Voltage Activation of Reconstituted Sodium Channels: Use of Bacteriorhodopsin as a Light-Driven Current Source†

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ABSTRACT: We present a general method for the development and control of transmembrane potentials ($\Delta\Psi$) in reconstituted vesicles. The light-driven proton pump bacteriorhodopsin (bR) from *Halobacterium halobium* is the current source in the system, and the intensity of light controls the magnitude of $\Delta\Psi$ at any given time. Transmembrane potentials were determined from the equilibrium distribution of hydrophobic ions, which was monitored by using either electron paramagnetic resonance spectroscopy and spin-labeled phosphonium ions or a tetraphenylphosphonium-selective electrode. A bias or holding potential was generated by using gradients of anions of limited permeability in the presence of an impermeable cation. Using *n*-methylglucamine or the polymer poly(ethylene imide) as the impermeable cation, the anions NO_3^- and SCN^- were most effective in producing large (>–60 mV) negative diffusion potentials in egg phosphatidylcholine vesicles. In the presence of a NO_3^--based negative holding potential (approx. –65 mV), bR is capable of depolarizing the membrane to at least the 0-mV level within a few seconds. More rapid depolarizations can be achieved by the application of a brief intense illumination preceding the preset illumination level (supercharging). The technique was successfully used to activate for the first time a population of unmodified voltage-dependent sodium channels purified from eel electroplax.

Voltage-dependent processes play a key role in membrane excitability and signaling. Mechanisms like the nerve impulse, excitation–contraction coupling, and excitation–secretion coupling respond to changes in transmembrane potential through the activation–deactivation of ion channels and other electrogenic transport systems. With the application of molecular cloning techniques, broad protein families have been defined for voltage-dependent ion channels and transport systems (Hille, 1989). Also, site-directed mutagenesis has allowed the investigation of specific structure–function relations in these proteins (MacKinnon & Miller, 1989; Stuhmer et al 1989; Hoshi et al, 1990; Hartman et al, 1991; Tanabe et al, 1991). However, very little is yet known about the mechanisms that confer the voltage dependence to these proteins.

The most direct evidence for a voltage-dependent conformational change is the gating current, measured from voltage-clamp experiments. First described in squid sodium channels (Armstrong & Bezanilla, 1973) and skeletal muscle fibers (Schneider & Chandler, 1973), this represents the movement of charge (or the rearrangement of dipoles) within the protein across the membrane voltage field. A specific region of the voltage-dependent ion channels, the S4 segment, has been implicated as the structural site for the voltage sensor (Noda et al, 1986). Site-directed mutagenesis of the S4 segment in sodium (Stuhmer et al., 1989) and potassium channels (Papazian et al., 1991; Liman et al., 1991) has indeed suggested a key role of the S4 segment in channel activation. The mechanism by which the S4 segment (or other associated voltage sensors) couples the changes in transmembrane electric field to the open-channel conformation remains to be determined.

Electrical measurements alone will not be enough to thoroughly study these processes. However, the use of other physical and biochemical techniques has been hampered by the lack of methods to control potentials in reconstituted systems when the properties of the whole population of reconstituted channels are under study. Specifically, purified and reconstituted voltage-dependent channels go into a deep, slow inactivation state due to the absence of a transmembrane electric potential and cannot be readily activated (Adelman & Palti, 1969; Tanaka et al., 1986). To observe voltage dependence in such systems, it is necessary to dynamically change the steady-state potential in such a way as to bring the channels out of the inactivated state before excitation.

Here, we present the development and first application of a method to actively control transmembrane voltage in reconstituted liposomes containing ion channels (or any other voltage-dependent protein). The method uses bacteriorhodopsin (bR),[1] the light-driven proton pump from *Halobacterium halobium* reconstituted vectorially inside-out as a variable current source. A stable holding potential can be generated to provide voltage control for both negative and positive ranges by asymmetrically controlling the ionic conditions of the liposome system. Co-reconstitution of bR with purified Na^+ channels in the presence of a negative holding potential results in the light-induced activation of the channel. This method, combined with an array of physical techniques, opens a wealth of opportunities to study voltage-dependent processes in purified and reconstituted molecules. A preliminary account of this work has been presented elsewhere (Perozo & Hubbell, 1993).

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[1] Abbreviations: bR, bacteriorhodopsin; $\Delta\Psi$, transmembrane electrical potential; ΔpH, transmembrane proton gradient; $\Delta\mu H^+$, transmembrane proton electrochemical gradient; PC, diacylphosphatidylcholine; NMG, *n*-methylglucamine; TTX tetrodotoxin; EPR, electron paramagnetic resonance; TPP^+, tetraphenylphosphonium ion.

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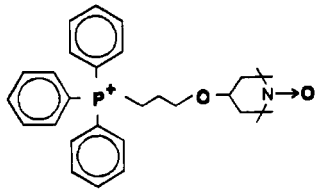
MATERIALS AND METHODS

Materials. Bacteriorhodopsin (bR) was a gift from Dr. Roberto Bogomolni (University of California, Santa Cruz). Crude purple membranes of *Halobacterium halobium* were obtained and separated from brown and plasma membrane fractions by centrifugation in a sucrose density gradient (Oesterhelt & Stoeckenius, 1974). The purple membrane was stored at 4 °C in 4 M NaCl, pH 7, and 2% sodium azide. Horse antisera against α-(2→8)-polysialic acid was a generous gift of Dr. J. B. Robbins (National Institutes of Health). Eels, *Electrophorus electricus*, were obtained from the Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela. Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). Carrier-free $^{22}NaCl$ was from New England Nuclear. Citrate-free TTX from Calbiochem was tritiated by the Wilzbach procedure (Benzer & Raftery, 1973). The (3-tempoxypropyl)triphenylphosphonium bromide spin label (I) was synthesized according to Cafiso and Hubbell (1978). All other reagents were obtained from Aldrich or Sigma.



(I)

Bacteriorhodopsin Reconstitution. The vectorial reconstitution of bR is based on the reverse phase evaporation method (Szoka & Papahadjopoulos, 1978) that simultaneously produces large, mostly unilamelar liposomes and incorporates bR in an inside-out configuration. Its application to bacteriorhodopsin reconstitution follows that of Rigaud et al. (1983). Briefly, 3 mL of an organic phase (diethyl ether) containing between 10 and 20 mg of egg PC was mixed with 1 mL of aqueous phase containing purple membrane patches and 200 mM NMG-SO_4 solution. Brief (30 s) sonication at 5 °C induced the formation of a reverse-phase emulsion. The organic phase was then slowly evaporated under reduced pressure in a rotary evaporator. These vesicles can be stored at 4 °C and are active for several days. The final bR/phospholipid ratio was between 1:80 and 1:500 (w/w). The sidedness of the reconstituted bR was investigated by controlled proteolysis with papain followed by polyacrylamide gel electrophoresis of the proteolysis reaction mixture (Gerber et al, 1977). According to estimates based on the relative areas of the two resolvable bands in the gel, more than 90% of all bR was reconstituted inside out.

Determination of Transmembrane Potentials. (A) Electron Paramagnetic Resonance (EPR) Spectroscopy. Spin-labeled phosphonium ions like I bind weakly to phospholipid bilayers and establish electrochemical equilibrium across the membrane of electrically polarized vesicles. As previously shown, the bound/free molar ratio of phosphonium at equilibrium, λ, may be determined from a single amplitude in the EPR spectrum according to

$$\lambda = \frac{A_f^0 - A}{A - (\beta/\alpha)A_f^0} \quad (1)$$

where A^0_f and A are the spectral amplitudes of the high-field line of the EPR spectrum in the absence and presence of polarized vesicles, respectively. The ratio β/α is a constant characteristic of the spin label (Cafiso & Hubbell, 1981). The transmembrane potential is related to λ by

$$\lambda = \frac{\lambda_0(1 + V_o/V_i)(1 + e^{F\Delta\Psi/RT})}{2(1 + V_o/V_i e^{F\Delta\Psi/RT})} \quad (2)$$

where λ_0 is the value in the absence of a transmembrane potential, and V_o/V_i is the ratio of outer to inner aqueous volumes for the vesicle preparation (Cafiso & Hubbell, 1981). This expression assumes that the inner and outer surface potentials are equal. In the present experiments, both surface potentials are assumed to be 0 since uncharged lipids are used, the density of bacteriorhodopsin does not exceed 1 molecule per 500 lipids, and the ionic strengths in 0.15 molar, inside and out.

EPR measurements were made in a Varian E-104 X-band spectrometer fitted with a loop–gap resonator (Froncisz & Hyde, 1982). Liposome samples of 5 μL with I at a final concentration of 1×10^{-4} M were placed in a sealed quartz capillary contained in the resonator. The microwave power was 2 mW with a modulation amplitude of 1 G peak to peak. In experiments where the sample was illuminated, a TE102 resonant cavity with a flat quartz cell was used (300-μL sample) and light was applied directly through the front of the cavity. Microwave power was 10 mW with a modulation amplitude of 1 G peak to peak. All measurements were obtained at room temperature (20–22 °C).

(B) Tetraphenylphosphonium-Selective Electrodes. The concentration of tetraphenylphosphonium (TPP^+) in solution can be determined electrochemically by using a PVC-based membrane containing the hydrophobic anion tetraphenylborate (TPB^-) (Kamo et al., 1979). The response of the electrode was near Nernstian in the range 10^{-2}–10^{-6} M TPP^+. Knowing the free initial concentration of TPP^+ and the ratio of the internal volume of the vesicles to the total bulk volume, the transmembrane potential (ΔΨ) is given by

$$\Delta\Psi = \frac{RT}{F}\ln\left(\frac{v}{V}\right) - \frac{RT}{F}\ln\left[\exp\frac{F(E - E_0)}{RT} - 1\right] \quad (3)$$

where v is the internal volume of the vesicles and V is the external bulk volume.

Electrical Recordings and Data Acquisition. All ΔΨ measurements using TPP^+ electrodes and ΔpH determinations were performed in a thermostated glass chamber that was continually stirred with a magnetic stirrer. The chamber was surrounded by a Faraday cage, and the whole apparatus was continuously illuminated with a dim red safety light. Illumination of the sample was provided by a 150-W halogen light through a fiber optic bundle directly coupled to two heat filters. Light intensity was controlled either with a set of calibrated neutral density filters or with a pair of tandem polarizing filters at different relative angles. Output from the pH electrode or the TPP^+ electrode was fed into a high-impedance amplifier (Keithley Intruments) and subsequently filtered at 50 Hz with a 4-pole Bessel filter. Time-dependent data was digitally acquired at 1 or 3 points/s. Data from the EPR spectrometer was acquired with 10 points/G for the steady-state data, and 4–8 scans were signal-averaged. Time-dependent data at a fixed magnetic field was acquired at 3 points/s.

Sodium Channel Purification and Reconstitution. The sodium channel from *Electrophorus electricus* electroplax was solubilized and purified following the methods of James et al (1989). Briefly, crude electroplax membranes were prepared

according to Rosenberg et al (1984), solubilized with 1% *n*-decyl-β-maltopyranoside, and combined with mixed micelles of egg PC/CHAPS in buffer containing 200 mM KCl, 50 mM phosphate buffer, pH 7.0, 5 mM EDTA, 0.02% sodium azide, and a cocktail of protease inhibitors (buffer A). An anti-polysialic acid IgM was purified from horse serum with a colominic acid-coupled affinity column and coupled to CNBr-activated Sepharose-4B (2 mg of antibody/mL of gel). The solubilized electroplax extract was loaded onto the anti-polysialic acid IgM column and washed extensively with buffer A, and the sodium channels were eluted with the same buffer containing 10 mM colominic acid. Fractions were tested for protein content (Lowry et al., 1958) and TTX binding activity (Agnew et al., 1978). The peak TTX-binding fraction showed a specific binding activity of ~2400 pmol of ^{3}H-TTX/mg. Fractions were pooled and concentrated by ultrafiltration on Amicon Centriprep 30 membranes, and dialyzed (three changes) against 1000 volumes of buffer B containing 200 mM NMG-SO_4 and 1 mM $CaSO_4$.

Co-Reconstitution of bR and Sodium Channels. Affinity-purified eel sodium channels reconstituted in egg phosphatidylcholine were fused with purple membrane sheets or with preformed reverse-phase liposomes containing bR using a freeze–thaw–sonication (FTS) procedure. One hundred fifty microliters of μL reverse-phase evaporation-resonstituted bR containing 20 mg/mL PC and 0.6 mg/mL bR, or 30 μL of a 4.1 mg/mL suspension of purple membrane, were mixed with 50 μL of reconstituted eel sodium channel vesicles containing 120 μg of sodium channel and 20 mg/mL PC. The mixture was rapidly frozen in liquid nitrogen and thawed 15 min at room temperature. This cycle was repeated five times. The mixture was then sonicated for 30 s in a bath-type sonicator. Under these conditions, the sidedness of bR did not change, as determined from ΔpH determinations or proteolytic assays.

Co-reconstitution of bR and sodium channels was followed by equilibrium ultracentrifugation. Reconstituted PC vesicles (100 μL, 20 mg/mL PC) containing sodium channels were mixed with purple membranes (50 μL, 4.2 mg/mL bR) and placed on top of a 5–30% linear sucrose density gradient, before and after the freeze–thaw procedure. The gradients were centrifuged at 45000*g* for 16 h at 4 °C and fractionated. Phospholipid in the fractions was located by phosphate analysis (Chen et al, 1956). bR was determined spectrophotometrically by its absorption at 570 nm. After the phospholipid peaks were detected among all the fractions, three fractions from each peak were assayed for TTX binding.

RESULTS

Proton Pumping by Reconstituted Bacteriorhodopsin. In order to be effective as a current source, bR has to be reconstituted vectorially, that is, more than 50% in one orientation. Reconstitution using the reverse-phase evaporation method results in more than 90% of the bR molecules being incorporated inside out (Rigaud et al., 1983; this study). We have tested the light-induced proton pumping of bR by measuring changes in the external pH (and therefore its ability to generate a transmembrane ΔpH; Figure 1). Vesicles suspended in an unbuffered solution of K_2SO_4 show a small response when illuminated maximally (150 mW; Figure 1A). The light-induced ΔpH corresponds to an alkalinization of the external solution, in agreement with the inside-out orientation of bR. Addition of valinomycin produces a large stimulation in the proton pumping (up to 8-fold). The presence of a significant stimulation by valinomycin suggests that the

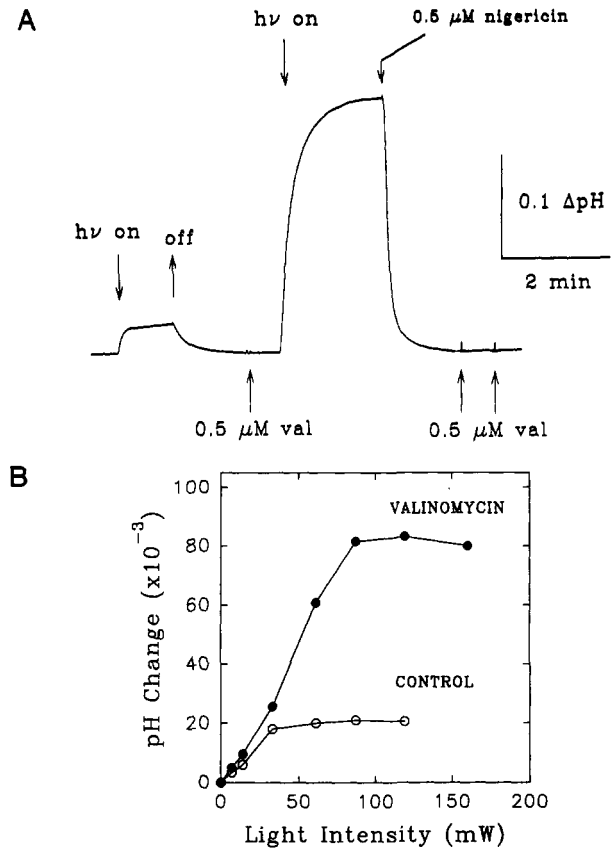


FIGURE 1: Light-dependent $\Delta\mu H^+$ generation in reconstituted bacteriorhodopsin. (A) Time-dependent changes in external pH. bR was reconstituted (1:100 bR/lipid, w/w) in a solution containing unbuffered 150 mM K_2SO_4. Arrows point at the beginning (hν on) and the end of illumination (off) (150 mW). A second illumination of the same intensity was applied after addition of 0.5 μM valinomycin. Subsequent addition of 0.5 μM nigericin completely abolishes $\Delta\mu H^+$. (B) Dependence of $\Delta\mu H^+$ on light intensity. To show $\Delta\Psi$ effects on the pH change, the light dependence was tested in the presence (●) and in the absence of 0.5 μM valinomycin (○).

vesicles are electrically tight, for in the absence of valinomycin there is a large, positive-inside potential that produces a back-pressure inhibition of the photocycle (Seigneuret & Rigaud, 1986; Manor et al., 1988). Figure 1B shows that the magnitude of the ΔpH of the external solution depends on the light intensity and that, by collapsing the $\Delta\Psi$ component of the electrochemical gradient ($\Delta\mu H^+$), valinomycin stimulates proton pumping at all light intensities.

Generation of Stable Holding Potentials. In vesicles with reconstituted inside-out bR, interior potentials generated by proton pumping can only range from 0 mV to positive values. Thus, a stable negative inside biasing potential is required to extend the control of transmembrane potentials to negative values. This bias potential has the important property of resetting some ion channels from a deep inactivated state to the closed state (Tanaka et al., 1986). A bias potential can be created by introducing a suitable transmembrane gradient of a permeable ion, with a highly impermeable counterion. However, the total ion permeability of the vesicle must be much less than the proton pumping rate of bR; otherwise, light control of the potential cannot be realized. Consequently, an ion must be found with sufficient permeability to generate the diffusion potential in a reasonable time, but with low enough permeability to permit potential control with bR. To this end, liposomes (20–50 mg/mL egg PC) were prepared in 200 mM NMG-SO_4, and their external solution was exchanged by gel filtration chromatography into a solution

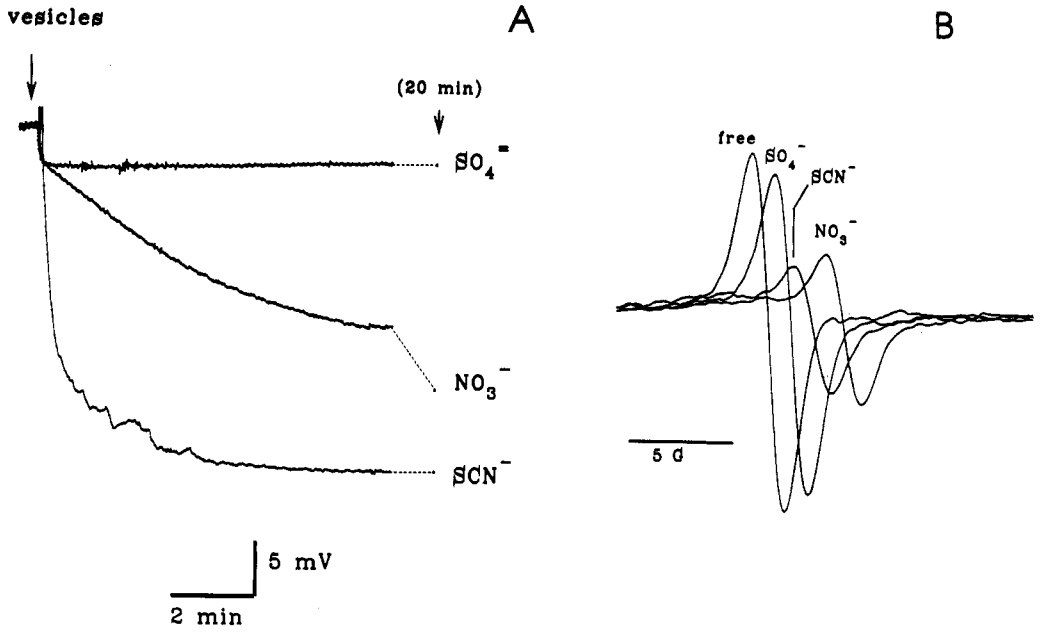


FIGURE 2: Generation of holding potentials. Anion-generated diffusion potentials were obtained by diluting egg PC liposomes (20 mg/mL egg PC) prepared in 150 mM NMG-SO_4 into solutions of 150 mM NMG-SCN, NMG-NO_3, and NMG-SO_4. (A) The traces are recordings of the output of the phosphonium ion electrode after generation of the ion gradients. Decreases in the electrode potential reflect increasing magnitude of potential, negative inside. The 5-mV calibration bar refers to the electrode potential. (B) Potentials were also monitored by the equilibrium distribution of spin-labeled phosphonium ions. Shown are the high-field resonance lines of 0.1 mM I in the vesicle suspension 1 h after creation of the ion gradient. Potentials computed from these signals are in agreement with the potentials at similar times obtained from the traces in (A).

containing 200 mM NMG-X, where X was any in a series of test anions (Br^-, Cl^-, F^-, NO_3^-, SCN^-, and SO_4^{2-}). The NMG cation is highly impermeable and is the cationic substitute of choice for electrophysiological experiments (Oxford & Yeh, 1985). Diffusion potentials were estimated by using both the phosphonium electrode and the spin-label methods as described in Materials and Methods. Of the anions tested, only NO_3^- and SCN^- are permeable enough to produce sizable (>–60 mV) holding potentials in a reasonable time. Figure 2A shows a time recording of the output from the phosphonium electrode in vesicles diluted into SO_4^{2-}, NO_3^-, and SCN^-. The dilution into SO_4^- provides a zero-potential reference since there are no ion gradients. The decreasing electrode potential corresponds to an increasing magnitude of potential, negative inside. The gradients generated with SCN^- achieved an equilibrium or steady-state potential of –140 mV within about 5 min, while those generated with NO_3^- reached –65 mV in about 20 min. The spin-label method gave similar maximum potentials (Figure 2B), and the EPR spectra were similar to those reported earlier for spin-labeled phosphonium ions in membranes (Cafiso & Hubbell, 1978) and showed no evidence of an immobilized population corresponding to protein-bound spin label. Thus NO_3^- and SCN^- are candidates for producing the bias potential, although SCN^- cannot be used in conjunction with bR due to its high permeability.

Light-Induced $\Delta\Psi$. In the presence of a negative holding potential, illumination of the bR-containing liposomes changes their transmembrane potential toward positive values. As the proton pumping rate is directly dependent on the light intensity (Figure 1), increased light intensities generate higher depolarizations. Figure 3A shows the high-field resonance line of a suspension of vesicles containing 1×10^{-4} M I. Vesicles prepared in a solution containing NMG-SO_4 and diluted into the same solution have no transmembrane potential and show the expected binding of I to the membranes as indicated by the reduction in amplitude relative to the spin label alone ($\Delta\Psi$ = 0 line). Vesicles diluted in an NMG-NO_3 solution show a negative-inside holding potential of approx. –60 mV in the dark, indicated by the further decrease in amplitude. Illumination of these latter vesicles drives $\Delta\Psi$ approximately to zero potential, indicating a light-induced depolarization of at least 60 mV. The transmembrane potential returns to the initial NO_3^- equilibrium potential when illumination ceases (not shown). This cycle of depolarization–repolarization can be repeated multiple times at different light intensities.

Figure 3B shows the dependence of transmembrane depolarization on light intensity. $\Delta\Psi$ was independently determined using TPP^+ electrodes (filled dots) or EPR spectroscopy (open dots). A simple Langmuir function used to fit both data points shows remarkable similarity between the two methods. In both cases the half-illumination point is 25 mW of light. The inset shows time-dependent TPP^+ electrode recordings of depolarizations of vesicles diluted into an NMG-NO_3 solution induced by various light intensities. All of the recordings were made with the same liposome preparation. Light pulses (1 min) of various intensities were separated by 5-min dark periods.

Although the steady-state level of $\Delta\Psi$ can be easily modulated by the light intensity, the time course of the development of each potential will depend on the kinetics of bR's photocycle. Consequently, at low light intensities the generation of $\Delta\Psi$ can be quite slow (Figure 4). This limitation can be circumvented by applying a brief but intense pulse of light before applying a given light intensity (supercharging). This protocol produces a dramatic effect on the kinetics of $\Delta\Psi$, particularly at low light intensities. The point is illustrated by comparing the time constant (from single-exponential fits) with and without supercharging (Figure 4B). There is an improvement of up to 3-fold for small (10 mV) depolarizations. The increased frequency response tends to diminish at higher light intensities as the rates converge to the maximum proton pumping rate. The concept of supercharging has been applied to improve the frequency response of patch clamp systems using an electronic current source (Armstrong & Chow, 1987).

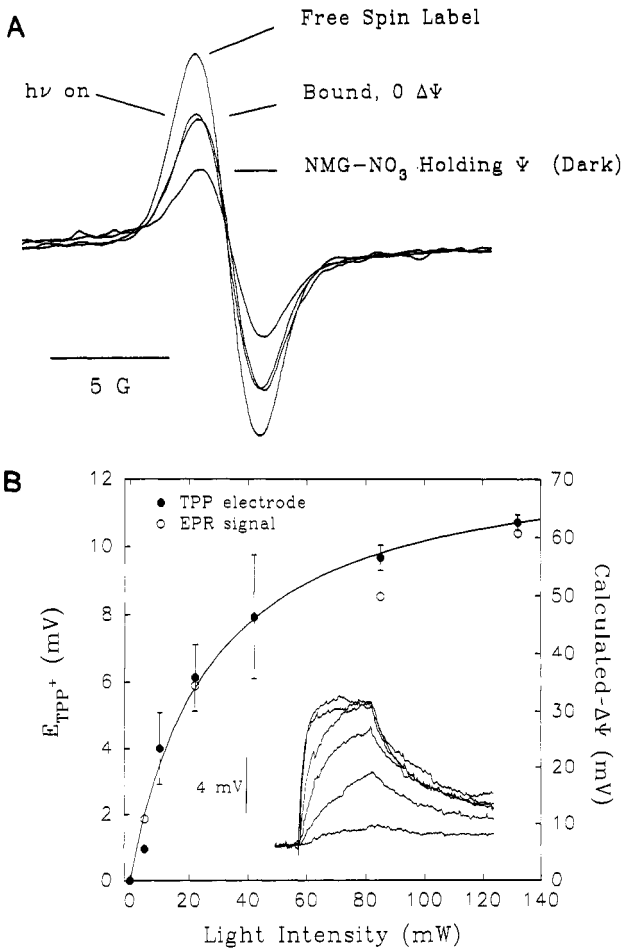


FIGURE 3: Light-induced $\Delta\Psi$. (A) High-field line of spin label I in a suspension of vesicles prepared in a solution containing NMG-SO_4. Vesicles diluted in an NMG-SO_4 solution have no potential ($\Delta\Psi$ = 0 line). Vesicles diluted in an NMG-NO_3 solution show a negative-inside holding potential of approx. –65 mV in the dark. Illumination of these vesicles displaces $\Delta\Psi$ to ≈0 mV ($h\nu$ on line). (B) Dependence of $\Delta\Psi$ on light intensity. Vesicles were diluted into an NMG-NO_3 solution and illuminated for 1 min at various intensities of light. Data from TPP^+ electrodes (●) and EPR spectra (○) are shown for comparison. A simple Langmuir function was used to fit the data points (—). The half-illumination point is 25 mW of light for both sets of data. Inset: Time-dependent recordings obtained at different light intensities using TPP^+ electrodes. All traces are from the same liposome suspension.

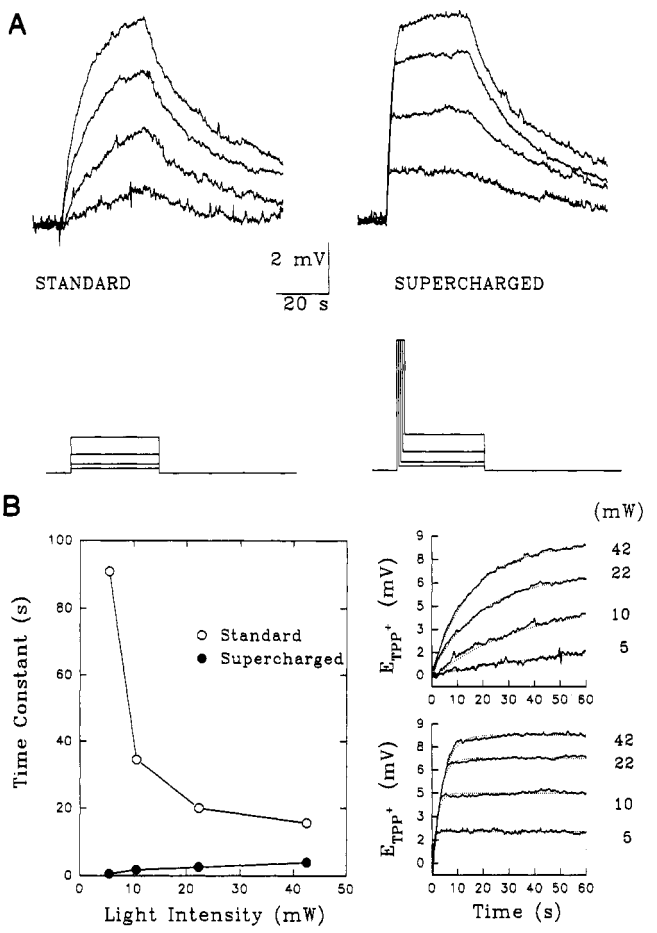


FIGURE 4: Optimization of the frequency response of the system (supercharging). (A) Time-dependent TPP^+ electrode recordings of light-induced $\Delta\Psi$ at different light intensities using standard square light pulses of 7, 12, 22, and 42 mW (left) or supercharged pulses, (right), where a 200-mW pulse precedes the standard pulse. (B) Comparison of the light-induced $\Delta\Psi$ development time constants (left) from single-exponential fits from experiments using standard square light pulses (top right) or supercharged pulses (bottom right).

Voltage Activation of Sodium Channels. To activate reconstituted sodium channels using bR as a current source, vesicles containing both sodium channels and bR must be prepared. Figure 5 illustrates a density gradient experiment to verify co-reconstitution of bR and sodium channels from a mixture of purple membrane and reconstituted Na channels by the freeze–thaw–sonication procedure. Fractions from the 5–30% density gradient were analyzed for phospholipids (organic phosphate), presence of bR (OD at 570 nm), and presence of sodium channels (^{3}H-TTX binding). The top panel shows the results of mixing the two preparations without freeze-thawing, where two distinct peaks originate from the purple membrane and the sodium channel vesicles. The bottom panel shows the same mixture after the freeze–thaw–sonication procedure, where a new vesicle peak has been generated with vesicles containing bR and sodium channels.

In order to determine sodium channel gating behavior in reconstituted vesicles from radiotracer flux assays, it is usually necessary to pharmacologically remove the slow inactivation using a battery of toxins (Catterall, 1980). It is possible to activate unmodified sodium channels by means of voltage only by applying the present method. The opening of purified and reconstituted voltage-dependent channels can be monitored by determining the light-dependent uptake of radionuclei ($^{22}Na^+$) in the presence and in the absence of channel blockers, following removal of the external cations with a strong cation-exchange resin, as originally proposed by Gasko et al (1976). A diagram of the assay is shown in Figure 6A. Vesicles containing bR and sodium channels were exposed to a bias potential for 1–1.5 h, in order to remove slow inactivation. The vesicles were mixed with $^{22}Na^+$ (5 μ Ci) in the dark and then illuminated with a halogen lamp through a heat filter. Samples were taken 30 s before and 30 and 90 s after illumination. Control samples were not illuminated. The isotope was separated from the vesicle by applying 30 μL of the sample to a Dowex 50 column and pressure-eluting with 1 mL of cold isotonic sucrose buffer containing 1 mg/mL BSA.

Figure 6B shows the results from several of these experiments. There is a light-dependent (and therefore voltage-dependent) $^{22}Na^+$ uptake (closed circles) relative to the $^{22}Na^+$ uptake in the dark (open circles). This uptake is sodium channel-specific, as it is clearly blocked by 500 nM externally applied TTX, a sodium channel-specific toxin that completely blocks ion conduction. An intrinsic advantage of this methodology is that channel activation occurs for right-side-

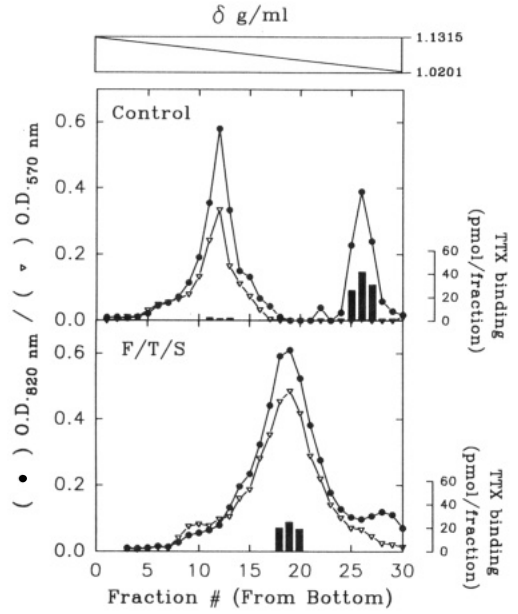


FIGURE 5: Sucrose gradient centrifugation of a purple membrane and reconstituted sodium channel vesicle mixture. Top panel, data from a control mixture. Bottom panel, data obtained after five cycles of freeze-thawing in liquid nitrogen and 30 s of sonication. Individual fractions (2 mL) were assayed for phosphate (●), bR (▽), and ^{3}H-TTX binding (filled bars).

reconstituted channels only. Channels incorporated in an inside-out configuration remain inactivated and do not respond to voltage. These results indicate that the reconstituted eel sodium channels can be activated by voltage alone and that they show normal voltage-dependent activity while apparently maintaining their pharmacology.

DISCUSSION

In order to assay for voltage dependence and to apply spectroscopic techniques such as spin and fluorescence labeling to the study of voltage-dependent ion channels in reconstituted systems, it is imperative to develop means of controlling transmembrane potentials in liposomes. While the simple method of using ion gradients to generate diffusion potentials can, in principle, provide some degree of control, it is highly limited and inconvenient. For example, changes in potential require modulation of the transmembrane ion gradient, and once such a change is carried out, the system cannot be easily reversed to its original state.

The technique reported here provides voltage control with a light-dependent current source, bR, and overcomes the limitations of the ion gradient approach. This work builds on the pioneering experiments of Racker and Stoeckenius (1974) in which a transient $\Delta\mu H^+$ created by bR was used to drive light-dependent ATP synthesis by the F_o-F_1-ATPase in a co-reconstituted system. $\Delta\mu H^+$ generation has found application in other systems that depend on proton gradients as energy sources (Driessen et al., 1985a; Maycox et al., 1990). Indeed, a widely used alternative $\Delta\mu H^+$ source is the reconstituted cytochrome oxidase complex (Hirata et al., 1977; Matsushita et al., 1983; Driessen et al., 1985b), which may also prove useful in providing voltage control for the purpose of channel activation. Control of transmembrane potential with a variable current source such as bR has a compelling advantage over simple ion gradients in that the potential can be dynamically controlled over a wide range in a single sample without modification of the ionic conditions. As shown above, the potential change is reversible and may be cycled over the

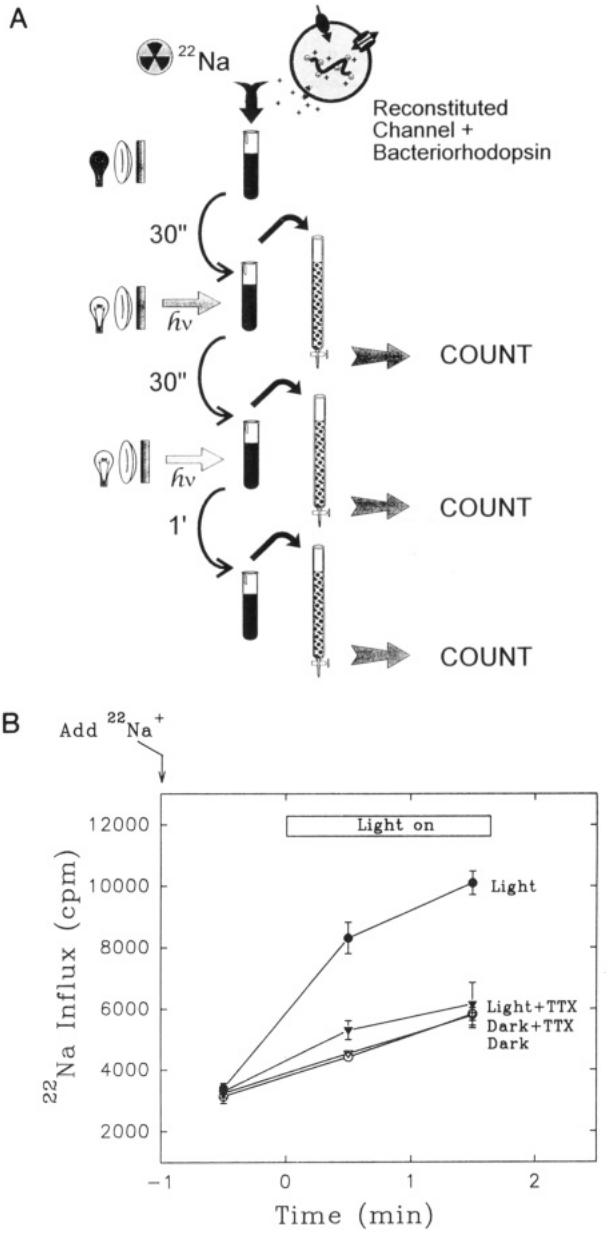


FIGURE 6: Voltage-dependent activation of unmodified sodium channels. (A) Schematic diagram of the functional assay for reconstituted voltage-dependent sodium channels. Vesicles containing purified eel sodium channels were fused with liposomes containing bR, and NO_3^--based holding potentials were generated. Fused vesicles were mixed with ^{22}Na in the dark and then illuminated. Samples were taken 30 s before and 30 and 90 s after illumination. (B) Light-induced $^{22}Na^+$ uptake in co-reconstituted sodium channels and bR. This uptake is not present in the dark and can be abolished by addition of 500 nM tetrodotoxin: (○) control uptake in the dark, (●) illuminated sample, (▽), dark plus 300 nM TTX, and (▼) illuminated plus 300 nM TTX.

potential range many times. Together with a bias potential supplied with an ion gradient, the method is capable of providing a range of potential changes sufficient to observe channel activation in vesicles.

An important component of the voltage-control system is a reliable method to monitor transmembrane potential. In the present experiments, this was accomplished by determination of the transmembrane distribution of hydrophobic phosphonium ions by both an electrochemical and an EPR method. The electrochemical method, while extremely convenient, monitors only the activity of extravesicular TPP^+ and assumes that changes in that activity reflect changes in the transmembrane ion distribution. However, direct binding

of the hydrophobic ion to membrane proteins such as bR or the protein being studied can complicate this simple analysis (Nakazato et al., 1988). The EPR method, on the other hand, detects both the bound and free populations of the hydrophobic ion and can directly reveal any significant interaction of the hydrophobic ion with proteins. The absence of such interactions in the systems described here and the agreement of the two methods for potential determination indicate that the electrochemical method may be employed with the bR current-generating system.

The use of anion-generated diffusion holding potentials imposes some performance limits on this technique. Since current injection by bR is always against a small but steady background current, the range of potentials (the magnitude of the depolarization) generated by light is limited by the absolute permeability of the anion generating the holding potential. This is the reason why SCN^-, being more effective in generating bias potentials than NO_3^-, cannot be used in conjunction with bR. The maximum bR-generated $\Delta\Psi$ in liposomes with a SCN^- bias potential was −22 mV (not shown). Additionally, this technique is limited to the study of ion-selective systems, those that do not allow voltage-activated proton fluxes or with channels so large that they are completely nonselective (like VDAC, porins, and colicins) and therefore are able to collapse $\Delta\mu H^+$.

Because of the lack of feedback control for the light intensity, this voltage control system is conceptually equivalent to a current clamp and not to a voltage clamp used in electrophysiological experiments (Bezanilla et al., 1982). However, given the use of solutions of nonconducting ions and the application of the supercharging principle, transmembrane voltages can be effectively applied (and maintained) within a few milliseconds (Helgerson et al., 1985). Although the use of a light intensity feedback loop is technically feasible (with an electronically controlled light attenuator), its application to the present system is severely limited due to the frequency response of the voltage-measuring methods and the relatively fast kinetics of the voltage-dependent systems under study (i.e., ion channel gating).

Biochemical and biophysical studies of reconstituted voltage-dependent ion channels to date have been limited by the fact that a reliable method to activate whole populations of reconstituted channels in vesicles was not available. One difficulty is the deep (slow) inactivation of the channels in the absence of a negative interior holding potential. There is a class of alkaloid toxins (among them batrachotoxin, veratridin, and garayanotoxin) that eliminate fast and slow inactivation in sodium channels (Catterall, 1980), and most of the functional studies in purified sodium channels have been performed with the use of these toxins (Weigele & Barchi, 1982; Talvenheimo et al., 1982; Rosenberg et al., 1984). Unfortunately, these toxins exert such dramatic effects in channel activation (Khodorov & Revenko, 1979; Quandt & Narahashi, 1982; Correa et al., 1992) and conduction (Khodorov & Revenko, 1979; Huang et al., 1979; Garber & Miller, 1987) that distinctions must be made when toxin-modified channels are studied.

Several groups have studied voltage-dependent activation in pharmacologically unmodified channels using patch clamp and bilayer techniques (Rosenberg et al., 1984; Shenkel et al., 1989; Correa et al., 1990), but the use of these techniques is intrinsically limited to the study of the microscopic, single-channel electrical properties of these proteins. In potentially heterogeneous reconstituted systems, it is desirable to monitor the macroscopic voltage-dependent activation of the entire population. This requires voltage control in proteoliposome suspensions containing a large number of channels. The system described here provides a means to eliminate slow inactivation and to provide voltage control sufficient to observe macroscopic channel activation in the vesicle population. Indeed, voltage activation of a population of unmodified eel sodium ion channels was observed for the first time using this system (Figure 6B). The reconstituted channels were recovered from the slow-inactivated state by incubation for several minutes in the presence of a preestablished bias potential. This step is critical since illumination of the bR and sodium ion channel-containing vesicles just after reconstitution results in no channel activation. Channel activation was promoted by illuminating the vesicle suspension. Due to the fast gating kinetics of sodium channels, most of the channels have opened and are again in the inactivated state by the time the steady-state potential is reached. This transient channel opening was enough to detect light-dependent ^{22}Na influx, but probably not to study conformational changes that occur on the millisecond time scale.

At this stage application of this technique is ideally suited to monitoring and assaying voltage-dependent activity when new purification methods are being developed. Also, it can be applied to systems like voltage-dependent carriers and ion pumps, in which the kinetics of $\Delta\Psi$ development is not critical. Potassium channel biochemistry is poorly developed because there are no alkaloid toxins that remove fast and slow inactivation in these channels, making difficult the use of traditional radiotracer fluxes as a functional assay. The use of this technique will provide the needed method to identify active fractions and, coupled with methods to determine the total number of channels, to determine the fraction of functional channels present in any preparation. In collaboration with L. Santacruz and D. M. Papazian, this method is being applied to monitor the purification of Shaker potassium channel from a high-expression system (unpublished results). This technique, combined with methods like site-directed spin labeling (Altenbach et al., 1990), will be useful in determining voltage-dependent conformations in channels and membrane proteins in general.

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