug binding or when only screening effects occur. Raising the concn of KCl from 1 to 5 mM caused a reduction of the mobility from 8 to 7 μm/sec/V/cm. At 50 mM KCl a mobility of μ = 5.63 μm/sec/V/cm was measured. In comparison, a mobility change from 8 to 7 is achieved by a concn of 70 μM propranolol, indicating that this drug is 70 times more potent in reducing mobility than is an increase in electrolyte concn. The drug effects are also stronger in phosphatidylserine liposomes ($\Delta\zeta$ = 77.9 mV for 1 mM propranolol in comparison to $\Delta\zeta$ = 64.3 mV for phosphatidylcholine) because negative surface charges favour the interaction of the positively charged amine drugs. The sequence of the four drugs according to their effectiveness corresponds to that found in nerves (blockade of nerve conduction) [20] or in heart tissues (negative inotropic effect) [35]. Calcium ions do not bind to liposomes made from phosphatidylcholine and hardly interfere with the interaction of the drugs with this phospholipid (Fig. 1). The slightly decreased drug effects in the presence of calcium can be explained by a screening effect of this ion on the lipid surface. When the liposomes are made from

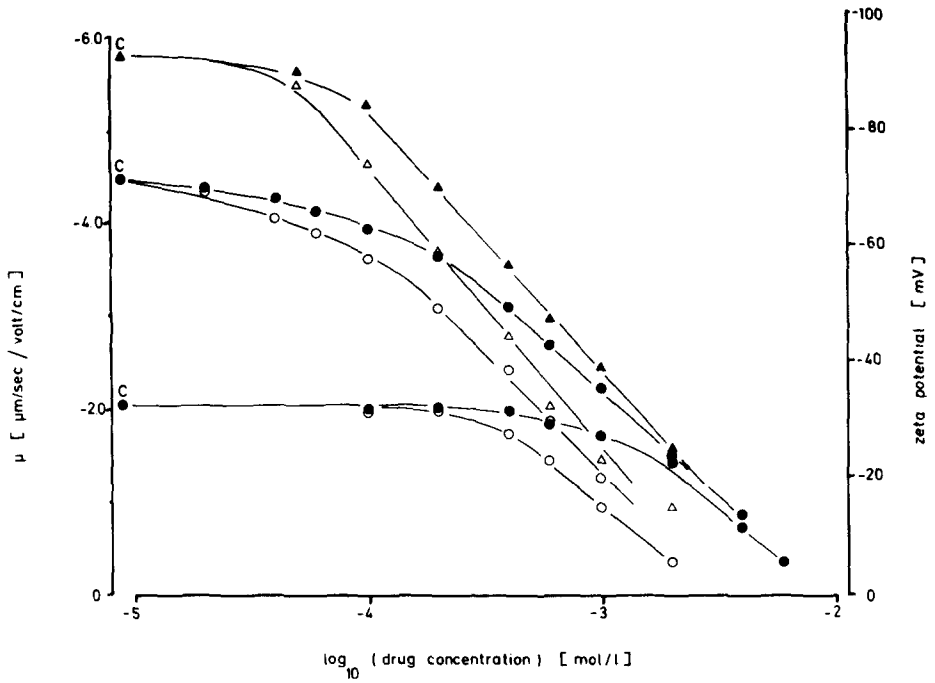

Fig. 4. Electrophoretic mobility (left ordinate) and zeta potential (right ordinate) of liposomes made from soy bean lipids as a function of drug concn. The experiments were performed in 1 mM KCl, 1 mM glycine, pH 7: (△) propranolol, (▲) tetracaine; and in buffers containing additional $CaCl_2$ (25 μM, control $\mu = 4.47$; and 1 mM, control $\mu = 2.03$): (○) propranolol, (●) tetracaine.

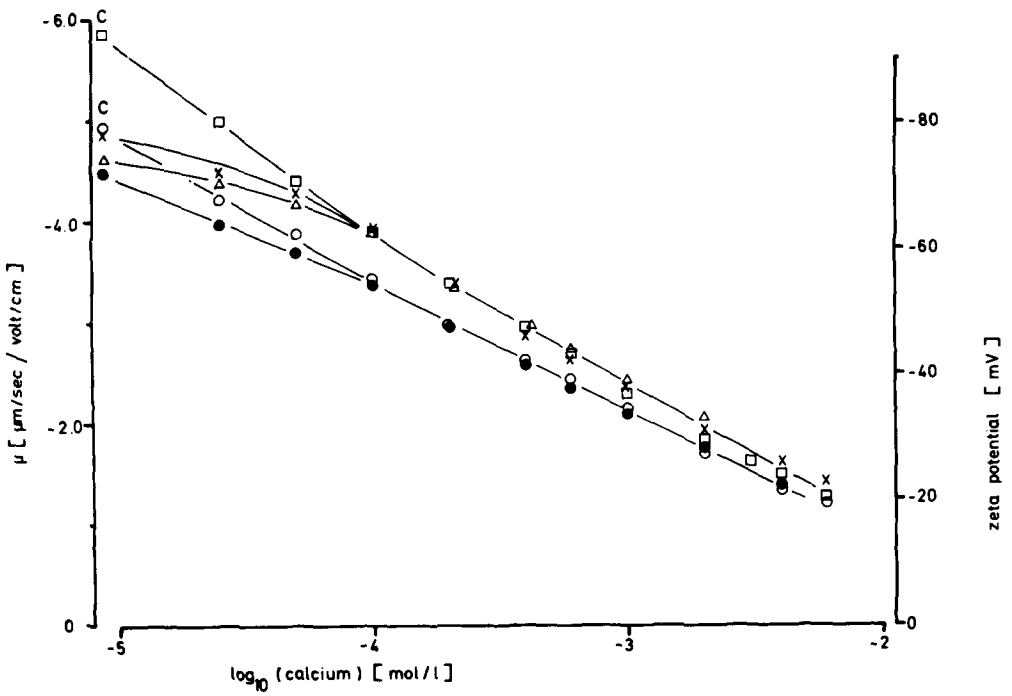

Fig. 5. Electrophoretic mobility (left ordinate) and zeta potential (right ordinate) of liposomes made from soy bean lipids and prepared in the presence of different drugs as a function of $CaCl_2$ concn. The buffer medium contained in addition to 1 mM KCl, 1 mM glycine, pH 7: (○) 50 μM propranolol, (●) 50 μM tetracaine, (x) 1 mM lidocaine, (△) 1 mM procaine, (□) untreated liposomes in 1 mM glycine, pH 7. Lipid concn = 0.33 mg/ml. C = control.

negatively charged phospholipids (phosphatidylserine or soy bean lipids), Ca^{2+} antagonizes the drug effect. Calcium is bound to the lipid surface and prevents the incorporation of the drugs. The antagonizing effect of Ca^{2+} is not the result of reduced negative surface charges. It is evident from Fig. 1 that the drugs also interact strongly with liposomes made from electrostatically neutral phosphatidylcholine. Furthermore, at a Ca^{2+} concn of 1 mM the drug effect is almost completely suppressed although there are still negative surface charges on the liposome. There is evidence that calcium chelates with two neighbouring phosphatidylserine molecules [36] or with four [9]. A possible explanation of the Ca^{2+} antagonism is that calcium occupies the intermolecular space between the phosphatidylserine molecules preventing the incorporation of the hydrophobic groups of the drugs into the hydrophobic region of the lipid bilayer.

Similar results are obtained in those experiments where the liposomes are prepared in the presence of the drug and then titrated with Ca^{2+}. The same interpretation holds for these results: the drug is incorporated between the phosphatidylserine molecules in such a way that its hydrophobic molecular group is located between the hydrocarbon chains and its protonated amine group points towards the hydrophilic polar region of the bilayer. Ion–ion interactions or proton bridges between the protonated amine group of the drug and the oxygen of the phosphate or the carboxylic group of phosphatidylserine lead to a compensation of negative surface charge and to a decrease in binding sites for calcium.

The linear dependence (Fig. 2a–d) of liposome mobility upon the log of drug concn demonstrates that no exchange mechanisms (one drug molecule to one Ca^{2+} or two drug molecules to one Ca^{2+}) are taking place. In such a mechanism a step-like dependence should be observed at a certain concn (replacement of one calcium by two amine molecules yields the same charge on the liposome surface and no further decrease in charge density). The amount of drug molecules in the liposome surface can be calculated from the change in surface potential. For phosphatidylserine liposomes in the absence of Ca^{2+} the drug density is calculated as 1 per 24 lipid molecules for 1 mM propranolol, 1 per 27 lipid molecules for tetracaine and for lidocaine and procaine as about 1 per 37 lipid molecules for 1 mM drug concn using 60 $Å^2$ per lipid molecule. This calculation, however, only takes into account the protonated drug molecules. Because of its amphipathic character a certain percentage of the free base form of the drugs is also dissolved in the hydrophobic interior of the lipid layer.

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