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Changes of the membrane potential profile induced by verapamil and propranolol

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Abstract

The effects of the organic calcium channel blocker verapamil and the β-receptor blocker propranolol on dipole (ϕ_d) and surface (ϕ_s) potentials of bilayer lipid membranes were studied. The boundary potentials ($\phi_b = \phi_d + \phi_s$) of black lipid membranes, monitored by conductance measurements in the presence of nonactin and by capacitive current measurements were compared with ϕ_s calculated from the electrophoretic mobility of lipid vesicles. It was shown that the increase of boundary potential, induced by the adsorption of the positively charged propranolol, was caused solely by an increase in surface potential. Although ϕ_s also increases due to the adsorption of verapamil, ϕ_b diminishes. A sharp decrease of the dipole potential was shown to be responsible for this effect. From Langmuir adsorption isotherm the dissociation constant K_d of verapamil was estimated. The uncharged form of verapamil ($K_d = (0.061 \pm 0.01)$ mM at pH 10.5) has a tenfold higher affinity to a neutral bilayer membrane than the positively charged form. The alteration of membrane dipole potential due to verapamil adsorption may have important implications for both membrane translocation and partitioning of small or hydrophobic ions and charged groups of membrane proteins.

Keywords: Lipid bilayer; Dipole potential; Surface potential; Liposome; Verapamil; Propranolol

1. Introduction

Verapamil (Fig. 1) is a calcium channel blocker and a well-established drug in the treatment of angina pectoris, cardiac arrhythmia, cardiomyopathies, hypertension [1] and migraine [2]. It is believed that the main site of its highly specific action is the voltage-dependent calcium channel in two tissues: the cardiac cell membrane and vascular smooth muscle [1,3]. Reducing intracellular free calcium concentration, verapamil causes coronary and peripheral vasodilation and depresses myocardial contractility and electrical activity in the atrioventricular and sinoatrial nodes [1]. In the last years the importance of verapamil as multidrug resistance (MDR) modulator is increased [4]. It is assumed that the mechanism of MDR reversal is based on inhibition of P-glycoprotein mediated drug efflux by binding at the enzyme at a different binding site or direct competition for drug efflux. Alteration of active as well as passive transport of an anti-cancer drug in the presence of verapamil was found [5–7]. Propranolol (Fig. 1) is a β-receptor blocker and is used in heart disease treatment similar to verapamil.

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The exact molecular mechanism whereby these two agents work is still unknown; the studies have focused on the characterization of binding sites [8] or on the effect on active anti-cancer drug efflux (for verapamil). The effects of these drugs on intrinsic membrane potentials were not investigated yet. Both substances are also frequently used in experiments in vitro as selective channel or receptor inhibitors. However, it was suggested earlier [9] that they are able to alter the potential distribution across biological membranes.

The existing model of a potential profile across the water-membrane boundary supposes the consideration of at least two parts of boundary potential ϕ_b. The biological significance and the origin of the surface potential (ϕ_s) is well known [10]. Lipid bilayers possess moreover a large membrane potential, positive inside, the so-called dipole potential (ϕ_d). It was found to be approx. 280 mV in phosphatidylcholine membranes [11,12]. This component of the total membrane potential arises from dipoles located, for the most part, in the transition region between the aqueous phase and the hydrocarbon-like interior of the membrane [13,14]. ϕ_d seems, however, to play a very important role because it strongly affects binding and transport of ions [15,16]. Consequently, caution is required when indicator substances are used as probes of electrical events in cells or model membrane systems. Contribution of the dyes themselves to the magnitude of membrane potentials must be excluded [17,18].

The aim of this study was to investigate the changes of the potential distribution across the membrane boundary induced by verapamil or propranolol with special attention to possible alterations of the dipole potential. The results of three different methods (membrane capacitance minimization technique, conductance measurements in the presence of nonactin and ζ-potential measurements) were critically compared. The first and second methods allow to determine the drop $\Delta\phi_b$ of the potential difference between the adsorption surface and some point in the bulk. With the last method only the potential at the surface of shear, the so-called zeta-potential, ζ, is measured. Our results indicate that the adsorption plane of verapamil is localized deeper than that of propranolol.

2. Materials and methods

2.1. Chemicals

Diphytanoylphosphatidylcholine (DPhPC) and diphytanoylphosphatidylserine (DPhPS) were obtained from Avanti Polars Lipids (Alabaster, AL, USA). Lipids were stored at −70°C and used without further treatment. For the preparation of buffer solutions Tris (Fluka, Buchs, Switzerland), MES (4-morpholineethanesulfonic acid; Boehringer, Mannheim, Germany), CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid; Sigma, St. Louis, MO) and KCl (Fluka, Buchs, Switzerland) were used. Verapamil hydrochloride and propranolol hydrochloride were obtained from Fluka (Buchs, Switzerland).

2.2. Formation of bilayer lipid membranes

The bilayer lipid membranes (BLMs) were formed by the conventional Mueller-Rudin technique in holes, 0.5–1 mm in diameter, of a diaphragm of a polytetrafluoroethylene (PTFE) chamber [19]. The membrane-forming solution contained 20 mg DPhPC or DPhPS, dissolved in 1 ml of *n*-decane (Merck, Darmstadt, Germany). The bilayers surrounding solution contained typically 20 mM Tris, 20 mM MES, 20 mM CAPSO and 10 or 100 mM KCl. It was agitated by magnetic bars. The experiments were carried out at room temperature (23–25°C).

Stock solutions of verapamil or propranolol were added bi- (conductance measurements) or unilaterally (capacitance minimization technique).

2.3. Preparations of liposomes

The procedures for preparing phospholipid liposomes were similar to those described in the literature [20]. DPhPC or DPhPS was dissolved in chloroform. After removing the solvent by evaporation, a thin film of a lipid on the wall of a round bottom flask was formed. An appropriate volume of the buffer (20 mM Tris, 20 mM MES, 20 mM CAPSO and 100 mM KCl) was then added into the flask and the solution was vortexed for 3 min. The resulting multilamellar vesicles were sonicated in order to re-

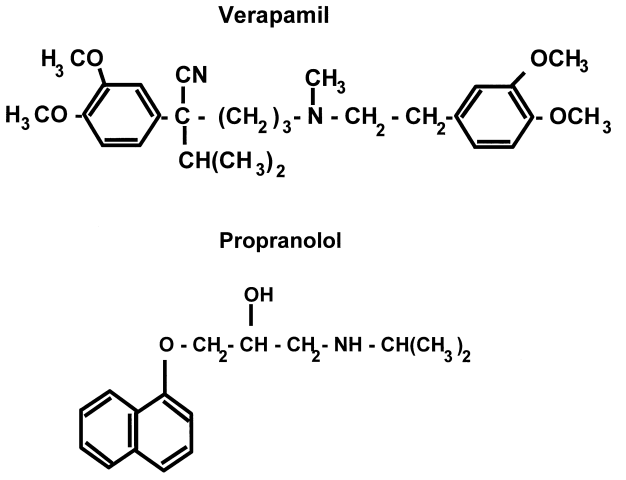

Fig. 1. Chemical structure of the studied drugs.

duce vesicle dimensions. The diameter of liposomes, a, was determined from light scattering, using Coulter DELSA 440. Typically, it was equal to 300 nm.

2.4. Conductance measurements

Current-voltage relationships were measured with a current amplifier (Model 428, Keithley Instruments, Cleveland, OH, USA) using the built-in voltage source. The effect of verapamil and propranolol on the nonactin-induced conductance was used to study their adsorption behavior at different pH values [21]. The change of ϕ_b was deduced from the relation of the initial conductance G_0 and the conductance G in presence of substances studied [22].

$$\frac{G}{G_0} = \exp\left[\frac{zF\phi_b}{RT}\right] \quad (1)$$

2.5. Monitoring of the boundary potential drop between the lipid layers using the inner field compensation method (IFC)

The method is based on the dependence of the capacitance on the inner membrane field, i.e., the electric potential gradient within the membrane, and allows to measure the potential difference between the boundaries of a bilayer [23–26]. Membrane capacitive current contains a signal harmonic to the applied fundamental frequency (418 Hz) which vanishes if a DC signal coinciding with the boundary potential difference is transferred to the reference electrodes additionally to the AC signal. The magnitude of the DC signal V_c is given directly by

$$V_c = -(\Delta\phi_s + \Delta\phi_d) \quad (2)$$

where $\Delta\phi_s$ is the difference in surface potentials between the two bilayer surfaces and $\Delta\phi_d$ is the dipole potential difference between the two monolayers.

Both drugs studied are weak bases with pK_a values between 8 and 9. It was shown previously that the permeation of weak acids and bases is accompanied by the formation of a transmembrane pH gradient [27]. Passing in their uncharged form across the membrane, verapamil and propranolol dissociate at one side of the membrane and associate with a proton at the other side (Fig. 2). A local pH shift in the unstirred layers is produced [28]. Diminishing the steady state concentration of the neutral drug, it counteracts the diffusion. In our experiments, we have preestablished a large, similar directed pH difference across the membrane to ensure that a negligible transmembrane diffusion occurs [29]. In this case, the measured potential difference is identical with the boundary potential drop of one monolayer due to the adsorption of substances on the lipid-solution boundary.

2.6. ζ-Potential measurements

Measurements of the electrophoretic mobility of

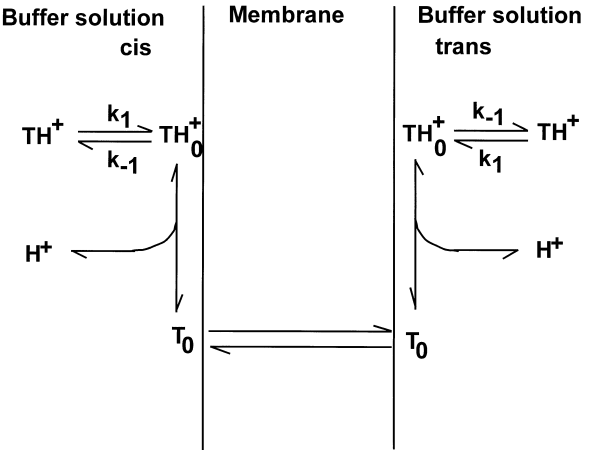

Fig. 2. Membrane transport of verapamil and propranolol. Only the neutral form of both substances, T_0, is able to diffuse across the bilayer.

liposomes were carried out with the Coulter DELSA 440 (Langley Ford Instruments, Coulter Electronics of New England). The velocity of liposome movement in an electrical field was deduced from the Doppler shift of a scattered laser beam.

In order to determine the ζ-potential from the electrophoretic mobility we have used Eq. 3, derived by Smoluchowsky [30]:

$$\zeta = \frac{\eta U}{\varepsilon\varepsilon_0} \tag{3}$$

where U is the electrophoretic mobility of liposomes, η is the viscosity of the solution and ε is the dielectrical constant of the medium. This equation is applicable for values of $\kappa a \gg 1$, where κ is the Debye constant (Eq. 6) and a is the diameter of liposomes [31]. Additionally, we have made control experiments measuring the ζ-potential of PC/PS liposomes at different concentrations of the electrolyte as described in [32]. Our experimental data were in good agreement with data shown therein.

3. Results

3.1. Measurements of the surface potential

The binding of verapamil and propranolol to bilayer membranes was monitored in terms of ζ and boundary ϕ_b potential changes. ζ was measured as a function of the concentration of verapamil and propranolol, pH, the lipid composition and the ionic strength of the buffer solution. It was shown that adsorbing to the neutral membrane both verapamil (Fig. 3A) and propranolol (Fig. 3B) increase the ζ-potential. Both drugs show an enhanced binding in the presence of negatively charged phospholipids such as DPhPS (Fig. 4, ▲), indicating that electrostatic interactions are involved.

From measured ζ-potentials ϕ_s was calculated, according to Eqs. 4–6 [33]:

$$\phi = \frac{2kT}{ze} \ln \frac{1 + \alpha \exp[-\kappa\delta]}{1 - \alpha \exp[-\kappa\delta]} \tag{4}$$

where

$$\alpha = \frac{\exp[ze\phi_s/2kT] - 1}{\exp[ze\phi_s/2kT] + 1} \tag{5}$$

and

$$\kappa = \sqrt{\frac{2e^2z^2CN_A}{\varepsilon\varepsilon_0 kT}}, \tag{6}$$

where C, $1/\kappa$, k, δ are, respectively, the concentration of the electrolyte, the Debye length, the Boltzmann constant and the distance of the shear plane from the surface; e, N_A, z, T, ε have their usual meanings. Assuming $\delta = 0.2$ nm [31], we obtain $\phi = \zeta$.

The Gouy-Chapman theory of the diffuse double layer predicts that the potential due to charge adsorption at the surface of a membrane ϕ_s is related to the total concentration of a monovalent electrolyte in the bulk solution, C:

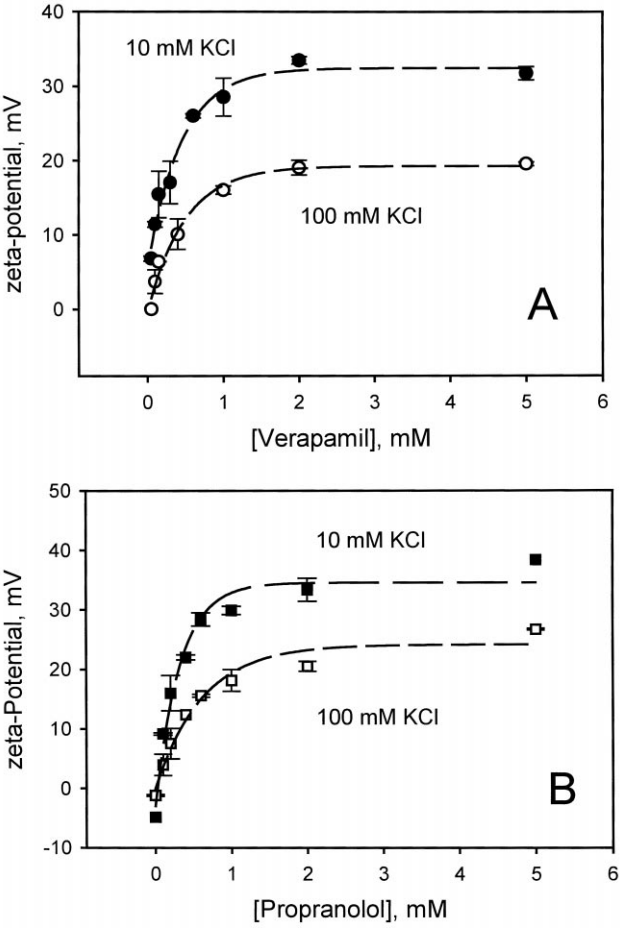

Fig. 3. Dependence of ζ-potential on drug concentration and ionic strength of buffer solutions for verapamil (A) and propranolol (B). Liposomes were made from DPhPC, pH of buffer solution was 6.5.

$$\frac{\sigma_a + \sigma_0}{\sqrt{8\varepsilon\varepsilon_0 RTC}} = \sinh\frac{\phi_s e}{2kT} \quad (7)$$

where $(\sigma_a+\sigma_0)$ is surface charge density due to the adsorption of charged substances and the initial charge of the lipid. As expected, a decrease of ϕ_s in the presence of verapamil or propranolol with an increase of ionic strength of buffer solution was found (Fig. 3). To relate the aqueous concentration, c_0, at the surface of the membrane to the bulk aqueous concentration, c, we have used the Boltzmann equation (Eq. 8) [34] assuming $z=+1$ for verapamil and propranolol:

$$c_0 = c \cdot \exp\frac{-zF\phi_s}{RT} \quad (8)$$

Since the membranes ($\sigma_0 = 0$) were initially neutral, the surface charge was produced by the adsorption of cations σ_s. Consequently, ϕ_s was not constant during the titration and it follows that the dissociation constant K was not constant for a given ionic concentration.

3.2. Measurements of the dipole potential

The measurements of boundary potential alteration due to the adsorption of verapamil and propranolol were carried out with the IFC method. Obtained data show that $\Delta\phi_b$ decreases after the addition of verapamil (Fig. 5A). With respect to the increase of the surface potential obtained, we have made the conclusion that the decrease of the boundary potential occurs due to the strong decrease of the dipole potential. On the contrary, the increase of the boundary potential induced by propranolol (Fig. 5B) is similar to the surface potential change.

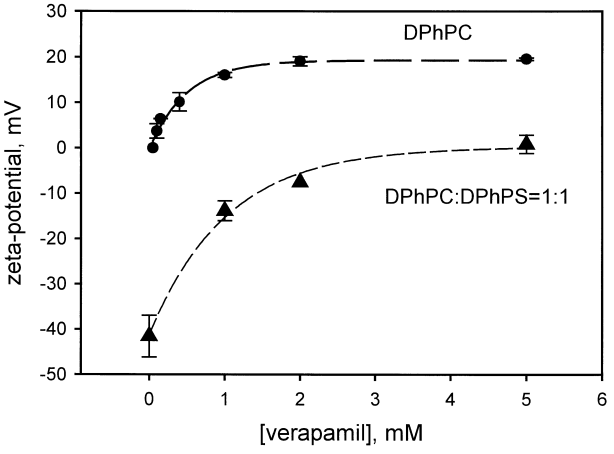

Fig. 4. Dependence of ζ-potential on concentration of verapamil and on lipid composition. The buffer solution contained 100 mM KCl, pH 6.5.

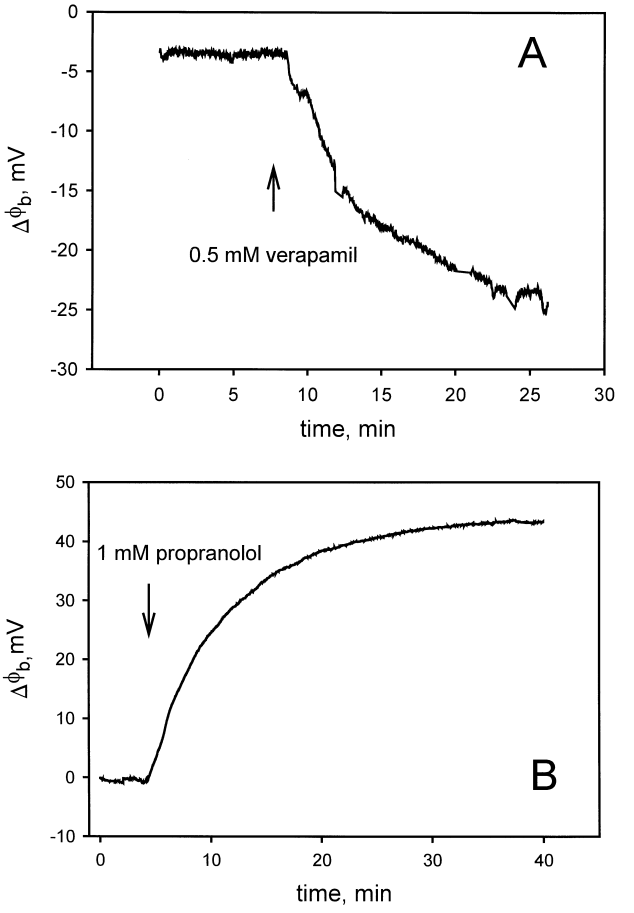

Fig. 5. Boundary potential alteration after the addition of verapamil (A) or propranolol (B) measured with the IFC method. BLMs were made from DPhPC (20 mg/ml).

From the knowledge that verapamil alters the dipole potential, some further predictions can be made. It is already well established that the membrane dipole potential is responsible for differences in permeability of hydrophobic ions [16], charged carriers, some potential sensitive dyes, charged spin label probes [35] and non-electrogenic carriers [36]. Verapamil was expected to increase the permeability for the nonactin-K^+ complex, because a decrease of the positive inner membrane potential is accompanied by an increase of the permeability for positively charged hydrophobic ions [37,38]. The adsorption of propranolol, on the contrary, should increase the energy

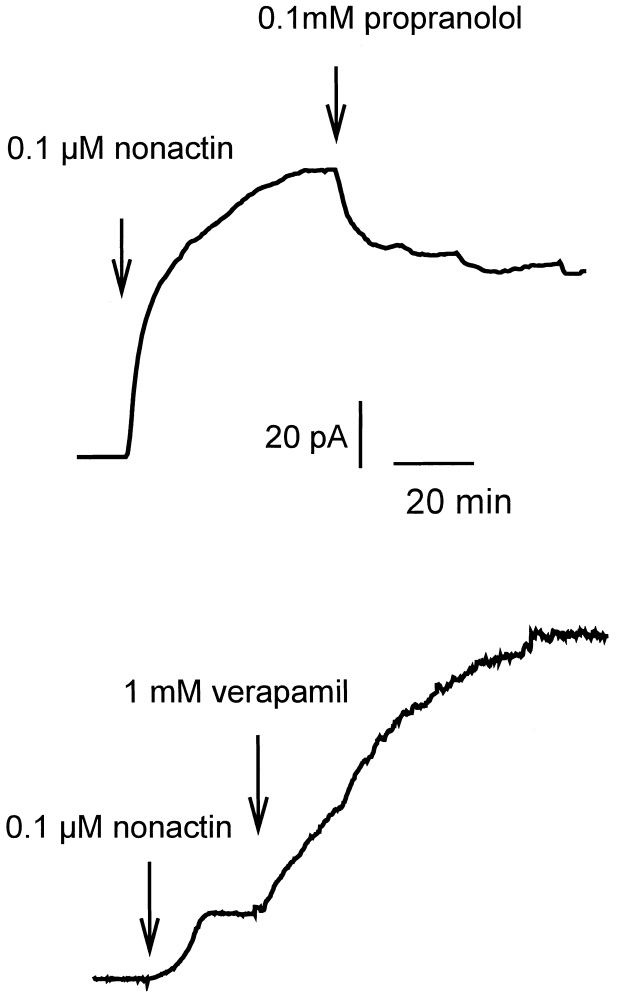

Fig. 6. Typical record of the effect of verapamil and propranolol on the transmembrane current.

barrier and consequently diminish the permeability for the positive charged carrier. To prove our assumption, we have monitored changes of the conductance of a BLM doped with nonactin. In fact, propranolol decreases the permeability for nonactin, whereas it is increased by verapamil (Fig. 6).

Based on the experimental values for the surface and the boundary potentials, calculated using Eq. 1, the dipole potential was estimated according to Eq. 9:

$$\Delta\phi_d = \Delta\phi_b - \Delta\phi_s \quad (9)$$

The use of this equation is justified by the assumption that the amount of drugs bound to liposomes is negligible in comparison to the total amount of drugs in solution. We have checked the concentration of verapamil in buffer solutions with a spectrophotometer [5] and concluded that it was not altered by the addition of lipid (data not shown). In Fig. 7 both substances are compared. It can be seen that the boundary potential $\Delta\phi_b$ induced by verapamil (●,○) differs from the surface potential $\Delta\phi_s$. For propranolol they are rather similar (■,□).

The changes of the dipole potential of lipid bilayers due to the adsorption of verapamil saturate with increasing concentrations of the compound. The experimental data are well described by a Langmuir adsorption isotherm. Fig. 8 shows this type of plot for verapamil. Quantitatively similar adsorption characteristics were found for phloretin [22,39]. It was shown that the adsorption isotherm can be rewritten in terms of dipole potential changes [40]:

$$\phi_d = \phi_d^{max} \frac{c}{K_d + c}, \quad (10)$$

where c is the drug concentration in the aqueous phases. The maximal change of the dipole potential, ϕ_d^{max}, is reached when all binding places are occupied. From Eq. 10 the dissociation constant K_d can be estimated.

Because 99.9% of verapamil exists in its neutral form at pH 10.5 and in its positively charged form at pH 6.4 (Eq. 11)

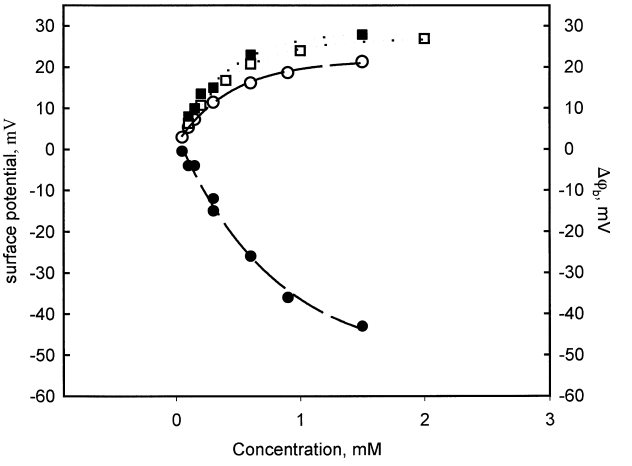

Fig. 7. Comparison of the boundary potential (●,■) deduced from the membrane conductance in the presence of nonactin and the surface potential (○,□). The latter was calculated from ζ-potential measurements at different concentrations of verapamil (●,○) and propranolol (■,□). The buffer solution contained 100 mM KCl. pH was 6.5.

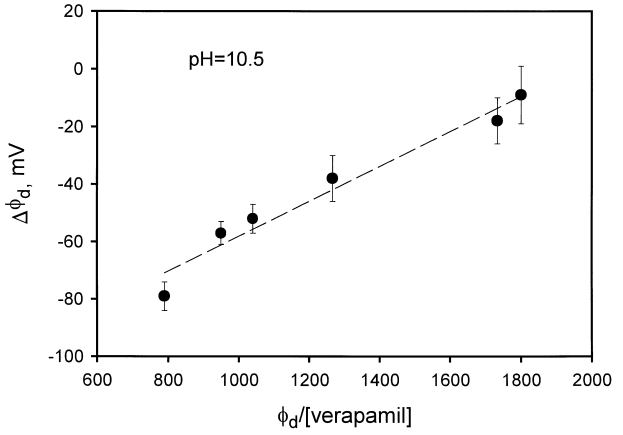

Fig. 8. Dependence of membrane dipole potential on verapamil concentration at pH 10.5 of the buffer solution. The buffer solution contained 100 mM KCl. BLM was made from DPhPC.

$$\frac{[T^+]}{[TH]} = 10^{pH-pK} \quad (11)$$

the binding constant was determined for each form separately. K_d and ϕ_s at pH 10.5 can be easily estimated from the Langmuir isotherm, because $\Delta\phi_s = 0$. They equal (0.061 ± 0.01) mM and (122 ± 5) mV respectively (Fig. 8). At pH 6.4 K_d is a function of $\Delta\phi_s$. Knowing $\Delta\phi_d^{max}$ and $\Delta\phi_s$, we determined K_d for each verapamil concentration (Eqs. 8,10). The calculated values of K_d varied in the range from 0.5 to 1 mM.

4. Discussion

Verapamil and propranolol are positively charged at physiological pH and induce, as expected, an increase in the surface potential of the BLM. Their contribution to $\Delta\phi_s$ is rather similar.

On the contrary, $\Delta\phi_b$ found after verapamil and propranolol adsorption were opposite in sign (Figs. 5 and 6). The boundary potential drop induced by propranolol is based entirely on the change of the surface potential due to the adsorption of the positively charged substance. In the case of verapamil, the comparison of $\Delta\phi_b$ and ϕ_s shows that $\Delta\phi_b$, obtained by capacitive current and conductance measurements, are significantly smaller than the values of ϕ_s, calculated from ζ-potential values (Figs. 7 and 9). From these differences it is obvious that the boundary potential drop is based not only on the change of the surface potential positive in sign but also on a simultaneous large dipole potential drop, negative in sign (Fig. 9).

Supposing that at pH 6.4 verapamil is positively charged and it is uncharged at pH 10.5, we were able to compare the adsorption constants for both forms. The affinity of the neutral form of verapamil is tenfold higher. This result seems to be reasonable, because repulsive ion-ion interactions take place only at the lower pH.

The dissociation constant of the uncharged verapamil was calculated from Langmuir isotherms (Fig. 8), because, both experimentally and theoretically, the dipole-dipole interactions are so weak that they have only a minor effect on the adsorption [22]. On monolayers Cseh and Benz have shown recently that alterations of the intrinsic membrane potential introduced by phloretin have an influence on the adsorption of subsequent molecules with dipole moments [41]. The authors have also seen a minor impact of electrostatics on the adsorption on bilayers [41] that, however, has not been observed before [22,39,40,42]. There is no complete understanding why the adsorption parameters are different between monolayers and bilayers [41]. The most obvious difference is the orientation of the adsorbing dipole. In bilayers the dipole moment of phloretin was shown to make an angle to the membrane surface [12] that is different from 90° usually assumed for monolayers [41].

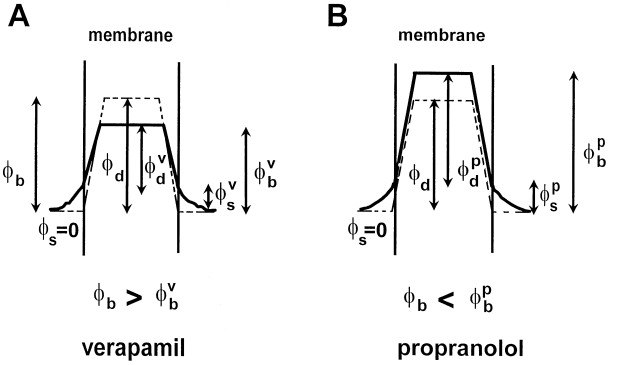

Fig. 9. Changes of the total potential profile for the interactions of charged molecules with lipid bilayers due to the adsorption of verapamil (A) and propranolol (B). ϕ_s, ϕ_d, ϕ_b are surface, dipole and boundary potentials respectively; ϕ_s^v, ϕ_d^v, ϕ_b^v and ϕ_s^p, ϕ_d^p, ϕ_b^p are the same potentials after addition of verapamil or propranolol.

Moreover, the large deviations in the dipole potential measured for monolayers and bilayers [10] may also influence, at least in part, the adsorption behavior. With respect to these considerations, it is more straightforward to evaluate drug-induced dipole potential changes from the nonactin mediated bilayer conductance than from boundary potential changes of lipid monolayers.

To separate potential changes from drug induced mobility changes within the membrane, an indicator free method for boundary potential measurements was also used. Because the same results have been obtained with the inner field compensation method and with conductance measurements, an influence of the drugs on the membrane fluidity is extremely unlikely. Additionally, Shi and Tien [9] have shown that the verapamil concentration required to produce the detectable fluidizing effect is tenfold (0.01 M) the highest concentration used in our experiments.

Our findings might have important implications for drug and ion transport studies in intact cells. Conformational changes of ion channels or receptors that involve the movement of charges or dipoles across the membrane interface could be strongly influenced by the dipole potential [43]. Rokitskaya et al. have found that phloretin, known to lower the dipole potential, affects the process of channel dissociation [44]. Fluorescent potential-sensitive dyes of the RH series, shown to increase the dipole potential [18], are supposed to affect the electrogenic transport performed by the sodium pump. Extrapolated to clinically relevant verapamil concentrations (μM range), the observed changes of the dipole potential are rather small. However, at essentially higher concentrations (mM range) verapamil is widely used in patch-clamp experiments (e.g. [45]). With respect to our results, it cannot be excluded that artifacts are introduced due to the non-specific alteration of membrane characteristics.

Acknowledgements

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