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03

Volume Editors Bernard Fermini · Birgit T. Priest

Ion Channels

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3
Topics in Medicinal Chemistry

Ion Channels

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Preface to the Series

Medicinal chemistry is both science and art. The science of medicinal chemistry offers mankind one of its best hopes for improving the quality of life. The art of medicinal chemistry continues to challenge its practitioners with the need for both intuition and experience to discover new drugs. Hence sharing the experience of drug discovery is uniquely beneficial to the field of medicinal chemistry.

The series *Topics in Medicinal Chemistry* is designed to help both novice and experienced medicinal chemists share insights from the drug discovery process. For the novice, the introductory chapter to each volume provides background and valuable perspective on a field of medicinal chemistry not available elsewhere. Succeeding chapters then provide examples of successful drug discovery efforts that describe the most up-to-date work from this field.

The editors have chosen topics from both important therapeutic areas and from work that advances the discipline of medicinal chemistry. For example, cancer, metabolic syndrome and Alzheimer's disease are fields in which academia and industry are heavily invested to discover new drugs because of their considerable unmet medical need. The editors have therefore prioritized covering new developments in medicinal chemistry in these fields. In addition, important advances in the discipline, such as fragment-based drug design and other aspects of new lead-seeking approaches, are also planned for early volumes in this series. Each volume thus offers a unique opportunity to capture the most up-to-date perspective in an area of medicinal chemistry.

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Dr. John Lowe
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Preface to Volume 3

The history of ion channel research is one that is rich and fascinating. It spans many different disciplines (biology, physiology, biophysics, bioelectricity, etc.), extends over more than two centuries, and today represents a mature and exciting field. Because of their prevalence and the critical role they fulfill in virtually all tissue types and organs, ion channels play a vital role in basic physiological functions, including muscle contraction, CNS signaling and hormone secretion among many. Not surprisingly, ion channels are drug targets for a number of therapeutic agents. At the other end of the spectrum, several human diseases have been linked to mutations or dysfunction of ion channels. In some cases, elucidating the specific ion channel dysfunction underlying the disease phenotype may provide a target for therapy. The recognized importance of ion channels in health and disease, combined with the potential to develop a broad range of new drugs for the treatment of ion channel-related diseases, has fueled the need to develop more suitable screening technologies accounting for their complex structure and function, and has led to a dramatic increase in the number of medicinal chemistry programs directed at ion channel targets.

Accordingly, this volume was written to introduce medicinal chemists to the field of ion channels. Its aim is to review recent advances in the field of ion channel-related diseases, and is meant to be accessible to graduate students, teachers, biologists, chemists and many other disciplines. Following an overview chapter summarizing the current state of ion channel screening technologies, five topics covering areas such as cancer, cardiac arrhythmias, cystic fibrosis, and pain have been selected, and the current state of knowledge is presented by leading experts in their field in a way that is accessible to all. Each chapter is structured to cover the biological rational for the target, the current status in the development of agents to treat the disease, and future perspective and challenges facing each therapeutic area. Hopefully, this effort will help to foster enhanced communication and collaborations between chemists and ion channel experts. Whether we are at the verge of a golden age for ion channel drug discovery remains to be determined, but what unfolds over the coming years should be of utmost interest for anyone involved in drug development.

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Contents

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Recent Advances in Ion Channel Screening Technologies

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Abstract Ion channels play a vital role in basic physiological functions such as generation of electrical activity in nerves and muscle, control of cardiac excitability, intracellular signaling, hormone secretion, cell proliferation, cell volume regulation, and many other biological processes. Because of their prevalence and the critical role they play in virtually all tissue types and organs, ion channels are also involved in a number of pathophysiological conditions. The recognized importance of ion channels in health and disease, combined with the potential to develop new drugs targeting ion channels in a broad range of diseases, has fueled the need to develop more suitable screening technologies accounting for their complex structure and function. Ion channels have been neglected as drug discovery targets because of the inability to study large number of compounds or validate large numbers of unknown or mutant ion channel genes using traditional ion channel screening technologies. Therefore, several efforts were undertaken to automate and improve the throughput of electrophysiological methods. In this chapter, we will review a number of the more standard ion channel screening technologies currently used, including: (1) radioligand binding assays, (2) fluorescent assays using membrane potential dyes, and (3) ion flux assays, and emphasize some of the advantages and shortcomings of

these different approaches. We will then discuss automated patch clamp technologies that aim to automate and dramatically increase the throughput of the standard voltage clamp method, and offer a true archetype shift in ion channel drug discovery.

Keywords Automated electrophysiology · HTS electrophysiology · Ion channels · Patch clamp · Planar patch · Screening technologies

1 Introduction

Ion channels play a vital role in basic physiological functions such as generation of electrical activity in nerves and muscle, control of cardiac excitability, intracellular signaling, hormone secretion, cell proliferation, cell volume regulation, and many other biological processes. Because of their prevalence and the critical role they play in virtually all tissue types and organs, ion channels are also involved in a number of pathophysiological conditions. Diseases involving dysfunction of ion channels in humans and animals are termed channelopathies, defined as inherited diseases caused by mutations in the genes encoding a multitude of ion channels. Channelopathies can arise in a number of different ways, ranging from complete loss of function of the channels through dominant-negative suppression, to discrete disturbances in channel function, often resulting in major health issues. They manifest themselves as prominent genetic and phenotypic heterogeneity related to the mutations affecting the different channels. Today, a multitude of human disorders have been linked to ion channel dysfunction including epilepsy, episodic ataxia, long QT syndrome, Bartter's syndrome, and cystic fibrosis (see the chapter from Dr. Van Goor et al. in this volume). A summary of a number of channelopathies associated with human disease can be found elsewhere [1].

Ion channels are also drug targets for a number of therapeutic agents developed for the treatment of various diseases (covered in this volume). Several of these agents are ion channel modulators that provide beneficial effects in the treatment of diseases, not by attenuating the effects of mutated channels, but rather by having direct effects on the channels in a more physiological context. For example, Na^+ channel blockers such as lidocaine and lamotrigine are used as local anesthetic and to treat epilepsy, respectively. Calcium channel blockers such as verapamil and nifedipine are used for the treatment of cardiac arrhythmias and hypertension, while ATP-dependent K^+ channel inhibitors such as tolbutamide and glibenclamide are effective in the treatment of type II diabetes. The recognized importance of ion channels in health and disease, combined with the potential to develop new drugs targeting ion channels in a broad range of diseases, has fueled the need to develop more suitable screening technologies accounting for their complex structure and function. In this chapter, we will review some of the recent advances in ion

channel screening technologies that offer a true paradigm shift in ion channel drug discovery.

2 Ion Channels as Drug Targets

Ion channels are broadly described in two major groups (Table 1): (1) voltage-gated channels, such as sodium, calcium, and potassium channels that respond to changes in membrane potential and are found in many different tissues including nerves and the heart; and (2) ligand-gated channels, which are regulated by various extracellular or intracellular ligands. These channels are often named according to the ligand they bind. For example, the nicotinic acetylcholine receptor (nAChR) is a prototypic ligand-gated channel. It is activated by the endogenous ligand, acetylcholine, and the drug nicotine. Stretch-activated, stretch-gated and/or mechano-sensitive channels represent another class of ion channels that open and close in response to mechanical stimuli, changes in membrane tension, or hypo-osmotic shock. Although our understanding of these channels has increased significantly over the past few years [2, 3], relatively little is known about them when compared to voltage-gated or ligand-gated channels, and they will not be covered in this section.

Table 1 Classification of the two major groups of ion channels found in the plasma membrane of mammalian cells

Ion channel	Selectivity	Activator
Voltage-gated channels		
Potassium	K^+	Membrane potential
Sodium	Na^+	Membrane potential
Calcium	Ca^{2+}	Membrane potential
Chloride	Cl^-	Membrane potential
HCN	Na^+, K^+	Membrane potential
Ligand-gated channels		
nAChR	Na^+, K^+, Ca^{2+}	Ach, nicotine
$GABA_{A,C}$	Cl^-	GABA
Glycine	Cl^-	Glycine, strychnine
$5\text{-}HT_3$	Na^+, K^+, Ca^{2+}	Serotonin
AMPA	Na^+, K^+, Ca^{2+}	Glutamate, AMPA
Kainate	Na^+, K^+, Ca^{2+}	Glutamate
NMDA	Na^+, K^+, Ca^{2+}	Glutamate, NMDA
CNG	Na^+, K^+, Ca^{2+}	cAMP
IP_3R	Ca^{2+}	IP_3
P2X, P2Z	Na^+, Ca^{2+}, Mg^{2+}	ATP

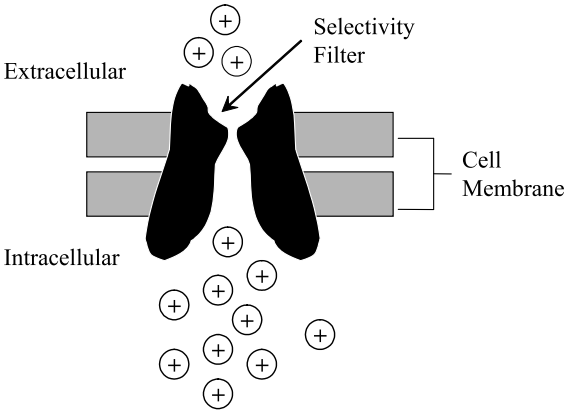

Fig. 1 Schematic of a voltage-gated ion channel

Tremendous progress has been achieved in elucidating the structure of several ion channels, and such discoveries have increased our understanding of the properties underlying their ionic selectivity, conductance, and gating [4]. Ion channels are protein complexes that span the cell membrane lipid bilayer, allowing charged ions to pass across a naturally impermeant barrier (Fig. 1). The core membrane-spanning domain of the channel forms the pore, allowing ions such as Na^+, Ca^{2+}, K^+, or Cl^- to flow through down their electrochemical gradient (Table 2). Ion channels are usually formed of an α-subunit that comprises the pore and the selectivity filter for that channel, as well as the gating apparatus that allows for channels to open and close in response to various stimuli. Often associated with α-subunits are multiple auxiliary subunits that have been shown to play a number of roles, including but not limited to modifying the function of the α-subunit, or playing a role in trafficking of the channel to the plasma membrane [5]. Most ion channels show selectivity, allowing only some inorganic ions of appropriate size and charge to pass through them. In order to do so, permeating ions need

Table 2 Typical concentrations of five major ions inside and outside mammalian cells

Ion	Ionic concentration (mM) Extracellular	Intracellular
Na^+	145	12
K^+	4	155
Ca^{2+}	1.8	0.001 (rest)–0.1 (active)
Mg^{2+}	1.5	0.8
Cl^-	123	4.2

to shed most of the water molecules associated with them in order to pass in single file through the narrowest portion of the channel, often referred to as the selectivity filter [6]. Most ion channels allow ions to move through them in either direction, but the direction of the current flow through a specific channel is dictated by the electrochemical gradient (Table 2). Moreover, channels are not continuously open, but rather undergo conformational changes from closed to open states. These properties distinguish channels from aqueous pores, transporters, and pumps. Once open, channels allow the passage of ions at rates of about 100 million ions per second. Yet some, like sodium channels, stay open for less than a millisecond at a time before closing again. Ion channels operate in virtually every cell, whether electrically excitable or not.

The diversity of ion channels is significant, especially in excitable cells of nerves and muscles. Of the more than 400 ion channel genes currently identified in the human genome [7], about 170 encode potassium channels, 38 encode calcium channels, 29 encode sodium channels, 58 encode chloride channels, and 15 encode glutamate receptors. The remaining are genes encoding other channels such as inositol triphosphate (IP_3) receptors, transient receptor potential (TRP) channels and others [8]. Currently, the potential for ion channel-directed drugs is relatively untapped. Only about 5% of marketed drugs target ion channels [9]. Given the number of genes identified in the human genome project it would appear that there remains a significant pool of unexploited targets within the ion channel field for successful discovery projects. Moreover, taking into consideration specific distribution of ion channel expression within and between tissues, heterogeneity in channel assembly and association, "splice variations" of the basic subunits, and potential state-dependent drug actions (i.e., open vs. closed), it is easy to envision the tremendous opportunities for selective modulation of ion channels and the re-emergence of drug discovery programs for the treatment of ion channel-related diseases. Yet, in spite of their historical success as therapeutic targets, and the considerable investment in this target class by the pharmaceutical industry, very few small molecule ion channel drugs have been submitted, or approved, by regulatory agencies in the past 10 years [10], and none were submitted for approval in 2007 [11]. A major constraint in developing new ion channel drugs has been the difficulty in screening ion channels in a cost-effective manner with validated functional screens that are relevant to clinical outcome at the throughput currently required by modern chemistry efforts. However, with the recent development of automated ion screening technologies, this is about to change.

The aim of this volume is to review, for the non-specialist, recent advances in the field of ion channel-related diseases. Six topics covering diseases such as cancer, cardiac arrhythmias, cystic fibrosis, and pain have been selected to reflect a number of different therapeutic areas, and each chapter is structured to cover the biological rational for the target, the current status in the development of novel agents to treat the disease, and the potential status of

clinical trials and future prospects. This first chapter provides an introduction into the world of ion channels, and covers new technologies that have allowed breakthroughs in the field of ion channel screening.

3 Historical Perspective

In order to fully appreciate the current advances in ion channel screening technologies, it is important to highlight some of the major discoveries that have directly or indirectly led to the current state in this field. The history of electrophysiology is rich and fascinating. It spans many different disciplines and fields (biology, physiology, biophysics, bioelectricity, etc.), extends over more than two centuries, and cannot be appropriately covered in this short review. Therefore, readers are referred to more comprehensive material on the subject matter [12–16].

The concept of ions and cell membranes dates back to the late 1800s. Carlo Matteucci (1811–1868) was an Italian physicist and neurophysiologist who was a pioneer in the study of bioelectricity. Using a galvanometer, an instrument that can detect and measure small amounts of currents, Matteucci was able to prove that injured excitable biological tissues generated direct electrical currents. He was the first to demonstrate that it was possible to induce muscle contraction by means of an action potential, and that action potentials were associated with depolarization of the muscle resting potential. His work in bioelectricity influenced directly the research developed by Emil du Bois-Reymond (1818–1896), a nineteenth century German physiologist, described by some historians [13] as the founder of modern electrophysiology. Using a galvanometer, du Bois-Reymond detected the flow of charges through all muscular and nervous tissue. He developed the view that a living tissue, such as muscle, might be regarded as composed of a number of "electric molecules" having certain electric properties, and that the electric behavior of the muscle as a whole in varying circumstances was the outcome of the behavior of these native electric molecules. We now know that these are sodium, potassium, and other ions that are responsible for electric membrane phenomena in excitable cells. His research established electrophysiology as a scientific discipline.

Following the discoveries by du Bois-Reymond, Sidney Ringer (1836–1910), a British clinician and pharmacologist, serendipitously discovered that Ca^{2+} was active in the heart, and performed a completely novel function: it carried the signal that initiated heart contraction. Ringer was able to show that adding small amounts of potassium chloride to a normal solution of sodium chloride allowed isolated organs to stay functional for longer periods of time. Ringer's papers published in the *Journal of Physiology* in the early 1880s are rightly acknowledged as the starting point for the development

of modern understanding of the role of calcium in the contraction of the heart [14]. In 1902, a German physiologist named Julius Bernstein (1839–1917) correctly proposed that excitable cells were surrounded by a membrane selective to K^+ ions at rest, and that during excitation the membrane became permeable to other ions. His hypothesis and research laid the foundation for understanding conduction of the nerve impulse and electrical transmission of information in the nervous system. In 1907, the British physiologist John Newton Langley (1852–1925) introduced the concept of receptor molecules on the surfaces of nerve and muscle tissue, in an attempt to explain the specific and potent actions of certain chemicals on muscle and nerve cells. Langley's theories were much debated at the time, and receptors remained theoretical until their discovery in the 1940s [15]. Then in 1937, John Zachary Young (1907–1997) was one of the first to make use of squid neurons to study ionic currents. The ease of working with large neurons made important experiments possible for the first time, including the first intracellular recordings of the nerve cell action potential, as well as the first measurements of the underlying ionic currents that produce them. His discovery and work with giant squid axons eventually led to the award of the Nobel Prize to Alan Hodgkin and Andrew Huxley (see below).

The appearance of true cellular electrophysiology followed the introduction, in 1949, of intracellular glass microelectrodes by Gilbert Ling and Ralf Gerard [17]. With this invention, it became possible to detect and measure the resting potential of a cell by impaling its membrane, without destroying the cell. Microelectrodes quickly became the technique of choice for electrophysiological recordings from all types of tissues and cells. Not long after this, using squid neurons, Alan Lloyd Hodgkin and Bernard Katz (1949) removed sodium ions from outside the neuron and were able to conclude from their data that sodium ions were responsible for the formation of the action potential [18]. The next improvement in instrumentation took place around the same period when Kenneth Cole and George Marmont described the concept of the voltage clamp method [19, 20]. This approach consisted of placing a second glass electrode inside the cell in order to stabilize or "clamp" the membrane potential of neurons for experimental purposes. Voltage clamping allowed measurements of the effect of changes in membrane potential on the conductance of this membrane to various individual ion species.

In the early 1950s, Hodgkin and Huxley characterized the time and voltage dependency of the ionic conductances that underlie an action potential in the squid giant axon, using the voltage clamp technique, and developed a mathematical model that accurately predicted the waveform of the action potential [21]. For this determining work on neuronal excitability, Hodgkin and Huxley received the Nobel Prize in Physiology or Medicine in 1963.

However, the ionic currents measured using the voltage clamp technique were the result of fluxes through an ensemble of membrane channels. Until the 1970s, it had only been possible to study ion channels as macroscopic cur-

rents, because there was simply no experimental method that could be used to isolate and characterize individual ion channels. Then, in 1976, Erwin Neher and Bert Sakmann pressed a smooth electrode tip on the surface of an isolated skeletal muscle fiber, electrically isolating a patch of membrane and reducing extraneous electrical noise so low that picoampere currents flowing through a single ion channel could be measured directly [22]. The patch clamp technique was born, and it quickly became the backbone of modern electrophysiology. It has since been referred to as the single most important development in ion channel research in the last half of the twentieth century. In 1991, Neher and Sackmann were rewarded the Nobel Prize for Physiology or Medicine for the development of the patch clamp technique.

However, this technique still had limitations because of the relatively low seal resistance (MΩ) between the recording pipette and the cell membrane. But all of this changed in 1980 with the discovery of the high resistance seal (GΩ) between the micropipette tip and the cell membrane [23]. This discovery turned out to be one of the most important revolutions in the world of electrophysiology. The incredible stability and tightness of the gigaseal's interaction between the pipette and the cell membrane allowed a complete isolation of a patch of cell membrane and an entirely new type of electrophysiological experiment (Fig. 2, part A). The gigaseal formed also allowed for three different configurations of the technique to be used:

1. Cell-attached patch, where the electrode remains sealed to the patch of membrane, allowing for the recording of currents through single ion channels in that patch of membrane
2. Inside-out patch, where the electrode is quickly withdrawn from the cell when the gigaseal is formed, thus ripping the patch of membrane off the

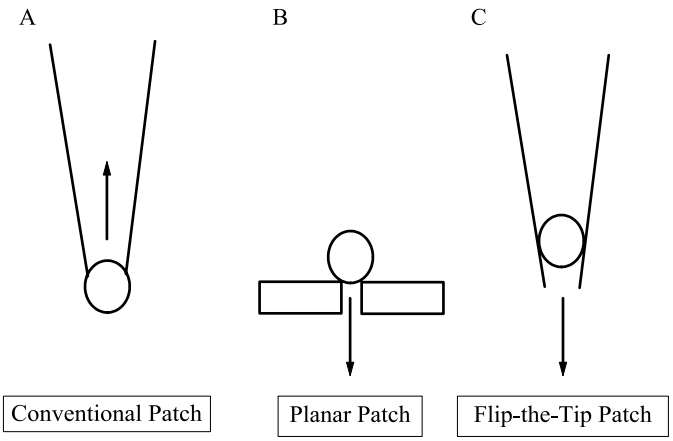

Fig. 2 Configuration of the different patch clamp methods. The *arrows* indicate the direction of suction

cell, leaving the patch of membrane attached to the electrode, exposing the intracellular surface of the membrane to the external media

3. Whole-cell recording or whole-cell patch, where the electrode is left in place, but more suction is applied to rupture the portion of the cell's membrane that is inside the electrode, thus allowing access to the intracellular space of the cell, and allowing the long-sought full control of the ionic driving forces, even with very small cells [24].

The patch clamp technique suddenly became the workhorse of modern electrophysiology, and the whole-cell configuration became one of the most crucial and popular techniques for the biophysical and pharmacological study of ion channels.

Breakthroughs in other scientific areas allowed further development of the ion channel field. For example, even though there would be no known sequence of an ion channel until the 1980s, Clay Armstrong in 1973 proposed that the structure of sodium channels in the squid neuron allows for opening and closing of their pores by a "ball and chain" model. He showed that there was a portion of the channel that could be cleaved off by a protease enzyme injected directly into the squid giant axon, acting as a kind of "ball", which was present just below the channel and could come up and plug the pore when the voltage on the cell membrane changed. When the ball was not present, the channel remained in the open configuration at all times [25].

The next major revolution in ion channel research occurred because of the advent of recombinant DNA technology in the mid to late 1970s. This approach provided the means for obtaining sequence information about genes and therefore the proteins they coded for, as well as the production of large amounts of the protein in easy-to-grow organisms such as the bacteria *E. coli*. With the amino acid sequences known, it was possible to begin predictions of what ion channels should look like in three-dimensional space. Another important advantage of being able to work with the gene of an ion channel was that the sequence could be changed deliberately (site-directed mutagenesis) in order to understand how it worked. Cloning of the first ion channel (nicotinic acetylcholine receptor, nAChR) occurred in 1982 [26], and the first channel to be sequenced was the Na^+ channel from the electric organ of the electric eel *Electrophorus electroplax* [27], followed by the Ca^{2+} channel from rabbit skeletal muscle [28]. Soon thereafter, the first K^+ channel was characterized from the *Shaker* behavioral mutant in *Drosophila* [29–32]. Introduction of recombinant expression of ion channels provided a well-defined, replenishable source of cells expressing a variety of human ion channels, opening the way to a number of biochemical assays to study ion channel function.

The first high resolution crystal structure of an ion channel (3.2 Å resolution), the potassium channel *KscA* from a bacteria, was provided by