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# Effect of Phospholipid Surface Charge on the Conductance and Gating of a $Ca^{2+}$-Activated $K^+$ Channel in Planar Lipid Bilayers

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**Summary.** A Ca-activated, K-selective channel from plasma membrane of rat skeletal muscle was studied in artificial lipid bilayers formed from either phosphatidylethanolamine (PE) or phosphatidylserine (PS). In PE, the single-channel conductance exhibited a complex dependence on symmetrical $K^+$ concentration that could not be described by simple Michaelis-Menten saturation. At low $K^+$ concentrations the channel conductance was higher in PS membranes, but approached the same conductance observed in PE above 0.4 M KCl. At the same $Ca^{2+}$ concentration and voltage, the probability of channel opening was significantly greater in PS than PE. The differences in the conduction and gating, observed in the two lipids, can be explained by the negative surface charge of PS compared to the neutral PE membrane. Model calculations of the expected concentrations of $K^+$ and $Ca^{2+}$ at various distances from a PS membrane surface, using Gouy-Chapman-Stern theory, suggest that the $K^+$-conduction and $Ca^{2+}$-activation sites sense a similar fraction of the surface potential, equivalent to the local electrostatic potential at a distance of 9 Å from the surface.

**Key Words** surface charge · potassium channel · calcium · phosphatidylserine · planar bilayer

## Introduction

The cell plasma membrane contains about 20% negatively charged phospholipids, such as phosphatidylserine (PS), which produces a negative electrostatic surface potential in the aqueous phase adjacent to the membrane. This negative surface potential causes the local accumulation of cations and depletion of anions in solution and results in a diffuse double layer of charge at equilibrium (McLaughlin, 1977). An important question in membrane biology concerns the effect of surface charge on the function of proteins responsible for the transport of electrolytes. In particular, one aspect of this problem that has received recent attention is the effect of surface charge on the gating and conduction properties of $Na^+$, $K^+$ and $Ca^{2+}$ channels of electrically excitable membranes (Fohlmeister & Adelman, 1982; Hahin & Campbell, 1983; Wilson et al., 1983).

Frankenhauser and Hodgkin (1957) first invoked surface charges and hence, surface potential, to explain the shift in the activation of $Na^+$ and $K^+$ currents of squid axon toward a more depolarizing voltage range when the external $Ca^{2+}$ concentration was increased. Thus, they attributed a possible physiological role to this membrane property. Since this early study, it has become apparent that the interpretation of such results requires the ability to distinguish between a variety of possible effects. For example, the total ionic current for a given channel in the membrane is determined by gating and conduction properties of the channel, which may be modulated by local ion activities and electrostatic potential in the vicinity of certain protein sites. Surface charge effects on conduction and gating can be mediated by local fixed charges on the lipids or the channel protein itself and can be modified by either direct binding of counterions to these sites or local screening by the unbound counterions in solution. One approach to resolving the individual contribution of these variables is to measure single-channel currents in artificial membranes of well-defined lipid composition. Such an approach has shown that the conducting pore of the small peptide channel, gramicidin A, senses most of the surface potential of the lipid in a PS bilayer (Apell, Bamberg & Läuger, 1979). In contrast to this finding, similar experiments with hemocyanin show that the conductance of this channel is insensitive to the surface potential (Cecchi, Alvarez & Latorre, 1981). Also, Bell and Miller (1984) have recently shown that conduction of $K^+$ through the sarcoplasmic reticulum $K^+$ channel senses less than the full lipid surface potential, as expected if the pore mouth is located some distance away from the bulk lipid.

In this paper, we compare the $K^+$-conduction and $Ca^{2+}$-dependent gating characteristics of a $Ca^{2+}$-activated $K^+$ channel (Latorre, Vergara & Hidalgo, 1982) in bilayers formed from the zwitterionic lipid, phosphatidylethanolamine (PE) or the negatively charged lipid, PS. In PE bilayers we find that the conductance of the channel is not a simple saturable function of $K^+$ concentration, expected for a one-site channel. The biphasic titration curve that we observe is consistent with several possibilities such as a conduction pathway with multiple occupancy sites for $K^+$, or a single-site channel that can exist in multiple slowly relaxing conformations. We also find that both the $K^+$-conduction and $Ca^{2+}$-activation processes are able to sense the lipid surface charge of a PS membrane. These effects can be conveniently interpreted as an increased local $K^+$ and $Ca^{2+}$ concentration in the vicinity of the conduction and activation sites as predicted by the Gouy-Chapman double-layer theory.

## Materials and Methods

### Experimental

Planar bilayers were formed from a 20 mg/ml solution of phosphatidylethanolamine (PE) or phosphatidylserine (PS) in decane by the technique of Mueller and Rudin (1969). The lipid solution was spread on a 200-$\mu$m diameter hole in a polystyrene partition separating two aqueous compartments. Channel incorporation was observed after the addition of 10 to 100 $\mu$g protein/ml of a rat muscle plasma membrane preparation to one side of the bilayer (*cis*). Incorporation at KCl concentrations lower than 50 mM required the use of at least a twofold KCl gradient across the planar bilayer, higher on the *cis* side. After channel incorporation, the *cis* chamber was extensively perfused with a KCl solution identical to that of the *trans* chamber. The plasma membrane fraction was purified from rat muscle microsomes according to Moczydlowski and Latorre (1983*a*).

The voltage-clamp circuit for measuring single-channel current was as previously described (Latorre et al., 1982). The sign of the applied voltage refers to the $Ca^{2+}$-sensitive side of the channel and is the same convention as normally used for cells: positive (depolarizing voltages) favor the activation of $Ca^{2+}$-activated $K^+$ current. All measurements were made at ambient temperature, 20 to 22°C.

Single-channel conductance was measured as the slope of the current-voltage relation of the open channel at voltages less than 50 mV and at various symmetrical KCl concentrations. Plots of current *vs.* voltage were linear in this voltage range (e.g. Fig. 2). The $K^+$ concentration was varied by addition of a stock solution of 3 M KCl to 5 mM MOPS-KOH, pH 7.0 buffer containing various initial KCl concentrations. For KCl concentrations below 0.2 M, the conductance was measured in the presence of 2 $\mu$M symmetrical $CaCl_2$. At higher KCl concentrations, up to 50 $\mu$M symmetrical $CaCl_2$ was used. Single-channel current was measured either by hand from records on chart paper or from the amplitude distributions of records digitized by a pulse-height analyzer. Values obtained by either method were similar.

The relative probability of channel opening as a function of voltage was measured in symmetrical solutions of 100 mM KCl, 10 mM MOPS-KOH, pH 7, and various $CaCl_2$ concentrations. For single-channel membranes, the time-averaged probability of the open state $P_o$ was obtained from digitized records as the time spent in the open current level divided by the total time of the record, usually 20 sec. The discrimination level was set at half the amplitude of open current level. For membranes containing more than one channel, $P_o$ was obtained by recording the current directly on a chart recorder by heavily filtering channel fluctuations with 1 Hz *R/C* passive circuit. Both methods of measuring $P_o$ gave similar activation curves. Total $Ca^{2+}$ concentrations were determined by atomic absorption spectroscopy.

Bovine brain PE and PS were obtained from Avanti Polar Lipids (Birmingham, Ala.). Samples of PE were found to contain less than 1% PS or phosphatidic acid as analyzed by high pressure liquid chromatography. Decane was obtained from Eastman Organic Chemicals (Rochester, N.Y.). Ultrapure KCl was from Alfa Division, Ventron Corp. (Danvers, Mass.).

### Analysis

The effect of lipid surface charge on channel conduction properties was analyzed using the Gouy-Chapman-Stern double-layer theory (McLaughlin, 1977; McLaughlin et al., 1981). The pure PE bilayer is assumed to have no surface potential under the conditions we have studied.[1] $Ca^{2+}$ binding to PE membranes is negligible, since the reported binding association constant is 3 $M^{-1}$ (McLaughlin et al., 1981) and our experiments involve less than 1 mM $Ca^{2+}$. The surface potential of the PS bilayer was calculated at different bulk $Ca^{2+}$ and $K^+$ concentrations using the measured binding association constants of these ions to PS liposomes of $K_1 = 0.15$ $M^{-1}$ for the $K^+$-$PS^-$ complex and $K_2 = 12$ $M^{-1}$ for the $Ca^{2+}$-$PS^-$ complex (McLaughlin et al., 1981). The surface potential $\psi_o$ may be calculated numerically from the relationship between $\psi_o$ and the surface change density $\sigma$, using the following three sets of equations:

$$\sigma = 2\varepsilon_r\varepsilon_o RT\Sigma_i C_i[\exp(-z_i F\psi_o/RT) - 1]^{1/2} \quad (1)$$

$$\sigma = \frac{[P^-]^{tot}[1 - K_2 C_o^{2+}]}{1 + K_1 C_o^{+} + K_2 C_o^{2+}} \quad (2)$$

$$C_o^{+} = C_b^{+} \exp(-F\psi_o/RT) \quad (3a)$$

$$C_o^{2+} = C_b^{2+} \exp(-2F\psi_o/RT). \quad (3b)$$

Equation (1) is the Grahame (1947) equation for solutions of mixed salts where $C_i$ is the concentration of ions of valence $z_i$ in the bulk aqueous phase, $\varepsilon_r$ is the dielectric constant of water, $\varepsilon_o$ is the permittivity of free space, $R$ is the gas constant, $F$ is the Faraday and $T$ is the absolute temperature. Equation (2) relates the net surface density of negative charge to the total surface density of PS ($[P^-]^{tot}$), the $Ca^{2+}$ and $K^+$ binding constants ($K_2$ and $K_1$, respectively) and the surface concentrations of $Ca^{2+}$ and $K^+$

[1] Bell and Miller (1984) found that bilayers and monolayers made from a mixture of 80% PE and 20% phosphatidylcholine have a slight negative charge (~4%). Our own measurements indicate that pure PE monolayers contain ~3% negative charge at pH 7. For the purpose of the present study, this amount of charge is negligible.

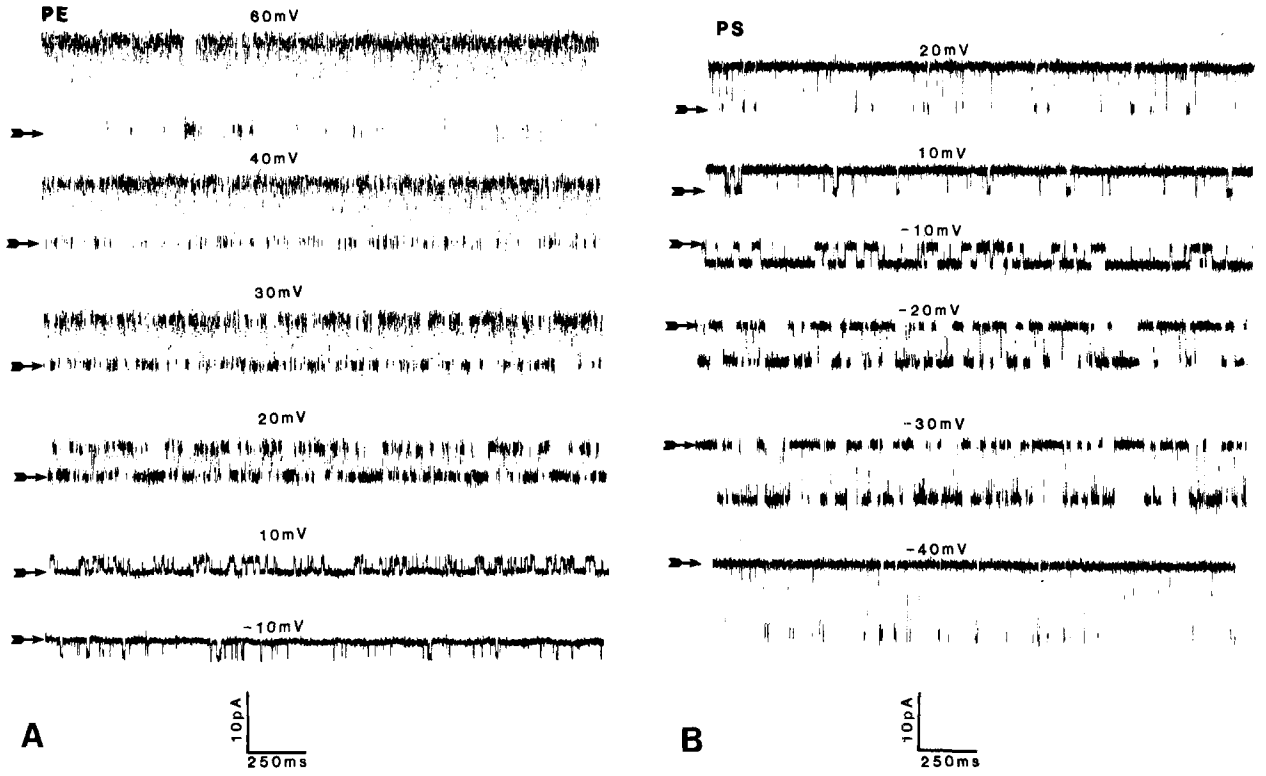


**Fig. 1.** Steady-state current fluctuations of single $Ca^{2+}$-activated $K^+$ channels in a neutral and negatively charged bilayer. Either PE (*A*) or PS (*B*) was used to form the bilayer. The buffer on both sides of the membrane was 5 mM MOPS-KOH, 100 mM KCl, 12 $\mu$M $CaCl_2$, pH 7.0. The arrow to the left of each record indicates the zero current level. The holding voltage was as indicated above each record. 1 kHz filter

($C_o^{2+}$ and $C_o^+$, respectively). The total surface density of the PS bilayer is calculated by assuming an area of 70 Å$^2$ per phospholipid (Loosley-Millman, Rand & Parsegian, 1982). The Boltzmann relations of Eqs. (3) must be used in Eq. (2) to relate the bulk aqueous concentrations of $K^+$ and $Ca^{2+}$ ($C_b^+$ and $C_b^{2+}$) to their surface concentrations ($C_o^+$ and $C_o^{2+}$). Once the surface potential is known, the electrostatic potential at a distance $x$ from the membrane can be estimated from:

$$\psi(x) = (2RT/F) \ln \frac{1 + \alpha \exp(-DX)}{1 - \alpha \exp(-DX)} \tag{4}$$

$$\alpha = \frac{[\exp(F\psi_o/2RT) - 1]}{[\exp(F\psi_o/2RT) + 1]} \tag{5}$$

where $D$ in Eq. (4) is the reciprocal of the Debye length. Lastly, the local $K^+$ or $Ca^{2+}$ concentration at a distance $x$ from the membrane can be calculated by:

$$C^+(x) = C_b^+ \exp(-F\psi(x)/RT) \tag{6a}$$

$$C^{2+}(x) = C_b^{2+} \exp(-2F\psi(x)/RT). \tag{6b}$$

Equation (4) applies strictly to the case where the solution only contains monovalent ions. For cases we consider, bulk calcium concentrations are generally less than 0.1 mM and the counterions in the double layer are mainly potassium. In this situation, Eq. (4) provides a valid estimate of the decay of potential with distance.

## Results

Figures 1*A* and 1*B* show records at various applied voltages of single-channel fluctuations measured in a PE and PS bilayer under identical ionic conditions (0.1 M KCl, 12 $\mu$M $CaCl_2$). These records summarize our main findings regarding the behavior of the $Ca^{2+}$-activated $K^+$ channel in a neutral *vs.* a negatively charged phospholipid environment. At the same $Ca^{2+}$ concentration and voltage, the channel exhibits a greater probability of the open state in PS compared to PE. Also at KCl concentrations below 0.4 M, we find that the single-channel conductance is higher in PS vs. PE bilayers. For example, the channel conductance is 340 pS in PS and 240 pS in PE in the experiments of Fig. 1 at 0.1 M KCl. Figure 2 shows current *vs.* voltage curves for the same single channels of Fig. 1, showing that under these conditions the channel is ohmic in the range of voltage studied (−60 to +60 mV).

The effects shown in Figs. 1 and 2 may be explained by the fact that the $K^+$ and $Ca^{2+}$ concentration near the surface of a negatively charged membrane is greater than the concentration of these ions in the bulk solution. If the binding sites for these

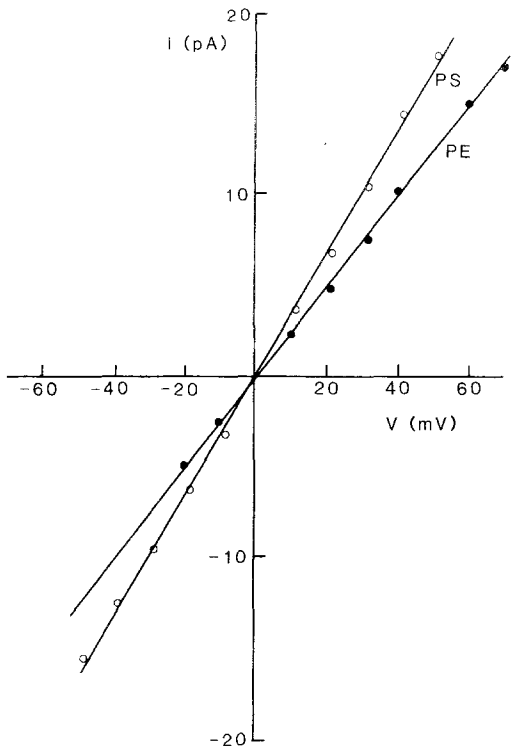


**Fig. 2.** Current-voltage relation of the open channel in PE and PS bilayers. The measured current for the same channels studied in the experiment of Fig. 1*A* and 1*B* is plotted at different applied voltages. The unit conductance corresponding to the slope of the solid lines is 244 pS for PE and 337 pS for PS

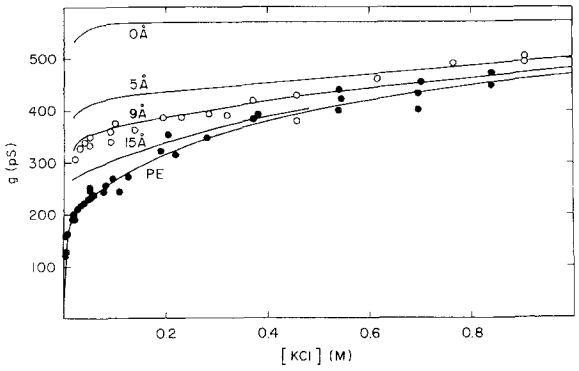


**Fig. 3.** Single-channel conductance as a function of KCl concentration. Unit conductance was measured for channels incorporated into PE (●) or PS (○) bilayers as described in Materials and Methods and as shown in Figs. 1 and 2. The solid lines are computer fits to Eq. (7) for the curve labeled "PE," which assumes that there are no surface charge effects in this lipid. The solid lines labeled "0 Å" to "15 Å" are computer fits using Eqs. (1) to (7), which allow $K^+$ concentration in Eq. (7) to be expressed as the local $K^+$ concentration at various distances from the surface of a PS bilayer, according to Gouy-Chapman-Stern theory

ions on the channel protein are located in the region of this increased concentration, then the gating equilibrium will be shifted toward open states by the higher $Ca^{2+}$, and the $K^+$ current through the open channel will be larger if the pore is not saturated with $K^+$. In order to determine whether this simple interpretation is adequate, we measured the conductance as a function of $K^+$ concentration and the probability of opening as a function of $Ca^{2+}$ and voltage in PE and PS bilayers.

## Conductance Behavior

In a previous discussion of the conductance *vs.* $K^+$ concentration behavior of the $Ca^{2+}$-activated $K^+$ channel in bilayers, Latorre and Miller (1983) suggested that this channel operates by a simple, single-ion mechanism based on the "Michaelis-Menten" behavior of the unit conductance. However, we have since found that the cited experiments were complicated by the presence of trishydroxymethylaminomethane as a buffer, which is a blocker of the channel and gives rise to artifactual Michaelis-Menten behavior with respect to $K^+$ (Vergara, 1983). To avoid this artifact in the present study, we measured the single-channel conductance in solutions where $K^+$ is the only cation, besides the 2–50 $\mu$M $Ca^{2+}$ needed for channel activation. The results for pure PE bilayers (Fig. 3) depart markedly from a single-site saturation curve, as noted previously in a preliminary report (Vergara, Moczydlowski & Latorre, 1984). The PE data exhibits biphasic behavior, showing a high-affinity region at low concentration, where the conductance rises steeply as a function of $K^+$, and a low affinity region, where the conductance increases in a gradual fashion. In the present context, we are not concerned with the mechanism responsible for this behavior, although several possible explanations are given in the Discussion. Rather, we address the effect of negatively charged phospholipids, indicated by the increased conductance in PS *vs.* PE observed at low $K^+$ concentrations (Fig. 3). Such an effect is expected according to the Gouy-Chapman theory of surface charge, since the negative surface potential of a PS membrane increases as the ionic strength is lowered.

To apply this theory, the results in PE (neutral) membranes were first fit empirically as a sum of two Langmuir isotherms:

$$g = \frac{G_1}{1 + K_1/[K^+]} + \frac{G_2}{1 + K_2/[K^+]}. \qquad (7)$$

Equation (7) expresses the observed conductance $g$ as a function of $K^+$ concentration, where the maximum conductance and concentration at half-saturation are $G_1$, $K_1$ and $G_2$, $K_2$ for the respective high-affinity and low-affinity terms. The PE data of Fig. 3 were fit to Eq. (7) by a nonlinear least-squares method and the following best-fit parameters were obtained: $G_1$ = 206 pS, $K_1$ = 2.5 mM, $G_2$ = 404 pS and $K_2$ = 500 mM. The behavior of Eq. (7) using these values is shown as a solid line in Fig. 3 that closely follows the PE results.

The expected behavior including the additional effect of surface charge was explored by calculating the predicted $K^+$ concentration at a distance $x$ from the membrane surface as described in Materials and Methods. The bulk $K^+$ concentration in Eq. (7) is multiplied by the Boltzmann factors of Eq. (6a), which makes Eq. (7) a function of the surface potential at distance $x$, $\psi(x)$. It is worthwhile to note that this procedure is equivalent to replacing the $K_1$ and $K_2$ Michaelis constants of Eq. (7) by apparent constants which are functions of the surface potential. Thus, we may consider the rate enhancement caused by PS as due either to an increase in the local $K^+$ concentration from a microscopic viewpoint or due to a $K^+$ binding constant that is a function of surface potential from a macroscopic viewpoint.

The results of these calculations are presented in Fig. 3 as solid lines given for various distances 0, 5, 9 and 15 Å from the membrane. It is clear from this approach that the channel conductance senses less than the full surface potential indicated by the 0 Å curve. The PS data corresponds fairly well to the predicted behavior at a distance of 8 to 10 Å from the surface of the membrane as indicated by the 9 Å curve. This treatment provides no indication of the spatial relationship of the channel mouth to the membrane surface, other than to suggest that the mouth is insulated from the charged surface by a perpendicular or lateral distance of this magnitude. This insulation is presumably part of the structure of the channel protein.

## Gating Behavior

An inherent difficulty in studying the gating behavior of the $Ca^{2+}$-activated $K^+$ channel is heterogeneity of the $Ca^{2+}$ sensitivity among individual channels. This is indicated by the variation in the probability of the open state $P_o$ at fixed $Ca^{2+}$ and voltage for different bilayers shown in Fig. 4. This heterogeneity is observed both in PS (Fig. 4*A*, *B*) and PE bilayers (Fig. 4*C*, *D*). The spread of data along the voltage axis is as large as 50 mV for different channels. However, any single channel we have examined has a similar voltage dependence of the probability of opening which corresponds to an $e$-fold change in $P_o$ per 10 ± 2 mV. Despite this difficulty, we do observe a significant increase in the $Ca^{2+}$ sensitivity in PS compared to PE by comparing the average data compiled from many different bilayers. This effect is shown by comparison of Fig. 4*B* with Fig. 4*D*. The PS data at 2 μM $Ca^{2+}$ appears to be shifted along the voltage axis to more negative voltages by about 30 to 40 mV compared to the PE data. A similar effect is seen at 100 μM $Ca^{2+}$ for Fig. 4*A* *vs.* 4*C*.

To analyze such data, we measured the voltage at half-saturation, $V_o$, where $P_o$ = 0.5, for many bilayers at different fixed $Ca^{2+}$ concentrations. The results of this comparison are presented in Fig. 5. In this figure, the filled circles and error bars represent the mean $V_o$ and its standard deviation for 6 to 9 different PE bilayers at 2, 100 and 1000 μM $CaCl_2$, while the open circles correspond to the same measurement for PS bilayers at 2, 15 and 100 μM $CaCl_2$. These results show that the average gating behavior in PS exhibits about 10-fold higher $Ca^{2+}$ sensitivity than that in PE. Figure 4 also shows the measured $V_o$ for two experiments in PE bilayers for which the $Ca^{2+}$ concentration was varied from 1 μM to 1 mM for two different single-channel channels (filled triangles and squares). These data agree well with the experiments of Fig. 4 at fixed $Ca^{2+}$ concentration and show that the channel exhibits a 35-mV change in $V_o$ per 10-fold change in $Ca^{2+}$ concentration.

To evaluate the effect of surface charge, we calculated the surface potential at a distance $x$ from the membrane necessary to result in a ten-fold higher local $CaCl_2$ concentration at different bulk $Ca^{2+}$ concentrations of 2, 15 and 100 μM. These calculations used the Gouy-Chapman-Stern treatment described in Materials and Methods to take into account both binding of $Ca^{2+}$ and $K^+$ to PS and the screening effect of these ions. The results of these calculations indicate that the distance from the membrane surface at which the local $Ca^{2+}$ concentration is ten times higher than the bulk $Ca^{2+}$ is 8.8, 8.2 and 7.0 Å for 2, 15 and 100 μM bulk Ca. At higher $Ca^{2+}$ concentrations, one would eventually expect $Ca^{2+}$ binding by PS to result in convergence of the PS data to that of a neutral PE bilayer. However, at less than 100 μM $CaCl_2$ this effect is not severe. Also, the data of Fig. 5 have too large a standard deviation to determine if $V_o$ is shifted significantly less at 100 μM $Ca^{2+}$ than at 2 μM $Ca^{2+}$.

A different method of predicting the effect of surface potential was examined by including sur-

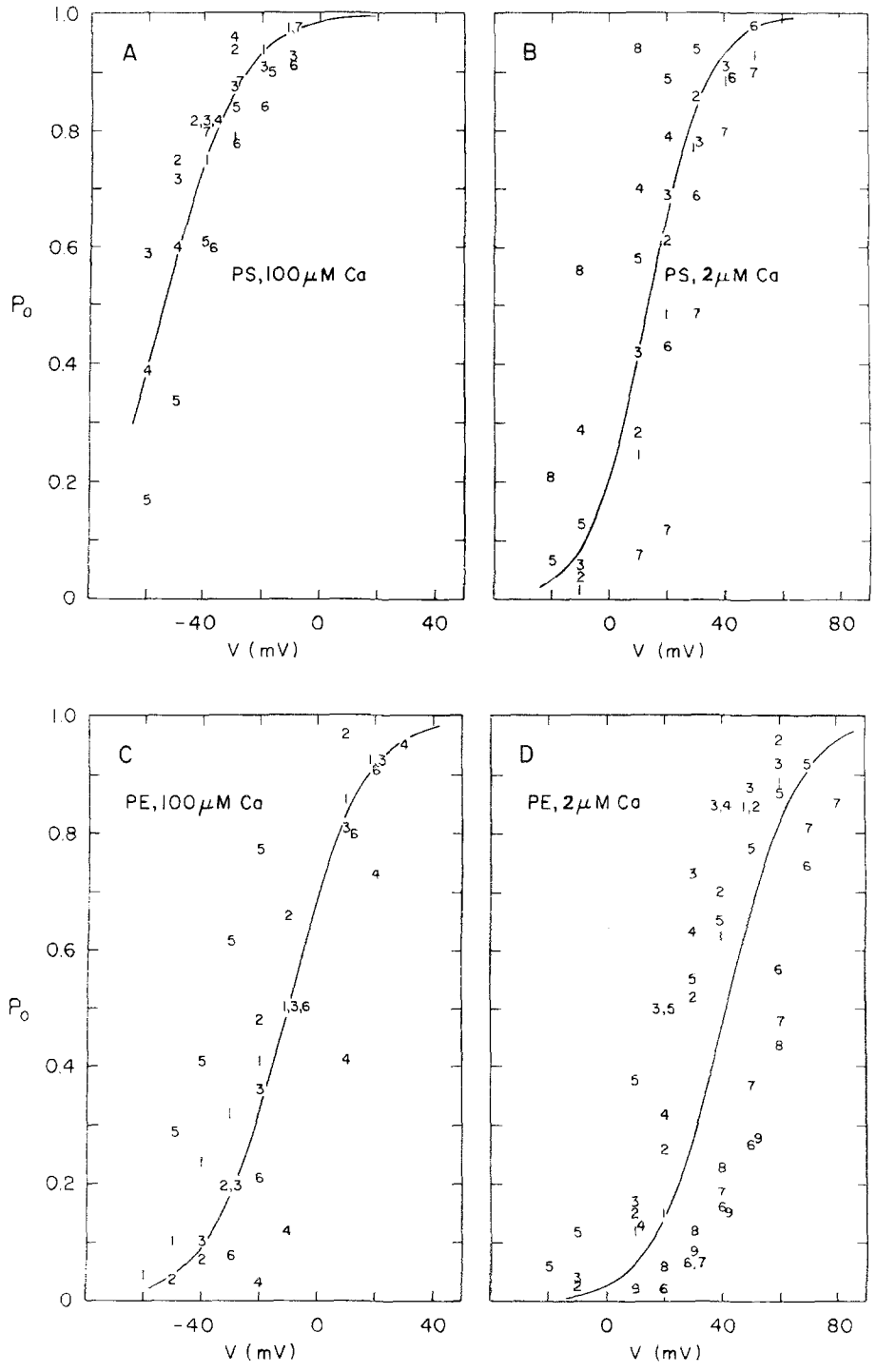


**Fig. 4.** Voltage dependence of gating behavior in PE and PS bilayers. The buffer was 10 mM MOPS-KOH, pH 7.4, 0.1 M KCl and either 2 μM (*B*, *D*) or 100 μM (*A*, *C*) $CaC_2$. After 1 to 4 channels had incorporated in either PS (*A*, *B*) or PE (*C*, *D*) bilayers, the time-average current at various holding voltages was measured using 1 Hz filtering. The ordinate parameter $P_o$ was calculated as the ratio of the measured current to the current expected if all channels in the membrane were fully open. Data points are indicated by numerals corresponding to different bilayers. Solid lines are drawn for purpose of comparison

face potential in the gating model of Moczydlowski and Latorre (1983*b*). This model proposed that the voltage dependence of gating is determined by two voltage-dependent $Ca^{2+}$ binding reactions expressed by equilibrium dissociation constants $K_1$ and $K_2$, where the equilibrium probability of channel opening is given by:

$$P_o([\mathrm{Ca}],V) = \frac{[\mathrm{Ca}]^2 + [\mathrm{Ca}]K_2}{[\mathrm{Ca}]^2 + [\mathrm{Ca}]K_2(1 + \alpha/\beta) + K_1K_2(\alpha/\beta)}. \quad (8)$$

To include the effect of surface potential in this gating model, we expressed $K_1$ and $K_2$ by the following relation:

$$K_D = K_D(0) \exp[-z\delta F(V + \psi)/RT] \quad (9)$$

where the observed $Ca^{2+}$ dissociation binding constant $K_D$ depends on an intrinsic binding constant $K_D(0)$ and a Boltzmann factor composed of the valence ($z$) of the $Ca^{2+}$ ion, the fraction of the applied field that is sensed by $Ca^{2+}(\delta)$, the applied potential ($V$) and the electrostatic potential ($\psi$). To relate this model to Fig. 5, Eq. (8) was solved for the $Ca^{2+}$ concentration at which the probability of opening is equal to 0.5. At this probability, $V$ is equal to $V_o$, referred to in Fig. 5. For the PE membrane, $\psi = 0$, and $Ca^{2+}$ concentrations were calculated as a function of $V_o$ from Eq. (8) using the average of gating parameters in Table II of Moczydlowski and Latorre (1983*b*) that were previously obtained by stochastic analysis of individual channels in PE bilayers. The results of the predicted dependence of $V_o$ on $Ca^{2+}$ for $\psi = 0$ and $\psi = -35$ mV are shown in Fig. 5. It can be seen that the prediction for zero surface potential is a reasonable fit to the PE data and the prediction for $\psi = -35$ mV corresponds closely to the PS data. Thus, a simple model of the gating behavior of the channel based on voltage- and surface potential-dependent $Ca^{2+}$ binding constants can be used to predict the observed behavior in PE and PS. Both the Gouy-Chapmann calculation and the gating model approach suggest that the $Ca^{2+}$ activation process senses less than the full surface potential of a PS membrane. By our analysis, the gating responds to only $-30$ to $-40$ mV of electrostatic potential, while the value at the surface of the membrane is expected to be about $-70$ to $-100$ mV.

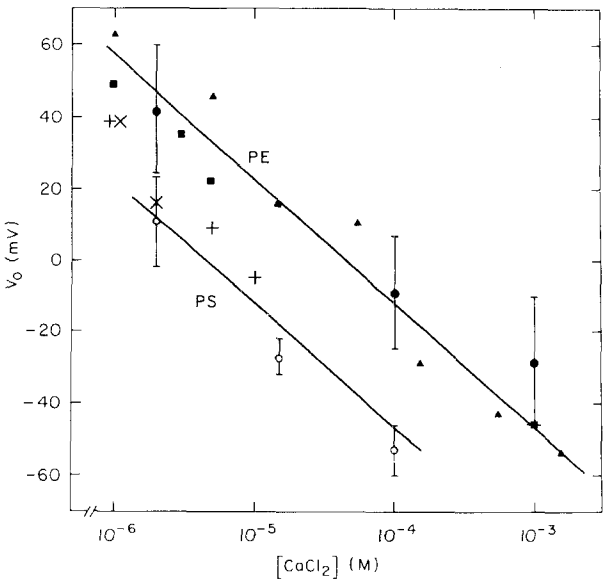


**Fig. 5.** $Ca^{2+}$ dependence of gating behavior in PE and PS bilayers. The ordinate parameter $V_o$ is the voltage at which channels are open half of the time. $V_o$ was determined for each bilayer from a plot of $\ln[P_o/1 - P_o]$ *vs.* $V$. Circles (●) PE, (○) PS, are the mean $V_o$ from macroscopic experiments as described in Fig. 3. Error bars on these points indicate the standard deviation of $V_o$ for a sample of 6 to 9 different bilayers. The filled points ▲, ■ are $V_o$ values from two different single-channel PE membranes, where the $Ca^{2+}$ concentration was varied for the same channel as described in Moczydlowski and Latorre (1983*b*). Other symbols are data taken from (×) Methfessel and Boheim (1982) and (+) Barrett et al. (1982) using patch-clamp recording in rat myotubes. Solid lines are theoretical fits using the gating model of Moczydlowski and Latorre (1983*b*) as described in the text

## Discussion

The purpose of this report is to document the different conductance and gating behavior of the $Ca^{2+}$-activated $K^+$ channel in planar bilayers formed from PE or PS. The observation of a lipid dependence for these two channel properties in an artificial membrane is evidence that the natural lipids surrounding the channel in native vesicles are replaced upon dilution by the exogenous planar bilayer lipids after channel incorporation. This is to be expected since the translational diffusion coefficients of lipids in biological and artificial membranes range between $10^{-8}$ to $10^{-7}$ cm$^2$/sec (Edidin, 1974). Although various physical techniques have detected a less mobile domain of lipids surrounding integral membrane proteins (Jost et al., 1973; Thomas et al., 1982), these boundary lipids are not bound so tightly that they cannot be removed and exchanged with exogenously added lipids (Warren et al., 1974). Our interpretation of the lipid effects is based only on the difference of surface charge of a PE and PS membrane. We caution that this interpretation assumes that there are no specific lipid-protein interactions due either to differences in the acyl chains or the headgroups of the lipids. We also assume that the observed lipid effects are mediated strictly by charge effects and that they are not due to differences in dipole potential between the two lipids.

The gating data of Figs. 4 and 5 in two different lipids provides a substantial body of information that may be compared with the results of other groups studying a similar channel by patch-clamp recording of rat myotubes in culture. We have previously noted the similarity in the voltage dependence of this $Ca^{2+}$-activated channel measured by the bilayer and patch-clamp methods (Moczydlowski & Latorre, 1983*b*), but here we focus more clearly on the $Ca^{2+}$ dependence. In Fig. 5, we

have also plotted the observed $V_0$ at $Ca^{2+}$ concentrations between 1 and 10 $\mu$M reported by Barrett, Magleby and Pallota (1982) (+) and Methfessel and Boheim (1982) (×) for the myotube channel. This comparison shows that the $Ca^{2+}$ sensitivity of the native myotube channel falls in between that of PE and PS bilayers. The slope of $V_0$ *vs.* $Ca^{2+}$ for the two methods is similar within the scatter of the experimental points. This comparison raises the possibility that negatively charged lipids are partly responsible for the greater $Ca^{2+}$ sensitivity observed in biological membranes compared to pure PE bilayers.

The finding of a non-Langmuir shape of the conductance *vs.* $[K^+]$ data in a pure PE bilayer for both the rat and the rabbit channel (Vergara et al., 1984), raises new questions regarding the structure and mechanism of the channel. Specifically, we wonder whether this channel can still be regarded as having single-ion occupancy of its conducting pore, since a multi-site $K^+$-occupancy mechanism could easily give rise to the observed shape of the conductance-concentration curve. For instance, if a pore can contain two $K^+$ ions simultaneously, electrostatic repulsion between the two ions could result in a lower binding affinity of the second ion (Levitt, 1978). Previous results on the blocking behavior of the channel by $Ba^{2+}$ (Vergara & Latorre, 1983) led us to believe that this channel could only contain one ion at a time, since the competition of the $Ba^{2+}$ block by $K^+$ was consistent with single-site occupancy. However, recent results for other blocking ions tend to contradict this simple view. We have observed that the voltage dependence of $Cs^+$ block from the *trans* side (extracellular) of a similar channel from smooth muscle is up to twofold greater than the maximum voltage dependence expected for a single monovalent ion (Cecchi, Wolf, Alvarez & Latorre, *unpublished results*). This may imply that two $Cs^+$ ions are able to occupy the channel. Also, in an independent study of the blocking reaction by $Na^+$ from the intracellular side of a similar channel from pituitary cells, Yellen (1984) found that $K^+$ competition from the extracellular side exhibits "knock-off" behavior, characteristic of a multi-ion pore. The final answer to the question of multi-ion occupancy must await more detailed analysis, but evidence appears to be mounting that large conductance $Ca^{2+}$-activated $K^+$ channels do not operate by a one-site mechanism.

In this report, we have chosen to fit the conductance data in PE using an empirical model that requires a minimum number of four free parameters. A model of two independent pores can give origin to this type of behavior, but it is probably unrealistic. Several alternative explanations could also account for the results. For instance, slow conformational transitions of a single-ion channel could result in a conductance-concentration relation similar to that of Eq. (7) (Läuger, Stephan & Frehland, 1980). Alternatively, local negative charge on the protein in the vicinity of the pore could explain the abnormally high conductance at low ionic strength. This type of behavior has been reported for a negatively charge derivative of gramicidin (Apell et al., 1979). As another example, we might propose a mechanism involving a low affinity modulation site for $K^+$, which is not involved in permeation, but exerts an allosteric effect. The simplest model of single pore with two identical, but interacting ion-binding sites has a similar theoretical expression for conductance *vs.* concentration as that of Eq. (7), except that it contains a cubed term in $K^+$ concentration in the denominator and includes an additional fifth constant (Finkelstein & Andersen, 1981). Although it is known that such models predict a decrease in the conductance at high concentrations, we have not observed such an effect up to 3 M KCl. This would imply that the fraction of the doubly occupied state of the channel is small if such a mechanism applies. Inasmuch as we have not observed a decrease in conductance at high $K^+$ concentrations this model cannot be used to fit the present data. Actually, we tried to use this model to fit the conductance-concentration data and the result was undetermined because of the excess of adjustable parameters.

If we accept the present results at face value, we would conclude that the $K^+$ conduction and $Ca^{2+}$ gating sites of the channel experience a similar electrostatic potential in a PS bilayer. This may mean that these sites are located in the same domain of the channel protein as proposed in one hypothetical model of the channel (Moczydlowski & Latorre, 1983*b*). The distance estimates that we calculated have little meaning in the absence of structural information regarding the location of ion binding sites with respect to membrane surface. It is perhaps not surprising that the conduction and gating properties do not sense the full value of the surface potential in PS. The dimensions of integral membrane proteins can be larger than the 40-Å thickness of the hydrocarbon region of the bilayer and binding sites could be insulated from the membrane lipid head groups, either by a distance lateral or normal to the membrane. For example, the available structural information on the acetylcholine receptor channel from electron microscopy (Kistler et al., 1982) indicates that the protein extends past the bilayer by 55 Å on one side and 15 Å on the other side of the membrane. The diameter of this protein in the plane of the membrane is as large as 85 Å. If the pore extends down the center of this structure,

we would expect the conduction pathway to be far enough from the surface to be insensitive to lipid surface charge down to salt concentrations as low as 50 mM. If these dimensions are typical of biological ion channels, one might expect conductance to be rather insensitive to surface charge effects. Indeed, this expectation has been the finding for biological channels that have been studied in native membranes. Previous studies of voltage-dependent $Na^+$ and $K^+$ currents indicated that the conductance of these channels sense very little surface potential (Begenisich, 1975; Fohlmeister & Adelman, 1982). On the other hand, the gating processes of these same channels appear to be quite sensitive to surface charge (Frankenhaueser & Hodgkin, 1957; Gilbert & Ehrenstein, 1969; Mozhayeva & Naumov, 1970; Begenisich, 1975; Hille, Woodhull & Shapio, 1975; Fohlmeister & Adelman, 1982). Until very recently, it has been difficult to distinguish whether these surface charge effects in cells originate from charge on the proteins or the lipids. With the present technique of transferring the channel to different lipid environments, it should be possible to answer this question.

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## References

Apell, H.J., Bamberg, E., Läuger, P. 1979. Effects of surface charge on the conductance of the gramicidin channel. *Biochim. Biophys. Acta* **552:**369–378

Barrett, J.N., Magleby, K.L., Pallota, B.S. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (London)* **331:**211–230

Begenisich, T. 1975. Magnitude and location of surface charges in *Myxicola* giant axons. *J. Gen. Physiol.* **66:**47–65

Bell, J.E., Miller, C. 1984. Effects of phospholipid surface charge on ion conduction in the $K^+$ channel of sarcoplasmic reticulum. *Biophys. J.* **45:**279–287

Cecchi, X., Alvarez, O., Latorre, R. 1981. A three-barrier model for the hemocyanin channel. *J. Gen. Physiol.* **78:**657–681

Edidin, M. 1974. Rotational and translational diffusion in membranes. *Annu. Rev. Biophys. Bioeng.* **3:**179–201

Finkelstein, A., Andersen, O.S. 1981. The gramicidin A channel: A review of its permeability characteristics with special reference to the single-file aspect of transport. *J. Membrane Biol.* **59:**155–171

Fohlmeister, J.F., Adelman, W.J., Jr. 1982. Periaxonal surface calcium binding and distribution of charge on the faces of squid axon potassium channel molecules. *J. Membrane Biol.* **70:**115–123

Frankenhaeuser, B., Hodgkin, A.L. 1957. The action of calcium on the electrical properties of the squid axon. *J. Physiol. (London)* **137:**218–244

Gilbert, D.L., Ehrenstein, G. 1969. Effect of divalent cations on potassium conductance of squid axons: Determination of surface charge. *Biophys. J.* **9:**447–463

Grahame, D. 1947. The electric double layer and the theory of electrocapillarity. *Chem. Rev.* **41:**441–501

Hahin, D.T., Campbell, D.T. 1983. Simple shifts in the voltage dependence of sodium channel gating caused by divalent cations. *J. Gen. Physiol.* **82:**785–805

Hille, B., Woodhull, A.M., Shapiro, T. 1975. Negative surface charge near sodium channels of nerve: Divalent ions, monovalent ions, and pH. *Philos. Trans. R. Soc. London B* **270:**301–318

Jost, P., Griffith, O.H., Capaldi, R.A., Vanderkooi, G. 1973. Evidence for boundary lipid in membranes. *Proc. Natl. Acad. Sci. USA* **70:**480–484

Kistler, J.R., Stroud, M., Klykowsky, M.W., Lalancette, R.A., Fairclough, R.H. 1982. Structure and function of an acetylcholine receptor. *Biophys. J.* **37:**371–383

Latorre, R., Miller, C. 1983. Conduction and selectivity in potassium channels. *J. Membrane Biol.* **71:**11–30

Latorre, R., Vergara, C., Hidalgo, C. 1982. Reconstitution in planar lipid bilayers of a $Ca^{2+}$-dependent $K^+$ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. USA* **79:**805–809

Läuger, P., Stephan, W., Frehland, E. 1980. Fluctuations of barrier structure in ionic channels. *Biochim. Biophys. Acta* **602:**167–180

Levitt, D.G. 1978. Electrostatic calculations for an ion channel. I. Energy and potential profiles and interactions between ions. *Biophys. J.* **22:**209–219

Loosley-Miilman, M.E., Rand, R.P., Parsegian, V.A. 1982. Effect of monovalent ion binding and screening and measured electrostatic forces between charged phospholipid bilayers. *Biophys. J.* **40:**221–232

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Trans.* **9:**71–144

McLaughlin, S., Mulrine, G.A.N., Gresalfi, T., Vaio, G., McLaughlin, A. 1981. Adsorption of divalent cations to bilayer membranes containing phosphatidylserine. *J. Gen. Physiol.* **77:**445–473

Methfessel, C., Boheim, G. 1982. The gating of single calcium-dependent potassium channels is described by an activation-blockade mechanism. *Biophys. Struct. Mech.* **9:**35–60

Moczydlowski, E., Latorre, R. 1983*a*. Saxitoxin and ouabain binding activity of isolated skeletal muscle membrane as indicators of surface origin and purity. *Biochim. Biophys. Acta* **732:**412–420

Moczydlowski, E., Latorre, R. 1983*b*. Gating kinetics of $Ca^{2+}$-activated $K^+$ channels from rat muscle incorporated into planar lipid bilayer membranes: Evidence for two voltage-dependent $Ca^{2+}$ binding reactions. *J. Gen. Physiol.* **82:**511–542

Mozhayeva, G.N., Naumov, H.P. 1970. Effect of surface charge on the steady-state potassium conductance of nodal membrane. *Nature (London)* **228:**164–165

Mueller, P., Rudin, D.O. 1969. Translocators in bimolecular lipid membranes: Their role in dissipative and conservative bioenergy transductions. *Curr. Top. Membr. Transp.* **3:**157–249

Thomas, D.D., Bigelow, D.J., Squier, T.C., Hidalgo, C. 1982. Rotational dynamics of proteins and boundary lipid in sarcoplasmic reticulum membrane. *Biophys. J.* **37:**217–225

Vergara, C. 1983. Characterization of a $Ca^{2+}$-activated $K^+$ channel from skeletal muscle membranes in artificial bilayers. Ph.D. Dissertation. Harvard University, Cambridge, Massachusetts

Vergara, C., Latorre, R. 1983. Kinetics of $Ca^{2+}$-activated $K^+$ channels from rabbit muscle incorporated into planar lipid bilayers: Evidence for a $Ca^{2+}$ and $Ba^{2+}$ blockade. *J. Gen. Physiol.* **82:**543–568

Vergara, C., Moczydlowski, E., Latorre, R. 1984. Conduction, blockade and gating in a $Ca^{2+}$-activated $K^+$ channel incorporated into planar lipid bilayers. *Biophys. J.* **45:**73–76

Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G., Meltcalfe, J.C. 1974. Reversible lipid titrations of the activity of pure adenosine triphosphatase-lipid complexes. *Biochemistry* **13:**5501–5507

Wilson, D.L., Morimoto, K., Tsuda, Y., Brown, A.M. 1983. Interaction between calcium ions and surface charge as it relates to calcium currents. *J. Membrane Biol.* **72:**117–130

Yellen, G.I. 1984. Ionic permeation and blockade in calcium-activated potassium channels of chromaffin cells. Ph.D. Dissertation. Yale University, New Haven, Connecticut

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