Topic Introduction

Patch-Clamp Recording of Voltage-Sensitive Ca^{2+} Channels

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In this article, we focus on a refinement of the traditional voltage-clamp methods that are used to measure current from whole cells, or relatively large areas of membrane, called the patch-clamp technique. Although this technique has extended the application of voltage-clamp methods to the recording of ionic currents flowing through single channels, in its whole-cell configuration it has become the most widely used method for recording ionic currents. We give particular attention to the study of voltage-gated (Ca_v) Ca^{2+} channels using the patch-clamp technique and discuss some aspects of the molecular physiology of these proteins.

INTRODUCTION

Much of what we know about the properties of ion channels in cell membranes has come from experiments using the voltage clamp, an experimental method that allows electrophysiologists to hold the voltage of the cell membrane at any preset potential and to measure the currents that flow through the membrane at that potential as a function of time. The first direct recordings of single ion channel currents in biological membranes were made by Neher and Sakmann using an innovative modification of the voltage-clamp method now called the patch-clamp technique (Neher and Sakmann 1976).

THE PATCH-CLAMP TECHNIQUE

Rather than penetrating the cell with sharp electrodes as is traditionally performed in voltage-clamp experiments, in the patch-clamp technique, blunt-tipped glass pipettes are used in such a way that, when pressed gently against the membrane of a cell, they isolate a small area of membrane. In this way, it is possible to trap or isolate one or a few ion channels in the membrane. The tip of the micropipette is heated to produce a smooth surface that helps in forming a high-resistance seal ($>1 \text{ G}\Omega$) with the cell membrane (Fig. 1A). Although the interior of the micropipette is filled with a solution matching the ionic composition of the bath solution, as in the case of cell-attached recordings, or the cytoplasm for whole-cell recordings, the composition of the recording solutions can be changed or drugs can be added to study the ion channels under different experimental conditions. The resistance of the gigohm seal (or gigaseal) and the use of low-noise devices allows the currents to be electronically isolated and measured across the membrane patch with little competing noise (Hamill et al. 1981). A

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FIGURE 1. Experimental setup for conducting the patch-clamp technique. (A) Sample cell immersed in external recording solution within a recording chamber attached via a glass micropipette that forms a gigaseal with the plasma membrane. The micropipette contains an internal recording solution connecting the cell interior to the feedback resistor and recording apparatus via a silver electrode. Current flow measured during a patch-clamp experiment is equal and opposite to the micropipette current. (B) Different configurations of the conventional patch-clamp technique allowing recordings to be made on excised patches: the inside-out mode (*top-right*), made by pulling the membrane patch off the cell into the bath solution, and the outside-out mode (*lower-right*), made by applying suction (*lower-left*) to destroy the membrane isolated by the patch pipette and then pulling the pipette away from the cell.

silver wire coated with silver chloride is placed in contact with the solution in the micropipette and conducts electric current to the amplifier.

Several variants of the basic patch-clamp technique can be applied according to the needs of the research plan (Hamill et al. 1981). Many studies have used patches in the cell-attached mode, but the resting potential of the cell is not known and neither the intracellular nor the extracellular ion concentrations can be changed easily. For these reasons, it is sometimes essential to work using a cell-free mode, with patches excised from the main body of the cell (Fig. 1B). There are two kinds of

recordings on excised patches: (i) the inside-out mode, made by pulling the membrane patch off the cell into the bath solution and (ii) the outside-out mode, made by applying suction to destroy the membrane isolated by the patch pipette and then pulling the pipette away from the cell. In this case, the membrane should reseal to give a patch of membrane whose intracellular face is in contact with the micropipette solution.

Finally, the whole-cell recording is achieved by destroying the membrane patch using suction so that the cell, whose interior then comes into contact with the solution in the micropipette, can be voltage- or current-clamped (Marty and Neher 1995). The cell contents equilibrate over time with the solution within the micropipette. In the perforated patch variant of the whole-cell recording, the experimenter forms a gigaseal but does not destroy the patch membrane. Instead, the electrode solution contains an antifungal or antibiotic agent (nystatin or gramicidin) that diffuses and forms small perforations in the membrane patch, providing electrical access to the cell interior (Fig. 1B). The whole-cell patch clamp has now largely replaced high-resistance microelectrode recording techniques to record currents across the entire cell membrane.

The main electronic components of a traditional patch-clamp setup include an operational amplifier, a personal computer, an analog-to-digital converter interface, a micropipette manipulator, a microscope, either upright or inverted, in most cases equipped for phase-contrast and fluorescence, a perfusion system, a vibration isolation table, and a Faraday cage. The single most important electronic component of a patch-clamp amplifier is the current-to-voltage converter, which is contained in the headstage and has a high gain, owing to the large feedback resistor. It can be arranged so that the potential inside the micropipette can either be held at a steady level or changed in a stepwise fashion.

In principle, current flow through the electrode across a resistor of high impedance causes a voltage drop that is proportional to the measured micropipette current. An operational amplifier is used to automatically adjust the voltage source to maintain a constant micropipette potential at the desired reference potential. As the response of the amplifier is fast, it can be assumed that, for all practical purposes, the micropipette potential is proportional to the reference potential. When current flows across the membrane through ion channels, the micropipette potential is instantaneously displaced from the reference potential. The amplifier alters the voltage source to generate a micropipette current that will exactly oppose the displacement of the micropipette potential from the reference potential. Thus, the current flow measured during a patch-clamp experiment is equal and opposite to the micropipette current. As discussed below, Ca^{2+} currents in many cells can be characterized in patch-clamp experiments by the mode of activation and the unitary conductance of the channels, as well as by selective blockade by drugs, ions, or toxins.

FUNCTIONAL DIVERSITY OF Ca_v CHANNELS

Ca_v channels are a family of transmembrane proteins widely distributed in excitable cells and also found at low levels in many nonexcitable cells. These channels open when the plasma membrane becomes depolarized and mediate Ca^{2+} entry in response to action potentials and subthreshold depolarizing signals. Ca^{2+} entering the cell through Ca_v channels serves as the second messenger of electrical signaling, initiating a variety of cellular events, including neurotransmitter release, muscle contraction and gene expression, among many others (Catterall 2011).

Soon after the first recordings of Ca^{2+} currents, it was apparent that there were multiple types of Ca^{2+} currents which have been defined subsequently by physiological and pharmacological criteria. In cardiac, smooth, and skeletal muscle, the major Ca^{2+} currents are distinguished by high voltage of activation, large single-channel conductance, slow voltage-dependent inactivation, and specific inhibition by antagonist drugs including dihydropyridines (Tsien et al. 1988). As these currents show slow voltage-dependent inactivation and therefore are long lasting, they have been designated L-type (Table 1). These currents are also recorded in endocrine cells, where they initiate release of hormones, and in neurons where they are important in the regulation of gene expression, integration of synaptic input, and initiation of neurotransmitter release at some synapses (Tsien et al. 1988; Catterall 2011).

TABLE 1. Different types of voltage-sensitive Ca^{2+} channels

Current	Channel	Specific antagonists	Localization
L	$\text{Ca}_v1.1$ (formerly α_{1S})	Dihydropyridines	Skeletal muscle
L	$\text{Ca}_v1.2$ (formerly α_{1C})	Dihydropyridines	Cardiac muscle
L	$\text{Ca}_v1.3$ (formerly α_{1D})	Dihydropyridines	Neurons, smooth and cardiac muscle, inner ear, pancreatic islets
L	$\text{Ca}_v1.4$ (formerly α_{1F})	Dihydropyridines	Retina
P/Q	$\text{Ca}_v2.1$ (formerly α_{1A})	ω -Agatoxin IVA	Nerve terminals
N	$\text{Ca}_v2.2$ (formerly α_{1B})	ω -Conotoxin GVIA	Nerve terminals
R	$\text{Ca}_v2.3$ (formerly α_{1E})	SNX-482	Neurons
T	$\text{Ca}_v3.1$ (formerly α_{1G})	None	Neurons, heart
T	$\text{Ca}_v3.2$ (formerly α_{1H})	None	Neurons, heart
T	$\text{Ca}_v3.3$ (formerly α_{1I})	None	Neurons

Subsequent electrophysiological studies revealed Ca^{2+} currents that had properties different from those of L-type (Hagiwara et al. 1975), which were then characterized in dorsal root ganglion (DRG) neurons (Carbone and Lux 1984). These novel currents activated at more-negative membrane potentials, inactivated rapidly, and had small single-channel conductance (Tsien et al. 1988; Perez-Reyes 2003). They were designated T-type for their transient openings or low-voltage-activated (LVA) currents for their negative voltage dependence (Table 1).

Patch-clamp recordings from DRG neurons revealed an additional Ca^{2+} current, designated N-type (neither) by their intermediate voltage dependence and rate of inactivation: more negative and faster than L-type but more positive and slower than T-type (Nowycky et al. 1985; Tsien et al. 1988). This current is also distinguished by its high sensitivity to the cone snail peptide ω -conotoxin GVIA (Tsien et al. 1988; Catterall 2011). Likewise, the use of other peptide toxins revealed additional Ca^{2+} current types (Table 1). P-type currents, first recorded in Purkinje neurons (Llinás et al. 1989), are distinguished by high sensitivity to the spider toxin ω -agatoxin IVA (Mori et al. 1996). Q-type currents, first identified in cerebellar granule neurons (Randall and Tsien 1995), can be distinguished from the P-type currents by their fast inactivation kinetics and by their significantly lower affinity for ω -agatoxin IVA. Finally, a residual (R-type) Ca^{2+} current, characterized by its insensitivity to blockade by most subtype-specific organic and peptide Ca^{2+} channel blockers (Ellinor et al. 1993; Randall and Tsien 1995), can be recorded in neuronal tissues.

MOLECULAR STRUCTURE OF Ca_v CHANNELS

Ca_v channels have been grouped into low-voltage-activated (LVA or T-type) channels and high-voltage-activated (HVA) channels, a class that includes the L, N, P/Q, and R types (Table 1). The molecular bases for these physiological types were clarified after the identification of several proteins that give rise to the channels. This era started with the purification of the skeletal muscle Ca^{2+} channel complex, which consisted of four different proteins or subunits (Campbell et al. 1988).

The α_1 subunit protein is the permeation pathway of all Ca_v channels and is also responsible for voltage sensing and binding of channel-specific drugs and toxins. The sequence of the $\text{Ca}_v\alpha_1$ subunit shows repeats comprising four transmembrane domains (Fig. 2A). Each domain contains the canonical arrangement for voltage-gated ion channels—that is, six transmembrane α helices (S1–S6) surrounding a central pore (Catterall 2011). Domains are connected by linkers that are located in the intracellular milieu, as are both the amino- and carboxy-termini. The ion-selectivity locus in the HVA channels comprises four glutamate residues (or two glutamates and two aspartates in the case of the LVA channels) within the channel pore (Varadi et al. 1999). The highly conserved S4 transmembrane α -helix acts as the primary voltage sensor of the channels (Bezannila 2008).

Biochemical studies have facilitated the identification of diverse proteins associated with Ca_v channels in different tissues. In HVA channels, three of these proteins ($\alpha_2\delta$, β , and γ) meet the criteria

FIGURE 2. Molecular structure and gating mode transitions of voltage-sensitive Ca^{2+} channels. (A) Putative structure of the ion-conducting $\text{Ca}_v\alpha_1$ subunits. The sites for binding of β auxiliary subunits and the Ca^{2+} -binding signaling protein calmodulin are the α interaction domain (AID) and the IQ region, respectively. P indicates the pore region. (B) Subunit structure of high-voltage-activated (HVA) Ca^{2+} channels, showing the arrangement of the primary α_1 channel and the auxiliary $\alpha_2\delta$, β , and γ subunits. (C) Diagram of gating transitions of a Ca^{2+} channel showing the main open, closed, and inactivated states. (D) Example of whole-cell currents. The letters C, O, and I represent the closed, open, and inactivated channel states, and arrows indicate the direction of the gating transitions.

to be considered auxiliary subunits (Fig. 2B). In contrast, LVA channels require only the $\text{Ca}_v\alpha_1$ subunit to give rise to fully functional channels.

The $\text{Ca}_v\alpha_2\delta$ auxiliary subunit is a glycosylated protein formed by two peptides, α_2 and δ , linked by a single disulfide bond (Felix 1999; Klugbauer et al. 2003; Calderón-Rivera et al. 2012) (Fig. 2B). There are four known $\text{Ca}_v\alpha_2\delta$ subunits, each encoded by a unique gene and all possessing splice variants (Klugbauer et al. 1999; Dolphin 2012). Topological analysis supports a model for the protein in which α_2 is entirely extracellular and δ has a single transmembrane α -helix with a very short intracellular carboxy-terminal region (Arikkath and Campbell 2003). There is evidence indicating a role for $\text{Ca}_v\alpha_2\delta$ in trafficking and in the modulation of channel properties (Felix 1999; Arikkath and Campbell 2003; Klugbauer et al. 2003). The mechanisms for these effects are as yet incompletely defined, although the increase in current density that these subunits convey could be explained by improved targeting of channels to the membrane and significant reduction in the rate of entry of surface channels into degradative pathways (Canti et al. 2005; Bernstein and Jones 2007).

The $\text{Ca}_v\beta$ auxiliary subunit interacts within cytosolic regions of $\text{Ca}_v\alpha_1$ (Pragnell et al. 1994) (Fig. 2A). There are four subfamilies of $\text{Ca}_v\beta$ subunits, each with splice variants (Buraei and Yang 2010). Structurally, the $\text{Ca}_v\beta$ subunits have a modular structure consisting of five regions. The first (amino-terminal), third and fifth (carboxy-terminal) regions are variable, whereas the second and fourth regions are highly conserved and are homologous to the Src-homology 3 domain (SH3, which

binds to proline-rich motifs) and guanylate kinase (GK, a phosphopeptide binding motif) domain, respectively. Most research indicates that the increase in current density brought about by the $\text{Ca}_v\beta$ subunits can be attributed to effects on the biophysical properties of the channel, as well as an influence in trafficking and a protective action against proteasomal degradation (Bichet et al. 2000; Altier et al. 2011; Fang and Colecraft 2011; Waithe et al. 2011). At the functional level, $\text{Ca}_v\beta$ subunits hyperpolarize the voltage dependence of channel activation and inactivation (Walker and De Waard 1998; Buraei and Yang 2010).

The $\text{Ca}_v\gamma$ subunit family comprises eight tetraspan membrane glycoproteins with intracellular amino and carboxyl termini. The first member of the family was identified in skeletal muscle (Sharp et al. 1987; Takahashi et al. 1987), but subsequent work revealed a $\text{Ca}_v\gamma$ variant in the brain, whose lack of expression was associated with the epileptic and ataxic phenotype of the stargazer mouse (Letts et al. 1998). Although there has been controversy about the function of the $\text{Ca}_v\gamma$ subunits, inhibitory effects of skeletal muscle and neuronal $\text{Ca}_v\gamma$ subunits on channel activity have been consistently showed by electrophysiological studies (Kang and Campbell 2003). Interestingly, $\text{Ca}_v\gamma$ subunits have more-diverse biological functions beyond their regulation of Ca_v channel activity, which include the trafficking of AMPA receptors to the cell membrane (Jackson and Nicoll 2011).

GATING TRANSITIONS OF VOLTAGE-SENSITIVE CHANNELS

Typically, Ca_v channels open (or activate) within one or a few milliseconds after the membrane is depolarized from rest and close (deactivate) within a fraction of a millisecond following repolarization (Fig. 2C). Activation of Ca_v channels is steeply voltage dependent: channels open more quickly and with higher probability on larger depolarizations (Fig. 2D). However, in response to a prolonged membrane depolarization, Ca_v channels enter a nonconducting inactivated gating state (Fig. 2C). Inactivation of HVA Ca_v channels occurs by two independent mechanisms initiated by either membrane depolarization or an increase of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) (Stotz et al. 2004; Morad and Soldatov 2005). This prevents the breakdown of Ca^{2+} gradients, ensures the temporal and spatial precision of Ca^{2+} signals in response to membrane depolarization, and is an important mechanism by which the accumulation of excessive, cytotoxic levels of $[\text{Ca}^{2+}]_i$ is prevented. The closed channels are available for immediate reopening, whereas inactivated channels must recover from inactivation before being able to reopen (Fig. 2C).

In the accompanying protocols, we present a basic description of a biological preparation to enable readers to perform electrophysiological recordings of whole-cell currents through Ca_v channels in neonatal rat neurons acutely dissociated from the DRG [see **Whole-Cell Patch-Clamp Recordings of Ca^{2+} Currents from Isolated Neonatal Mouse Dorsal Root Ganglion (DRG) Neurons** (Gandini et al. 2014a)]. We also describe the use of whole-cell patch-clamp recording to study recombinant Ca_v channels heterologously expressed in a mammalian cell line lacking endogenous Ca_v channel activity [see **Whole-Cell Patch-Clamp Recording of Recombinant Voltage-Sensitive Ca^{2+} Channels Heterologously Expressed in HEK-293 Cells** (Gandini et al. 2014b)].

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