# Transduction of Membrane Tension by the Ion Channel Alamethicin

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ABSTRACT Mechanoelectrical transduction in biological cells is generally attributed to tension-sensitive ion channels, but their mechanisms and physiology remain controversial due to the elusiveness of the channel proteins and potential cytoskeletal interactions. Our discovery of membrane tension sensitivity in ion channels formed by the protein alamethicin reconstituted into pure lipid membranes has demonstrated two simple physical mechanisms of cytoskeleton-independent transduction. Single channel analysis has shown that membrane tension energizes mechanical work for changes of conductance state equal to tension times the associated increase in membrane area. Results show a $\approx$40 Å$^2$ increase in pore area and transfer of an 80-Å$^2$ polypeptide into the membrane. Both mechanisms may be implicated in mechanical signal transduction by cells.

## INTRODUCTION

Mechanically activated (MA) ion channel molecules are thought to serve universally as mechanoelectrical transducers for biological systems. Since reports of MA single channel recordings by Guharay and Sachs in 1984 MA channels have been found in a multitude of cells and organisms, their ubiquity being attributed to a universal requirement for osmotic and turgor regulation (Gustin et al., 1988; Christensen, 1987). MA channels are implicated in the mechanoelectrical transduction of numerous sensory stimuli, including the sensation of sound, acceleration, touch, pressure, and gravity (Corey and Hudspeth, 1979; Howard et al., 1988; Zhou et al., 1991; Lansman et al., 1987; Olesen et al., 1988). In the case of sound transduction by specialized cells of the inner ear, mechanoelectrical transduction by MA channels has been clearly established (Howard et al., 1988; Denk et al., 1992), and many properties of the channels have been identified (Corey and Hudspeth, 1979) in spite of the inaccessibility of the MA channel complex. However, in other systems controversy continues to build concerning MA channels (Morris and Horn, 1991a; Gustin, 1991; Sachs et al., 1991; Morris and Horn, 1991b) largely due to difficulties in identifying the effective mechanisms. Early studies proposed channel gating through an unidentified elastic coupling to the cytoskeleton implying second order tension dependence (Guharay and Sachs, 1984). Recent studies of adaptation in the MA channels of frog oocytes show mechanical sensitivity that does depend on cytoskeletal coupling (Hamill and McBride, 1992). Such cytoskeleton interactions have created controversy about MA channel mechanisms and function and have complicated quantitative study of their behavior in cells (Ruknudin et al., 1991).

We report here the discovery and single channel recording analysis of mechanoelectrical transduction of membrane tension by the channel forming polypeptide alamethicin reconstituted into pure lipid membranes in the absence of cytoskeletal proteins and the determination of underlying molecular mechanisms for its mechanical sensitivity (Opsahl et al., 1990). Partition of the channel protein among its accessible states in the membrane and the aqueous solution is modulated by mechanical work driven by membrane tension. Such changes of partition coefficient between phases comprise a mechanism of transduction that is broadly implicated in biochemical transmembrane signalling.

Alamethicin is a channel forming peptide of 20 amino acids which is produced as an antibiotic by the fungus *Trichoderma veride*. It forms channels on inserting into membranes by aggregating to form barrel shaped pores which switch among multiple conductance states (Sansom, 1991). Switching occurs only between adjacent conductance states with the conductance jumps increasing nearly linearly with conductance level. Conductance state switching has been thought to occur through either an enlargement of the pore size by incorporation of an additional peptide into the pore-forming annulus as if another stave were added to the barrel (Baumann and Mueller, 1974; Schwarz et al., 1987) or by peptide rearrangement within the channel aggregate (Fox and Richards, 1982; Boheim, Hanke and Jung, 1983; Hall et al., 1984). Our study of the tension dependence of alamethicin switching distinguishes between these models and identifies general physical mechanisms responsible for MA channel behavior.

## THEORY

Signal transduction by ion channels occurs if the stimulus alters the free energy differences between conductance states of the channel, thereby shifting the equilibrium statistical distribution between these states of the active system. In the case of voltage-gated channels the application of transmembrane voltage $V_m$ energizes work $W_{ji} = qV_m$, where $q$ is the transmembrane charge transfer which accompanies channel switching from state $i$ into state $j$. Similarly, the application of membrane tension $t$ energizes mechanical work $W_{ji} = t\Delta A_{ji}$ for changes of state $i \rightarrow j$ that differ in effective membrane area by $\Delta A_{ji} = A_j - A_i$. The resulting

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change in differences between the state free energies $\Delta G_{ji}$ with change of tension $t - t_0$ is

$$\Delta G_{ji}(t) - \Delta G_{ji}(t_0) = -\int_{t_0}^{t} \Delta A_{ji}(\tau)\, d\tau \qquad (1)$$

The equilibrium partition functions express the effect of membrane tension change $t - t_0$ on the ratios of occupation probabilities of each pair $\rho_{ji} = P_j/P_i$ of states $j$ and $i$ in terms of the tension dependence of the free energy changes, without need to know the nontensioned state energies. The result to first order in $t - t_0$ is simply

$$\rho_{ji}(t - t_0) \equiv \frac{\rho_{ji}(t)}{\rho_{ji}(t_0)} = \exp[(t - t_0)\Delta A_{ji}/kT] \qquad (2)$$

Omitted from Eq. 1 are second order terms of the form

$$\frac{1}{2}[\Delta A_{ji}(K_j + K_i)/2 + \Delta K_{ji}(A_j + A_i)/2](t^2 - t_0^2)/kT \qquad (3)$$

where $K_j$ and $K_i$ are the elastic compliances of the system in states $i$ and $j$. Elementary estimates indicate that the second order terms $t^2\Delta A_{ji}(K_j + K_i)/2$ and $t^2\Delta K_{ji}(A_j + A_i)/2$ are negligible compared with the large first order terms $t\Delta A_{ji}$, justifying our approximations except for conditions where the changes $\Delta A_{ji}$ are small (Sachs and Lecar, 1991).

Elementary molecular modeling of alamethicin state switching distinguishes two possible contributions to the area changes $\Delta A_{ji}$ between conductance states. Conductance changes between adjacent channel states identify the changes in the pore area $\Delta A^{\mathrm{P}}_{i\pm1,i}$ caused by the gain or loss of one peptide unit from the channel perimeter, independent of any peripheral, channel associated peptides. Transfer of a monomer with intramembrane cross sectional area $A^{\mathrm{M}}$ between the channel and the membrane would not contribute directly to $\Delta A_{i+1,i}$. However maintenance of equilibrium by repartition of a monomer into the tensioned membrane from its surroundings would entail mechanical work imparting an additional tension sensitivity

$$W^M = tA^M \qquad (4)$$

Since that equilibration of peptide monomer with the surrounding aqueous solution occurs rapidly compared to measurements of $\rho_{\mathrm{ji}}$ (Schwarz et al., 1987) the area changes add leading to $\Delta A_{i+1,i} = \Delta A^{\mathrm{P}}_{i\pm1,i} + A^{\mathrm{M}}$ so that Eq. 2 becomes

$$\begin{aligned} \ln[\rho_{i\pm1,i}(t - t_0)] &= (t - t_0)\Delta A_{i\pm1,i}/kT \\ &= (t - t_0)(\Delta A^{\mathrm{P}}_{i\pm1,i} \pm A^M)/kT \end{aligned} \qquad (5)$$

Therefore measuring the tension dependence of the occupation probability ratios will enable us to measure the total effective area change of the channel as it switches between adjacent conductance states.

Although the derivation above assumes for simplicity that the intramembrane cross-sectional area occupied by a free monomer in the membrane is the same as that of a monomer incorporated into the channel aggregate, the result of Eq. 4 does not require this assumption, nor is the assumption necessarily valid. The net work done in transferring the monomer from the aqueous solution to an ion channel in the tensioned membrane is dependent only on the cross-sectional membrane area occupied by the channel-bound monomer. Thus Eq. 4 is valid regardless of the pathway taken by the monomer from the aqueous solution to the channel aggregate.

## MATERIALS AND METHODS

Standard patch clamping procedures are used to obtain high resistance ($R \geq 10$ GΩ) seals to pure phospolipid membranes at the tip of patch pipettes (TW150-6; World Precision Instruments, Inc., Sarasota, FL). Bilayer membranes are formed at the tips of patch pipettes using the tip-dip technique of Coronado and Latorre (1983). The lipid monolayer is formed by adding 5 μl of a 10 mg/ml pentane solution of lipids to the bath surface in a 2-ml petri dish. After allowing 5–10 min for pentane evaporation, a patch pipette is twice dipped through the surface to form a bilayer across the tip. A solvent free method is also used in which 25 μl of a 40 mg/ml suspension of lipid vesicles in a 1 M NaCl aqueous solution is added to the 2-ml petri dish (Hanke et al., 1984). Similar results are obtained with both methods. Lipids used are dioleoyl phosphatidylethanolamine:dioleoyl phospatidylserine: cholesterol at a concentration ratio of 3:1:1 (Avanti Polar Lipid, Inc., Pelham, AL). All experiments are conducted at room temperature. Alamethicin (Sigma Chemical Co., St. Louis, MO) is high-performance liquid chromatography-purified, retaining the major fraction following the procedure of Balasubramanian et al. (1981). The major fraction of alamethicin produced by Sigma is an uncharged polypeptide at pH 7.0, for which mass spectrometry has shown to have the primary sequence Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phol, where Aib is α-amino isobutyric acid (Archer et al., 1991). Purified alamethicin is added to the pipette buffer at a concentration of 150 ng/ml, and channels were nucleated by applying a large voltage $V_i > 100$ mV across the lipid membrane. Stable, single channels are obtained by lowering the voltage to $-60 < V_e < 30$ mV. Under these conditions switching of an individual alamethicin channel can be observed for several hours.

Voltage clamping and current-to-voltage conversion is done by an EPC-7 amplifier from Medical Systems, Greenvale, NY. The current signal is sent to a VR-10 pulse code modulator (Instrutech Corp., Mineola, NY) which filters the data at 37 kHz, digitizes it at a sampling frequency of 94.4 kHz, and converts the digital signal to video format for storage on a SONY betamax VCR. The data is played back through the pulse code modulator where the video formatted data is converted back into an analog signal. The analog data is filtered using an eight-pole Bessel filter (Frequency Devices) at 0.8 kHz and digitized by a D/A converter in a Labtech-DMA (Axon Instruments, Inc., Foster City, CA) at a frequency of 2 kHz with a sampling time of 500 μs. The digitized data is analyzed on an IBM-AT using in house software.

Microscopic measurement of the radius of curvature of the membrane $R_{\mathrm{m}}$ allows determination of the membrane tension $t$ from transmembrane pressure $\Delta p$ and $t = R\Delta p/2$. The hydrostatic pressure difference $\Delta p$ across the membrane is controlled by applying suction to the patch pipette. Suction pressure $0 \leq \Delta p \leq 3.0$ kPa is stepped at intervals of 30–600 s by a computer-controlled piezoelectric valve (MaxTeck, Torrance, CA) with a 5-ms rise time (Denk and Webb, 1992). Average alamethicin conductance increased to a new equilibrium value within 3 s after the pressure was stepped, indicating that any equilibration of alamethicin between aqueous phase and the membrane occurred rapidly as expected from previous stopped flow kinetics measurements (Schwarz et al., 1987). Microscopic measurements of the membrane radii of curvature $R_{\mathrm{m}}$ are obtained from image analysis of high contrast images of the membrane profile. Membrane images are obtained by using buffers with different indices of refraction on either side of the membrane and imaging with Hoffman Modulation Contrast optics which create contrast based upon index of refraction gradients (Hoffman and Gross, 1975). The index of refraction gradient is produced by using a pipette buffer of 1 M NaCl, 5 mM $CaCl_2$, 10 mM 4-morpholinepropanesulfonic acid

(MOPS), pH 7.0, and an equiosmotic bath buffer of 0.4 M sucrose, 0.8 M NaCl, 5 mM $CaCl_2$, 10 mM MOPS, pH 7.0. Reversal of the bath and pipette buffers has no observable effect on channel activity. Images are recorded with a CCD video camera (Dage-MTI, Inc., Michigan City, IN), processed as digitized images in an RCI Trapix, edge-enhanced, and the inner 80% of the membrane arc is fit using a nonlinear least-squared fitting routine. Membranes remained sectors of spheres but not hemispherical during pressure cycles (Opsahl and Webb, 1994).

## RESULTS

Equation 4 provides the basis for determining the molecular mechanisms of mechanical transduction by ion channels. By measuring the occupation probability ratios $\rho_{i+1,i}(t)$ of open channel conductance states, it is possible to obtain the cross-sectional area changes which are responsible for tension sensitivity in the alamethicin channel. Single channel recordings determine the probability of finding the channel in each conductance state as a function of membrane tension and thus provide a quantitative measure of mechanical transduction, as illustrated in Fig. 1. The total time $T_i$ spent in each conductance state at a fixed membrane tension is found by summing the occupation times for each conductance level in the amplitude histograms and the occupation probability ratios are given by $\rho_{i+1,i}(t) = T_{i+1}(t)/T_i(t)$. Data taken from separate patches at various membrane potentials $V_e$ are analyzed to set an upper limit for the magnitude of the second order terms. Second order polynomial fits to individual data sets show that second order terms are equal to zero to within the standard error of the estimate and are less than 1% of the first order terms at the highest tensions. The linear dependence of the data on tension confirms that first order area changes are responsible for the mechanical sensitivity of the alamethicin channel, ruling out second order effects such as membrane thinning or curvature energy (Huang, 1986). Further analysis reveals no significant dependence of $\Delta A_{ji}$ on $V_e$ nor tension-dependent hysteresis. Therefore all of the data is binned into values of $t - t_0$ equal within $\pm$ 0.2 dyne/cm, typically including 100 min of recording for each tension bin with $10^2$–$10^3$ transitions per level. Precise values of $\Delta A_{i\pm1,i}$ are obtained from the slope of $\ln[\rho_{i+1,i}(t - t_0)]$ as plotted versus $t - t_0$ in Fig. 2. The values for $\Delta A_{i+1,i}$ are approximately constant to within the experimental errors of $\pm 10$ Å$^2$ for each of the state pairs plotted in Fig. 2, suggesting that $\Delta A_{i+1,i} = 120 \pm 10$ Å$^2$ for conductance changes between adjacent states $i$, $i + 1$ for $i = 1$, 2, and 3.

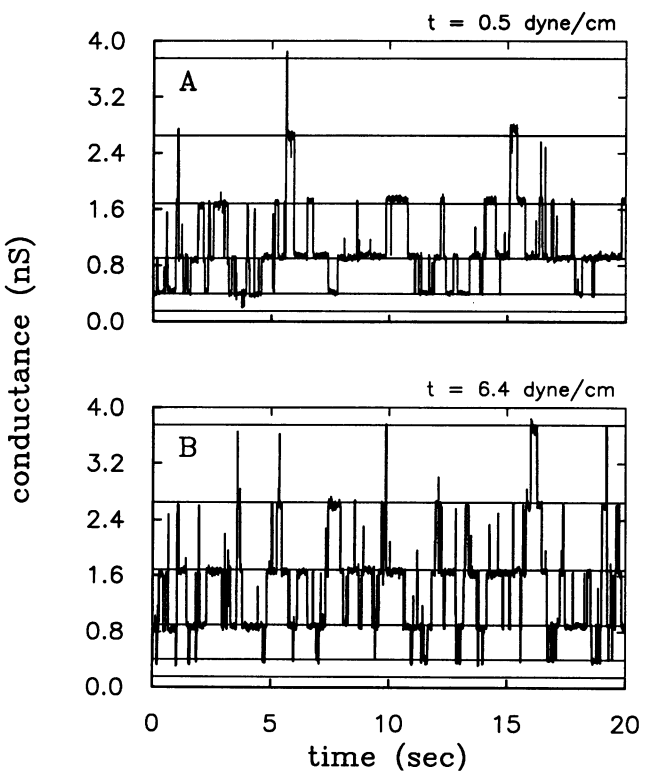


FIGURE 1 Single channel recordings demonstrating tension dependence of conductance state occupation probabilities of the alamethicin channel. Raising the membrane tension from $t = 0.5$ dyne/cm ($A$) to $t = 6.4$ dyne/cm ($B$) increases the probability of finding the channel in its higher conductance states. The multiple conductance levels are designated by horizontal lines with the lowest open level named state $i = 1$.

## DISCUSSION AND CONCLUSIONS

Measurements of $\Delta A_{i+1,i}$ differentiate between putative models for alamethicin conductance state switching. Fixed-aggregate models which invoke internal rearrangement of an aggregate containing a fixed number of polypeptides predict no change in protein area within the membrane and limit $\Delta A_{i+1,i}$ to the increase in pore area $\Delta A^{P}_{i\pm1,i}$ (Fox and Richards, 1982; Hall et al., 1984; Boheim, Hanke and Jung, 1983), which conductance data limits to 30–50 Å$^2$ for states $i = 1$–3 (Hanke and Boheim, 1980). Alternatively, the barrel-stave model, which invokes switching between states through ad-

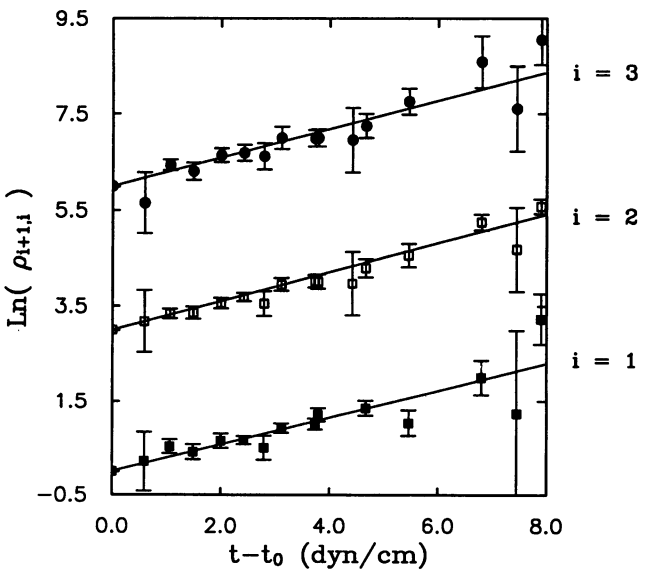


FIGURE 2 Tension dependence of open channel conductance state occupation probability ratios $\ln(\rho_{i+1,i})$ vs. $t - t_0$ shown with arbitrary ordinate offsets and slopes $d[\ln(\rho_{i+1,i})]/d[t - t_0] = \Delta A_{i+1,i}/kT$ for adjacent state pairs $i + 1$, $i$. Best fit values of $\Delta A_{i+1,i}$ from linear least squared fits are given; the error bars represent the Poisson statistical uncertainties.

dition of a peptide stave into the pore-forming barrel perimeter, includes the cross-sectional area of one alamethicin monomer provided the peptides are free to partition at equilibrium between the membrane, the aqueous bath, and a metastable channel forming aggregate (Baumann and Mueller, 1974; Schwarz et al., 1987). The monomer area of $A^M \approx 80$ Å$^2$ (Rizzo et al., 1985) makes the total predicted area change for the barrel stave model consistent with our experimental value for $\Delta A_{i+1,i}$ of $120 \pm 10$ Å$^2$. Therefore, two physical mechanisms are implicated in alamethicin's tension sensitivity: 1) the change in pore area between conductance states, and 2) the peptide transfer between membrane and surrounding phases that accompanies changes of channel state.

These measurements of mechanoelectrical transduction of membrane tension by alamethicin channels in pure lipid membranes demonstrate that membrane tension $t$ directly energizes mechanical work $t\Delta A_{i\pm1,i}$ associated with the changes of membrane area $\Delta A_{i\pm1,i}$ accompanying changes of conductance state. These simple physical mechanisms for mediation of mechanosensitivity and mechanoelectrical transduction by transmembrane channels are thereby established as a paradigm for the often controversial phenomenon. In cells the mechanism of mechanical sensitivity is likely to be complicated by cytoskeletal coupling (Howard et al., 1988; Hamill and McBride, 1992; Assad et al., 1989).

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## REFERENCES

Archer, S., J. Ellena, and D. Cafiso. 1991. Dynamics and aggregation of the peptide ion channel alamethicin, measurements using spin-labelled peptides. *Biophys. J.* 60:389–398.

Assad, J., N. Hacohen, and D. Corey. 1989. Voltage dependence of adaptation and active bundle movement in bullfrog saccular hair cells. *Proc. Natl. Acad. Sci. USA.* 86:2918–2922.

Balasubramanian, T., N. C. Kendrick, M. Taylor, G. R. Marshall, J. E. Hall, I. Voydanoy, and F. Reusser. 1981. Synthesis and characterization of the major component of alamethicin. *J. Am. Chem. Soc.* 103:6127–6132.

Baumann, G., and P. Mueller. 1974. A molecular model of membrane excitability. *J. Supramol. Struct.* 2:538–557.

Boheim, G., W. Hanke, and G. Jung. 1983. Alamethicin pore formation: voltage dependent flip-flop of alpha helix dipoles. *Biophys. Struct. Mech.* 9:181–191.

Christensen, O. 1987. Mediation of cell volume regulation by $Ca^{2+}$ influx through stretch-activated channels. *Nature (Lond.).* 330:66–68.

Corey, D., and A. Hudspeth. 1979. Ionic basis of the receptor potential in a vertebrate hair cell. *Nature (Lond.).* 281:675–677.

Coronado, R., and R. Latorre. 1983. Phospholipid bilayers made from monolayers on patch-clamp pipettes. *Biophys. J.* 43:231–236.

Denk, W., R. Keolian, and W. Webb. 1992. Mechanical response of frog saccular hair bundles to the aminoglycoside block of mechano-electrical transduction. *J. Neurophysiol.* 68:927–932.

Denk, W., and W. Webb. 1992. Forward and reverse transduction at the limit of sensitivity studied by correlating electrical and mechanical fluctuations in frog saccular hair cells. *Hear. Res.* 60:89–102.

Fox, R., and F. Richards. 1982. A voltage-gated ion channel model inferred from the crystal structure of alamethicin at 1:5-Å resolution. *Nature (Lond.).* 300:325–330.

Guharay, F., and F. Sachs. 1984. Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. *J. Physiol.* 352: 685–701.

Gustin, M. 1991. Single-channel mechanosensitive currents. *Science (Wash. DC).* 253:800.

Gustin, M., X.-L. Zhou, B. Martinac, and C. Kung. 1988. A mechanosensitive ion channel in the yeast plasma membrane. *Science (Wash. DC).* 242:762–765.

Hall, J., I. Vodyanoy, T. Balasubramanian, and G. Marshall. 1984. Alamethicin, a rich model for channel behavior. *Biophys. J.* 45:233–247.

Hamill, O., and D. McBride, Jr. 1992. Rapid adaptation of single mechanosensitive channels in *xenopus* oocytes. *Proc. Natl. Acad. Sci. USA.* 89:7462–7466.

Hanke, W., and G. Boheim. 1980. The lowest conductance state of the alamethicin pore. *Biochim. Biophys. Acta.* 596:456–462.

Hanke, W., C. Methfessel, U. Wilmsen, and G. Boheim. 1984. Ion channel reconstitution into lipid bilayer membranes on glass patch pipettes. *Bioelectrochem. Bioenerget.* 12:329–339.

Hoffman, R., and L. Gross. 1975. Modulation contrast microscope. *Appl. Opt.* 14:1169–1176.

Howard, J., W. Roberts, and A. Hudspeth. 1988. Mechanoelectrical transduction by hair cells. *Ann. Rev. Biophys. Biophys. Chem.* 17:99–124.

Huang, H. W. 1986. Deformation free energy of bilayer membrane and its effect on gramicidin channel lifetime. *Biophys. J.* 50:1061–1070.

Lansman, J., T. Hallam, and T. Rink. 1987. Single stretch-activated ion channels in vascular endothelial walls as mechanotransducers? *Nature (Lond.).* 325:811–813.

Morris, C., and R. Horn. 1991a. Failure to elicit neuronal macroscopic mechanosensitive currents anticipated by single-channel studies. *Science (Wash. DC).* 251:1246–1249.

Morris, C., and R. Horn. 1991b. Single-channel mechanosensitive currents. *Science (Wash. DC).* 253:801.

Olesen, S.-P., D. Clapham, and P. Davies. 1988. Haemodynamic shear stress activates a $K^+$ current in vascular endothelial cells. 331:168–170.

Opsahl, L. 1994. Lipid glass adhesion in gigasealed patch-clamped membranes. *Biophys. J.* 66:75–79.

Opsahl, L., D. Mak, and W. Webb. 1990. Tension dependence of the ion channel alamethicin in artificial lipid membranes. *Biophys. J.* 57:321*a*. (Abstr.)

Rizzo, V., G. Schwarz, K.-P. Voges, and G. Jung. 1985. Molecular shape and dipole moment of alamethicin-like synthetic peptides. *Eur. Biophys. J.* 12:67–73.

Ruknudin, A., M. Song, and F. Sachs. 1991. The ultrastructure of patch-clamped membranes: a study using high voltage electron microscopy. *J. Cell Biol.* 112:125–134.

Sachs, F., and H. Lecar. 1991. Stochastic models for mechanical transduction. *Biophys. J.* 59:1143–1145.

Sachs, F., W. Sigurdson, A. Ruknudin, and C. Bowman. 1991. Single-channel mechanosensitive currents. *Science (Wash. DC).* 253:800–801.

Sansom, M. 1991. The biophysics of peptide models of ion channels. *Prog. Biophys. Mol. Biol.* 55:139–235.

Schwarz, A., H. Gerke, V. Rizzo, and S. Stankowski. 1987. Incorporation kinetics in a membrane, studied with the pore-forming peptide alamethicin. 52:685–692.

Souvignet, C., J. M. Pelosin, S. Daniel, E. M. Chambaz, S. Ransac, and R. Verger. 1991. Activation of protein kinase c in lipid monolayers. *J. Biol. Chem.* 266:40–41.

Zhou, X.-L., M. Stumpf, H. Hoch, and C. Kung. 1991. A mechanosensitive channel in whole cells and in membrane patches of the fungus *Uromyces. Science (Wash. DC).* 253:1415–1417.