Bioelectrochemistry III
Charge Separation Across Biomembranes

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Edited by

G. Milazzo

Bioelectrochemical Society
Rome, Italy

and

M. Blank

College of Physicians and Surgeons
Columbia University
New York, New York

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PREFACE

This book contains a series of review papers related to the lectures given at the Third Course on Bioelectrochemistry held at Erice in November 1988, in the framework of the International School of Biophysics.

The topics covered by this course, "Charge Separation Across Biomembranes," deal with the electrochemical aspects of some basic phenomena in biological systems, such as transport of ions, ATP synthesis, formation and maintenance of ionic and protonic gradients. In the first part of the course some preliminary lectures introduce the students to the most basic phenomena and technical aspects of membrane bioelectrochemistry. The remaining part of the course is devoted to the description of a selected group of membrane–enzyme systems, capable of promoting, or exploiting, the processes of separation of electrically charged entities (electrons or ions) across the membrane barrier. These systems are systematically discussed both from a structural and functional point of view.

The effort of the many distinguished lecturers who contributed to the course is aimed at offering a unifying treatement of the electrogenic systems operating in biological membranes, underlying the fundamental differences in the molecular mechanisms of charge translocation.

As is usual in multiauthored books, a certain lack of homogeneity in length and depth of each subtheme is also present here, but the abundance of quoted papers and reviews at the end of every chapter should be helpful to readers wishing to deepen their understanding of the topics treated.

SYMBOLS AND ACRONYMS

For the sake of consistency and to ensure immediate understanding, the symbols of the most frequently occurring quantities and the acronyms of the organic chemicals are collected here. Consulting this list, attention must be given to the following points:

1. Only the most common symbols are included. Some, only seldom used, are not included to avoid confusion. Their meaning is given in the text.
2. Since the number of all quantities symbolized in chemistry, physics, biology, *etc.*, and officially accepted by the corresponding International Unions, even using different characters (roman, boldface, italic, *etc.*) is remarkably larger than the number of available letters, it occurs that the use of the same symbols for different quantities becomes sometimes unavoidable, and was accepted by the International Unions (for example the symbol *G* for the free enthalpy and for the electric conductance, or the symbol *A* for the area and for the optical absorbance). But the quantity to be correctly considered unambiguously results from the text.

Latin alphabet

[...]	concentration of the species (mol / dm^3)
A	absorbance, area, preexponential factor
A	ampere
a	activity
a.c.	alternating current
AcCh	acetyl choline
AcChR	acetyl choline receptor
ADP	adenosine diphosphate
ANS	anilino–naphthalene sulphonate
a.p.	action potential
ATP	adenosine triphosphate
Bgt (Btx)	bungarotoxin
BLM	bilayer membrane, black lipid membrane
BR	bacteriorhodopsin
C	capacitance
C	coulomb
°C	CELSIUS degree
c	concentration
	centi
cyt	cytochrome
F	farad
F	FARADAY's constant
f(...)	function of...

f	femto
FCCP	carbonylcyanide – *p* – trifluoro- – methoxy – phenyl hydrazone
FPLC	fast protein liquid chromatography
FTIR	FOURIER transformed infrared spectroscopy
G	free enthalpy (GIBBS free energy); conductance
GTP	general insertion protein
H	enthalpy
h.p.l.c.	high performance liquid chromatography
HR	halorhodopsin
Hz	hertz
I	current intensity; light intensity
i.c.p.a.e.s	inductively coupled plasma atomic emission spectroscopy
I.R.	infrared
J	joule
j	current density
K_m	MICHAELIS MENTEN constant
K	KELVIN degree
k	BOLTZMANN constant, rate constant
lg	logarithm, decadic
ln	logarithm, natural
LDAO	dodecyldimethyl amine
M	concentration (mol / dm^3, molar)
m	meter, milli
m.c.d.	magnetic circular dichroism
MES	morpholino – ethane sulfonic acid
min	minute
MIT	monoiodotyrosine
MOPS	N – morpholino – propane sulfonic acid
MPP	mitochondrial processing peptidase
m.w.	molecular weight
N,n	number of...
N	newton
NEM	N – methyl maleinimide
n.m.r.	nuclear magnetic resonance
P	permeability
p	pico
p.a.g.e.	polyacryl amide gel electrophoresis
PEP	processing enhancing protein
PERS	protein electric response signal
p.i.x.e.	proton induced X ray emission
pm	purple membrane
Q	electric charge
R	electric resistance, gas constant
r	radius
RNA	ribonucleic acid
RR	resonance Raman
S	siemens
s	second
sat	(subscript) saturated
SDS	sodium dodecyl sulfate
SKL	serine – lysine – leucine
SR	sarcoplasmic reticulum; sensory rhodopsin
SRP	signal recognition particle
SV	slow vacuolar
T	KELVIN temperature
t	time
TEMED	tetramethylethylene diamine
TMPD	tetramethyl – *p* – phenylene diamine
$TPMP^+$	triphenylmethyl phosphonium
TPP^+	tetraphenyl phosphonium
U	electric potential difference
UV	ultraviolet
V	volt
v	velocity, reaction rate
VDAC	voltage dependent anion channel

W	watt
w	weight
z	ionic charge

Greek alphabet

α	polarizability
Δ	difference
ε	dielectric constant
ζ	zeta potential
θ	angle
λ	wavelength
μ	dipole moment
$\tilde{\mu}$	electrochemical potential
ϱ	resistivity
Σ	sum
τ	time (as special quantity)
φ	internal electric potential

CODES FOR AMINO ACIDS

Amino acid	Three – letter abbreviation	One – letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CONTENTS

MOLECULAR MECHANISMS OF ION TRANSPORT: NEW INSIGHTS BY PATCH-CLAMP STUDIES

R. HEDRICH*, W. STÜHMER• and B.U. KELLER•

** Pflanzenphysiologisches Institut, Universität Göttingen*
• Max Planck Institut für biophysikalische Chemie
3400 Göttingen, West Germany

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Bioelectrochemistry III
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1. Introduction

New insights into the molecular processes involved in ion and nutrient transport across membranes of animal and plant cells were obtained since the application of the patch–clamp technique to isolated cells, protoplasts (wall–free plant cells) and organelles. While excitable electrical behaviour was first observed in plant cells about a century ago, the underlying mechanisms are only now being directly studied at the molecular level. Ion channels are integral transmembrane proteins which, when open, allow the movement of ions and some non–electrolytes down their electrochemical potential gradients. Although ion currents in plant cells were among the first to be studied in detail, the electrophysiological characterization of plant ion channels has been somewhat slower compared to their animal counterparts. This has been due to problems specific to plants, such as the presence of the cell wall, having the plasma membrane and vacuolar membrane in series separated by only a relatively small cytoplasmic compartment.

These difficulties are rapidly being overcome with the application of the patch–clamp technique. The patch–clamp technique is a revolutionary electrophysiological method allowing high resolution recording of ion currents from biological membranes, both at the single channel level and from whole cells or organelles. The various applicabilities of the patch–clamp technique were used to investigate active and passive mechanisms of solute fluxes across cell membranes and their regulation. In this chapter the role of ion channels in important cellular processes such as osmoregulation, turgor control and the transduction of external and internal signals will be discussed. Electrophysiological methods, including the patch–clamp technique, and their application to record ion fluxes from whole tissues, cells and membrane patches will be described.

2. Electrophysiological techniques

2.1. Vibrating probe

Extracellular recorded potential differences, or currents are a reflection of transmembrane ion transport being resolved as current flowing across an extracellular series resistor. Although these types of measurements are relatively easy to perform, they suffer

from lack of resolution. A large body of earlier work on whole tissues using extracellular recording electrodes concerned the stimulation and propagation of action potentials.

More recent examples of this approach were the studies from L.F. JAFFE's group on sea urchin eggs and plant pollen using the vibrating probe. The vibrating probe technique is based on the rapid, extracellular measurement of potential difference at two different locations close to one another. Any spatial difference in potential is attributed to the presence of net ion current flow, representing the sum of currents arising from membrane conductances. These currents result in a characteristic topology of the electric field along single cells or whole tissues. The results obtained using this approach led to the suggestion that ionic currents are required as triggers for the establishment of cellular polarity or developmental responses. It has been shown that changes in the intensity of external electrical fields precede changes in cell polarity, and that certain types of tissue growth are associated with transcellular current flow. Nevertheless, the vibrating probe technique is limited because the contribution of individual ion species (*e.g.* K^+) to the overall current is not easily distinguishable, and direct access to the source of ion currents, the membrane, is constrained because of the physical size of the probe.

2.2. *Standard microelectrodes*

If the composition on either side of a membrane is different, and if the membrane is semipermeable, an electrical potential difference will exist across the membrane, the size of which is a function of the membrane conductance for the permeant ion species. This is the ***transmembrane potential***, the difference between the intracellular and extracellular potentials. It can be measured directly using fine intracellular glass microelectrodes or more indirectly using potential-sensitive dyes. Subsequently, with the application of voltage-clamp techniques (clamping the voltage to the zero-potential level by measuring the injected current needed), the measurement of transmembrane ionic current flow became possible. The best example of such an application is the characterization of the ionic basis for the action potential in the squid giant axon by COLE, HODGKIN, HUXLEY and KATZ roughly 40 years ago.

In animal cells, following the macroscopic description of cellular ion currents and their activating mechanisms, much effort was expended in trying to study the elementary characteristics of individual ion channels. At that time, the 1970's, noise analysis was the method of choice and was used to study the fluctuations in current arising from a varying number of open channels in the population present in a single cell. Ion channels are now accepted as the main transporters for ion movement down electrochemical potential gradients. Questions of current interest concern notably the regulation and modulation of

ion channel activity by external and internal signals (such as neurotransmitters and second messengers). Thus the physiologist wanted access to living cells to effectively control these signals.

To study small cells of 10–20 micrometer in diameter (a normal cell size), blunt, low resistance microelectrodes were used to penetrate the cell wall (in plant cells only) and/or the plasma membrane, thus leading unavoidably to high leak conductances. These may give rise to uncontrolled shifts in intracellular ion concentrations, and therefore poorly defined equilibrium potential differences, due to excessive leakage into the cell of the electrolyte used to fill the microelectrodes. Thus the inevitable leakage conductances associated with intracellular microelectrode impalements are too large with respect to the inherent membrane conductances to allow reliable interpretation of potential clamp data from small cells. However, many of these limitations have been circumvented in recent years, following the development of the patch–clamp technique.

2.3. Patch clamp

The patch–clamp technique was first used to obtain direct measurements of the elementary current passing through a single ion channel. Recent advances allow the patch–clamp technique to be used to record ionic currents from membrane patches and from entire small cells.In a typical patch–clamp experiment, measurements are performed in physiological buffer solutions. Cells or organelles are usually allowed to settle on the surface of a petri dish for patch–clamp measurements. A heat–polished glass pipette with a tip diameter on the order of 1 micrometer is pressed against the membrane surface. When suction is applied to the interior of the pipette, a seal forms between the pipette tip and the membrane (cell–attached configuration, Fig. 1). The high seal resistance and small membrane area reduce background noise and ensure that currents passing the channels in the membrane patch will flow into the pipette.

This cell–attached measuring configuration, as well as excised–patch configurations (inside–out and outside–out patch, see Fig. 1), allows the resolution of single–channel currents of less than one picoampere. Figure 2 shows current recordings from the vacuolar membrane of suspension–cultured plant cells. Single–channel currents consist of rectangular pulses of random duration reflecting conformational changes of a macromolecule. Each downward current step represents the opening, and each upward current step the closing, of a single ion channel. As long as the channel is open, ions pass through it driven by their gradients of electrochemical potential. The current amplitude indicates the number of ions passing through the channel within a given time. The duration of the mean open and closed–times can depend on the applied potential difference and on a

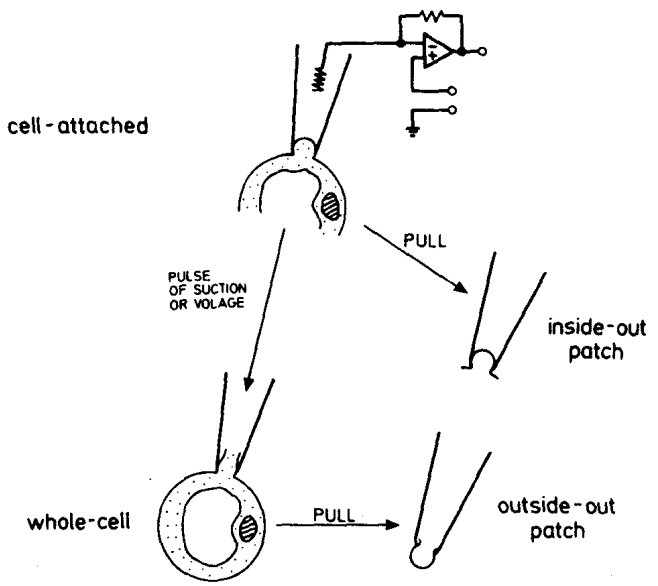

Fig. 1. – Patch–clamp configurations for animal and plant cells.

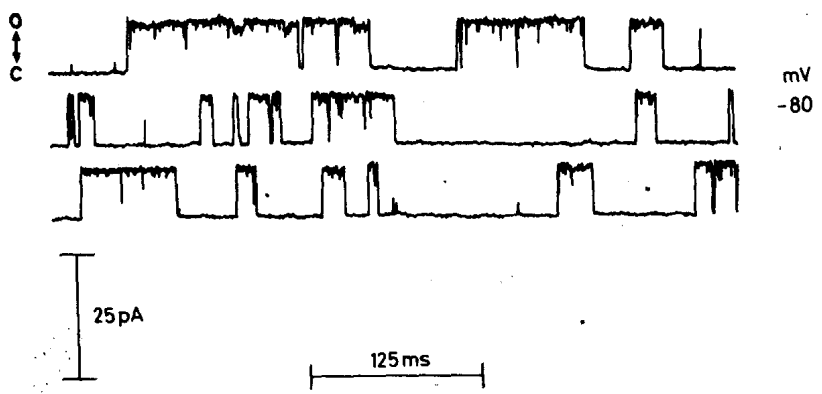

Fig. 2 – Single "SV–type" channels in the vacuolar membrane of *Chenopodium rubrum* recorded at a transmembrane potential of –80 mV inside the vacuole.

variety of chemical interactions with the channel protein (*e.g.* Ca^{2+}, pH, second messengers, phosporylation). A statistical analysis of open and closed time intervals gives insights into the molecular dynamics of the channel protein.

The mechanical stability of the *pipette–to–membrane seal* allows a membrane patch encircled by the pipette tip to be excised (inside–out or outside–out orientation), or to be ruptured without destroying the seal to provide access to the interior of the cell or organelle (*whole–cell* or *organelle* configuration, Fig. 1). With these patch–clamp configurations, the transmembrane potential and the composition of the media on either side of the membrane are well–defined and easy to control. Nevertheless, *wash–out* of cellular factors (into the pipette) essential for ion transport or for membrane–associated processes has to be taken into account. On the other hand, *wash–in* (from the pipette interior) of essential compounds may prevent loss of activity (*rundown*) or even reactivate processes under investigation, thus leading to an understanding of ion transport regulation. The whole–cell configuration enables experiments concerning overall current flow arising from a population of channels or other transporters distributed over the entire membrane surface. Using the whole–cell configuration, it is possible to study the properties of pumps or carriers, even though the current arising from a single pump protein is too small to be detected. Specific stimuli (such as pulses of substrate, light or hormones) can elicit activation of these *low–turnover* transporters in the entire cell, resulting in a summed whole–cell current that can be readily measured and studied. Whereas patch–clamp recordings of pump currents from several animal and plant systems have already been obtained, direct measurements of ion currents produced by carriers are still restricted to a few cases in animal systems. Ionic currents through single channels in animal and plant cells measured with the patch–clamp technique ([1], Figs. 1 and 2) will be discussed in the following sections.

3. Channels in the plasma membrane

Patch–clamp techniques have traditionally been used to measure ionic currents through the plasma membrane. Observations of ion channels in the plasma membrane of animal and plant cells is currently increasing. Particular types of ion channels can be classified by the ion species able to permeate the open channel (*e.g.* K^+, Cl^- and Ca^{2+}).

3.1. K–channels from rat brain: Molecular structure and biological functions

Injection of mRNA corresponding to a K^+ channel from rat brain (BAUMANN *et. al.*, [2]) into *Xenopus* oocytes leads to the functional expression of a K^+ current with

delayed rectifier properties (STÜHMER *et al.*, [3]). The pharmacological characterisation of this channel reveals that it has all the properties attributed to voltage–dependent delayed rectifier channels. The single channel conductance obtained from cell–attached patches of oocytes gives a conductance of 9.3 pS in the main open state. The outward K^+–current does not inactivate in the ms scale. Since this channel is derived from cDNA, it is possibile to introduce site–directed mutations in order to study structure–function relations. This example shows how molecular genetics, in combination with patch–clamp techniques, reveals insights into the molecular processes involved in ion channel gating.

3.2. *K^+ channels in plants*

Following the initial characterization of K^+ channels in guard cells (SCHROEDER *et al.*, [4]), the presence of a variety of K^+ channels has been reported in the plasma membrane of other higher plant tissues. K^+ channels in guard cells and other motor tissues (SCHROEDER *et al.*, [5]; SCHROEDER [6]) are strongly regulated by the potential difference across membrane, allowing K^+ influx or efflux upon activation [4–8]. Outward K^+ current is carried by approximately two hundred K^+ channels in the plasma membrane of a guard cell (SCHROEDER *et al.*, [5]). The K^+ channels found in various higher plant protoplasts have properties very similar to the outward K^+ conductance in algal cells (TAZAWA *et al.*, [9]; SOKOLIK and YURIN , [10]; HEDRICH and SCHROEDER, [11]). The magnitude of K^+ fluxes through K^+ channels in guard cells can account for physiological K^+ fluxes of 0.7 fmol s^{-1} per guard cell during stomatal closing ([5], OUTLAW [12]). Properties of K^+ channels in guard cell protoplasts [6] agree with K^+ fluxes observed in guard cells embedded in their original environment of the epidermis [12]. It may be concluded that K^+ channels represent a major pathway for K^+ uptake and release in guard cells, and possibly in plant cells in general.

3.3. *Chloride channels*

Volume decrease in plant cells is mediated by a reduction of turgor through the release of potassium salts. The detection of K^+ selective channels in guard cell plasma membrane has provided evidence that turgor regulation is achieved by potassium efflux through potential–difference–dependent K^+ channels. However, the question of how accompanying anion fluxes are mediated across the plasma membrane remained largely unresolved until now. To address this question, the anionic permeability of the plasma membrane was studied with the patch–clamp technique. Experiments from KELLER, HEDRICH and RASCHKE (personal communication) demonstrate the existence of strongly potential–difference–dependent anion channels in guard cells. These channels are pre-