EFFECT OF DIVALENT CATIONS ON THE ASSEMBLY OF NEUTRAL AND CHARGED PHOSPHOLIPID BILAYERS IN PATCH-RECORDING PIPETTES

ROBERTO CORONADO
Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

ABSTRACT Monolayers of the negatively charged phospholipid phosphatidylserine (PS) and of the amphoteric phospholipid dioleoylphosphatidylethanolamine (DOPE) were used to assemble bilayers at the tip of patch-recording pipettes. PS bilayers, with seal resistances in the range of gigaohmns (gigaseals), could only be generated when millimolar concentration of divalent cations, Ca^{++}, Mg^{++}, or Ba^{++} were present in the pipette and bath solutions. In contrast, gigaseals of DOPE were independent of divalent ion concentration in the pH range where DOPE is predominantly neutral (pH 6.5) or positively charged (pH 1.5). At pH 10.0, when most DOPE molecules bear a net negative charge, gigaseals became divalent cation dependent, in a manner quantitatively similar to that of PS at neutral pH. The results indicate that divalent cations play an important role in stabilizing gigaseals of negatively charged lipid but are of no consequence in neutral or positively charged seals.

INTRODUCTION

Two phospholipid monolayers deriving from lipids spread at an air-water interface can be apposed at the tip of a patch-recording electrode to form a bilayer (6, 10, 17, 18). Recording of ion channels of cellular origin and peptide ionophores only active in bilayers have confirmed that the bulk of the film is truly bimolecular (6, 7, 10, 17, 18, 20).

The nature of the phospholipid-glass interaction seems to be critical for the assembly of stable phospholipid films in patch electrodes. Many phospholipids such as phosphtidylethanolamines (PE) purified from brain or egg, or synthetic PE (dioleoyl 18:1; dimiristoyl 14:0) can be consistently recognized as good "seal-formers." Sealed electrode resistance for these phospholipids, for several variants of the technique, is in the range of 1–20 G Ω (6, 20). Many others such as phosphatidylcholines (PC) with the noticeable exception of diphytanoyl-PC (6) are extremely poor "seal formers" (Coronado, R., unpublished results).

There are several clues towards the understanding of lipid-glass bonding since the chemical reactivity of glass surface has been well recognized (5, 11). Specifically, the adsorption of fatty acids, hydrocarbons, and long-chain organic cations has been known for some time (3, 4, 9, 23). Another consideration relevant to phospholipid seals is the charged character of the glass surface. Contributions to the negative surface charge of glass arise from dissociation of surface silanol groups $\equiv$ Si-OH, which have an estimated pK of 9.8 (9) and a surface density of 5–8 groups per 100 $Å^2$ (1, 11, 22); from adsorption of hydroxyls, bicarbonate, and other inorganic anions in solution (2, 14, 15); and from cation exchange (14, 15).

In this paper I describe gigaseal formation using the dipping technique in pure lipid systems composed of monolayers of the negatively charged lipid phosphatidylserine (PS) and of the amphoteric lipid dioleoylphosphatidylethanolamine (DOPE). The advantage of measuring gigaseals in an amphoteric lipid such as PE resides in the changes in net charge that can be induced in the polar groups of the lipid by varying solution pH (12). Thus, the electrostatic contributions of phospholipid polar group charge to gigaseal formation can be approached experimentally. The significant finding is that while neutral DOPE (pH 6.5) or positively charged DOPE (pH 1.5) can readily form gigaseals in monovalent salt solutions without addition of divalent cations, stabilization of negatively charged DOPE (pH 10.0) or PS (pH 7.0) gigaseals require millimolar concentration or divalents in the solutions bathing the bilayers. It is suggested that the physical nature of negatively charged DOPE and PS seals, albeit unknown, is probably the same and that differences in seal resistance for these lipids are a consequence of the charge of the phospholipid at a given pH.

MATERIALS AND METHODS

Glass and Pipettes

Soft glass capillaries made from soda-lime class II glass (Sherwood Medical Industries, Inc., St. Louis, MO) or Kimble R6 glass (Fredrich and Dimmock, Mellville, NY) were used with similar results. Pipettes were pulled at the moment of use from a vertical pipette puller (DKI 700C; David Kopf Instruments, Tujunga, CA) using the standard two-pull method. Heater current was adjusted to produce a tip diameter of 2–4 μm. Pipettes were heat polished using 25 μm Pt wire covered with melted soft glass from the same sources given above. The tip diameter of polished pipettes was always in the range of 1–2 μm. Heat polishing was found useful to generate a homogeneous population of pipettes and to reduce the scattering of the data. However, gigaseals could be formed with unpolished pipettes with equal success. The open tip resistance of each pipette was measured in 0.12 M KCl, 10 mM Tris-Cl, pH 7.0. Pipettes with tip resistance $R > 15$ MΩ and $R < 5$MΩ were rejected. The mean resistance of all pipettes used in experiments ($n = 195$) was 8.9 ± 2 MΩ standard deviation (SD). Glass capillaries were used as shipped without additional cleansing.

Solutions and Glassware

Solutions were prepared at the moment of use from solid salts (reagent grade; Fisher Scientific Co., Allied Corp., Pittsburgh, PA). The use of stock solutions was avoided. For preparing solutions, the distilled water provided through the building pipeline was filtered in the laboratory using Barnstead Ultrapure ion exchange resin DO809 (Barnstead Co., Sybron Corp., Boston, MA) and distilled again in an all-glass still (MPI; Corning Glass Works, Corning Science Products, Corning, NY). All glassware used was of the sterile disposable type made from polystyrene usually used in tissue culture. All determinations were done in 0.1 M KCl + X M C^{++} Cl_2 + 10 mM Tris Cl, pH 7.0, where C^{++} was Ca^{++}, Mg^{++}, or Ba^{++}. Pipette and bath solution was always the same.

Lipids, Monolayers, Bilayers

Lipids were purchased from Avanti Polar Lipids (Birmingham, AL) and were stored at all times in chloroform at −80°C. PS corresponds to mixed fatty acid chain purified from brain (Cat. No. 850032) and DOPE is prepared by synthesis (Cat No. 850725). Monolayers were spread by addition of 1–2 μl of phospholipid dissolved in chloroform (10 mg/ml lipid) to the surface of a polystyrene dish (Corning 35 mm, tissue culture; Corning Glass Works, Corning Science Products) containing 5 ml of buffer. The chloroform droplet that deposits against the bottom of the dish was carefully removed with a micropipette. To form bilayers, the pipette was introducted into the bath through the monolayer, with constantly applied positive pressure. After releasing pressure with the pipette inside the solution, seals were formed by moving the pipette out into the air and back into the solution. A total of 10 trials (out and in movements through the monolayer) were performed with each pipette. The highest seal recorded in the 10 trials was entered in the averages. In between trials, the previous seal was broken with positive pressure. Seals were obtained without applying suction through the pipette holder, since this manipulation was not always reproducible. To insure that seals formed were actually bilayers, the seals were routinely checked by the recording of alamethicin channels (6). The pipette holder and head stage amplifier were mounted on a coarse manipulator (Narishige M-2; Narishige Scientific Instrument Laboratory, Tokyo, Japan). The same monolayer was used during a period of 1–4 h without changes in the success of bilayer formation provided that the surface of the solution was not disturbed. The head-stage amplifier was constructed from an FET-input design using a 10 GΩ Cobra feed-back resistor (K & M Electronics, Inc., West Springfield, MA). Resistance was measured with a 10-mV voltage pulse from a holding of 0 mV.

RESULTS

Fig. 1 *A* shows that in the range of tip diameter of 1–2 μm selected here to measure seals, open pipette resistance and seal resistance are uncorrelated. The lack of correlation holds for gigaseals and seals of lower value. Thus, pipette size is not a determinant factor in the present study. The data correspond to PS monolayers in high and low divalent (Fig. 2). For the same set of pipettes, Fig. 1 *B* shows that the number of passes through the monolayer required to establish seals is variable, usually, 1 to 5.

The basic observation concerning seals of acidic lipid is shown in Fig. 2. It is not possible to form PS gigaseals at neutral pH unless the monovalent salt solution in the bath and pipette contains divalent cations. The same effects are observed with either Ca^{++}, Mg^{++}, or Ba^{++}. Fig. 2 *B* shows that in the range of 10^{-3} to 10^{-1} M divalent cation, the resistance increases monotonically, 4-fold per every 10-fold change in divalent concentration. Seals below 10^{-4} M are independent of divalent with an average of only 80 MΩ. Changes in PS seal conductivity for monovalent and divalent salts are mostly cationic with a cation/anion, P(+)/P(−) conductivity ratio >40. For DOPE, however, P(+)/P(−) is ~3.5 at neutral pH (not shown).

Fig. 3 *A* shows gigaseals of DOPE over a wide range of solution pH. Seals are independent of pH and divalent cation in the range of 7.0 to 1.5 pH. The solid line in this range corresponds to 8.5 GΩ, the average resistance for data <6.0 pH. Thus, under similar conditions to that used in PS experiments, DOPE can readily form seals that are two orders of magnitude higher with or without divalents. Above pH 7.0, in parallel with the increase in PE negative charge, formation of gigaseals drops sharply, ~20-fold per 1 pH unit. Fig. 3 *B* shows that divalent cations restore DOPE gigaseals at basic pH. The slope of the titration curve in the range of 10^{-3} to 10^{-1} M corresponds to 3-fold change in conductance per 10-fold change in divalent concentration. Thus, the divalent effect in both acidic DOPE at pH 10.0 and PS at pH 7.0 is in quantitative agreement.

Contributions to the divalent cation effect in seals may arise from the lipid surface potential and the screening produced by divalent cations (12, 13). A complete calculation would require, however, detailed knowledge of the geometry of the seal space, which is entirely unknown in this system. Nevertheless, a simple correlation can be established by calculating surface potentials from the conductance of neutral and charged seals and by comparing these with surface potentials solved from Grahamme equation (12) at constant monovalent and varying divalent concentration in the solution. This is shown in Fig. 4 for seals of PS pH 7.0, DOPE pH 10.0, and two additional acidic lipids, phosphatidylglycerol (PG) pH 7.0, and phosphatidylinositol (PI) pH 7.0. The solid lines correspond to Grahamme surface potential curves solved for negative surface charge densities in the range of 1 charge

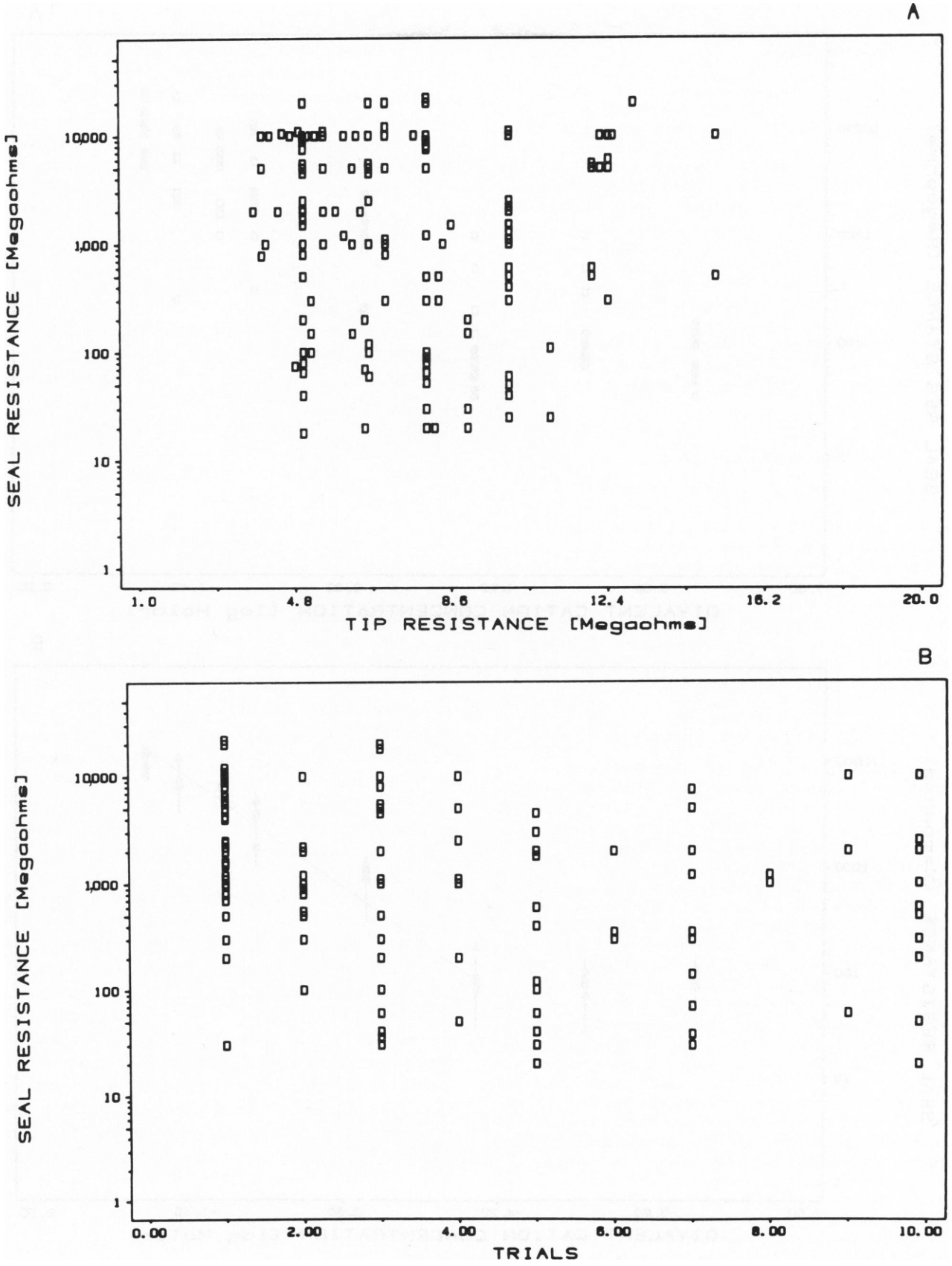

FIGURE 1 Relation between pipette size and seal resistance. For the set of 133 pipettes used in PS monolayers, *A* shows the lack of correlation between open pipette resistance and seal resistance. Open pipette resistance was measured in all cases in 0.1 M KCl, 10 mM Tris-Cl, pH 7.0. *B* Corresponds to the number of trials that were needed to form a stable seal (seal lasting >5 min). Gigaseals were usually established within five trials. Each point in *A* and *B* is a single pipette.

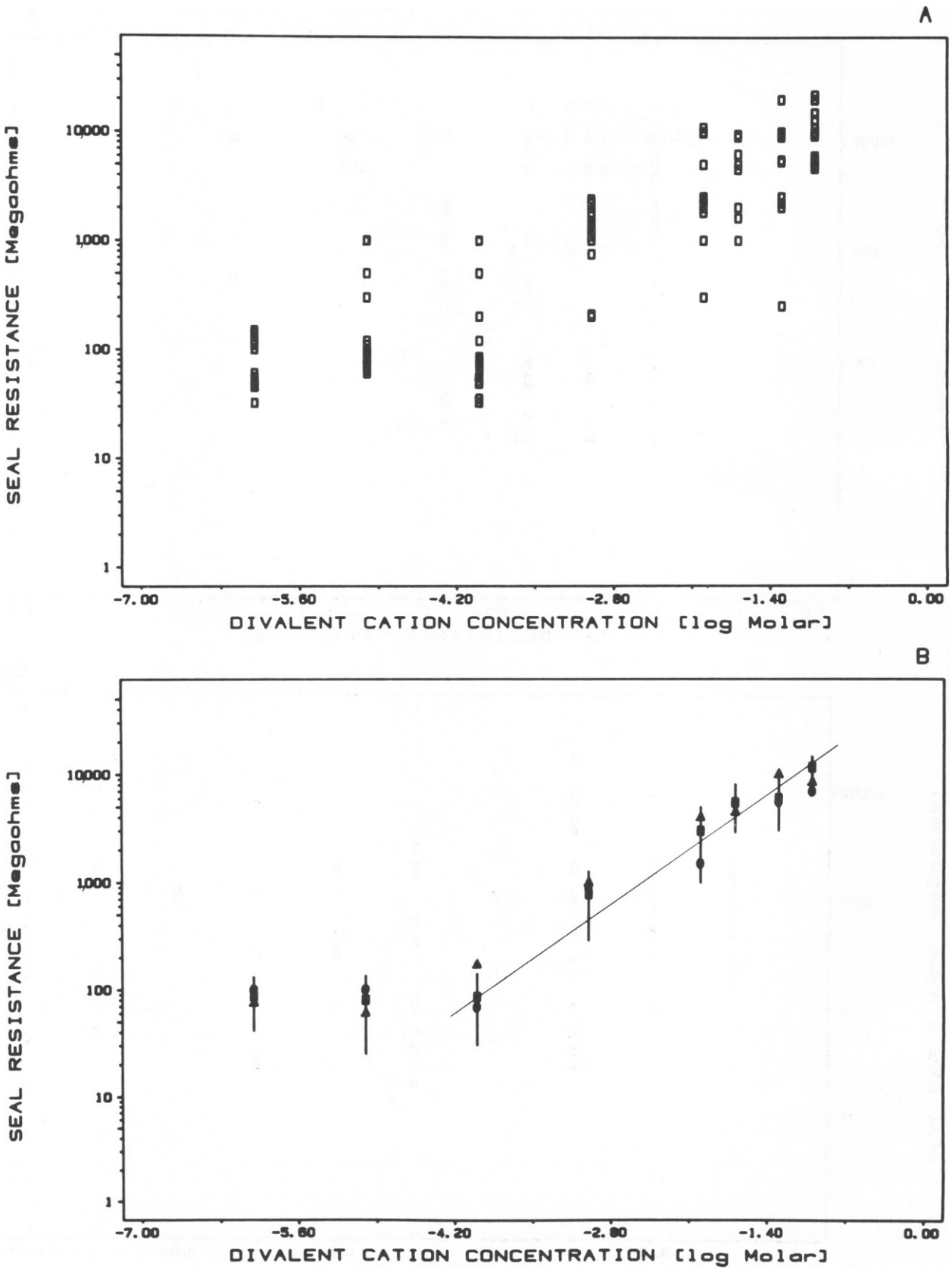

FIGURE 2 Divalent cation dependence of phosphatidylserine seals. *A* and *B* correspond to plots of seal resistance in units of megaohms vs. log molar concentration of divalent cation. Measurements were made in 0.1 M KCl, 10 mM Tris-Cl, pH 7.0, plus the indicated concentration of divalent; *A* shows seal resistance of each pipette ($n = 133$); *B* corresponds to average seal resistance for each divalent plotted separately, Ca^{++} (■), Mg^{++} (●), Ba^{++} (▲). Vertical bars correspond to SD of data collected for the three divalents. The solid line is a linear fit in the range of 10^{-3} to 10^{-1} M divalent. The slope is equivalent to a 4-fold change in resistance per 10-fold change in divalent concentration.

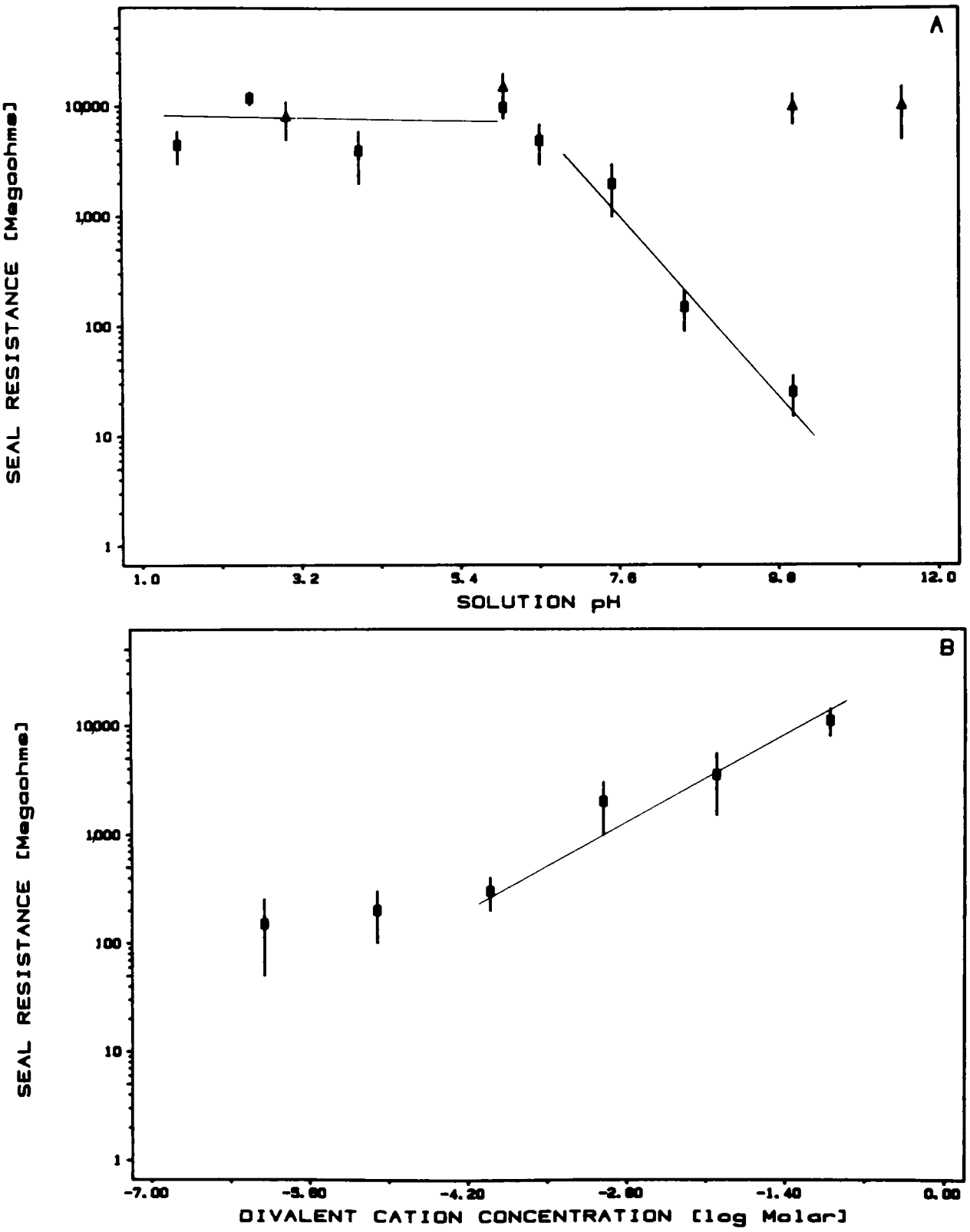

FIGURE 3 pH dependence of dioleoylphosphatidylethanolamine (DOPE) seals. (*A*) Solutions were adjusted to desired pH with 10 mM citrate-(HCl-KOH) for pH <6.0, or 10 mM Tris-(HCl-KOH) for pH ≥6.0. Entries correspond to mean ± SD of seals in 0.1 M KCl, 10 mM buffer (■) or 0.1 M KCl, 10 mM buffer + 0.1 M $CaCl_2$ (▲). The solid line drawn from 1.5 to 6.5 pH corresponds to 8.5 G Ω, the average resistance of all seals in that interval. Solid line from 10.0 to 7.0 pH is a linear fit of data in the absence of divalent ion in that pH interval. Each point is the average of 5–10 pipettes. (*B*) Log plot of DOPE seal resistance at pH 10.0 vs. divalent cation concentration. Measurements were made in 0.1 M KCl, 10 mM Tris-KOH, pH 10.0, plus C^{++} Cl_2 at the indicated concentration. Entries correspond to mean and SD of data obtained in Ca^{++}, Mg^{++}, and Ba^{++}. Solid line corresponds to a 3-fold change in resistance per 10-fold increase in divalent ion concentration. Each point is the average of 15–20 pipettes.

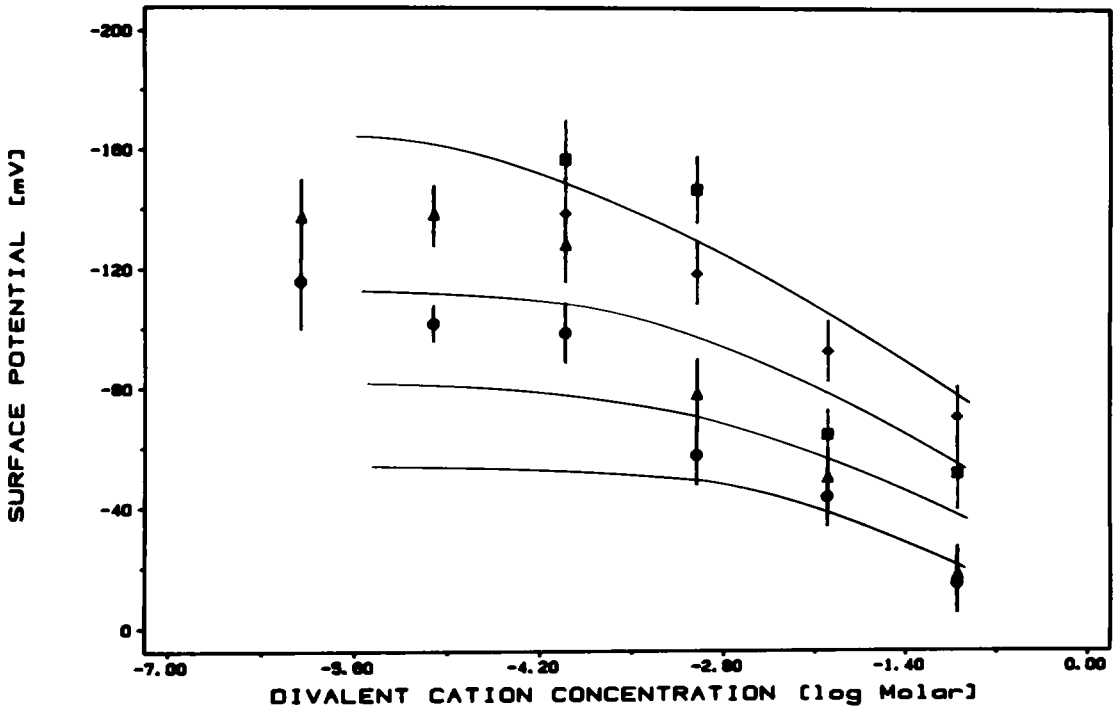

FIGURE 4 Correlation between seal surface potential and Grahamme curves. Solid lines correspond to solutions to Grahamme equation relating membrane surface charge, surface potential, and bulk concentration of monovalent and divalent ions (13). Curves correspond to numerical solutions in 0.1 M monovalent salt (1:1) plus indicated divalent salt (2:1), for negative surface charges of 1/38, 1/100, 1/200, 1/330, charges per Å^2, from *top* to *bottom*, respectively. Data correspond to PS pH 7.0 (▲), PG pH 7.0 (•), PI pH 7.0 (■), and DOPE, pH 10.0 (●), calculated as described in the text. Entries correspond to mean ± SD.

per 35 Å^2 (top curve) to 1 charge per 330 Å^2 (bottom curve). Data are transformed into surface potential, $\Psi(o)$, according to reference 13, $\Psi(o) = RT/F \log (Gcharged/Gneutral)$, where Gcharged is the seal conductance of a charged lipid at a given divalent concentration, Gneutral = 1/20 GΩ^{-1}, which was arbitrarily chosen as the conductance of a fully neutral seal, and $RT/F = 58.3$ mV. This equation assumes that the seal conductance is directly proportional to the local cation concentration. Concentration of cations follow the surface potential through the Grahamme equation (12, 13). The significance of this calculation is that in the range of 10^{-3} to 10^{-1} M divalent the seal surface potentials and the Grahamme curves decrease with the same slope of ~30 mV per 10-fold change in divalent. This suggests, although without proof, that in the millimolar range of divalent cation, the seal conductance of negatively charged lipid is dominated by the local conductivity of the solutions nearby the seal space. This would explain the similarities in slopes in the plots of seal resistance vs. divalent for PS and DOPE (Figs. 2 *B*, 3 *B*) as for PG and PI (not shown).

DISCUSSION

The results shown attempt to identify one of the many possible variables that can influence seal formation in the dipping technique. Some of these variables may be unique to this technique since unlike gigaseal formation onto cell surfaces (5, 16), seal formation and bilayer assembly occur here at the same time. A notable difference in seal-forming characteristics was found when neutral and negatively charged phospholipids of different chemical composition were compared under similar conditions. PS seals without divalents average 80 MΩ (Fig. 2), while DOPE seals average 11 GΩ at neutral pH or 150 MΩ at pH 10.0 (Fig. 3). Three observations, which I restate below, suggest that the two log unit differences between neutral and charged seal conductance can be interpreted qualitatively in terms of the lipid charge and its effect on the conductivity of the solutions adjacent to the bilayer and seal surfaces (12, 13). First, PS seals reach the same value of DOPE seals when Ca^{++}, Mg^{++}, or Ba^{++} are added to the supporting medium. Second, DOPE seals are gradually lost as PE molecules become negatively charged at alkaline pH (12). The approximate *pK* of the conductance drop shown in Fig. 2 *A* (*pK* = 9.3) correlates well with the *pK* of the ethanolamine group of the lipid (*pK* = 9.8). Third, titration of the divalent effect for DOPE pH 10.0 and PS pH 7.0 shows that gigaseals can be recovered with comparable slopes. This agreement strongly suggests that DOPE and PS seals differ mostly on the net charge of the polar group of the lipid at a given pH. Hence, no additional chemical differences are necessary to invoke in order to explain the marked differences in seal-forming characteristics of these

two lipids. At acid pH, however, the results do not bear out a simple electrostatic interaction between lipid polar groups and the pipette wall as the cause of seal conductance. If the lipid charge were the only variable, positively charged DOPE should have increased the local concentration of anions near the membrane surface (12) and this should have produced a drop in seal resistance in the acid side of the seal vs. pH curve. Fig. 2 *A* shows that on the contrary, DOPE seals are independent of solution pH in the range of 6.5 to 1.5. This can only indicate that specific interactions, probably in this case via direct bonding of the positive lipid to the negative silanols, might dominate gigaseal formation in the acid range. A complete explanation of pH and divalent effects in glass pipettes would have to consider, in addition to a precise knowledge of the seal geometry, the degree of ionization of glass silanols (9, 10) and silanol-divalent cation binding (8, 19). None of these variables can be presently considered in quantitative terms. In silica, for example, Ca^{++} ions bind strongly, with association constants in the order of 10^3 l/mol depending on temperature and pH (8). Likewise, based on measurements in silica (19 and references therein), high pH ionizes silanol groups ≡SIOH, which are then transformed into pseudosilicate groups ≡SIO—C^+. There are many measurements that indicate, however, that silica is not a good model for the surface of glass capillaries (11, 21). Thus, it becomes difficult to know the direction in which glass surface groups might influence seal formation.

Finally, it should be emphasized that my observations pertain to pure lipid systems. The behavior of mixed monolayers of neutral PE and PS is quite complex. Gigaseals in DOPE/PS bilayers are roughly independent of divalents until the molar fraction of PS in the monolayer exceeds 0.5 (not shown). This result could explain why seals of lipid mixtures such as asolectin, which contain usually <10% PS have no requirement for divalent cation (Eisenman, G., personal communication).

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