Resealing dynamics of a cell membrane after electroporation

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The membrane of a living cell consists of a bilayer of amphipolar lipid molecules as well as much larger proteins. Transmembrane potentials of up to 120 mV are physiologic and well tolerated, but when the potential is more than 300 mV, this lipid bilayer is unstable. Pores are then formed through which measurable flow of ions can occur. We follow currents through frog muscle cell membranes under 4-ms pulses of up to 440 mV. We present a theory that allows us to describe the relaxation of the current back to zero after the pulse in terms of membrane parameters. We obtain a line tension of 3.6×10^{-6} N, which is similar to that found in artificial lipid bilayers.

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The membrane of a living cell is about 5 nm thick, and is primarily made up of a bilayer of phospholipid molecules. A cytoskeletal network gives structural reinforcement, and embedded proteins carry out the necessary exchange of material between the inside and outside of the cell. The polar heads of the phospholipid molecules stick out into the aqueous intracellular and extracellular solution. In the middle of the bilayer the apolar tails, with a length of 16–18 carbons, are directed towards each other. Even though the membrane is only kept together by hydrophilic and hydrophobic forces it is very resistive and generally able to withstand fields of several tens of MV/m. Transmembrane potentials up to 120 mV are normal and essential for the functioning of the cell, but under more than 300 mV, the bilayer becomes unstable. Pores are then formed through which measurable flow of ions can occur. Electroporation is commonly utilized to ''open'' a cell so that certain ''foreign'' material can be brought in. In this context it is very important in the development of genetic engineering and gene therapy [1,2]. Electroporation is also partly responsible for the damage that a high voltage electric shock can do to living tissue, it is assumed to play a role in the effect of electroshock therapy in psychiatry, and it is the reason that cardiac defribrillators can damage the heart. A lot of experimental and theoretical work has been done on electroporation [1,2] (see also references in Ref. [2]). In this paper we describe the electroporation of a membrane in terms of elementary membrane physics.

To study electroporation experimentally, we use a frog muscle cell in a double vaseline gap (Fig. 1). With the double vaseline gap voltage clamp setup it is possible to control the transmembrane potential and measure the current. For a complete description of the materials and methods, we refer to Ref. [3]. Figure 2 shows how the experiments were conducted. The transmembrane potential is elevated for 4 ms. Under the increased transmembrane potential, pores form and expand. The conductivity of the membrane thus increases and we measure this as an increasing current. Once the pores form a stable stationary distribution, the current remains constant. Upon returning from the pulse potential to the 90 mV resting potential, a relaxation back to an almost zero current takes place. Figure 3 shows the currents obtained in an actual experiment.

All of the ion channels in our experiment were blocked. Capacitative currents were subtracted. So the currents in Fig. 3 derive solely from the behavior of a population of pores in the membrane. Below we describe quantitatively the dynam-

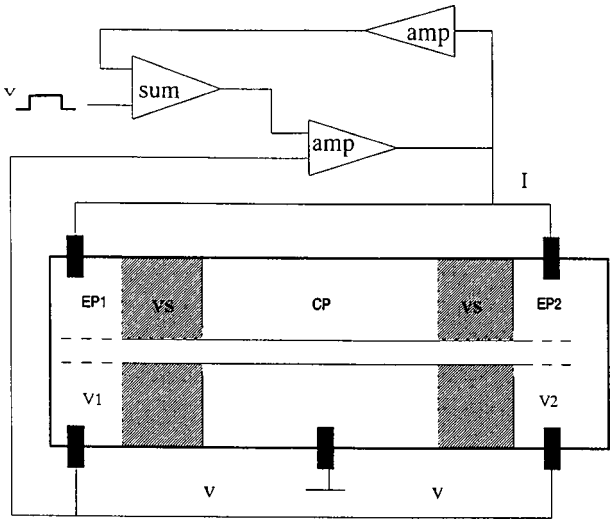

FIG. 1. The double vaseline gap voltage clamp. A muscle cell goes through two notches in a bath. Vaseline seals divide the extracellular space into three electrically isolated pools. In the end pools (EP), the membrane is permeabilized with a detergent. A muscle cell has a length of 3–5 mm and a diameter of 50–100 μm. The central pool is about 300 μm long. The applied electrical potential between the edge pools and the central pool is also the transmembrane potential over the membrane in the central pool (CP).

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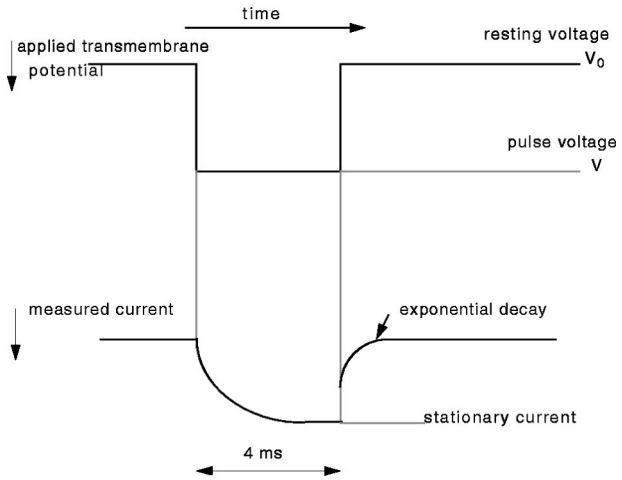

FIG. 2. Time course of the applied transmembrane potential (top) and the measured current (bottom) in our double vaseline gap experiments. We apply a pulse of duration 4 ms and follow the transmembrane current that results. Ion channels have been blocked and capacitative currents have been subtracted, so the ensuing current is due solely to electroporation.

ics of this population of pores. The creation of a pore requires the formation of a new edge. For the lining of the edge to consist of hydrophilic heads a number of lipid molecules have to reposition themselves and form a highly curved surface [4]. This requires an energy γ per unit length of pore edge. This γ is called the line tension. Line tension has the dimension of force. The formation of a circular pore of radius r requires $2\pi\gamma r$ of energy. A variety of ingenious methods has been employed to obtain estimates for the line tension for different kinds of artificial and biological lipid bilayers [5].

In many experiments with artificial bilayers, a piece of membrane is stretched across a small hole in a thin plate [4]. In such experiments the membrane is under some tension, and the appearance of a pore in that case means a decrease of

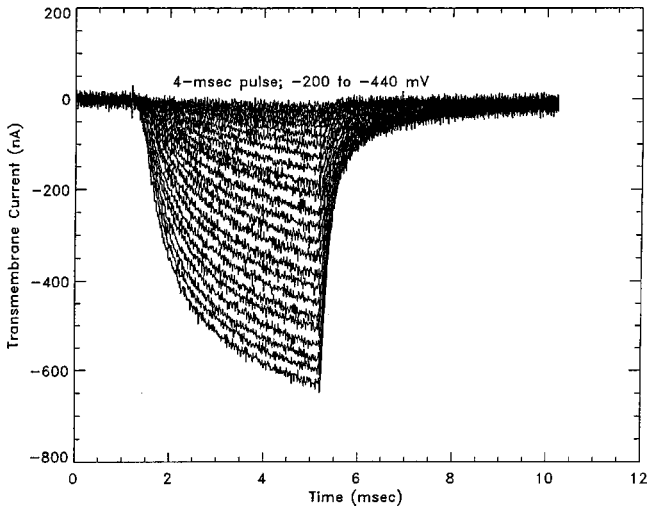

FIG. 3. A series of 4-ms pulses of amplitude 200–440 mV (in steps of 10 mV) is applied. The transmembrane currents that result from these pulses are shown.

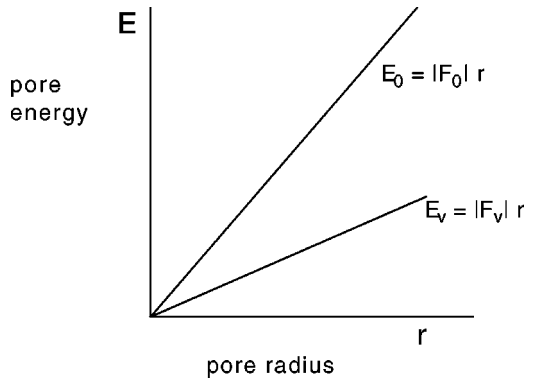

FIG. 4. In our model, the energy to create a pore depends linearly on the pore radius. Due to thermal noise a population of pores forms a Boltzmann distribution on the potential. The force driving the pores back to zero radius equals $F=-2\pi\gamma+\varepsilon_0\varepsilon_w V^2$, where γ represents the line tension and V represents the transmembrane potential. After the pulse the large number of pores forming a Boltzmann distribution on the E_v pulse profile "slides down" the E_0 profile of the resting potential V_0 and disappears.

the total membrane area and thus a decrease of energy. For the intact muscle cell in our setup, there is a constant surface area and no mechanical tension on the membrane. We therefore do not have the frequently used surface energy term $-\Gamma\pi r^2$. There will be a small quadratic term in r due to the osmotic pressure, but this term will be hard to estimate [2]. Our assumption will be that the pores are sufficiently small for the linear term in r to dominate.

Next, we make a quantitative assessment of the effect of the transmembrane potential. It is erroneous to think of the membrane as positioned in between two capacitor plates and to conceive of a pore as a piece of lipid bilayer material (with a dielectric permittivity of $\varepsilon_l=2$) that is replaced by water (with $\varepsilon_w=80$). We measure the presence of pores via the electric current that they conduct. If a pore is conductive, the applied voltage will collapse in the region of the pore. In such a case the force exerted on the pore by a field V has to be derived from the Maxwell stresses. After some algebra [6], a leading order linear term in the pore radius r can be derived. This term can be interpreted as a correction on the line tension:

$$\gamma_v=\gamma-\frac{\varepsilon_0\varepsilon_w V^2}{2\pi}. \tag{1}$$

Figure 4 shows the pore energy as a function of the pore radius r. E_0 represents the energy at the resting potential and E_v represents the energy at the pulse potential. When the pulse is applied or upon returning to the resting potential, the population of pores relaxes to a new Boltzmann distribution. The relaxation of the current during the pulse and after the pulse (Figs. 2 and 3) reflects this behavior of the pore population. For a pore to be conducting, i.e., to let water and ions through, its radius needs to be larger than the minimum radius r_{min}. This minimum radius is estimated to be around 0.3 nm [2]. The force driving the pore back to r_{min} (i.e., the

slope in Fig. 4) is independent of r, so it is no problem to identify $r=0$ in Fig. 4 with r_{min}. The exponential factor in the Boltzmann distribution does not depend on r_{min}.

The evolution of a population of pores can be described by the Fokker-Planck equation. The Fokker-Planck equation takes the forces due to the energy profile into account as well as the thermal noise (Brownian motion), and it is the appropriate way of description in the microscopic realm. Let $n(r,t)\Delta r$ be the number of pores that at time t have a radius in between r and $r+\Delta r$. Then the Fokker-Planck equation that describes the time evolution of a distribution $n(r,t)$ is

$$\beta\partial_t n(r,t) = -(F - kT\partial_r)\partial_r n(r,t), \tag{2}$$

where β is the coefficient of friction ''experienced'' by the expanding or contracting pore, k is the Boltzmann constant, and T is the temperature. The variable F represents the macroscopic force towards $r=0$ and equals F_0 at the resting potential and F_v during the pulse. The diffusion coefficient D of a pore in radius space is related to β by Einstein's fluctuation-dissipation theorem, i.e., $D=kT/\beta$. We assume that D (and thus β) is independent of the pore radius r. We will come back to this point later. One phospholipid head occupies about 0.6 nm^2 [7], and this means that the surface of the inside lining of a torus shaped pore with a 0.3 nm radius contains about 100 phospholipid heads. Brownian motion of the phospholipid heads and of the surrounding water implies that the radius of a pore will also fluctuate. The term with the second derivative in r represents the diffusive effects (i.e., the net effect of the Brownian motion on the population of pores). The equation predicts exactly the behavior that is depicted in Fig. 3. It is, for instance, easily seen in Fig. 3 that the relaxation to the stationary value during the pulse is much slower than the relaxation back to zero current after the pulse. This makes sense after the realization that during the pulse the pores first have to be formed and that the subsequent growth of pores during the pulse is diffusion driven and against a force F_v. The relaxation after the pulse involves the population of pores being ''pushed'' to $r=0$ by a large force F_0.

Because there is no measurable current at V_0, the resting potential, we assume zero pore creation at V_0. When the pulse is applied, the slope in Fig. 4 drops from $|F_0|$ to $|F_v|$. We assume that during the 4-ms pulse, pores are formed at $r=0$ at a rate S_{in} that depends on the pulse potential V. Pores can also seal when they reach the minimum radius. The rate of pore sealing is proportional to the pore density at $r=0$. So in every time unit $S_{out}n(0,t)$ pores seal and disappear from the pore population. Once the pores have formed a Boltzmann distribution, just as many pores are created as are destroyed. In that case we have a stationary state with a constant current.

The Boltzmann distribution when the pulse potential V is on is given by

$$n_v(r) = \frac{S_{in}}{S_{out}}\exp\left(-\frac{2\pi\gamma_v}{kT}r\right). \tag{3}$$

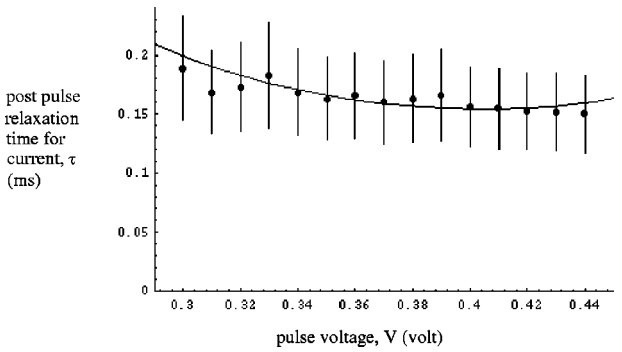

FIG. 5. The characteristic time of the relaxation back to zero current after the pulse has ended as a function of the pulse potential. Data points and error bars are from eight different experiments like the one depicted in Fig. 3. The curve derives from Eq. (5) with $\gamma = 3.6\times10^{-11}$ N for the line tension and $D=9.0\times10^{-18}$ m^2/s for the diffusion coefficient of the pore in radius space.

One easily checks that for this exponential distribution the pore sealing equals the pore creation. When the pulse ends, we go back to the steeper slope F_0 and an absorbing barrier at $r=0$. At the beginning of the relaxation, we have for the flux $J(r)=(1/\beta)(F_0-kT\partial_r)n_v(r)=(1/\beta)(F_0-F_v)n_v(r)$, i.e., the distribution $n_v(r)$ ''slides down'' towards $r=0$ with a constant and uniform speed $|F_0-F_v|/\beta$. This implies for the pore density right after the shock ends:

$$n(r,t)\approx\frac{S_{in}}{S_{out}}\exp\left\{-\frac{|F_v|}{kT}\left(r+\frac{|F_0-F_v|}{\beta}t\right)\right\}. \tag{4}$$

We see from this formula that the characteristic time for the relaxation is $\tau=[(F_v/kT)(F_0-F_v)/\beta]^{-1}$. The electroporation current will be proportional to the total surface area occupied by all pores together, i.e., $\int n(r,t)r^2\,dr$. Upon substituting Eq. (4) into this integral, the time dependence separates out and $\exp[-t/\tau]$ becomes a prefactor before the integration over r. The current will therefore also have the same relaxation time τ. If we let γ_0 represent the line tension at the $V_0=90$ mV resting potential, then we have in terms of more basic parameters:

$$\frac{1}{\tau}=\frac{4\pi^2D(\gamma_0-\gamma_v)\gamma_v}{(kT)^2}=D\left(\frac{\varepsilon_0\varepsilon_w}{kT}\right)^2\left(\frac{2\pi\gamma}{\varepsilon_0\varepsilon_w}-V^2\right)(V^2-V_0^2). \tag{5}$$

Figure 5 shows the experimentally measured postpulse relaxation times and a best fit of τ as a function of V according to Eq. (5). It appears from Fig. 3 that for the lower voltage pulses the relaxation during the pulse to a stationary current is slower. In order to be sure that we start the postpulse relaxation with a distribution $n_v(r)$ [cf. Eq. (3)], we only included pulses of $V\geqslant300$ mV. The best fit (according to a least squares criterion) to the experimental data occurs for $\gamma=3.5(\pm1.5)\times10^{-11}$ N and $D=1.0(\pm0.4)\times10^{-17}$ m^2/s. The theoretical curve is still within the experimental error bars of Fig. 5 for values of γ and D within the indicated margins of error. The value that we find for the line tension of the membrane is in the range of what has been

found with other methods (often involving much larger pore radii) for other artificial and biological membranes (see, e.g., Ref. [5]). The result of our fit is orders of magnitude smaller than the highly speculative values reported elsewhere [2,8].

The experimentally obtained relaxation time τ in Fig. 5 shows almost no variation when the pulse potential V is changed. It is possible to derive estimates for γ and D from the almost constant value of τ without the use of a computationally laborious least squares fit. Taking away the additive and multiplicative constants, we see that the V dependence in Eq. (5) looks like $f(V^2)=V^2\{[(2\pi\gamma/\varepsilon_0\varepsilon_w)+V_0^2]-V^2\}$. If the relaxation time varies little in response to changes in V, then V must be around the value, where $1/f(V^2)$ has its minimum. This occurs for $V_{min}^2=\frac{1}{2}[(2\pi\gamma/\varepsilon_0\varepsilon_w)+V_0^2]$. In our experiments, the values of V_{min}^2 were more than ten times as large as V_0^2. So neglecting V_0^2 relative to V_{min}^2, we find a simple formula for the line tension γ. Substituting this back into Eq. (5), a simple relation between D and τ is obtained.

$$\gamma \approx \frac{\varepsilon_0\varepsilon_w V_{min}^2}{\pi}, \quad D \approx \frac{(kT)^2}{\tau\varepsilon_0\varepsilon_w V_{min}^4}. \tag{6}$$

It is possible to analytically solve the Fokker-Planck equation [Eq. (2)] for the situation that arises after flipping from F_0 to F_v, and thus describe how the current evolves during the pulse. But the solution is complicated and involves the pore sealing rate S_{out} in a nontrivial way. The time and radius dependences do not separate like they do in the description of the postpulse relaxation of the current. So the analysis of the current during the pulse will not help us to get estimates for γ and D.

When pulses larger than 440 mV are applied, we find on occasion that the pulse current ''jumps off the scale'' and does not return to zero after the pulse. Within our model, this can be explained as follows. For a high V, the slope $|F_v|$ in Fig. 4 will be small and some pores may expand to a size where the quadratic term in r is significant. If the quadratic term is negative, then there will be a critical value for the radius above which the net force expands the pore. Only one pore has to reach the critical radius for such a rupture to occur. It is possible for the cell to seal such a rupture on a time scale of minutes [9,10] through a mechanism that probably involves the cytoskeletal network.

Based on macroscopic physics, a conducting pore with the minimum radius of 0.3 nm can be estimated to have a conductivity of about 300 pS [11]. Even for a 440-mV pulse, the characteristic radius $kT/(2\pi\gamma_v)$ for the exponential distribution in Eq. (3) is such that 90% of all pores have a radius between r_{min} and $r_{min}+0.1$ nm. So the microampere currents that we see correspond to at most 10^5 pores. The total membrane area in the central pool of the double vaseline gap is about 10^5 μm^2. The pores thus occupy a negligible fraction (10^{-8}) of the total area. Finally, with almost all pores in a narrow range between $r=0.3$ nm and $r=0.4$ nm the ansatz of an r-independent D appears to be a valid approximation.

Even though cell membranes consist of different kinds of phospholipid and contain up to 30% protein, the electroporation properties are well described by simple phospholipid dynamics. We obtain an estimate of the line tension, which is comparable to that found in chemically pure artificial bilayers. Equations (5) and (6) express the easily measurable relaxation time in terms of basic membrane parameters. These equations can, for instance, be useful in assessing effects of drugs on membrane integrity.

ACKNOWLEDGMENTS

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