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Membrane potential difference of isolated plant vacuoles: positive or negative? I. Evidence for membrane binding of cationic probes

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The binding of membrane potential cationic probes was studied on phospholipidic liposomes by equilibrium dialysis and microelectrophoresis. Surface binding of lipophilic cations (benzyltributylammonium or tetraphenylphosphonium) appears to be the major accumulation mechanism in liposomes and simulates the existence of a negative transmembrane potential (E_m), in absence of any transmembrane ionic gradient. Furthermore, this apparent negative potential has a classical response with regard to common E_m effectors, namely a depolarization induced by KCl or FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone). The relevance of these results to the study of transtonoplast potential difference on isolated vacuoles was investigated. Tetraphenylphosphonium was shown to bind to the tonoplast, the essential features of binding and interaction with E_m effectors being similar in vacuoles and liposomes. Therefore the assumption of negligible binding of cationic probe to vacuoles, classically admitted in determinations of vacuolar E_m using lipophilic cations, is untenable.

Introduction

Lipophilic cations such as tetraphenylphosphonium (TPP^+) or triphenylmethylphosphonium ($TPMP^+$) have been extensively used as membrane potential probes on prokaryotic and eukaryotic cells as well as on isolated organelles (for a review, see Ref. 1). Caution must be exercised in using these cation probes, as exemplified in the literature.

Ritchie [2] demonstrated active transport of $TPMP^+$ across the vacuolar membrane of giant-celled algae. Various examples show that lipophilic cations can be strongly adsorbed on membranes of bacterial cells [3,4] and *Chlorella* cells [5]. Extensive binding of $TPMP^+$ on dead cells, together with little penetration into living cells, renders this probe unsuitable for potential measurements on runner bean cells [6]. Binding of $TPMP^+$ on bovine chromaffin granules has also been demonstrated [7]. Similar criticisms have been raised against fluorescent cationic probes (cyanine dyes) [8–10].

Such binding phenomena have been claimed to be negligible on vacuoles isolated from higher plant cells [11,12]. However, values for the transtonoplast potential, E_m, have been in systematic conflict: with cationic probes apparent E_m is negative [11–15] and with microelectrodes, it is positive [16–20]. This controversy stimulated this

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TPP, tetraphenylphosphonium; TBBA, benzyltributylammonium; TPMP, triphenylmethylphosphonium; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DiS-C_2-(5), 3,3′-diethylthiadicarbocyanine iodide.

reevaluation of the degree of binding of cationic probes to vacuoles.

Phospholipidic liposomes were used to study lipophilic cations on a well-defined membrane system. Trapping of either tetraphenylphosphonium (TPP^+) or benzyltributylammonium ($TBBA^+$) in liposomes was determined simultaneously in equilibrium dialysis and electrophoresis experiments. The results show that surface binding of lipophilic cations is a major accumulation mechanism in liposomes in absence of transmembrane potential, and this phenomenon simulates the existence of negative E_m. Furthermore, this apparent negative potential has a classical response with respect to common E_m effectors. The relevance of these results to tonoplast polarization studies was then tested, and indeed cationic probes bind to isolated vacuoles, as to liposomes.

Materials and Methods

Liposomes were prepared according to Uso and Rossignol [21] from equimolar amounts of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) dispersed in the various experimental media.

Vacuoles from *Acer pseudoplatanus* cells were isolated from a cationic treatment of protoplasts as described elsewhere [22,23]. Protoplasts of *Catharanthus roseus* were isolated according to Ref. 24 in medium A (0.55 M sorbitol/10 mM Hepes (pH 7.4)). Vacuoles were isolated from protoplasts by diluting 1 ml protoplast suspension with 1.75 ml medium B (7.9 mM EDTA-Na_2/10 mM Hepes (pH 7.1)). After 30 min at 4°C, nearly 99% of the protoplasts have burst. Vacuoles were purified by floatation on a two-step gradient: 10.25 ml 0.36 M sorbitol/5 mM EDTA/8.5% Nycodenz/10 mM Hepes (pH 7.1) were added to the 2.75 ml of burst protoplasts so that the final concentrations of sorbitol and Nycodenz were 0.31 M and 7.7%, respectively. The upper layer consisted of 1 ml 0.42 M sorbitol/2% Nycodenz/10 mM Hepes (pH 7.1). Vacuoles were recovered in the uppermost 0.6 ml by flotation at 4°C, either at $1 \times g$ for 30 min, or at $160 \times g$ for 3 min, and were counted in a Malassez haemocytometer. Their purity, assessed by enzymatic measurements, was higher than 95% (to be detailed elsewhere).

Equilibrium dialysis experiments were performed with 2 ml Teflon cells (Dianorm) and cuprophane membranes. Each dialysis was run for 45 min (approx. 9-times the half-equilibration time). The tetraphenylphosphonium (TPP^+) and benzyltributylammonium ($TBBA^+$) concentrations were assayed by absorbance at 268 nm and 262 nm, respectively.

The fluorescence of diS-C_2-(5) was assayed with a Jobin-Yvon fluorimeter JY3D at 670 nm in 3 ml cells (excitation at 650 nm; slit width, 10 nm; temperature, 25°C).

The electrophoretic mobility of liposomes and vacuoles was measured with a Rank Mark II apparatus, maintained at 25°C. Constant currents of 2 mA were applied, corresponding to voltage gradients ranging from 200 to 1000 $V \cdot m^{-1}$. Under a given ionic condition, 15–20 vesicles were timed in each direction of the field, and measurements were repeated twice with different vesicle preparations. Under control conditions, velocities of migration were about 8 and 2 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$ for DOPG-DOPC liposomes and vacuoles, respectively. Standard errors for the values of electrophoretic mobilities were in the range 2–5% for liposomes and 10–15% for vacuoles. Zeta potentials were calculated from the electrophoretic mobility with the Helmholtz-Smoluchovski relation (see Ref. 25). The surface potentials (ψ_0) were identified to the zeta potentials. The Gouy-Chapman model, which conveys the basic concepts for the electrostatic interactions at the surface of membranes, was used in the analysis of these data. In the case of 1 : 1 electrolytes, the surface charge density (σ) is predicted to be related to ψ_0 by:

$$\psi_0 = 2RT/F \text{ arc sin } h(\sigma/B\sqrt{C})$$

where C is the bulk concentration of the salt, $B = \sqrt{8\epsilon RT}$, and R, T and F have their usual meanings; ϵ represents the dielectric constant of the medium [26]. The surface ionic concentrations (C_s) were calculated from the bulk concentrations (C_b) according to the Boltzman law:

$$C_s = C_b\, e^{-ze\psi_0/kT}$$

where z is the valency of the ion.

Results and Analysis

Binding of cationic probes to phospholipidic membranes

Liposomes were used as a membrane model for studying lipophilic cation binding. As they were prepared in the media used for experiments, there was no ionic gradient across their membrane. The DOPC : DOPG 1 : 1 mixture conferred to the vesicles a negative surface potential, which decreased upon addition of KCl (Fig. 1). This depolarization was correctly described by the Gouy-Chapman model (Fig. 1, theoretical curve). It was totally accounted for by the screening effect, since the calculated surface charge density was constant (mean value = $-3.3 \cdot 10^{-3}$ elementary charge per Å^2, corresponding to a mean molecular areas of about 150 Å^2 per phospholipid head).

The amounts of TPP^+ and $TBBA^+$ disappearing from the bulk medium in dialysis experiments were compared to the amounts of these probes bound on the surface of liposomes (as determined from the variations of the external surface charge) (Fig. 2). To aid comparison, the data from dialysis experiments (mol probe absorbed by liposomes per mol phospholipid) were expressed in the same unit as the microelectrophoretic data (elementary charges per Å^2), using 150 Å^2 as mean molecular

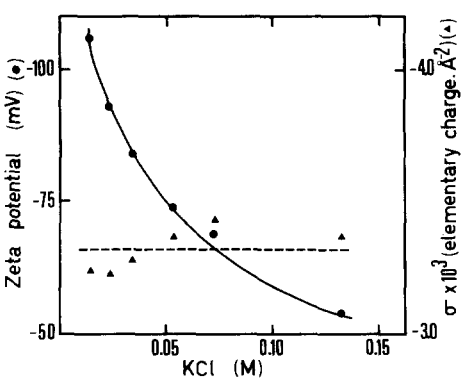

Fig. 1. Effect of KCl concentration on zeta potential and surface charge density (σ) of DOPC-DOPG liposomes, as determined from microelectrophoretic mobilities. The medium comprised 1 mM Na_2-EDTA/2 mM Hepes (pH 7.4). Experimental data, ●. The theoretical curve calculated using the Gouy-Chapman model is also shown (continuous line). The dashed line indicates the mean value of surface charge density: $-3.3 \cdot 10^{-3}$ elementary charge $\cdot$ Å^{-2}.

area per phospholipid head. In both cases the binding to the membrane surface accounted for the major part of the total probe absorption by the liposomes as estimated by equilibrium dialysis.

The surface concentrations of free probes were calculated from the Boltzman law applied to bulk concentrations and measured surface potentials. The amounts of bound probes (expressed as variations of surface charge) were analyzed as a function of surface concentrations. This analysis indicated that for both probes the binding obeys a simple first-order law with a finite number of binding sites (Scatchard plots, Fig. 2, insets) and gave the intrinsic binding parameters (dissociation constant, K_i, and maximum number of binding sites, N, expressed as the maximum probe-induced variation of surface charge density). With this set of data, the experimental results from electrophoresis were simulated as described elsewhere for the binding of the anion 8-anilino-1-naphthalenesulphonate [27]. The Gouy-Chapman relation was used for the description of the ionic environment, the Boltzman law for describing the effect of surface potential on surface probe concentrations and the mass action law for binding. The masking effect of probe binding on the surface charge was taken into account in addition to the screening effects described by the Gouy-Chapman relation. As shown by Fig. 2 the experimental results are correctly fitted with this procedure.

Increasing the KCl concentration reduced TPP^+ or $TBBA^+$ binding (Fig. 3). The effects of KCl on TPP^+ and $TBBA^+$ binding were analyzed as indicated above, using the Gouy-Chapman model for describing the screening effect of KCl, and the intrinsic binding parameters determined from the analysis of Fig. 2. For both probes, the correct fitting to the results (calculated curves on Fig. 3) indicated that KCl reduced their binding via classical electrostatic surface interactions.

The anionic uncoupler FCCP increased the negative surface charge of DOPC liposomes at pH 7.4 (Fig. 4A), indicating a binding to the membrane (such a binding has been described for black films of phosphatidylethanolamine-chlorodecane and egg phosphatidylcholine multilayers [28,29]). The binding of FCCP to liposomes was analyzed in the same way as that of the lipophilic cations, by calculating the surface concentrations from

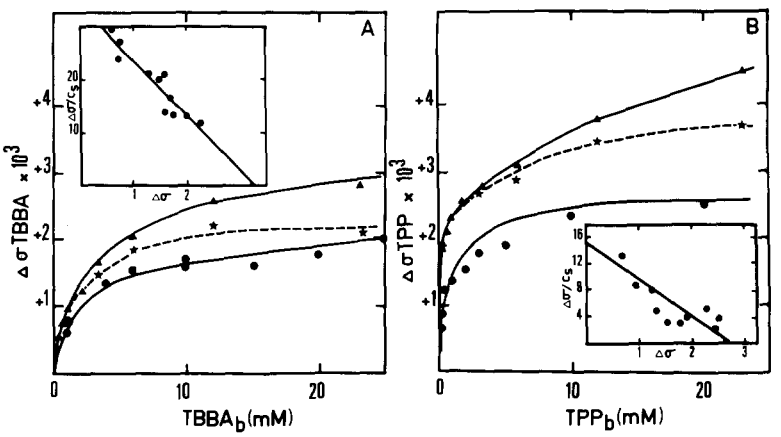

Fig. 2. Binding of $TBBA^+$ and TPP^+ to 1:1 DOPC-DOPG liposomes, determined by equilibrium dialysis (▲) or by microelectrophoresis (●) as a function of the bulk concentration of cations ($[TBBA]_b$ or $[TPP]_b$, mM). The microelectrophoretic data were expressed as the variation of surface charge density ($\Delta\sigma$ TBBA or $\Delta\sigma$ TPP, elementary charge·$Å^{-2}$). For comparison, the dialysis data (mol cation per mol phospholipid) were expressed also as $\Delta\sigma$, using a phospholipid mean molecular area of 150 $Å^{-2}$. Dashed lines represent the true binding of cations on membranes, after correction of dialysis data for the amount of free probe trapped in the internal volume of liposomes. This amount was calculated assuming no transmembrane gradient and a maximal specific volume of 5 l/mol lipid. Scatchard plots of microelectrophoresis data are presented in insets ($\Delta\sigma$, variation of surface charge density, elementary charge·$Å^{-2}$; C_s: surface concentration of cation, mM). The medium comprised 10 mM KCl/1 mM Na_2-EDTA/2 mM Hepes (pH 7.4). The theoretical curves shown were calculated with the Gouy-Chapman model as described in the text (intrinsic charge density of liposomes $-3.3 \cdot 10^{-3}$ elementary charge·$Å^{-2}$). (A) Binding of $TBBA^+$. Intrinsic binding parameters: $K_i = 95.5$ mM, $N = 3.3 \cdot 10^{-3}$ elementary charge·$Å^{-2}$. (B) Binding of TPP^+. Intrinsic binding parameters: $K_i = 23.2$ mM, $N = 2.7 \cdot 10^{-3}$ elementary charge·$Å^{-2}$.

Boltzman's law applied to bulk concentration and surface potential. The Scatchard plot was linear and indicated an intrinsic affinity constant of about 2 μM (Fig. 4B). The mechanism of this effect is unknown.

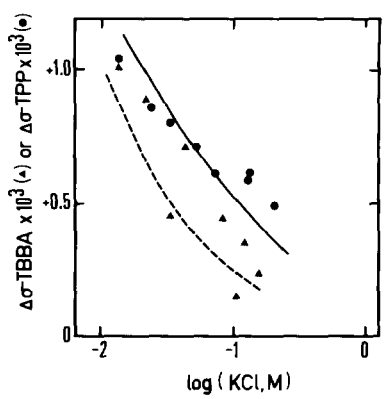

Fig. 3. Effect of KCl concentration on $TBBA^+$ and TPP^+ binding on DOPC-DOPG liposomes. The binding of lipophilic cations was estimated from the experimental variations of surface charge density ($\Delta\sigma$TBBA or $\Delta\sigma$TPP, elementary charge ·$Å^{-2}$). The theoretical curves shown were calculated using the Gouy-Chapman model and the binding parameters given in Fig. 2. The medium contained various concentrations of KCl, 1 mM Na_2-EDTA, 2 mM Hepes (pH 7.4) and 1 mM lipophilic cation ($TBBA^+$ or TPP^+).

In presence of FCCP, TPP^+ was no longer able to depolarize the membrane surface (Table I). Neutral FCCP (pH 5.5) had the same inhibitory effect as the ionized form (pH 8.5) on the TPP^+-induced surface depolarization (Table I). This inferred that the effect of FCCP was not due to a cancelling of the positive charge of bound TPP^+ by the negative charge of bound FCCP, and that the binding of FCCP reduced that of TPP^+, in spite of its hyperpolarizing effect on the membrane surface (Table I).

The quenching of the fluorescence of the cyanine dye diS-C_2-(5) has been interpreted as indicating an accumulation of this cationic probe into beet vacuoles driven by negative E_m [13,15]. This could, in fact, be due to the binding of the probe to the membrane. This possibility was first checked by measuring the quenching of diS-C_2-(5) fluorescence by liposomes, in absence of any transmembrane ionic gradient, as a function of the dye concentration. The amount of dye bound to the external surface of liposomes was determined in

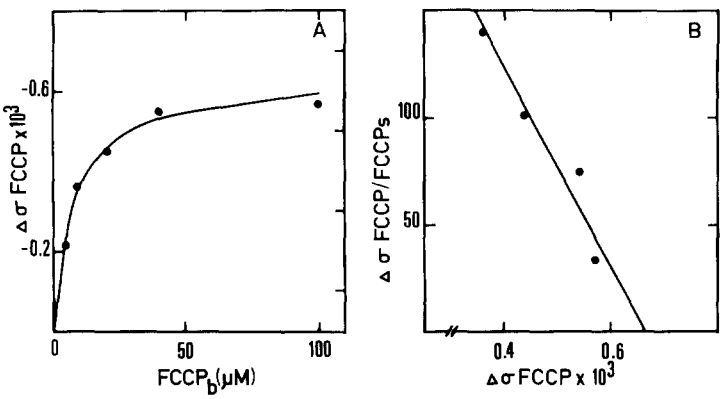

Fig. 4. Binding of FCCP on DOPC liposomes as determined from microelectrophoresis data. The medium comprised 10 mM KCl/1 mM Na_2-EDTA/2 mM Hepes (pH 7.4). The theoretical curve in (A) was calculated using the Gouy-Chapman model and the intrinsic binding parameters given in (B). (A) Variation of surface charge density ($\Delta\sigma$FCCP, elementary charge·$Å^{-2}$) as a function of the bulk concentration of FCCP ($FCCP_b$). (B) Scatchard plot of microelectrophoresis data. Intrinsic binding parameters: $K_i = 2.1$ μM; $N = 6.64 \cdot 10^{-4}$ elementary charge·$Å^{-2}$.

TABLE I

EFFECT OF FCCP ON TPP^+ BINDING ON DOPC-DOPG LIPOSOMES, DETERMINED FROM MICROELECTROPHORESIS EXPERIMENTS

The medium contained 10 mM KCl, 0.1 mM Na_2-EDTA and 2 mM Hepes-NaOH. The bulk concentrations of FCCP and TPP^+ were 100 μM and 1 mM, respectively. The changes of surface charge density ($\Delta\sigma$, elementary charge per $Å^{-2} \times 10^3$) were estimated at pH 8.5 and pH 5.5 (n.d., not determined).

Medium	$\Delta\sigma$ (FCCP)	$\Delta\sigma$ (TPP^+)	$\Delta\sigma$ (TPP^+ + FCCP)
pH 8.5	−0.50	+0.91	+0.03
pH 5.5	n.d.	+0.91	+0.10

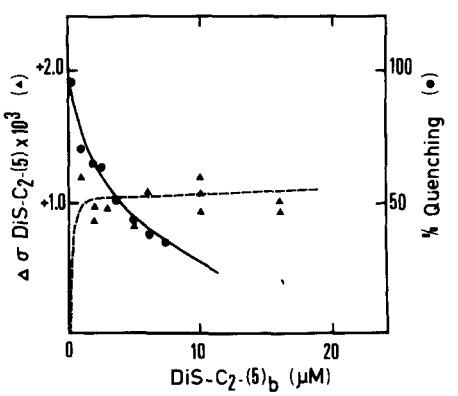

Fig. 5. Binding of the fluorescent probe diS-C_2-(5) on DOPC-DOPG liposomes, as determined from the variation of surface charge density ($\Delta\sigma$ DiS-C_2-(5)), elementary charge·$Å^{-2}$) in microelectrophoresis measurements (▲), or of fluorescence intensity (% quenching, ●), as functions of the bulk concentration, diS-C_2-$(5)_b$. The medium comprised 10 mM KCl/1 mM Na_2-EDTA/2 mM Hepes (pH 7.4).

parallel runs from the changes in the surface charge density. Liposome membranes exhibited a high affinity for the probe. They were saturated at low diS-C_2-(5) concentrations (less than 1 μM), in which condition the negative net surface charge was reduced by 30% and the quenching reached 95% (Fig. 5). Increasing the diS-C_2-(5) concentration above 1 μM led to a progressive decrease of the quenching, reflecting a fall in the ratio of bound to free probe.

Binding of cationic probes to vacuoles

TPP^+ binding on the external surface of tonoplast was studied by microelectrophoresis on *Acer pseudoplatanus* and *Catharanthus roseus* vacuoles. TPP^+ reduced the surface charge of isolated vacuoles (Fig. 6) as already observed on liposomes, indicating a binding of the probe on the tonoplast.

Analysis of the amounts of TPP^+ bound to the external surface of vacuoles as a function of surface TPP^+ concentration indicated first-order saturation kinetics with intrinsic affinity constants in the mM range (Fig. 6A and B, insets). As in the case of liposomes, it was possible to simulate the experimental results with the Gouy-Chapman model and the mass action law (calculated curves on Fig. 6A and B).

Increasing KCl concentration depolarized the tonoplast surface potential (Fig. 7A), and consequently reduced TPP^+ binding (Fig. 7B), as already demonstrated for liposomes. Again, it was

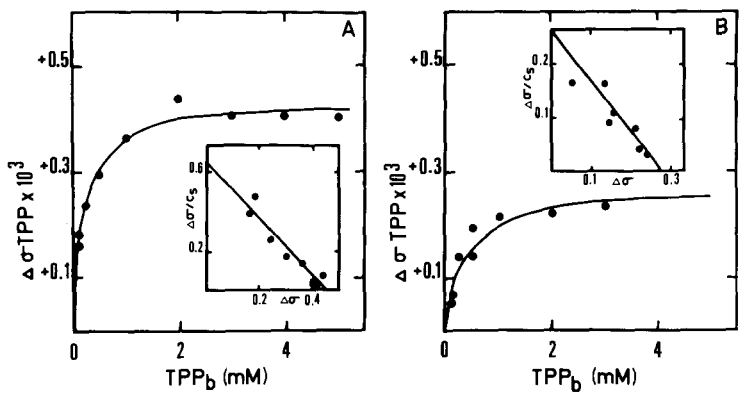

Fig. 6. Binding of the lipophilic cation TPP^+ on isolated vacuoles, as estimated from the variation of surface charge density ($\Delta\sigma$TPP, elementary charge·Å^{-2}) as a function of the bulk concentration of TPP^+ (TPP_b). The medium comprised 10 mM KCl/0.7 M mannitol/1 mM Na_2-EDTA/10 mM Hepes (pH 7.4). The theoretical curves shown were obtained using the Gouy-Chapman model and the intrinsic binding parameters. (A) Binding of TPP^+ on vacuoles isolated from *A. pseudoplatanus* cells. Intrinsic surface charge density: $-0.872 \cdot 10^{-3}$ elementary charge·Å^{-2}. Inset: Scatchard plot of microelectrophoresis data. Intrinsic binding parameters: $K_i = 0.68$ mM; $N = 0.446 \cdot 10^{-3}$ elementary charge·Å^{-2}. (B) Binding of TPP^+ on vacuoles isolated from *C. roseus* cells. Intrinsic surface charge density: $-0.708 \cdot 10^{-3}$ elementary charge·Å^{-2}. Inset: Scatchard plot of microelectrophoresis data. Intrinsic binding parameters: $K_i = 1.2$ mM; $N = 0.281 \cdot 10^{-3}$ elementary charge·Å^{-2}.

found that this effect could be correctly described by the Gouy-Chapman model and the intrinsic binding parameters obtained from the TPP^+ binding isotherm (Fig. 7B, calculated curve).

The cyanine dye diS-C_2-(5) reduced the surface charge of *A. pseudoplatanus* vacuoles as estimated from microelectrophoresis experiments (Fig. 8), indicating a binding of the probe on tonoplast. As for TPP^+, it was possible to describe the binding of diS-C_2-(5) by a first-order law (Scatchard plot, Fig. 8, inset) and to simulate the experimental results with the Gouy-Chapman model and the intrinsic binding parameters (calculated curve on Fig. 8).

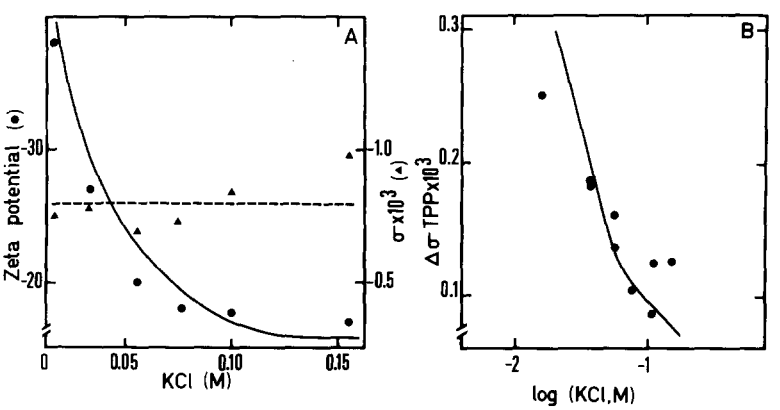

Fig. 7. Effect of KCl concentration on electrostatic properties of *A. pseudoplatanus* vacuoles, as determined from microelectrophoresis data. (A) Effect of KCl on zeta potential (mV, ●) and surface charge density (σ, elementary charge·Å^{-2}, ▲). The medium comprised 0.7 M mannitol/1 mM Na_2-EDTA/10 mM Hepes (pH 7.4). The theoretical curve was calculated using the Gouy-Chapman model. The dashed line indicates the mean value of surface charge density: $-0.796 \cdot 10^{-3}$ elementary charge·Å^{-2}. (B) Effect of KCl on TPP^+ binding, as estimated from the variation of surface charge density ($\Delta\sigma$TPP, elementary charge·Å^{-2}). The medium comprised 0.7 M mannitol/1 mM Na_2-EDTA/10 mM Hepes (pH 7.4)/2 mM TPP^+. The theoretical curve was calculated using the Gouy-Chapman model and the intrinsic binding parameters given in Fig. 6A.

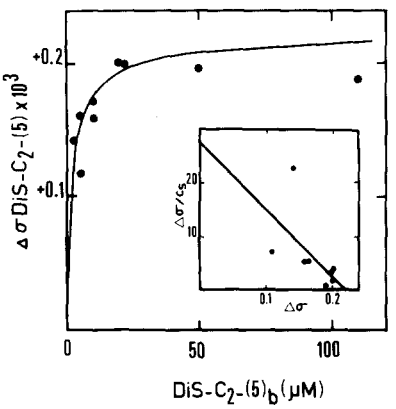

Fig. 8. Binding of the fluorescent probe diS-C_2-(5) on *A. pseudoplatanus* vacuoles, determined from the variation of surface charge density ($\Delta\sigma$ diS-C_2-(5), elementary charge·Å^{-2}) as a function of the bulk concentration, DiS-C_2-$(5)_b$. The medium comprised 10 mM KCl/0.7 M mannitol/1 mM Na_2-EDTA/10 mM Hepes (pH 7.4). The intrinsic charge density of vacuoles was $-0.694 \cdot 10^{-3}$ elementary charge·Å^{-2}. The theoretical curve was obtained using the Gouy-Chapman model and the intrinsic binding parameters. Inset: Scatchard plot of microelectrophoresis data. Intrinsic binding parameters: K_i = 7.6 μM, $N = 0.22 \cdot 10^{-3}$ elementary charge·Å^{-2}.

Discussion

Importance of binding of lipophilic cations on phospholipidic membranes

In the above study, the importance of binding in total probe accumulation by liposomes was estimated from the difference between microelectrophoresis and dialysis results. In microelectrophoretic experiments, only the binding at the interfaces of the membrane is determined. If the probe binds inside the bilayer, the microelectrophoretic data would underestimate the total binding. Assuming a maximal specific volume of 5 l per mol lipid [30], the maximal amount of the free probe trapped inside the vesicles would represent about 20% of the total amount of absorbed probe for a bulk concentration of 20 mM $TBBA^+$ or TPP^+. This amount of free probe was deduced from the total vesicular probe measured by dialysis (Fig. 2, dashed lines), Such a correction would entirely account for the difference between the two sets of experimental data in the case of $TBBA^+$. On the other hand, in the case of TPP^+, this correction suggests that electrophoretic measurements could underestimate the true binding. Nevertheless, microelectrophoresis appears to be a good tool for appreciating the binding of lipophilic cations to membranes. Another important conclusion of the theoretical analysis of Figs. 1–6 is that the Gouy-Chapman model correctly describes electrostatic interactions, as classically accepted [26,27].

It is clear that the binding to the membrane represents the major part of the lipophilic cation accumulation by liposomes. Assuming that the amounts of accumulated probes measured by dialysis (Fig. 2) were homogeneously distributed in the internal volume of liposomes (2.5 l/mol phospholipid [30]), the accumulation ratios would vary from 5 (for 20 mM external TPP^+) to 2800 (for 0.2 mM external TPP^+). These ratios would correspond to apparent Nernst potentials from −40 to −200 mV. Hence, apparent negative potentials may be simulated by cation binding on membranes, in absence of a true transmembrane potential.

Effect of classical E_m effectors on the binding of lipophilic cations on phospholipidic membranes

The effects of KCl on probe binding (Fig. 3) were analyzed in terms of apparent Nernst potentials. The bound probes were assumed to be freely distributed in an internal volume of 2.5 l/mol phospholipid, and the corresponding Nernst potentials were calculated (Fig. 9). The central conclusion is that the Nernst potentials are in reasonably good linear correlation with log[KCl], as if they were true transmembrane potentials.

The results of Fig. 3 were obtained at 1 mM external probe concentration. This relatively high lipophilic cation concentration was used for technical reasons. As could be expected from the binding law, micromolar TPP^+ concentrations (classically used for E_m estimations) did not induce measurable modifications of the surface charge density. Nevertheless, the influence of KCl on the apparent Nernst potential at low TPP^+ concentration was studied by simulation with the model described above. The results of these calculations (Fig. 9, inset) predict a linear relation, with a slope of −51 mV for 1 μM TPP^+.

The mechanism of the interaction between FCCP and TPP^+ at the external membrane surface is unknown. Nevertheless, it is clear from these

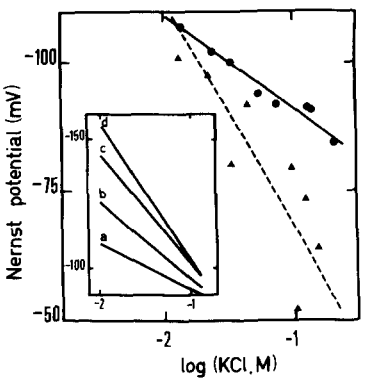

Fig. 9. Theoretical Nernst potentials calculated from the apparent concentrations of $TBBA^+$ (▲) or TPP^+ (●) accumulated by the liposomes as a function of KCl concentration (data of Fig. 3; bulk concentration of TPP^+ 1 mM). The curves were calculated admitting identical binding properties for both surfaces of the membranes. Slopes: $TBBA^+$ 44 mV, TPP^+ 18 mV per unit log[KCl]. Inset: Theoretical curves of Nernst potentials as a function of KCl concentration, determined for various bulk concentrations of TPP^+. The amounts of TPP^+ associated with the liposomes were calculated by assuming that the internal concentration was equal to the external one, and that the binding of TPP^+ to the membrane obeyed the experimentally determined kinetics (Fig. 2B). (a) $[TPP]_b = 1$ mM, slope 18.0 mV; (b) $[TPP]_b = 5 \cdot 10^{-1}$ mM, slope 31.6 mV; (c) $[TPP]_b = 10^{-2}$ mM, slope 43.4 mV; (d) $[TPP]_b = 10^{-3}$ mM, slope 51.2 mV.

results that FCCP at concentrations classically used for collapsing pH gradients is able to bind at the interfaces of membranes and simultaneously to impede the binding of TPP^+.

These results show that lipophilic cation binding responds to classical effectors in such a way that it simulates an accumulation of free ions driven by transmembrane potential.

Binding of cationic probes to isolated vacuoles

The experimental results of Fig. 6 and 7, and their theoretical analysis, show that the essential features of TPP^+ binding and KCl interaction are similar in liposomes and vacuoles: in both cases, the binding of the lipophilic cations obeys first-order kinetics, and is affected by the ionic strength, as predicted from the Gouy-Chapman screening effect. Although less documented, the binding of diS-C_2-(5) is evident (Fig. 8). In summary, this study shows that the assumption of negligible cationic probe binding, classically made in determinations of vacuolar E_m, may not be realistic. The relative degrees of binding and free probe accumulation need to be quantified on vacuolar preparations.

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