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Curvature-electric effects in artificial and natural membranes studied using patch-clamp techniques

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Abstract. Methods for applying sound pressure to membrane patches formed at the tips of patch-clamp pipettes have been developed. Artificial membrane patches were formed from diphytanoyl phosphatidylcholine using a pipette dipping technique. Natural membrane patches were excised (inside-out mode) from collagenase-treated locust muscle membrane. Curvature-electric signals were registered under both voltage clamp and current clamp conditions. The phenomenon of flexoelectricity in membranes has previously been attributed to curvature-induced polarization originating from the liquid crystalline properties of membranes. The estimated magnitude ($2 \cdot 10^{-18} C$) of the flexoelectric coefficient of the artificial lipid bilayers is consistent with previous findings while that of the muscle membrane was in certain cases several times larger. The present study is the first to report on flexoelectricity in a natural membrane and raises the question of the biological significance of this phenomenon.

Key words: Membrane curvature, flexoelectricity, patch-clamp technique, self-assembled lipid bilayers, locust muscle membranes, inside-out patches

1. Introduction

The proposal that biomembranes in their liquid crystalline state possess flexoelectric properties was first advanced many years ago (see Petrov 1975). This phenomenon, and some of its biological consequences, has recently been reviewed (Petrov 1984; Petrov and Bivas 1984). The curvature-induced (or flexoelectric) surface polarization (P_S) per unit area of a membrane is proportional to the total membrane curvature ($1/R_1 + 1/R_2$):

$$P_S = f\left(\frac{1}{R_1} + \frac{1}{R_2}\right), \tag{1}$$

where R_1 and R_2 are the principal radii of curvature and f is the flexoelectric coefficient (units of charge). f is assumed positive if the polarization P_S points outwards from the centre of curvature. Earlier theoretical estimates gave a value for f of approximately -10^{-19} C (Petrov 1984), but black lipid membrane (BLM) experiments with ethanolamine and lecithin membranes (Petrov and Sokolov 1986; Derzhanski et al. 1987) yielded values for f as large as -2.10^{-18} C. The larger values were thought to originate mainly from the contribution of the weakly-charged lipid heads in the bilayer resulting from the curvature-induced shift of the surface charge equilibrium.

The curvature-induced P_S is equivalent to a step in the electric potential distribution (ΔU) across the membrane, equal to (in SI units):

$$\Delta U = \frac{P_S}{\varepsilon \varepsilon_0}, \tag{2}$$

wher ε is the membrane dielectric constant and ε_0 is the absolute dielectric permittivity of vacuum. If one assumes that the membrane surface is part of a sphere of radius R, then total membrane curvature is simply $2/R$. If the membrane curvature oscillates according to $(2/R) \sin \omega t$, where $2/R$ is the curvature amplitude, then under current clamp conditions (open external circuit) voltage oscillations are registered as follows:

$$\Delta U = \frac{f}{\varepsilon \varepsilon_0} \frac{2}{R} \sin \omega t. \tag{3}$$

Under voltage clamp conditions (transmembrane voltage difference clamped to zero) a displacement current in the outer electric circuit is obtained. This current is given by the time derivative of the charge of

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** To whom offprint requests should be sent the membrane capacitor $Q = C\Delta U$ produced by the voltage difference (3). Its fundamental harmonic is essentially given by the time derivative $\Delta\dot{U}$ ($\dot{C}$ brings about higher harmonics):

$$I_\omega = \frac{S}{d} f \frac{2}{R} \omega \cos \omega t\,, \tag{4}$$

where S and d are the membrane area and thickness respectively and $\omega = 2\pi\nu$ where ν = frequency. Equations (3) and (4) hold if P_S instantaneously follows the oscillations of the curvature, which is true for the so-called blocked lipid exchange regime, which is presumably realized at higher frequencies ($\nu > 200$ Hz; see Petrov and Sokolov 1986).

Until now, there have been no direct demonstrations of the existence of flexoelectricity in native membranes. This paper reports on the results of studies made to determine whether it exists in patches of membrane excised from locust skeletal muscle fibres.

2. Materials and methods

Patch pipettes were pulled from hard borosilicate glass (GC 150-10, Clark Electromedical Instruments) on a horizontal micro-pipette puller model M1 (Industrial Science Assoc., Inc.) in two successive pulls. By varying the temperature of the second pull tip radii of 3 to 10 μm could be produced (Fig. 1).

Model bilayer membranes were formed at the tip of the pipettes using the method of Coronado and Latorre (1983). Diphytanoyl lecithin (Avanti Polar Lipids, Birmingham, Alabama) dissolved in pentane (1 mg/ml) was pipetted onto the surface of a buffer solution containing 0.5 *M* KCl, 10 m*M* HEPES (pH 7.0). However, in some cases unbuffered 0.1 *M* KCl (pH 2.0) was used to introduce partial positive charges on the lipid head groups. In some other cases this was achieved by mixing negatively charged lipids (dipalmitoyl phosphatidyl serine, Sigma), molar ratio 1:10 with diphytanoyl lecithin. Formation of stable seals (>10 GΩ) required millimolar quantities of calcium ions in the buffer (Coronado 1985).

Inside-out patches of native membranes were formed from the surface membrane of locust extensor tibiae muscle. The muscle was treated with collagenase (Sigma 1 A, 2 mg ml^{-1}) for 1 h before patching. Both the patch pipette and muscle bath contained standard locust saline (180 m*M* NaCl, 10 m*M* KCl, 2 m*M* $CaCl_2$, 10 m*M* HEPES, pH 6.8). Typically, gigaohm seals with >1 GΩ resistance were obtained.

Several methods were developed for producing oscillating curvatures of the membrane patches. They included a loudspeaker-driven Alga syringe in the low frequency region (<100 Hz) and a piezoelectric sounder at higher frequencies (500 to 1000 Hz). The outputs of either the syringe or sounder were connected via a three-position valve to the plastic tube commonly used to supply a suction pressure to the patch pipette holder for giga-seal formation. The sound sources were fed by a sweep generator (Model 126 VCF, EXACT). The sound pressure at the pipette holder end of the plastic tube was measured by a sound level meter (Type 1400 H; DAWE Instruments). Oscillating sound pressure was also applied to the patch via the bath. In this case the sounder was fixed in the base of the bath and decoupled from the bath saline by wax. In all cases care was taken to minimize mechanical and electrical pick-up.

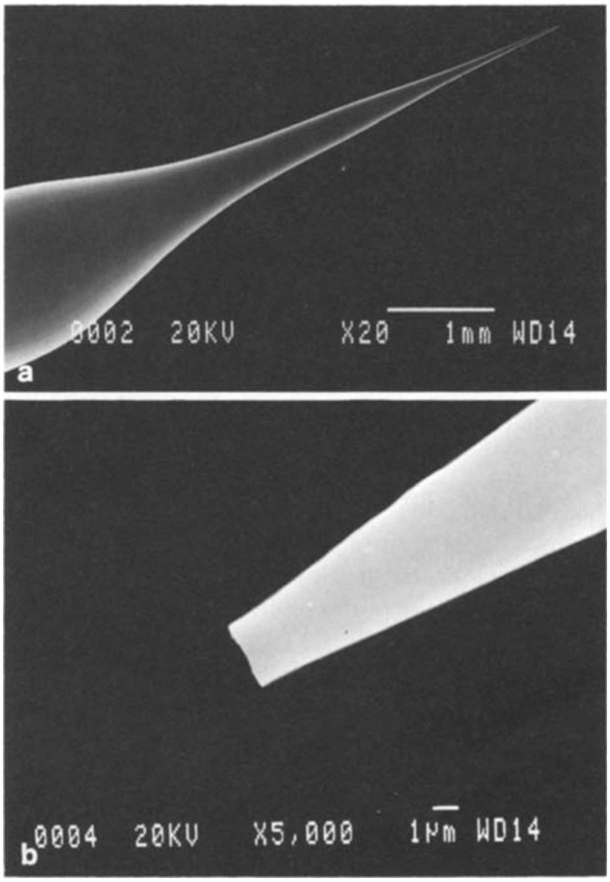

Fig. 1 a and b. Patch pipettes viewed by scanning electron microscope (JEOL GSM-840). **a** general view; **b** pipette tip

The recording system consisted of a List EPC 7 patch clamp amplifier with the output directed to a SR 510 single phase lock-in amplifier (Stanford Research Systems) referenced by the sweep generator and to a two channel Sony PCM linked to a standard video recorder. A Textronics scope was used to visualize the various outputs.

3. Results

Examples of the curvature-induced electric responses of model and native membranes under voltage clamp

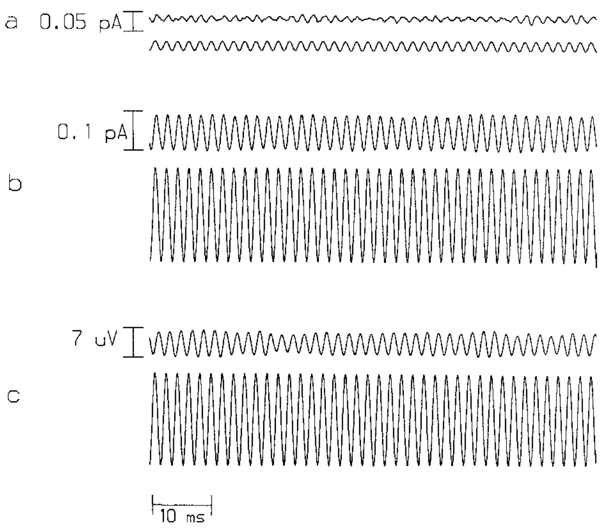

Fig. 2 a–c. Signal averaged (500 sweeps; 80 ms per sweep) curvature-electric responses of artificial lipid bilayers. Diphytanoyl lecithin plus 10 mol% dipalmitoyl phosphatidyl serine. 10 µm patch pipette tip diameter. 44 GΩ seal. Standard locust saline (see text). Stimulus frequency 500 Hz. Responses, top trace; stimuli lower trace. **a** Stimulus amplitude 2 V peak-to-peak, response 7 fA rms; **b** Stimulus amplitude 20 V peak-to-peak, response 35 fA rms; **c** Current clamp. Diphytanoyl lecithin. Locust saline. 10 µm tip diameter 1.7 GΩ seal. Stimulus amplitude 20 V peak-to-peak, response 2.3 µV rms

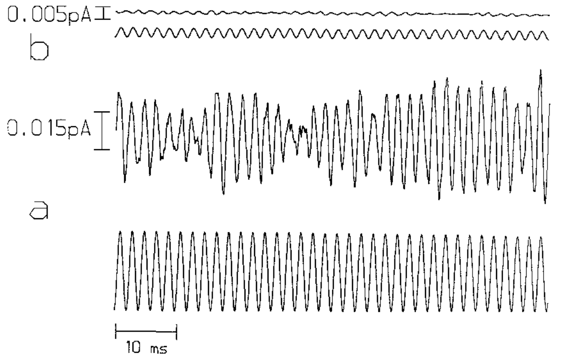

Fig. 3 a and b. Signal averaged (500 sweeps; 80 ms per sweep) curvature-electric response of muscle membrane of locust extensor tibiae muscle. 3 µm patch pipette tip diameter. 7 GΩ seal. Standard locust saline in both patch pipette and dish. Stimulus frequency 500 Hz. Patch pipette potentil held at 0 mV, i.e. at the equilibrium potential of potassium channels in patch. Responses, top trace; stimuli, lower trace. **a** Stimulus amplitude 20 V peak-to-peak, response 15 fA rms, noise 6 fA rms; **b** Stimulus amplitude 1 V peak-to-peak, response 1–2 fA

and current clamp are shown in Figs. 2 and 3. Typically, the responses were recorded at and all measurements were taken at 500 Hz, where the piezoelectric sounder was particularly powerful according to its own frequency characteristics. The pressure at the pipette holder was 91 dB or 0.6 N/m^2 at the maximum output amplitude (20 V peak-to-peak) of the sweep generator. The resulting curvature radius is calculated to be 5 mm (see below). The variations of the patch capacitance under these conditions was negligible with respect to the total patch pipette capacitance. This precluded use of the patch itself as a pressure sensor through application of the so-called "capacitance microphone effect" (Petrov and Sokolov 1986). Because of this, comparisons of the flexoelectric responses at various frequencies are not meaningful and determination of the detailed frequency characteristics of the flexoelectric responses of the natural and artifical membranes was not attempted.

Instead we choose to compare the flexoelectric response of various bilayers at one frequency (500 Hz) and amplitude (20 V peak-to-peak). The noise at the List EPC 7 amplifier was 300–400 fA but at the lock-in amplifier, due to its phase sensitive detection (time contant = 30 s), it was reduced to 1 to 2 fA under voltage clamp and 0.2 to 0.5 µV under current clamp. The signal-to-noise ratio was better than 3. The typical flexoelectric responses of the model membranes were 5 to 10 fA rms or 7 to 14 fA amplitude under zero voltage clamp and about 8 µV rms or 11 µV amplitude under zero current clamp. These values were obtained after subtracting the pick-up amplitudes measured in controls without a patch, with the patch pipette tip either outside (voltage clamp) or inside (current clamp) the buffer solution or muscle bath. They were typically 5–10 times lower than the recorded response and were phase shifted by almost 90°. The flexoelectric responses of the artificial bilayers are consistent with a value for the flexoelectric coefficient of $f = 2.10^{-18} C$, i.e. similar to that previously obtained for weakly charged zwitterionic lipid bilayer membranes (Petrov and Sokolov 1986). Variations of the surface charge obtained either by lowering pH or by introducing negatively charged lipids led to an increase of the flexoelectric response.

Of special interest was the effect of transmembrane voltage on the flexoelectric response under voltage clamp. It was found to be polarity dependent, with a weaker enhancing effect at positive ΔV (inside of the pipette positive), and sometimes a drastic effect at negative ΔV, which at voltages as high as −200 mV led a to 5–10 times increased amplitude of the response and a shift of the phase by about 30°. Breakdown of the patch often resulted during application of high pipette potentials (ca 190 mV). If the patch was still intact following such a treatment, high pipette potentials had a long-term enhancing effect on the flexoelectric response even after the pipette potential was reduced to zero. This is probably connected with the initial membrane curvature and the converse flexoelectric deformation introduced by the voltage (Petrov 1984). Another possibility is that electroporation occurs with high pipette potentials. This is indicated by the increased current noise amplitude which occurs at these potentials. Inverted pores in lipid bilayers increase lipid flip-flop and enhance the flexoelectric coefficient (Derzhanski et al. 1981).

The flexoelectric behaviour of natural membrane patches was similar to that of artificial lipid bilayers. In the case of the natural membranes pipettes of maximum 3 μm tip diameter were used. Despite this limitation, peak-to-peak amplitudes up to 100 fA and 20 μV were registered in some instances. Unfortunately, systematic investigation of the transmembrane voltage influence was not possible because of the activity of voltage sensitive potassium channels in the patches, which strongly interfered with the phase-sensitive detection. The response of the natural membrane patches was characterized by a damped oscillating behaviour with time. As the patch aged a 2–3 fold decrease of the flexoelectric response was observed. Sometimes artificial patches also displayed a gradual decrease of the response with time, which was correlated with an increase of the seal resistance.

4. Discussion

The membrane curvature ($2/R$) induced by the sound pressure ΔP can be evaluated by using the Laplace law if one assumes that the patch pipette holder and patch pipette do not damp the stimulation:

$$\Delta P = \sigma \frac{2}{R}, \tag{5}$$

where σ is the membrane tension. For bilayers of diphytanoyl lecithin σ is 1.5 ± 0.1 mN/m (Redwood et al. 1971). Our measurements give $\Delta P = 0.6$ N/m^2. Equation (5) gives $2/R = 400$ m^{-1} or $R = 5$ mm. With this value of R, with $f = 2 \times 10^{-18}$ C, and taking $\varepsilon = 2$ and $\varepsilon_0 = 8.85 \times 10^{-12}$ F/m one can obtain from Eq. (3) the frequency independent and tip radius independent, voltage amplitude, viz:

$$U_0 = \frac{f}{\varepsilon\varepsilon_0} \cdot \frac{2}{R} = 45\ \mu\mathrm{V}.$$

U_0 is larger than the experimentally measured value by a factor of 2 to 4, most probably because part of the flexoelectric response is shorted out by the input capacitance of the patch clamp amplifier (~3 pF).

With $S = \pi r^2$, where r is the tip radius, for $r = 3$ μm and $d = 5$ nm the current amplitude at $\nu = 500$ Hz is from Eq. (4):

$$I_0 = \frac{\pi r^2}{d} f \frac{2}{R}\, 2\pi\nu = 14\ \mathrm{fA}.$$

This value of I_0 closely corresponds to the measured amplitude of the flexoelectric current response of artificial patches, suggesting that the flexoelectric coefficient is close to that obtained in previous BLM measurements (Petrov and Sokolov 1986; Derzhanski et al. 1987).

The large flexoelectric response of the locust muscle patches suggests a larger flexoelectric coefficient for this natural membrane, which may be due to the presence of proteins (see Petrov and Bivas 1984, for a flexoelectricity theory of lipid-protein membranes).

The effect of transmembrane voltage ΔV could be discussed in terms of the voltage dependent membrane tension $\sigma_\varepsilon = \sigma_0 - \frac{1}{2} C_0 \Delta V^2$ where C_0 is the specific membrane capacitance (Kruglyakov and Rovin 1978). With decreasing σ Eq. (5) predicts a curvature increase at the same ΔP and, thereby, an increase in the flexoelectric response. However, this effect is polarity independent and may be partly accounted for by the positive voltage influence. The larger effect of negative voltage could be explained as a result of the initial patch curvature (Ω shaped) and the converse flexoelectric effect (voltage-induced curvature strains, Petrov, 1984). At a negative sign of the flexoelectric coefficient (Petrov and Sokolov 1986) and a negative ΔV the converse flexoelectric effect would act in the direction of decreasing the initial curvature of the Ω-shaped patch, thus decreasing the patch strains and activating in this way its direct flexoelectric the patch strains and activating in this way its direct flexoelectric response to an oscillating pressure difference. If it is really the case it means that the patch-clamp technique might provide a useful approach towards a study of the converse flexoelectric effect as well.

This idea was further supported by experiments on artificial patches with simultaneous application of static and dynamic pressure differences. When a positive static pressure was applied in the early stages of patch formation a more than two-fold increase of the flexoelectric response and a simultaneous increase of the noise was observed (not shown), which suggests the

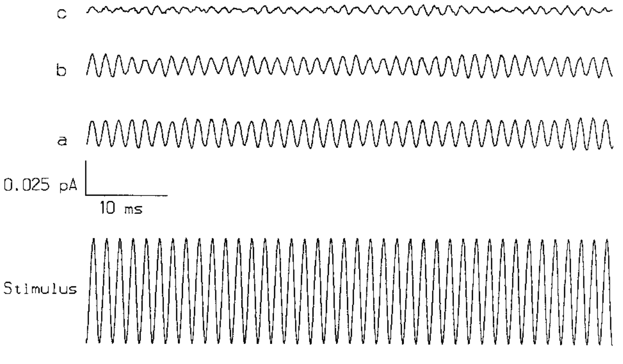

Fig. 4a–c. Signal averaged (500 sweeps: 80 ms per sweep) curvature-electric response of artificial lipid bilayer in the presence of static pressure difference. Diphytanoyl lecithin. 0.1 M KCl, pH 2.0. 4 μm tip diameter. 120 GΩ seal resistance. Stimulus amplitude 20 V peak-to-peak. Frequency 500 Hz. **a** Positive pressure, response 8.5 fA rms; **b** Zero pessure, response 5.3 fA rms; **c** Negative pressure, response 1.7 fA rms

appearance of pores (Derzhanski et al. 1981). In the later stages of patch formation positive pressure did not result in a substantial increase in noise, but a reversible increase of the response from, e.g. 4.5 fA rms to 8 fA rms was still observed (Fig. 4). With negative pressures a decrease of the response down to 1.7 fA was observed (Fig. 4). These findings support the idea that the patch is initially negatively curved, a curvature which is obviously decreased (or increased) by positive (or negative) pressure, thus decreasing (or increasing) membrane tension and, thereby, the flexoelectric response.

Increased membrane tension during patch ageing could result from a stronger adhesion of the patch to the inner walls of the pipette. This would explain the decrease of the flexoelectric response correlated with an increase in seal resistance.

5. Conclusions

Patch-clamp techniques together with devices producing oscillating pressure differences and phase sensitive detection proved to be an effective approach for investigating membrane flexoelectricity in natural and artificial membranes. It has resulted in the first experimental evidence for flexoelectricity in muscle membrane, with values for the flexoelectric coefficient comparable to, or larger than, that of model lipid membranes. This finding encourages one further to consider the possible role(s) of flexoelectricity in natural membranes. Such a basic mechanoelectric property, which binds together the two degrees of freedom (electrical and mechanical) of a membrane and results in a coupled mechanoelectric machine-like behaviour, could be directly involved in the processes of energy transformation and storage, shape transformation, passive and active ion transport, cell-cell contact etc. in natural membranes as previously predicted (e.g. Petrov 1975, 1984; Petrov and Bivas 1984). A comparison of the flexoelectric coefficients of other natural membranes should greatly increase our understanding of the biological significance of flexoelectricity.

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