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The strategy of this program of research on the molecular mechanisms of electric field interactions with vertebrate cells was based on experiments designed to observe directly the effects of applied fields on living vertebrate cells in culture. We focused first on two directly observable electric field induced phenomena: (1) Measurement of changes of the spatial distribution of local changes of transmembrane potential in perpendicular applied electric fields. (2) Electrophoresis of cell surface proteins in tangential applied electric fields.

In order to measure and map the local changes of transmembrane potential drops, we developed a high resolution optical imaging procedure.[1] Prof. Leslie Loew collaborated on this research during his sabbatical with us to work on this project. He brought along a new series of lipid-soluble electrochromic membrane soluble potential sensing dyes which were very successful beginnings of a series of studies which he continues. Application to cells in culture showed the expected polarization of a virtually insulating membrane up to a "breakdown" potential around V_m ~ 100 mV maximum transmembrane potential drop. Of course, the polarization increases V_m at one pole and decreases it at the opposite pole.[2, 3] Clusters of cells showed surprising inhomogeneities of membrane potential change that are still not understood. For really useful experiments in cell physiology sensitivities of ~1 mV with time resolution of 100 μsec and spatial resolutions of 1 μm in "one shot" experiments are desired. The present measurement technology cannot deliver this capability so some compromise is always required. Subsequent developments of one of our techniques by Dr. K. Kinosita in Japan has led to powerful technique for measurement changes of membrane potential in applied

[1] D. Gross, L. M. Loew and W. W. Webb, "Optical Imaging of Cell Membrane Potential: Changes Induced by Applied Electric Fields," Biophys. J. 50, 339 (1986).

[2] Ibid.

[3] D. Gross, L. M. Loew, T. A. Ryan and W. W. Webb, "Spatially-Resolved Optical Imaging of Membrane Potentials Induced by Applied Electric Fields," in *Ionic Currents in Development* (Alan R. Liss, Inc., 1986), pp. 263-70.

electric fields which have in turn led to recent interesting studies of membrane permeabilization mechanisms induced by very high (breakdown) fields.

Proteins on cell surfaces are electrophoresed along the cell surfaces in tangential electric fields of only a few volts per centimeter, well within the physiological range. Redistribution is measured by imaging fluorescent indicators attached to appropriate cell surface proteins. The early experiments of Nucatelli and Poo showed significant molecular migration along the surfaces of living cells in culture. It has been hypothesized that polarization of motile cells to set the direction of cell motion in electrotaxis and/or chemotaxis might be attributed to segregation of certain receptors or channels to the leading edge.

In our research we have turned the logic around to use electrophoretic receptor segregation as a measure of molecular mobility, diffusion and driven molecular motility on cell surfaces. A major objective in our research has long been the mechanisms that restrict molecular diffusion by as much as 4 orders of magnitude below the physical limit for a fluid lipid membrane and that regulate molecular distributions and traffic on the cell surface. Thus we electrophorese fluorescence labeled cell surface receptors and observe their segregation in applied fields and the subsequent relaxation of the segregated distribution after switching off the field. A series of experiments using this strategy continues to generate illuminating results in our laboratory.

In one of the first of our electrophoresis experiments David Tank[4] found that the low density lipoprotein receptor (LDL-R) appeared to redistribute faster after electrophoresis in post electrophoresis relaxation (PER) with diffusion coefficients about ten times larger than the diffusion coefficients we had previously determined by fluorescence photobleaching recovery (FPR).[5,6] At the time we attributed the difference to the absence of the large ligand (LDL) on the receptor during the PER experiments which were carried out by fixing many cells after PER for various periods and subsequently staining to observe the time course of the redistribution.

Eventually we realized that these two experiments measure different physical quantities so that the results should generally differ. FPR measures the tracer diffusion coefficient D_B^* which represents the effective molecular mobility of molecular species B as it diffuses randomly in a uniform material of any composition. PER measures the mutual diffusion coefficient D_m which represents

[4]D. W. Tank, W. J. Fredericks, L. S. Barak and W. W. Webb, "Electric Field-induced Redistribution and Postfield Relaxation of Low Density Lipoprotein Receptors on Cultured Human Fibroblasts," J. Cell Biol. 101, 148 (1985).

[5]L. S. Barak and W. W. Webb, "Fluorescent Low Density Lipoprotein for Observation of Dynamics of Individual Receptor Complexes on Cultured Human Fibroblasts," J. Cell Biol. 90, 595 (1981).

[6]L. S. Barak and W. W. Webb, "Diffusion of Low Density Lipoprotein-Receptor on Human Fibroblasts," J. Cell Biol. 95, 846 (1982).

the mixing of molecules of various species driven by the chemical potential gradient associated with a concentration gradient. In an ideal binary solution $D_m \approx D_A X_B + D_B(1-X_B)$.

In the course of our investigation of this fundamental difference in the diffusion process on cell surfaces,[7] we discovered a profound property of the equilibrium distribution of cell surface proteins.[8] We noticed that the equilibrium electrophoresis generated concentration distributions of the cell surface receptor for IgE antibodies did not obey the Boltzmann statistics expected of ideal or regular solutions. Instead the results fitted a Fermi distribution function implying that on the cell surface an exclusion principle was necessary. This work (see footnotes 7 and 8) had led to much subsequent discussion and has provided a fundamental basis for our continuing research on molecular mobility on the crowded surfaces of biological cells. This IgE Receptor is the signalling entity in the allergic response which is intensely studied in order to discern the molecular mechanism for the transmembrane signal for histamine release that is generated after the receptor is cross linked the by binding an allergen particle to the IgE.

Late in the program studies of the mechanisms of mechano-electrical transduction in sensory signal perception of sound and acceleration were added to our studies of response to electrical signals and became a significant aspect of the research. Mechano-electrical transduction occurs notably in hair cells of the inner ear where minuscule displacements of a "hair bundle" (a cluster of connected cilia) in the oscillatory flow of the surrounding fluid are transduced as changes of the membrane potential leading eventually to propagation of a signal along the auditory nerve. Seven senses are involved, notably the detection of sound, but often forgotten are the senses present for detection of three components of linear acceleration and three components of rotational acceleration. As bipeds who also pilot vehicles capable of strong accelerations, humans depend strongly on these seven senses--bringing our total number of senses to 11, not just the five that usually come to mind.

Our first experiments measured the spontaneous thermal fluctuations of the displacements of the hair bundle. The absolute displacement spectra provide details of the micromechanics of the transducting organelle.[9] We established that the fundamental physical limit for mechano-electrical sensory transduction that can sometimes be reached is the thermal noise limit, unlike sensory detection of light

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[7]T. A. Ryan, J. Myers and W. Webb, "Molecular Interactions on the Cell Surface Revealed by Electrophoresis," Biol. Bull. 176S, 164 (1989).

[8]T. A. Ryan, J. Myers, D. Holowka, B. Baird and W. W. Webb, "Molecular Crowding on the Cell Surface," Science 239, 61 (1988).

[9]W. Denk, W. W. Webb and A. J. Hudspeth, "Mechanical Properties of Sensory Hair Bundles are Reflected in their Brownian Motion Measured with a Laser Differential Interferometer," Proc. Natl. Acad. Sci. USA 86, 5371 (1989).

and odors which can be quantum limited to one photon or one pheromone molecule.[10] Direct measurements of the efficiency of mechano-electrical transduction[11] confirmed the implied molecular mechanism based on stretch-activated, voltage-sensitive transmembrane channels for ions and showed that mechano-electrical transduction resonant frequencies are associated with the molecular electrochemistry processes involved in transduction, not with mechanical resonances. Continuing work is further characterizing the molecular mechanisms, key pharmacological phenomena, and the process of accommodation in mechanical transduction. All of these experiments are based on our development of a microinterferometer[12] with at least picometer sensitivity in a one hertz bandwidth up to about 50 kHz. We had earlier developed a light scattering heterodyne interferometer for vibration measurements of insect eardrums[13] that achieved comparable sensitivity with larger specimens and much less convenience.

Since stretch sensitive ion channels are implicated, we subsequently began research on the ubiquitous stretch-sensing channels in nonauditory cell membranes where a variety of stretch sensitivity had been observed. Although many of these channels have been found, their observed properties are notoriously variable and the molecular mechanisms have escaped physically consistent description. To avoid this problem we have turned to studies of a channel from a fungus that can be reconstituted into a model membrane using techniques we had developed previously to study reconstituted ion channels.[14] Recent experiments have shown well-defined stretch sensitivity of all of the open channel levels of the alamethecin channel. Our earlier experiments on alamethecin had shown that the conductivity of the open channel states fluctuates orders of magnitude more strongly than the shot noise limit. These experiments which probe the mechanisms of

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[10]W. Denk and W. W. Webb, "Thermal-Noise-Limited Transduction Observed in Mechanosensory Receptors of the Inner Ear," Phys. Rev. Lett. 63, 207 (1989).

[11]W. Denk and W. W. Webb, "Simultaneous Recording of Fluctuations of Hair-Bundle Deflection and Intracellular Voltage in Saccular Hair Cells," in *Cochlear Mechanisms*, eds. J. P. Wilson and D. T. Kemp (Plenum, NY, 1989), pp. 125-133.

[12]W. Denk and W. Webb, "Optical Measurement of Picometer Displacements of Transparent, Microscopic Objects," Applied Optics (June 1990).

[13]P. R. Dragsten, W. W. Webb, J. A. Paton and R. R. Capranica, "Auditory Membrane Vibrations - Measurements at Sub-Angstrom Levels by Optical Heterodyne Spectroscopy," Science 185, 55 (1974); "Light Scattering Heterodyne Interferometer for Vibration Measurements in Auditory Organs," J. Acoust. Soc. Am. 60, 665 (1976).

[14]D. W. Tank, C. Miller and W. W. Webb, "Isolated-Patch Recording from Liposomes Containing Functionally Reconstituted Chloride Channels from *Torpedo* Electroplax," Proc. Natl. Acad. Sci. USA 79, 7749 (1982); D. W. Tank, R. L. Huganir, P. Greengard and W. W. Webb, "Patch-recorded Single-channel Currents of the Purified and Reconstituted *Torpedo* Acetylcholine Receptor," Proc. Natl. Acad. Sci. USA 80, 5129 (1983).

conformational changes of state of channel molecules have provided our local technological experience to work with this elegant molecular system as well as some new information about molecular fluctuations in this archetypal molecule.

The above research took advantage of enhanced digital electro-optical microscopy equipment used for high sensitivity, quantitative recording of the fluorescence microscopy images of cell surface receptor distributions, potential sensing dyes and calcium ion activity indicators. This equipment was funded by ONR Grant No. N00014-86-G-0120 (06/15/86 to 06/14/87). Therefore, this final report comprises a final report for that equipment grant as well.