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Final Report

Photochemical Observation of Ion Flows in Membrane Channels

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Major Objective

Laser Doppler velocimetry is applied to a study of the synchronous motions of ions in membranes and membrane channels. The technique provides direct measurements, for example, of local velocities within gramicidin channels and these measurements can be used to elucidate the kinetic mechanism of ion flows in such channels.

The laser Doppler experiments yield data which suggest that ion motions in the channel are regular, i.e. the ions move at a steady velocity with some fluctuations about this velocity due to the molecular structure of the channel. Standard electrochemical measurements give no detailed information about such motions within the channels and, because of this lack of information, many multiparameter mechanisms have been postulated to describe the intrachannel kinetics. Since the number of "fitting" parameters is often large with respect to the available data, many models can be fitted to the data and it is extremely difficult to prove a permeation mechanism. With the detailed velocity distribution for ions in the channel, it becomes possible to differentiate the various mechanisms for ion permeation and develop a consistent mechanism for ion permeation in a specific channel.

Instrumentation

When light is scattered from a moving particle, the light frequency is Doppler-shifted. This shift can be detected by mixing a reference beam and the scattered beam at a non-linear device such as a photomultiplier. Since detection is optimal when reference beam and scattered beam have comparable amplitudes, the system was designed using a crossed beam technique. The laser beam is split into two equal beams which are displaced equal and opposite distances from the optical axis (Figure 1). These two beams are then refocused on a common point on the optical axis. For velocity components perpendicular to the optical axis, the projections on each of these beams are equal and opposite to give a net Doppler frequency difference for light scattered from the intersecting beams.

Although back scattering is generally more intense than forward scattering, the initial system was designed for forward scattering because the photomultiplier could then be located directly on the optical axis behind the intersecting beams. A pinhole collected

scattered light only.

The photomultiplier signal was sent to a H/P spectrum analyzer with a 110 kHz bandwidth with no intermediate amplification or current to voltage conversion since the current to voltage converters produced additional noise with no improvement of the signal to noise ratio.

The membrane formation and total gramicidin conducting channel population were monitored initially using a triangle waveform. To permit a large spectrum acquisition time, a constant potential was applied to the membrane using Ag-AgCl electrodes. Gramicidin concentrations in the bathing solutions were selected so that the net membrane current was roughly 50-60% of the current observed in the absence of the membrane to maximize scattering centers. The entire cell was mounted to permit rotation so that the signal could be monitored for different angles between the optical axis and the membrane normal. A study of signal with orientation was used to establish that the observed signals arose from motions normal to the membrane surface.

The experimental chamber consisted of two cylindrical quartz sections which were separated by a teflon sheet containing the hole for the bilayer membrane. The hole was centered on the optical axis at the point of beam intersection using a micrometer-controlled hydraulic positioning system. Final optimization for maximal scattering amplitude was determined using a photodiode which was inserted into the chamber on the optical axis. After optimization of this signal, the diode was removed and replaced by the electrode.

Preliminary Experiments

Since laser Doppler spectroscopy had generally been applied to studies of larger scattering centers, the system was tested using such one systems. Water, seeded with latex spheres to provide large scattering centers, flowed through a tube at constant velocity. The intersecting beams of the velocimeter were directed into this solution perpendicular to the flow direction and the velocity of these spheres was determined using the velocimeter to verify the accuracy and consistency of such measurements.

In a second set of experiments, an electrophoretic cell drove charged spheres and, in addition, large charged proteins across the intersecting beams. Like the flow system above, the signal was an envelope of waves at the Doppler scattering frequency difference. The signals were stored on a Tektronix storage oscilloscope and the frequency differences were determined directly from the stored waveform.

Ion flow in Nucleopore membranes was also used to study the system in the configuration which was to be used for the bilayer

membranes. With the forward scattering configuration, no Doppler difference signal was observable.

Gramicidin Channels

Glycerol monooleate bilayer membranes were formed using the capillary method. Bilayer formation was monitored by observing the increase in capacitative current generated by a triangular electrical potential. Gramicidin was then added to the bathing solutions to produce currents approximately 50-60% of those observed for the pinhole without membrane. A constant applied electrical potential was used to provide sufficient data acquisition time for each experiment. The photomultiplier signal was routed directly to the input of the spectrum analyzer. Observable signals were detected over periods from 10 to 20 minutes. In some cases, the potential was held constant and the Doppler scattering was observed at a series of angles of the membrane normal relative to the optical axis. These experiments established that the observed signals were produced by motions normal to the membrane, i.e. in the expected direction of ion flow.

Univalent ions which are permeable in gramicidin channels were used in the laser Doppler experiments. However, only Tl^+ ion gave observable signals with the cylindrical cell. Tl^+ has the largest ionic radius of the permeant ions.

Some sample spectra for Tl^+ ion in gramicidin channels at different applied potentials are illustrated in Figure 2. The Doppler frequency and ion velocity increased monotonically with increasing transmembrane potential and the average frequencies and velocities for each potential are tabulated in Table 1. The Doppler difference frequencies ranged up to approximately 100 kHz for a transmembrane potential of 150 mV for the H/P spectrum analyzer with a bandwidth of 110 kHz. Additional studies with a Tektronix spectrum analyzer of 10 MHz bandwidth revealed no additional spectral peaks at higher frequencies. The velocities determined from these Doppler difference frequency range from 0.0375 m/s to 0.238 m/sec for transmembrane potentials from 10 to 150 mV respectively. Because the signals observed were small amplitude, there was considerable noise (Figure 2) and variation in the observed bandwidths. These bandwidths provide information about velocity fluctuations of the ions about the steady velocity as they move through the channel.

Interpretation

The Doppler system detects the net scattering from a large number of ions which are moving in a common direction in a small spatial volume, i.e. the membrane. The velocity distribution which is observed may represent motion through the entire channel or some

section of the channel where channel homogeneity leads to a steady velocity. Although the appearance of a single narrow spectral region indicates a single velocity within the channel, it cannot prove that there may be additional undetected velocities associated with motion to the channel or to binding sites in the channel. Such motion would be more likely to have a wider range of velocity components since such ion motions need not be normal to the membrane surface. The observable velocity components would then produce a broader, flatter difference spectrum which would be difficult to detect with the present system. This problem may be rectified with a monochromatic source of higher power. The experiments clearly show, however, that there is some region within the membrane channels where the ions move synchronously at a constant velocity.

The hypothesis that the ion moves at a steady velocity through the entire length of the channel can be tested by determining a transit frequency which is simply the length of the channel divided by the observed velocity. These transit frequencies are included in Table 1 and they are consistent with estimated transit frequencies for ions in the gramicidin channel (Andersen and Procopio, Acta Physiol. Scand. Supp 481, 27 (1980). If the steady velocity is observed for a shorter section within the channel, e.g. a motion between two binding sites, the transit frequency between these two sites will be larger. To reconcile the two sets of data, it is necessary to conclude that the rate determining kinetic step for ion permeation in the channel is the motion into the channel. This question can be resolved by more sensitive experiments which do permit observation of any spectra associated with an ion approach to the channel. The ions in bulk solution lack the synchronous motion produced by the short directed channels and will probably be undetectable as individual scattering centers.

As expected, the motion associated with the membrane channels is the overall rate determining step. The ion velocities calculated using the mobility of the Tl^+ ion in bulk water and an electric field comparable to that expected across a homogeneous membrane give mobilities which are approximately ten times larger than those observed for motions within the membrane channel.

The possibility that the permeation mechanism involves motions in addition to the steady velocity through the channel or a portion of the channel is emphasized by the non-linearity of a plot of intrachannel velocity versus transmembrane potential. The curvature at higher potential suggests a limiting velocity within the channel. Since the currents in this potential range are ohmic, additional rate determining permeation steps may be present. The rate determining step to produce the linear current would then be approach to the channel rather than motion within the channel. This hypothesis must be tested with additional higher sensitivity experiments.

The experiments described have been criticized in terms of the Heisenberg uncertainty principle since the transit times in the 10^7 s^{-1} regime imply a frequency uncertainty of 10 MHz ($\Delta t \Delta \nu = 1$). A more detailed calculation using the formal uncertainty principle (position and momentum) generates frequency uncertainties in the 10-100 kHz regime. However, the Tl^+ ion is not isolated in the channel. It is accompanied by a plug of water molecules and can include interactions with the channel itself. The uncertainty principle limitations hold only for an isolated Tl^+ ion at this velocity. The Doppler observations describe the ion in a "matrix" which should minimize effects associated with the uncertainty principle.

Other Experiments

The laser Doppler experiments provide evidence of a steady velocity within the channels but they raise new questions about channel entrance velocities and the full mechanism for ion permeation. The questions raised by the new information from the laser Doppler experiments prompted several related experiments to probe the kinetics of ion motions for a membrane system. Ca^{2+} ion, while impermeant in the gramicidin channel, does act to block the flow of univalent cations through the channel. The incomplete blockage by these ions is inconsistent with some models which suggest a rapid block and unblock of the channel by the ions. The system was tested using a potential ramp technique which permitted a scan of currents through a large number of channels. One of the baths contained only permeant, univalent cations and the linear currents from this region could be extrapolated to predict the expected currents in the absence of blockage. This information, coupled with the actual current produced by a bath containing the Ca^{2+} ion permitted definition of a fraction of blocked channels. The change in this fraction with voltage was consistent, not with a blockage model, but a concentration polarization model in which the polyvalent ions produce a charge barrier which restricts entry to the channel. The ramp technique with asymmetric bathing solutions permits experiments with a large concentration of gramicidin channels since it does permit the determination of observed current relative to predicted current in the absence of all blockage.

Concentration polarization by the polyvalent ion or binding of this ion to the membrane or the channels can be probed by a spectroscopic technique which distinguishes binding and concentration polarization models. The approach uses the special spectroscopic properties of the Eu^{3+} ion, a Ca^{2+} substitute. The narrow absorption bandwidth of the ion makes it possible to do laser spectroscopy to distinguish aqueous Eu^{3+} ions from ions bound to sites on the membrane or channel. The environment-sensitive laser excitation spectrometer which we have previously used to study binding to protein sites has been redirected toward such

experiments. Initial experiments on a vesicular membrane system appear that the concentration polarization model, i.e. no binding, is the more probable model. The system is now being refined for increased sensitivity.

The actual mechanism of Tl^+ ion in the gramicidin channel may be more complex than a single ion motion through the channel. The Tl^+ ion is known to function as a blocking ion in mixed ion systems, e.g. Na^+ and Tl^+. The ramp system has been used to study this phenomenon and the observed current-voltage curves display an Tl^+ dependence. The Tl^+ ion does indeed produce block at low mole fraction and low potential. However, as the ramp potential increases in a positive or negative direction, currents increase in a second, high-potential phase indicating a lifting of the ion block at these potentials. These data are most consistent with a displacement model in which an approaching Tl^+ ion forces an ion within the channel out to the opposite bath. Block occurs at low potentials when only a Tl^+ ion can displace the blocking Tl^+ from the channel. Electrochemical methods are now being used to test the displacement mechanism. However, the electrochemical results are important for elucidating the actual mechanism of ion permeation. Even though the Doppler experiments reveal a constant motion of the ion within the channel, additional experiments are required to determine if this motion is produced entirely by the effects of the local field on a single ion or by some combination of this field and ion-ion repulsion.

The nature of Tl^+ permeation is also probed using quenching techniques. The Tl^+ ion is forced through the channel to quench a dye on the opposite side using a hemispherical bilayer system. Since the total number of ions passing through the channels can be determined with a current measurement, it becomes possible to ascertain the fraction of the total permeant ions which are Tl^+ ions. This experiment has been limited by the long times required to produce an observable change in the fluorescence intensity. The long time required for a significant change in the fluorescence intensity is probably due to a lack of effective mixing in the interior solution of a hemispherical bilayer. The experiment is presently being reconfigured to use membrane permeable dyes to monitor quenching by ions in the interfacial region. The experiments will also provide evidence for a single ion permeation mechanism or an ion displacement mechanism. The experiments will permit determination of the fraction of current carried by the Tl^+ ion. If only Tl^+-Tl^+ ion displacement is permitted at low potentials in mixed ion systems, the ratio of Tl^+ ion to total current should approach 100% for these solutions. A single ion model, in which some gramicidin channels are permanently blocked to produce the observed decreased currents, should give a Tl^+ ion permeation fraction which reflects the mole fraction of this ion in the bathing solutions.

Publications

F. Macias and Michael Starzak, Laser Doppler Scattering for the Determination of Ionic Velocity Distributions in Channels and Membranes. American Chemical Society Advances in Chemistry Series 235, Membrane Electrochemistry (In press)

Resubmission:
F. Macias and Michael Starzak,Ion Velocity Distributions in Gramicidin Channels Determined with Laser Doppler Velocimetry. (Biophys. Biochim. Acta)

To be submitted:
S. Masserant and M. Starzak, Surface Concentration Polarization induced Block of Currents in Gramicidin Channels Studied with Potential Ramps and Asymmetric Solutions.

S. Masserant and M. Starzak, Potential-dependent Block of Gramicidin Channels by Tl^+ in Tl-Na Solutions Studied with Potential Ramps.

FIGURE LEGENDS

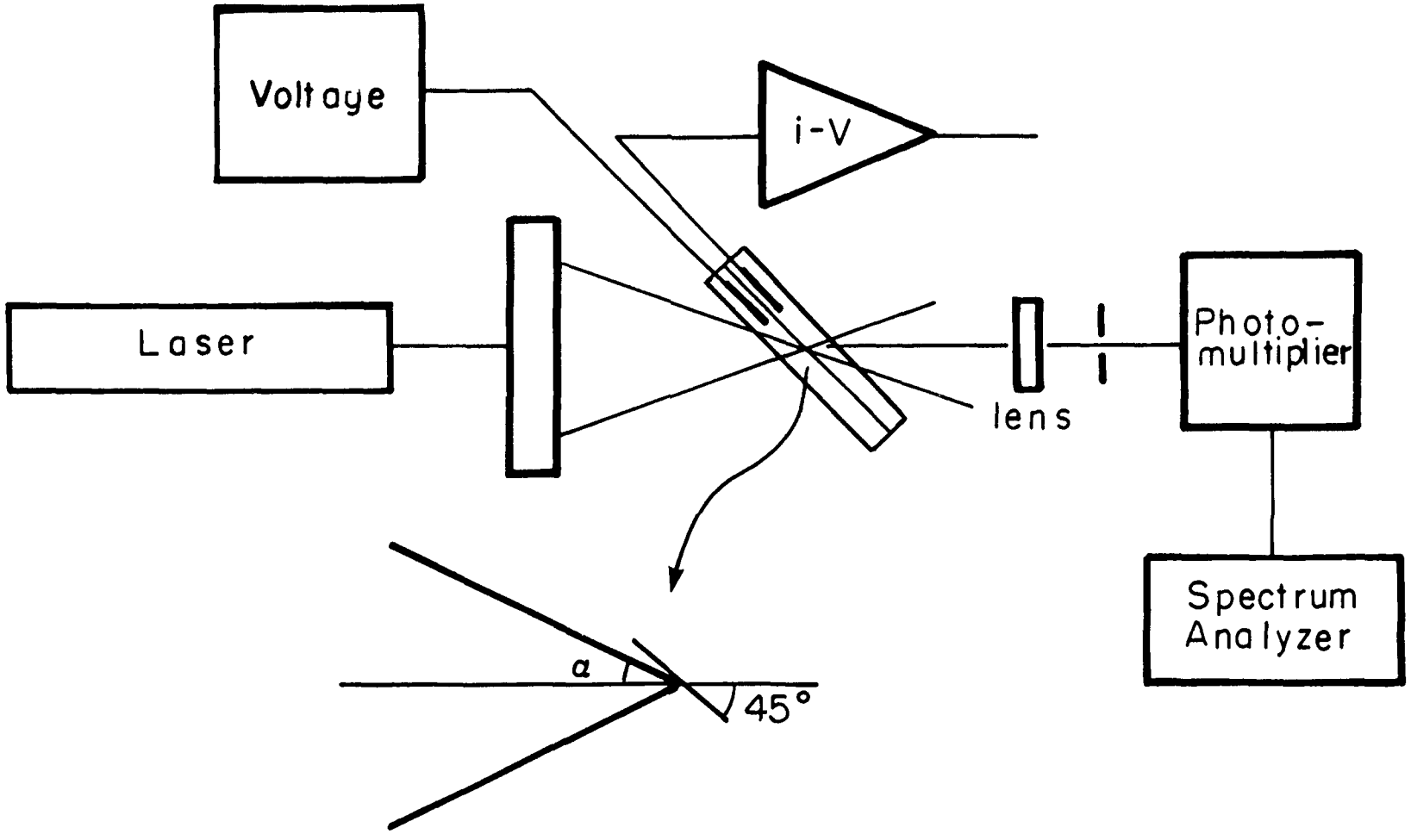
1. Crossed Beam Laser Doppler Velocimeter

2. Doppler Difference Spectra Observed as a Function of Transmembrane Potential. Average values for the spectra are listed below each spectrum.

Table 1

Tl(I) velocities and transit frequencies for transmembrane potentials in the ohmic regime.

potential mV	observations	Doppler shift kHz	velocity m/s x 10^{1}	transit frequency s^{-1} x 10^{-7}
10	2	15.6 ± 0.19	.375	1.44
15	1	19.5	.469	1.80
20	2	24.2 ± 1.0	.582	2.24
25	1	30.1	.724	2.79
30	2	32.8 ± 0.8	.788	3.03
40	3	40.8 ± 1.3	.981	3.78
50	2	44.5 ± 1.4	1.09	4.21
60	2	54.3 ± 0.1	1.31	5.01
70	2	61.2 ± 0.2	1.47	5.68
80	3	67.5 ± 0.5	1.64	6.30
90	3	73.5 ± 1.3	1.77	6.78
100	3	78.3 ± 0.6	1.88	7.21
110	3	83.8 ± 1.4	2.01	7.72
120	3	87.4 ± 1.2	2.10	8.10
130	3	92.3 ± 0.5	2.22	8.53
140	3	96.4 ± 0.6	2.32	8.93
150	1	99.0	2.38	9.14


Voltage
i-V
Laser
lens
Photo-
multiplier
Spectrum
Analyzer
α
45°

V_α	150mV	120mV	85mV
$\Delta\upsilon$	99.khz	89.2khz	71.7khz

V	50mV	40mV	25mV	15mV
$\Delta\upsilon$	44.5khz	41.7khz	30.1khz	19.5khzV