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Voltage-Dependent Translocation of R18 and DiI Across Lipid Bilayers Leads to Fluorescence Changes

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ABSTRACT We show that the lipophilic, cationic fluorescent dyes R18 and DiI translocate from one monolayer of a phospholipid bilayer membrane to the other in a concentration and voltage-dependent manner. When the probes were incorporated into voltage-clamped planar membranes and potentials were applied, displacement currents resulted. The charged probes sensed a large fraction of the applied field. When these probes were added to only one monolayer, displacement currents were symmetrical around 0 mV, indicating that the probes distributed equally between the two monolayers. Charge translocation required that the bilayer be fluid. When membranes were in a condensed gel phase, displacement currents were not observed; raising the temperature to above the gel-liquid crystalline transition restored the currents. Translocation of R18 was also shown by fluorescence measurements. When R18 was in the bilayer at high, self-quenching concentrations, voltage pulses led to voltage-dependent fluorescence changes. The kinetics of the fluorescence changes and charge translocations correlated. Adding the quencher I^- to one aqueous phase caused fluorescence to decrease or increase when voltage moved R18 toward or away from the quencher at low, nonquenching concentrations of R18. In contrast to R18, DiI incorporated into bilayers was a carrier for I^-, and hence I^- altered DiI currents. Voltage-driven translocations allow R18 and DiI to be used to probe membrane potential changes.

INTRODUCTION

The cationic fluorescent dye octadecylrhodamine B (R18) and the indocarbocyanine dyes (DiI) are commonly used to evaluate membrane continuity. They are employed, for instance, for tracing cell surfaces (Heffner et al., 1990), to follow the movement of lipids from one region of a membrane surface to another (Dragsten et al., 1981; van Meer and Simons, 1986; Winckler and Poo, 1996), and to monitor membrane fusion (Clague et al., 1990; Kaplan et al., 1991; Tse et al., 1993; Morris et al., 1989). When added to aqueous solutions, both R18 (with a single acyl chain) and DiI (with a double chain) incorporate into membranes. These are particularly useful probes because they remain within the membrane and transfer only slowly between membranes (Hoekstra et al., 1984). There have been indications that these probes translocate from one monolayer of a membrane to the other, which is referred to as "flip-flop" (Dragsten et al., 1981; van Meer and Simons, 1986; Wunderli-Allenspach et al., 1993; Stegmann et al., 1993; Arbuzova et al., 1994). Flip-flop has been explicitly demonstrated for R18 in liposomes (Leenhouts and De Kruijff, 1995). In contrast, it has been concluded that DiI does not flip-flop in liposomes (Wolf, 1985).

It is of practical importance to know whether a fluorescent probe remains confined to the monolayer of a membrane to which it is added or whether it distributes between both monolayers. This is significant, for example, when using fluorescent probes to assess whether hemifusion, the mixing of outer monolayers but not inner ones, is an intermediate of full membrane fusion, the merger of both inner and outer monolayers (Kemble et al., 1994; Melikyan et al., 1995b). Probes that flip-flop cannot be used to determine whether continuity is established through one or both lipid monolayers of the membranes. Moreover, the self-quenching properties of some fluorescent probes, notably R18, are routinely used to follow fusion (Stegmann et al., 1993; Clague et al., 1990). The self-quenching of R18 incorporated into one membrane at high concentrations is relieved when the dye redistributes into an unlabeled membrane as a result of fusion. These fluorescence increases have been analyzed to quantitatively determine physical-chemical parameters that characterize binding and rates of fusion (Stegmann et al., 1989; Ramalho-Santos et al., 1993). However, flip-flop would cause the dyes to distribute between inner and outer monolayers in different quantitative manners before and after fusion because of changes in membrane potentials or dissimilar lipid compositions between the fused membranes. Hence flip-flop would affect the measured fluorescence signal.

Flip-flop of charged molecules is more readily studied in planar membranes than in vesicular systems. Both sides of planar membranes are readily available, membrane potentials are unambiguously known, and currents are measured. When charged molecules are confined to a bilayer, their translocation from one monolayer to another in response to applied potentials results in transient currents, known as displacement currents. The currents decay as molecules are depleted in one monolayer and accumulate in the other. Such current transients are characteristic of lipid-soluble ions that translocate across membranes from one monolayer to the other (Ketterer et al., 1971; Andersen and Fuchs,

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1975). In this paper we show that R18 and DiI incorporated into planar bilayer membranes yield large transient currents in response to applied voltage steps, and we rigorously demonstrate that these displacement currents are due to flip-flop. We used the current transients to characterize the kinetics and voltage dependence of translocation of the probes at high time resolution. By simultaneously measuring the fluorescence and displacement currents of the probes, we characterized their concentration- and voltage-dependent fluorescence changes. These changes in fluorescence are due to flip-flop. These probes can be used to follow membrane potential changes with time constants greater than a second. We also evaluated the effects of I^-, a collisional quencher of fluorescence, on R18 and DiI translocation and fluorescence. I^- quenched R18 fluorescence as expected and did not affect flip-flop. However, DiI was a carrier for I^-, and hence I^- altered the flip-flop of DiI. I^- is therefore not a suitable quencher of DiI fluorescence.

MATERIALS AND METHODS

Fluorescent dyes, lipids, and reagents

Octadecylrhodamine B chloride (R18), 1,1′-dioctadecyl-3,3,3′3′-tetramethyl indocarbocyanine iodide (DiI-C18), and 1,1′-didodecyl-3,3,3′3′-tetramethyl indocarbocyanine perchlorate (DiI-C12) were purchased from Molecular Probes (Eugene, OR), dissolved in methanol or anhydrous dimethylsulfoxide (DMSO) (Aldrich Chemical Co., Milwaukee, WI), and stored under argon at −20°C. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), brain phosphatidylserine (PS), dinervonoylphosphatidylcholine (DNPC), and lissamine rhodamine B sulfonyl dioleoylphosphatidylethanolamine (RhoPE) were obtained from Avanti Polar Lipids (Alabaster, AL).

Formation of planar lipid bilayers and measurement of charge translocation

Simultaneous electrical and fluorescence measurements were performed with horizontal, solvent-free planar lipid bilayers formed by applying lipid solutions in squalene (Aldrich) through a 160-μm hole in a teflon partition (Melikyan et al., 1995b). When membranes with different lipid compositions in each monolayer were required or when fluorescent probes needed to be added to only one side of the membrane, vertical, solvent-free bilayers where formed by apposing two monolayers within a 150-μm hole pretreated with squalene as described (Niles et al., 1988). The probes were included in the membrane-forming solutions at the indicated mole fraction of total lipid. Planar bilayers were voltage-clamped with locally constructed amplifiers equipped with capacitance cancellation and frequency boosting circuitry. Silver-silver chloride electrodes were connected to the aqueous solutions bathing the membranes via agar bridges. Electrode polarization was negligible. Voltage pulses were generated and currents were collected by a computer with D/A, A/D boards. Currents were filtered by a 4-pole Bessel filter at 1 kHz and digitized using either custom-written software or PClamp 6.0 (Axon Instruments, Foster City, CA). Experiments were carried out at room temperature (23°C), except for experiments with DNPC bilayers, when the chamber was maintained at the indicated temperatures.

Current transients were fit with single exponentials to determine time constants (τ) of decay. The charge translocated (Q) for a 3-s voltage pulse was determined by subtracting the steady-state current, integrating the resulting current transient, and normalizing by the membrane area. Similar values of Q were obtained by setting $Q = I_0\tau$, where I_0 is the current amplitude at $t = 0$ and τ is the time constant of the fit exponential. The membrane area was calculated from the ratio of bilayer capacitance (measured with the capacitance cancellation circuitry) and the specific capacitance. The specific capacitance of DOPC/DOPE (2:1) membranes was measured in separate experiments as 0.81 ± 0.04 μF/cm^2 ($n = 24$) and was not affected appreciably by including up to 6 mol% of R18 or DiI in the membrane.

The translocation of charge was treated as movement between two energy minima located near the membrane-solution interfaces as formulated in models that describe the transport of lipophilic ions across membranes (Ketterer et al., 1971; Andersen and Fuchs, 1975). In these models Q and τ as a function of voltage are given by (Andersen and Fuchs, 1975; González and Tsien, 1995)

$$Q = Q_o\{\tanh(\alpha[V - V_o]) + \tanh(\alpha V_o)\} \quad (1)$$

and

$$\tau = \tau(V_o)\mathrm{sech}(\alpha[V - V_o]), \quad (2)$$

where $\alpha = \beta q/2kT$, Q_o is the mobile charge in a monolayer when the probes are symmetrically distributed (when $V = V_o$), $\tau(V_o)$ is the time constant of translocation at V_o, V is the applied voltage, V_o is the voltage across the membrane due to asymmetries (e.g., in surface charges), q is the number of elementary charges per molecule (one for both R18 and DiI), and β is the fraction (<1) of the applied electrical field to which the mobile charge responds. β, V_o, and $\tau(V_o)$ were obtained by fitting τ versus V; these values of β and V_o were used when fitting Q versus V to obtain Q_o. For symmetrical membranes, $V_o = 0$ mV and Eqs. 1 and 2 reduce to

$$Q = Q_o\tanh(\alpha V) \text{ and } \tau = \tau(0)\mathrm{sech}(\alpha V). \quad (3)$$

When the Gouy-Chapman-Stern equation was used (McLaughlin, 1989), the fixed surface charges of the lipid headgroups were assumed to be confined to a plane that defined the origin for the drop of the applied potential across the bilayer.

Fluorescence measurements

The horizontal bilayer chamber was mounted on a stage of an inverted fluorescence microscope (Diaphot; Nikon, Instrument Group, Melville, NY). A rhodamine filter cube (G2A; Nikon, ex 510–560 nm, dichroic mirror 580 nm, em > 590 nm) was used to monitor both R18 and DiI fluorescence. The excitation light was attenuated by neutral density filters to eliminate excessive photobleaching. The field diaphragm was almost closed, to produce a narrow illuminated spot with a diameter of about 70 μm on the lipid bilayer. This avoided directly exciting the supporting Gibbs-Plateau border (the torus), which was very bright because it was thick and therefore contained a large amount of dye. Fluorescence was monitored with either a video camera (SIT-66; Dage MTI, Indianapolis, IN) and recorded on VHS format videotape or with a photomultiplier tube (PMT) (model HC120-08; Hamamatsu Corp., Bridgewater, NJ). The PMT was used primarily to measure the kinetics of fluorescence changes upon stepping of voltages. The output of the PMT was low-pass filtered at 1 kHz and simultaneously digitized with the displacement currents. To improve the signal-to-noise ratio of the fluorescence at a given voltage, 10 consecutive fluorescence traces were averaged for R18 and 20 traces were averaged for DiI-C18. The fluorescence of the bilayer was obtained by subtracting the background fluorescence from the total signal. The background fluorescence (due to scattered light) was taken as fluorescence measured with the PMT after the bilayer was deliberately broken. The video camera was used to monitor the extent of voltage-dependent quenching of R18 fluorescence. Images from videotape were digitized off-line with a frame grabber (Meteor; Matrox Electronic Systems, Dorval, QC, Canada) and analyzed with custom-written software utilizing a commercial C library (Matrox Imaging Library, Matrox). Regions of interest were selected, and the average intensities within those areas were measured on a frame-by-frame basis. The camera was calibrated by measuring its output

to fixed light passing through different portions of a stepped neutral density filter whose optical densities varied by 10% between bands (Edmond Scientific Co., Barrington, NJ). In this way the output of the camera was made to span the range of brightnesses that occurred when the quenching of R18 fluorescence varied. The ratios of two digitized levels of brightness obtained at different voltages were thus converted to ratios of fluorescence intensities.

RESULTS

Current transients indicate that R18 and DiI translocate across bilayers

R18 or DiI-C18 were included in membrane-forming solutions of planar bilayers, and potentials of varied amplitudes and polarities were applied. Significant displacement currents were observed with both dyes. These displacement currents were similar to current transients displayed by hydrophobic ions such as tetraphenylborate (Andersen and Fuchs, 1975), but the transients with the dyes were about two orders of magnitude slower. The currents were larger for R18 (Fig. 1, *top*) than for DiI-C18 (*bottom*) for the same dye/lipid molar ratios. The current transients are consistent with the interpretation that the cationic dyes flip from one monolayer of the bilayer to the other in response to voltage, and move back in a concentration-dependent manner when the voltage is returned to 0 mV.

The total charge translocated (Q_{on}) upon stepping the voltage from 0 mV to V was calculated by integrating current transients with respect to time. For low concentrations of R18, Q_{on} saturated with increasing V (Fig. 2 *A*, *closed circles*), whereas the "on" time constant, τ_{on}, continued to decrease (Fig. 2 *B*, *closed circles*). Returning the voltage to 0 mV resulted in an approximately equal charge translocating in the opposite direction, Q_{off} (Fig. 2 *A*, *open circles*), with time constants τ_{off} (Fig. 2 *B*, *open circles*) virtually independent of V. The experimental dependence of Q_{on} and τ_{on} on V was fitted adequately by $\beta = 0.86$ (solid lines of Fig. 2 *A* and *B*), where β is the fraction of the applied field effective in translocating charge. That R18 responds to 86% of the applied electric field is consistent

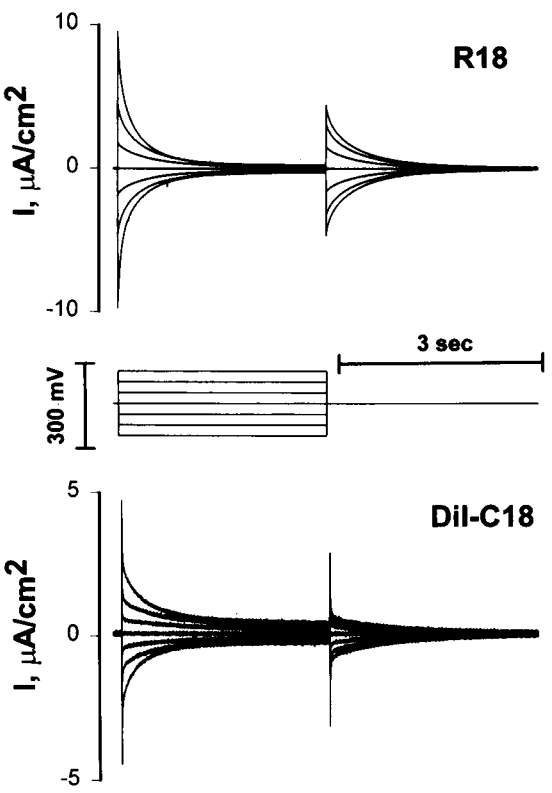

FIGURE 1 Displacement currents for planar lipid bilayers containing R18 (*top*) and DiI-C18 (*bottom*). The pulse protocol (potentials between −120 and +120 mV) applied to voltage-clamped membranes is shown in the middle panel. Planar bilayers were formed from DOPC/DOPE (2:1) with about 6 mol% of R18 or DiI-C18. When horizontal bilayers were used, somewhat more dye tended to translocate from the upper to the lower monolayer than in the opposite direction, for reasons we do not understand. Unless stated otherwise, all experiments were carried out at room temperature in 0.2 M NaCl, 5 mM Tris, pH 7.4.

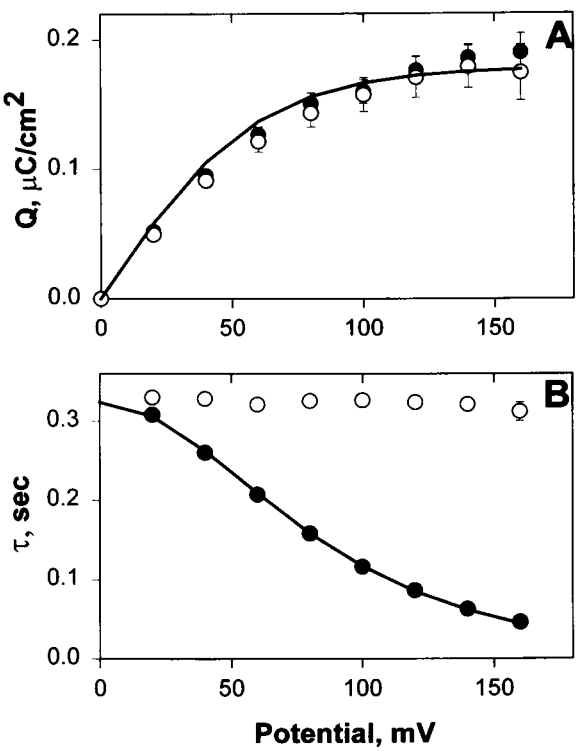

FIGURE 2 Potential dependencies of translocated charge (*A*) and time constants of displacement currents (*B*) for 0.9 mol% R18-containing planar bilayers. (*A*) "On" (●) and "off" (○) charges were calculated by time-integrating the displacement currents over a 3-s interval and normalizing by the membrane area. The bars show standard errors for six experiments. (*B*) "On" (●) and "off" (○) time constants for R18 translocation were determined by fitting the current transients with single exponentials. The "off" time constant was virtually independent of the amplitude of the previously applied potential and was taken as an estimate for $\tau(0)$ (Eq. 3). The standard error ($n = 6$) was usually smaller than the size of the symbols. Because both the charges and the time constants of R18 translocation were nearly symmetrical around 0 mV, the experimental points represent a mean value for voltage pulses of both polarities. The solid lines on *A* and *B* are nonlinear curve fits of Eq. 3, for $Q(V)$ and $\tau(V)$, to the experimental points. The fit parameters were $\beta = 0.86$, $\tau(0) = 0.32$ s, and $Q_o = 0.18$ $\mu C/cm^2$.

with its charged headgroup flipping between the two membrane-solution interfaces of the bilayer. The values of $\tau(0)$ obtained by the fit were close to the experimentally observed τ_{off}, also validating the description of the current transients as charge translocation. When the R18 content was increased to 6 mol%, the translocated charge increased proportionally, but did not completely saturate at high voltages (e.g., see Fig. 4 *A*). The calculated value of β decreased to 0.45, consistent with increased ion-ion interactions at high concentrations (Andersen et al., 1978; Tsien and Hladky, 1982).

At high concentrations, the relation of Q_{on} versus V for DiI showed less saturation than was the case for R18. Q_{on} saturated less for DiI-C18 (Fig. 3 *A*, *open circles*) than for the shorter chain DiI-C12 (*filled circles*). At high voltages, for both DiI dyes, Q_{on} was consistently greater than Q_{off} which saturated as V increased (not shown). The difference between Q_{on} and Q_{off} is anomalous and indicates that a process in addition to simple flip-flop was occurring (see Discussion). The current transients decayed more quickly for DiI-C12 (Fig. 3 *B*, *closed circles*) than for DiI-C18 (*open circles*), suggesting that the longer acyl chains hindered translocation. The τ_{on} versus V relations for DiI-C18 and DiI-C12 and the Q_{on} versus V relation for DiI-C12 were adequately fitted by Eq. 3 using $\beta = 0.67$. The absence of saturation of Q_{on} with voltage for DiI-C18 precluded fitting this curve with a reasonable β.

For both R18 and DiI, transient currents were observed only when membranes were fluid. DNPC forms a condensed subgel (L_c) phase below 27°C (Lewis et al., 1988). As expected for a flip-flop mechanism, there was virtually no R18-induced charge movement when the membrane was at 23°C (Fig. 4 *A*, *filled circles*). Transient currents were recovered after heating the DNPC/R18 membranes to 37°C (*open circles*). For comparison, Q_{on} versus V is shown for

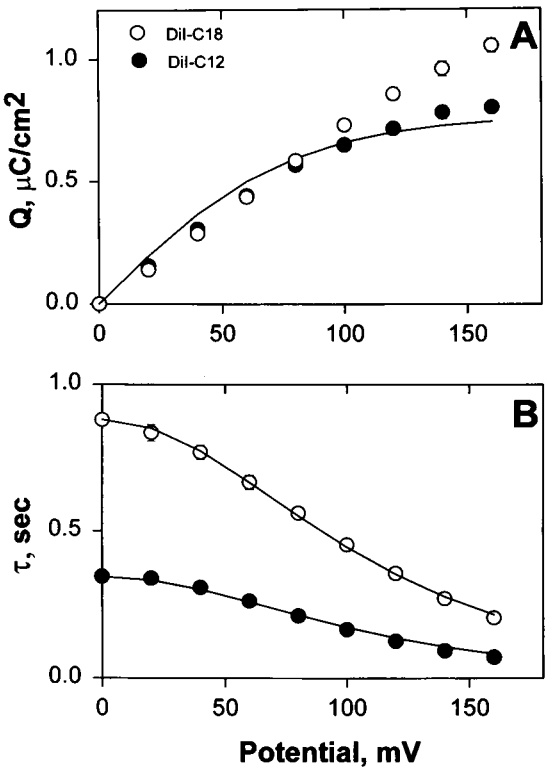

FIGURE 3 Potential dependencies of translocated charges (*A*) and the time constants of displacement currents (*B*) for bilayers containing 6 mol% DiI-C18 (○) or DiI-C12 (●). The standard errors were comparable to or smaller than the size of the symbols ($n = 4$ for DiI-C18 and $n = 3$ for DiI-C12). $\tau(0)$ was obtained for each dye by averaging the time constants of the "off" times after pulses of less than 80 mV. Solid lines were obtained by curve-fitting experimental dependencies of $\tau(V)$ and $Q(V)$ according to Eqs. 1 and 2. The optimal fits for both dyes were achieved for $\beta = 0.67$, $Q_o = 0.76$ μC/cm^2, $\tau(0) = 0.34$ s for DiI-C12, and $\tau(0) = 0.88$ s for DiI-C18. We did not fit the $Q(V)$ measured for DiI-C18 because Q did not saturate over the experimental voltage range (≤180 mV), leading to a low value of β. The translocated charge saturated better with DiI-C12. For both DiI-C12 and DiI-C18, after a voltage pulse was returned to 0 mV, the charges translocated (Q_{off}, not shown) were consistently smaller than those during the voltage pulse (Q_{on}).

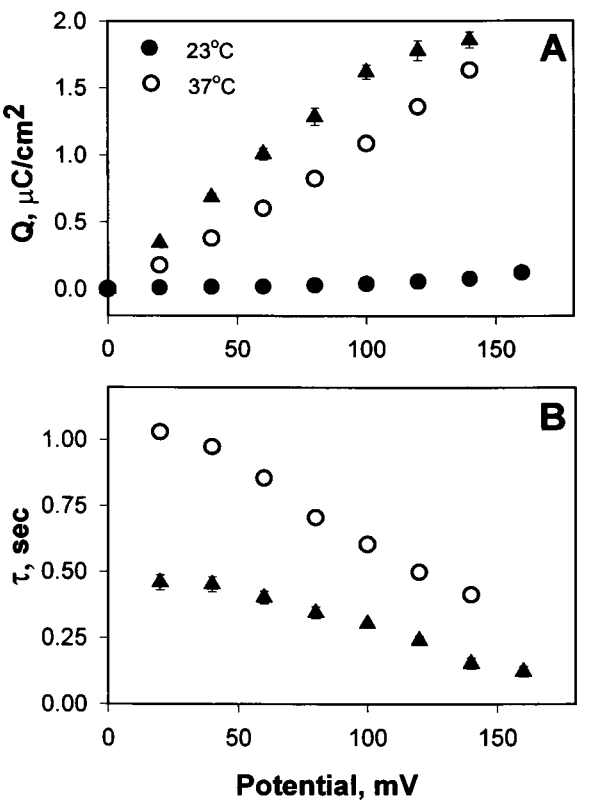

FIGURE 4 Charge translocation across DNPC and DOPC/DOPE bilayers containing 6 mol% R18. (*A*) Voltage dependence of charge translocation across a DNPC membrane at 23°C ($n = 3$, ●) and at 37°C ($n = 3$, ○). Charges translocated across DOPC/DOPE bilayers at 23°C are also shown for comparison ($n = 10$, ▲). Virtually no displacement currents were detected at 23°C for DNPC/R18 bilayers because the bilayer was in a crystalline state. At 37°C these membranes are liquid and current transients were present. However, the translocated charge did not saturate well with voltage. (*B*) Voltage dependence of τ for DNPC (○, 37°C) and DOPC/DOPE (▲, 23°C) bilayers. The time constants for "off" current transients are not shown to aid visual clarity. Because the current transients were negligible across DNPC bilayers at room temperature, we were unable to obtain reliable values of their translocation time constants.

R18 incorporated at room temperature in fluid DOPC/DOPE bilayers (*triangles*). Similar results were obtained for DNPC/DiI-C18 bilayers (not shown). The time constant of R18 flip-flop across DNPC bilayers at 37°C (Fig. 4 *B*, *open circles*) was about two times greater than that for DOPC/DOPE membranes (*triangles*). This is expected because DNPC bilayers with their 24 carbon acyl chains (specific capacitance 0.58 ± 0.04 $\mu F/cm^2$) are thicker than DOPC/DOPE membranes and lipophilic ions translocate more slowly across thicker bilayers (Benz and Läuger, 1977).

Asymmetrical incorporation of probes resulted in symmetrical displacement currents

If the probes readily flip-flop, they should distribute to equal concentrations in each monolayer even if explicitly incorporated into only one. To test this, we formed bilayers by apposing monolayers with R18 or DiI incoporated into only a single (*cis*) monolayer. The resulting displacement currents were symmetrical with respect to zero voltage for R18 (Fig. 5, *open symbols*) and DiI (not shown): both the charge translocated (Fig. 5 *A*) and the time constants of translocation (Fig. 5 *B*) were symmetrical around 0 mV. That is, the displacement currents were independent of the polarity of the applied potential, implying that the probes distributed equally into both monolayers.

R18 included into only the *cis* monolayer transferred into the *trans* monolayer by flip-flop and not as a result of lipid mixing during membrane formation. When a fraction of the lipids within one monolayer were negatively charged (DOPE/PS; 7:3) and only zwitterionic lipids were included in the other, charge translocation (Fig. 5 *A*, *closed circles*) and the time constants (Fig. 5 *B*, *closed circles*) were not symmetrical around 0 mV. This nonzero potential across the membrane is due to charge asymmetry. For membranes containing 1 mol% R18 and bathed by 200 mM KCl, fits to τ_{on} versus V and Q_{on} versus V were symmetrical around V_o = 31 mV (Fig. 5, *solid curves*). In other words, there was more R18 in the PS-containing monolayer at 0 mV. Because R18 responded to 67% of the transmembrane potential (i.e., $\beta = 0.67$), the surface potential at the PS-containing membrane-solution interface would be $-31/\beta = -46$ mV. This should be compared to −50 mV estimated from Gouy-Chapman-Stern theory (McLaughlin, 1989). Clearly, lipid asymmetry was preserved and lipid mixing was minimal (Hall and LaTorre, 1976).

Fluorescence quenching of R18 by iodide is consistent with R18 translocation across the bilayer

The time course of fluorescence changes of R18 in response to voltage was monitored and compared to the time course of the displacement current. When the dyes were placed symmetrically at about 1 mol% of total lipid, the fluorescence of R18 (Fig. 6 *A*, *dots*) did not change with voltage

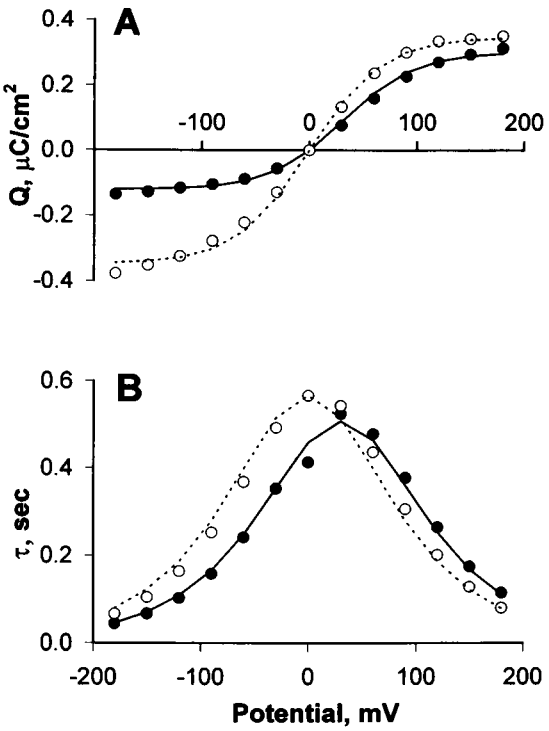

FIGURE 5 Translocation of R18 incorporated into only one monolayer of a planar bilayer. R18 (5 mol%) was included in one monolayer of a DOPC/DOPE (2:1) bilayer formed by the apposition of two monolayers. As soon as measurements could be performed, both the translocated charges (*A*, ○) and the time constants of current transients (*B*, ○) were symmetrical with respect to 0 mV. The dotted lines are the curve fits for $\beta = 0.76$, $\tau(0) = 0.57$ s, and $Q_o = 0.35$ $\mu C/cm^2$. The value of $\tau(0)$ was obtained from the "off" time constants as described in the legend to Fig. 3. A greater amount of dye was incorporated by the technique of painting membranes than by apposing monolayers, as judged by the smaller charge translocated in bilayers formed by apposing monolayers. Furthermore, smaller currents occurred when including probes asymmetrically rather than symmetrically. In separate experiments, 1 mol% of R18 was included in both monolayers of asymmetrical membranes formed by apposing two monolayers of different composition (DOPE/brain PS, 7:3 on *cis* side and DOPC/DOPE, 2:1 on *trans* side). We assumed that this low concentration of R18 did not alter the surface potential. The polarity of potentials applied to planar bilayers refer to the *cis* side that contains negatively charged PS. For these asymmetrical membranes $Q_{on}(V)$ (*A*, ●) and $\tau_{on}(V)$ (*B*, ●) were symmetrical with respect to 31 mV. R18 passively distributed according to the asymmetrical surface potentials. The data were fit (*solid curves*) by Eqs. 1 and 2 with $\beta = 0.67$, $V_0 = 31$ mV, $Q_o = 0.21$ $\mu C/cm^2$, and $\tau(V_o) = 0.51$ s.

pulses, although charge was translocated (*solid lines*). When the *cis* side of the bilayer was bathed with 200 mM NaI, the fluorescence was significantly reduced (not shown) because of I^- quenching the fluorescence of R18 in the *cis* monolayer. When potentials were applied to drive R18 away from I^- and toward the *trans* solution, fluorescence increased (Fig. 6 *B*, *dots* for positive fluorescence changes). Voltages that moved R18 toward I^- caused fluorescence decreases (*dots* for fluorescence decreases). The translocated charges (Fig. 6 *B*, *solid lines*) had time courses identical to that of the fluorescence changes and were not appreciably affected by the presence of I^-. However, there were slightly larger

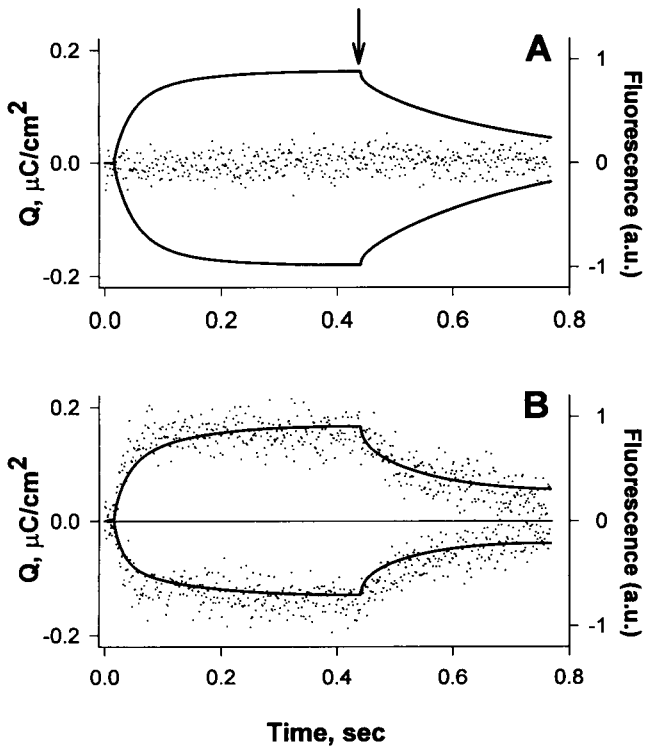

FIGURE 6 Correlation between the charge translocation and R18 fluorescence changes in bilayers in the absence (*A*) and in the presence (*B*) of the collisional quencher, NaI. (*A*) DOPC/DOPE (2:1) membranes containing a low fraction of R18 (0.9 mol%) included in both monolayers did not exhibit voltage-dependent fluorescence changes (*dots*). The charges translocated upon applying ±160 mV and after returning the voltage to 0 mV (arrow) are shown by solid lines. (*B*) The bilayer was bathed by 0.2 M NaI in the *cis* and 0.2 M NaCl in the *trans* solution. $Na_2S_2O_3$ (0.1 mM) was included in the *cis* solution to reduce contaminating I_2 and minimize the formation of the membrane-permeable polyiodide complexes, I_3^- and I_5^- (Finkelstein and Cass, 1968). Stepping the voltage to 160-mV *cis*-negative resulted in a decrease in R18 fluorescence. A 160-mV *cis*-positive voltage pulse resulted in a fluorescence increase. The time courses of fluorescence changes (*dots*) were virtually identical to those of charge translocation (*solid lines*). Fluorescence signals from the bilayer and displacement currents were averaged for 20 voltage pulses to improve the signal-to-noise ratio. The points on the fluorescence traces were decimated to aid visual clarity. The fluorescence changes are superimposed on the background fluorescence due to indirect excitation of the torus. R18 fluorescence changes were normalized and are shown as arbitrary units.

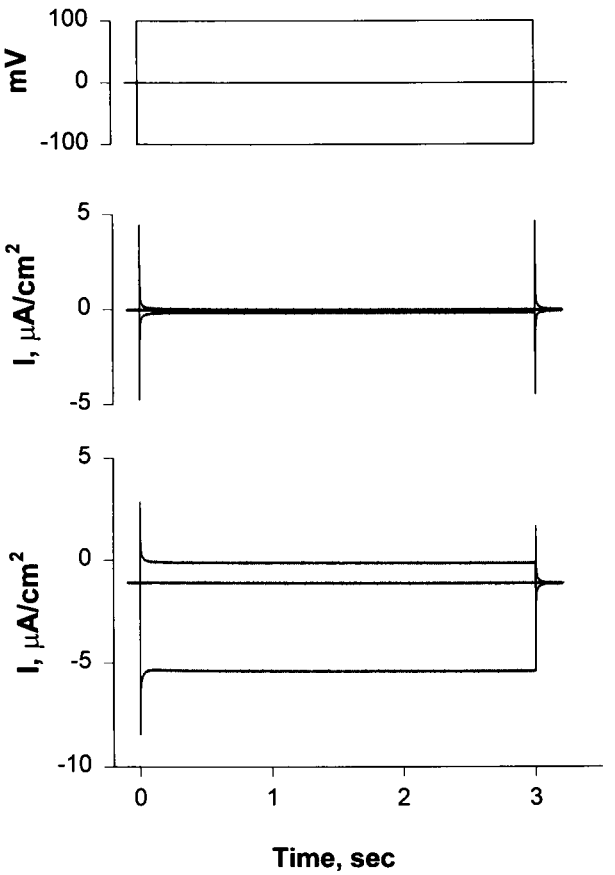

FIGURE 7 Currents induced by DiI-C18 in the presence of iodide. A DOPC/DOPE (2:1) bilayer was bathed by 0.2 M NaI *cis* and 0.2 M NaCl *trans*. In the absence of DiI, steady-state currents were small (*middle*). When DiI was included in only the *trans* monolayer, large steady-state currents occurred in response to a *trans*-positive potential (*bottom trace of bottom panel*) but not to a *trans*-negative one (*upper trace of bottom panel*). The displacement currents that occur for DiI in the absence of I^- were not present. DiI functions as a carrier of I^-. The residual capacitative transients had a time constant of 6 ms and were about 2% of the uncompensated bilayer capacitance. They arise from the membrane (they do not occur with model circuits) and are suggestive of membrane electrostriction.

steady-state currents for *cis*-negative voltages, corresponding to R18 movement *trans* to *cis*.

DiI currents were dramatically altered when iodide was added to an aqueous phase. DiI was included in only one (*trans*) monolayer bathed by 200 mM NaCl, and membranes were formed by apposing monolayers. The opposite, *cis*, monolayer was bathed by 200 mM iodide. A large steady-state current occurred when the *trans* solution was made positive, but not when it was made negative (Fig. 7, *bottom*). At 0 mV, a significant steady-state current was still present. The reversal potential was 125–130 mV, *trans*-negative. When DiI was not included in the planar bilayer, steady-state currents were negligible (Fig. 7, *middle*). The very fact that I^- placed on the opposite side of DiI affected currents shows that the dye and I^- made physical contact. The most likely explanation is that DiI is an iodide carrier (see Discussion). That is, DiI translocated to the *cis* side, I^- complexed with DiI on the *cis* side, the neutral DiI-iodide complex back-diffused and released I^- into the *trans* solution, and then the voltage flipped the charged DiI back to the *cis* side.

Charge translocation leads to fluorescence self-quenching at high concentrations of dye

At high concentrations the dyes self-quenched. The translocation of dye led to significant changes in their densities within the two monolayers and therefore to changes in fluorescence. For both R18 (Fig. 8 *A*) and DiI (Fig. 8 *B*) the time courses of charge translocation and fluorescence changes correlated, although there were quantitative differ-

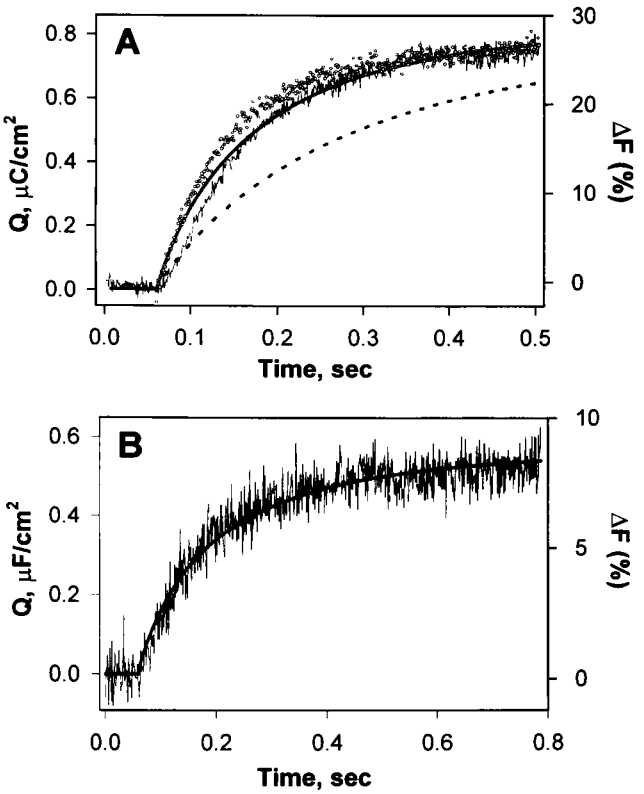

FIGURE 8 Temporal correlation between charge translocation and fluorescence changes for self-quenching concentrations (6 mol%) of R18 and DiI-C18 in planar bilayers. (*A*) Kinetics of the R18 fluorescence decreases ($\Delta F = (F_0 - F)/F_0$, *noisy solid trace*) and the charge translocation (Q, *smooth solid curve*) upon application of 100 mV. The rate of the fluorescence increase ($\Delta F = (F - F_0)/F_0$, *small circles*) after returning the voltage to zero was faster than the rate of increase in Q_{off} (*dashed curve*). Fluorescence and current traces were averaged for 10 voltage pulses. The steady-state fluorescence change for a 100-mV pulse was approximately 29% (not shown). (*B*) Decrease in DiI-C18 fluorescence (*noisy solid trace*) correlated well with the charge translocated (*smooth solid curve*) for 140-mV pulses. These curves were obtained by averaging the current transients and fluorescence signals for 20 pulses. Q_{off} and the corresponding fluorescence increase are not shown for visual clarity. The steady-state fluorescence change of DiI-C18 was only 9% (not shown). The background fluorescence was determined as described in Materials and Methods and was subtracted from the total fluorescence signal.

ences. Notably, the rate of charge translocating back at 0 mV, Q_{off} (Fig. 8 *A*, *dashed curve*), was significantly slower than the rate of the fluorescence increases (*open circles*). At 100 mV, some charge (Fig. 8 *A*, *smooth solid curve*) was translocated before the fluorescence decrease, which displayed a detectable lag (*noisy solid curve*). In control experiments, neither displacement currents nor fluorescence changes were recorded for membranes containing up to 12 mol% RhoPE (not shown).

The decrease in fluorescence depended on the amount of dye translocated in response to an applied voltage. Likewise, the increase in fluorescence when the voltage was returned to 0 mV was a function of the amount of charge that back diffused. Although both the increases and decreases depended on charge translocation, they did so with different quantitative relationships. For the experiment of Fig. 8, the fluorescence (F_{on}) relative to that when the membrane was at 0 mV (F_0) was compared to the value of Q_{on} over the time course of a 100 mV pulse. That is, F_{on}/F_0 and Q_{on} were determined for each time point. This yielded the quantitative dependence of the decrease in fluorescence as a function of charge translocation (F_{on}/F_0 versus Q_{on}) in response to a voltage (Fig. 9, *filled circles*). In the same fashion, the increase in fluorescence F_{off} relative to the steady-state fluorescence at 100 mV (F_{100}) was obtained as a function of Q_{off} (Fig. 9, *open circles*). These dependencies are clearly different. Similar phenomena were observed for DiI, although less charge was translocated and the changes in fluorescence were smaller. By 140 mV, the charge (Fig. 8 *B*, *smooth solid curve*) translocated as fast as the fluorescence decreases (*noisy curve*).

Steady-state R18 fluorescence quenching is voltage dependent

The steady-state fluorescence of R18 and DiI-C18 at high concentrations (6 mol%) was voltage dependent and was readily observed by video microscopy. Images clearly illustrate the increased self-quenching of R18 with voltage (Fig. 10, *top panels*) with the fluorescence approaching background level when voltages greater than 120 mV were applied. The brightness levels recorded from the video camera were converted to fluorescence units (see Materials and Methods), and F_{on}/F_0 was plotted as a function of voltage (Fig. 10, *lower panel*). For this membrane the fluorescence was reduced by 26% at 100 mV and further decreased to 32% at 180 mV. Equivalent amounts of fluo-

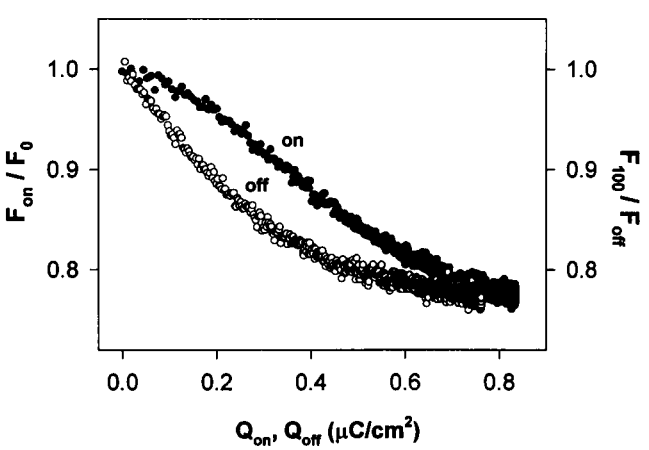

FIGURE 9 R18 fluorescence as a function of translocated charge. The fluorescence and translocated charge at the same times were obtained from Fig. 8 *A* to derive the fractional fluorescence changes versus Q. F_0 is the fluorescence at 0 mV and F_{on} is the fluorescence for a given Q_{on} translocated. F_{100} is the steady-state fluorescence at 100 mV, and F_{off} is the fluorescence when a charge Q_{off} has translocated. Both the fractional fluorescence decreases (F_{on}/F_0) versus Q_{on} (●) and the fractional recovery of fluorescence (F_{100}/F_{off}) versus Q_{off} (○) were nonlinear. The curve for fluorescence recovery was significantly steeper than that for fluorescence quenching.

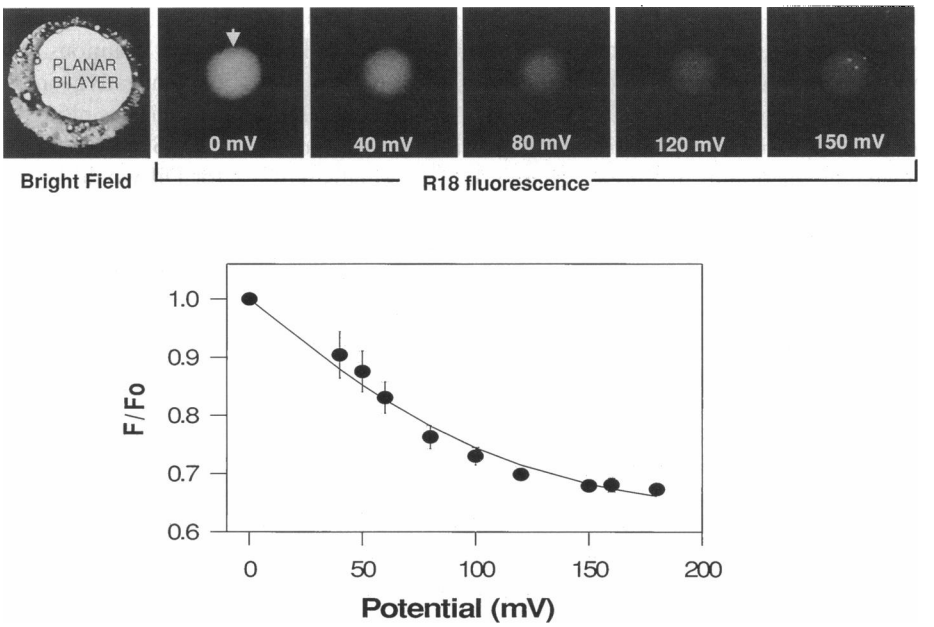

FIGURE 10 Voltage-dependent self-quenching of R18 fluorescence. (*Upper panels*) Series of images showing a planar lipid bilayer (bright field) and the fluorescence of the membrane area excited by a narrow light beam (*arrow*) as the voltage was increased from 0 to 150 mV (either polarity). The decreased fluorescence with voltage is apparent. (*Lower panel*) The brightness levels from the video camera were converted into fluorescence. The fluorescence signals for positive and negative voltages were averaged and plotted as a function of potential (●). The solid line was obtained by fitting the data by the equation analogous to Eq. 1: $F/F_0 = 1 - \tanh(\beta qV/2kT)$, where $\beta = 0.43$, which is nearly identical to $\beta = 0.45$ as determined electrically.

rescence reductions were obtained when a PMT was used (compare to Fig. 8 *A*), verifying the video camera calibration. The dependence of F_{on}/F_0 on voltage roughly paralleled the dependence of Q on voltage (Figs. 2 *A* and 5 *A*).

DISCUSSION

Phospholipids do not normally flip-flop across bilayers (Rothman and Lenard, 1977), because the polar headgroups do not dissolve well into the membrane interior (Ganong and Bell, 1984). Some headgroups can be rendered permeable, and in these cases the lipid readily translocates, with the acyl chains maintaining the lipids within the membrane. For example, the acidic lipid phophatidylglycerol (PG) is not transported across bilayers at neutral pH, whereas at low pH PG flip-flops with a half-time of several seconds (Redelmeier et al., 1990).

The headgroups of R18 and DiI are membrane permeable. Rhodamine 6G is positively charged and quite similar to the headgroup of R18. In the presence of 1 mM rhodamine 6G in the aqueous solution bathing a planar membrane, substantial currents are observed upon application of small voltages (data not shown), establishing that the dye is membrane-permeable, as has been concluded by using biological preparations (Aiuchi et al., 1982). The positive charges of rhodamine 6G and of the R18 headgroup delocalize over their ring structures. In contrast, the negative charges of the sulfonyl and phosphate of the headgroup of RhoPE, which does not flip-flop, should not delocalize.

The use of short-tailed DiI (C-3 and C-5) and other related probes to monitor membrane potentials of organelles (Cohen and Salzberg, 1978; Kinnally et al., 1978) and vesicles (Loew et al., 1985; Cabrini and Verkman, 1986) derives from the dyes' ability to passively distribute across membranes (Waggoner, 1979). Displacement currents are observed for planar bilayers containing short-chain cyanine and oxonol dyes (Waggoner et al., 1977). However, significant steady-state currents also result (Szabo, 1974) because the dyes readily exhange between the solution and bilayer. With long hydrophobic acyl chains, the permeable headgroups are effectively confined to the membrane-solution interfaces and steady-state currents are negligible. The flip-flop of charged species can be readily studied through their displacement currents.

Experimental evidence for translocation of R18 and DiI across membranes

Previously it has been concluded that R18 translocates across liposomal membranes in a voltage-dependent manner (Leenhouts and De Kruif, 1995), but it has been argued that DiI does not flip-flop (Wolf, 1985). There are multiple lines of evidence that R18 and DiI in our study translocate across bilayers: 1) Displacement currents are described by large values of β, implying that the charged headgroup senses a major fraction of the applied field. 2) Displacement currents are symmetrical when dyes are placed into only one monolayer. 3) I^- within one aqueous phase causes voltage-dependent fluorescence of R18 at low concentrations and large steady-state currents in the presence of DiI. 4) Fluid membranes are required to obtain displacement currents. We do not understand why DiI did not appear to flip-flop when incorporated in liposomes (Wolf, 1985). We now discuss 1)–4) in greater detail.

1. The displacement currents were best fitted by assuming that the delocalized positive charges of R18 and DiI moved across a large fraction of the applied potential difference. The values of β were lowered when the probes' concentrations were increased, characteristic of translocation across low dielectric bilayer interiors. Lowered β occurs because

lateral electrostatic repulsion between dye molecules at high surface densities requires that more work be expended to move additional charges into a monolayer (Andersen et al., 1978; Tsien and Hladky, 1982). In other words, increased ion-ion repulsion causes a given voltage difference to move a smaller fraction of the mobile charge. In the formalism of Eqs. 1 and 2, this appears as a reduced fitted β. The calculated values of β may be underestimated. The total amount of dye, and therefore charge, within the bilayer is not absolutely fixed, as there is a reservoir of dye within the Gibbs-Plateau border (torus) that connects the planar membrane to the teflon partition. As dye translocates from one monolayer to the other, dye from the torus would tend to diffuse into the depleted monolayer, whereas dye accumulated in the enriched monolayer would tend to move into the torus. (The slower the translocation the more pronounced will be this effect.)

2. When either R18 or DiI was added to only a single monolayer of an otherwise symmetrical bilayer, the displacement currents were symmetrical around 0 mV (Fig. 5). This is expected if the probes translocated (at a rate given by $1/\tau_{off}$) and became distributed with equal concentrations in the two monolayers. In contrast, if the dyes remained within a single monolayer and the displacement currents were due to reorientation of headgroups, rather than flip-flop, the displacement currents would, in general, be symmetrical around a nonzero value of voltage, the exact value given by the orientation of the headgroup.

3. The quencher, I^-, placed in only the *cis* aqueous phase caused the fluorescence of low, non-self-quenching concentrations of R18 to become voltage dependent. The rhodamine headgroup of R18 had to physically reach the iodide-containing solution for its fluorescence to be quenched by *cis*-negative voltage and be physically removed by *cis*-positive voltage for its fluorescence to increase. The identical time courses of fluorescence changes and charge translocation (Fig. 6) demonstrate that voltage-driven movement of the charged rhodamine headgroup was responsible for the fluorescence changes.

In contrast, I^- placed in the *cis* solution caused large steady-state currents in only one direction when DiI was placed in only the *trans* monolayer. We propose that DiI is an anion carrier, with greater affinity for I^- than Cl^-: in response to a *trans*-positive potential, the positively charged DiI translocates *trans* to *cis* in a voltage-dependent manner, complexes with iodide at the *cis* membrane-solution interface, back-diffuses in a neutralized form to the *cis* solution, and releases iodide, and the charged form repeats the classical carrier cycle. The absence of DiI current relaxations when I^- was present (Fig. 7) shows that the back-diffusion of the neutral species and the association-dissociation of I^- with DiI were sufficiently rapid to sustain the concentration of the charged form of DiI: the translocation of the positively charged DiI was the rate-limiting step. We did not attempt to determine the stoichiometries of the DiI-anion complexes of the carrier cycle.

With equal concentrations of Cl^- and I^-, placed in opposite solutions, the zero-current potential was 125–130 mV, negative on the iodide-containing side. This implies that DiI has more than a 100-fold greater affinity for I^- than Cl^- (Szabo et al., 1969). A low, but nonzero, affinity of Cl^- for DiI would lead to $Q_{on} > Q_{off}$, as is observed (legend to Fig. 3): Q_{on} would increase as a result of carrier recycling; Q_{off} would be somewhat reduced because the dye would back-diffuse in its complexed, neutral form as well as in its charged form. I^- had a much smaller, but similar and measurable, effect on R18 currents than it did on DiI currents (note slight asymmetry in charge translocation in Fig. 6 *B*). (R18 did not carry any Cl^-: steady-state currents with R18 returned to baseline.) The greater affinity of Cl^- for DiI than for R18 would explain why Q_{on} of R18 saturated with voltage better than did DiI. Binding of I^- to the probes could lead to static quenching, in addition to its well-known dynamical quenching of fluorophores (Lakowicz, 1983).

4. Bilayers must be fluid for lipid-soluble carriers to translocate across them (Krasne et al., 1971). When the temperature was below the $L_c \rightarrow L_\alpha$ transition of DNPC bilayers, displacement currents were not present. Increasing the temperature to place the bilayer in the fluid state resulted in normal current transients. The time course of the current transients of R18 and DiI were appreciably slower than would be expected if they were due to headgroups reorienting, with the acyl chains remaining in the same monolayer. Displacement currents were slower in fluid DNPC membranes than in DOPC/DOPE membranes, consistent with DNPC membranes being the thicker of the two, as expected from its longer chain length and verified by its lower specific capacitance: the work required for a permeating charge to polarize the low dielectric bilayer interior (the image charge force) increases somewhat with bilayer thickness (Neumcke and Läuger, 1969).

Linear self-quenching does not account for voltage-dependent fluorescence changes

R18 self-quenches as a linear function of concentration between 1 and 10 mol% when liposomes are composed solely of phosphatidylcholine (PC) (Hoekstra et al., 1984; MacDonald, 1990), but quenches nonlinearly when cholesterol is included in the membrane (MacDonald, 1990). The concentration dependence of R18 quenching has not been studied with other phospholipids (e.g., PE and PS) included in bilayers. If linear quenching occurs, at high concentrations of R18, translocation in response to voltage should not alter the fluorescence of the enriched monolayer: the increased fluorescence due to the greater number of probes would exactly cancel the decreased fluorescence per probe. Thus the decreases in R18 fluorescence would reflect the depletion of probe from the other monolayer. The fluorescence of the depleted monolayer would not change while in the linear self-quenching range. Only when R18 became sufficiently dilute would further removal lead to fluores-

cence decreases. The fluorescence decreases should therefore lag behind charge translocation. This was observed (Fig. 8 *A*, noisy trace lags smooth solid curve). However, too little charge moved to explain the onset of fluorescence changes according to the above linear quenching paradigm (Fig. 8 *A*). There were additional phenomena that were not expected if linear quenching were occurring. When a voltage was returned to 0 mV, the rate of the fluorescence increase was greater than that for Q_{off} (Fig. 8 *A*, *open circles* vs *dotted line*) and greater than the rate of Q_{on} at low voltages (not shown). Also, the fluorescence decreases and increases that depended on Q_{on} and Q_{off}, respectively, took different functional forms (Fig. 9). Furthermore, if all of the R18 moved into one monolayer, the fluorescence of the bilayer should have been one-half of the initial fluorescence when the probe was equally distributed between the two monolayers. However, the fluorescence decreased by only 30% at high voltages (F/F_o, Fig. 10). A 30% quenching of R18 was also observed at high potentials when liposomes were used (Leenhouts and De Kruiff, 1995). This smaller-than-expected change in fluorescence would be accounted for if not all the dye translocated at high voltages, in agreement with the incomplete saturation of Q_{on} for high concentrations of R18 (Fig. 4 *A*). Alternatively, a linear quenching versus concentration relation may not hold under our experimental conditions.

Dimer formation leads to nonlinear quenching

Potential-dependent quenching of R18 and DiI ultimately depends on the redistribution of dye between two monolayers. Several processes quench the fluorescence of xanthene dyes such as fluorescein and rhodamine in general, and R18 in particular. Monomers as well as nonfluorescent dimers (Massari et al., 1988; MacDonald, 1990; Johansson and Niemi, 1987) and trimers (Arbeloa, 1981) of dye should quench fluorescent monomers, probably by both static and dynamic complex formation (Plant, 1986). Quenching by resonance energy transfer between monomers also occurs (Arbeloa, 1981; MacDonald, 1990). The very formation of nonfluorescent dimers (and higher order complexes) would cause nonlinear quenching with R18 concentration. Quenching of monomers by nonfluorescent oligomers predicts additional nonlinear quenching. Linear quenching should therefore be a fortuitous and not a general situation.

Formation and dissociation of nonfluorescent dimers would account for the observed larger percentage increase in fluorescence than in charge movement when voltage was returned to 0 mV. The fluorescence would rise both because the monomer concentration would increase in the *cis* monolayer as monomers back-diffused from the *trans* to *cis* monolayer and because a larger percentage of the dye in the *trans* monolayer would be in the monomeric form as dimers dissociated by mass action. The existence of dimers would also qualitatively account for the steeper dependence of fluorescence increases as a function of Q_{off} than of fluorescence decreases as a function of Q_{on} (Fig. 9), because a greater percentage of R18 would be in the nonfluorescent dimer form at high than at low concentrations. For Q_{off} the dye is initially at high concentration within one monolayer, and a significant amount of dimer would dissociate into fluorescent monomer during charge movement, whereas for Q_{on} the dye is initially distributed equally between both monolayers and relatively less dimer would form with charge translocation.

Use of R18 and DiI as voltage-dependent dyes

Our results indicate that DiI self-quenches, albeit to a lesser extent than R18, and undergoes voltage-dependent quenching. The time constants for fluorescence changes of R18 and DiI with changing voltages are rather slow, from tens to hundreds of milliseconds. These dyes are suitable for measuring slow changes in membrane potentials as occur, for instance, in mitochondria (Kinnally et al., 1978; Emaus et al., 1986). Furthermore, because the dyes distribute across the bilayers in accordance with the membrane potential, they can be used to monitor differences in potentials at the two membrane-solution interfaces. The voltage that must be applied to establish maximum fluorescence should yield the difference. Of course, any toxic and other undesired effects that might be caused by the high concentrations of dye required will have to be addressed.

Voltage-dependent fluorescence changes that were completed within 2 s have been noted for R18, and similar behavior in DiI has been inferred when it is incorporated in erythrocyte membranes (Tse et al., 1993; Tse, personal communication). A clever application of this voltage dependence has been to detect formation of fusion pores connecting DiI-labeled red blood cells (RBCs) to influenza hemagglutinin (HA)-expressing cells (Morris et al., 1995). Upon fusion, the RBC potential moved to that of the HA-expressing cell, and the DiI fluorescence changed. However, it is unlikely that the observed time constant of about 600 ms for fluorescence changes reflects the time for current through the fusion pore to charge the RBC membrane to its new potential, as interpreted (Morris et al., 1995), because the fusion pore in series with the 1-pF capacitance of the RBCs would have to have a conductance of about 1.6 pS. This is more than an order of magnitude less than directly measured conductances of fusion pores (Spruce et al., 1991; Monck et al., 1990; Zimmerberg et al., 1994; Lollike et al., 1995). We suggest that the observed time constant for fluorescence changes reflects the time for DiI to flip-flop across the RBC membrane. It remains to be determined whether R18 and DiI flip-flop across biological membranes in general. R18 appears to be the more suitable dye for monitoring potential changes because its fluorescence quenching is significantly larger than that of DiI.

In summary, our data demonstrate that DiI and R18 translocate across bilayers over a subsecond time scale. This translocation results in potential-dependent fluorescence

self-quenching. Thus these dyes can be used to follow changes in membrane potentials.

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