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From Action Potential-Clamp to "Onion-Peeling" Technique – Recording of Ionic Currents Under Physiological Conditions

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1. Introduction

Upon stimulation, excitable cells generate a transient change in the membrane potential called Action Potential (AP). The AP is governed by numerous ionic currents that flow in or out of the cell membrane. The goal of cellular electrophysiology is to understand the role of individual ionic currents and the interplay between currents in determining the profile and the time course of AP. A critically important question of the field is how different ionic currents behave individually and interact collectively during the AP cycle in an excitable cell. To answer this question we need to know the dynamic behavior of ionic currents during AP and how these currents work in concert to determine the cell's membrane potential at every moment.

Ionic currents are studied with voltage-clamp technique. Since the introduction of this method, intensive research has been conducted to characterize the kinetic properties of ionic currents. Various versions of the method were used to determine the charge carrier, voltage gating, ligand gating, activation, inactivation, recovery, etc. of individual ionic currents. One variation of the voltage-clamp is the Action Potential-clamp (AP-clamp) which can record the ionic currents during the AP cycle. In this chapter we will review the principles and variations of the AP-clamp technique and discuss the advantages and limitations of the technique. We will discuss and demonstrate how AP-clamp can help us to understand the ionic mechanisms underlying AP by using the experimental data obtained from cardiac cells where these techniques are extensively used to study the fundamental role of the ionic currents and AP dynamics in governing the cardiac function and heart diseases.

2. The principles of the AP-clamp technique

In order to determine the profile of ionic currents during AP, extensive efforts have been made in two different approaches: one is to use mathematical models based on the traditional voltage-clamp data to simulate the current profile during AP; another is to experimentally measure the ionic currents under AP-clamp.

The modeling approach uses the method pioneered by Hodgkin-Huxley (Hodgkin & Huxley, 1952) to describe the currents using ordinary differential equations and parameters derived from the voltage-clamp data. The standard voltage-clamp experiments were designed to isolate each individual ionic current, and to investigate its kinetic properties including activation, inactivation, recovery etc. Later, more detailed single channel kinetic state models (Mahajan et al., 2008) and molecular structure models (Silva et al., 2009) were also used to describe some channels. However, standard voltage-clamp experiments often used non-physiological conditions. The ion concentrations in the internal and external solutions were usually different from the physiological ionic milieu in order to isolate a particular current. Rectangular voltage pulses, instead of the AP waveform, were used to characterize the biophysical properties of the channel/transporter. In most cases, the intracellular Ca^{2+} was buffered with chelants and the currents were measured without the Ca^{2+} transients. These artificial conditions can bring inaccuracies into the data and cause the resultant models to deviate from the physiological reality, as evidenced by the differences between model simulations and experimental measurements. (Decker et al., 2009; Mahajan et al., 2008)

The experimental approach uses the AP-clamp technique to directly record the current profile during AP. The AP-clamp technique is quite simple in principle, although can be challenging in practice. (The technical aspects are discussed below.) The experimental protocol mainly involves the following steps (Figure-1) with some variations. (1) Record the steady state AP of the cell under current-clamp mode (I=0). (2) Apply this AP waveform as the voltage command onto the same cell under voltage-clamp. After reaching steady state, the net current output, I_{Ref} should be zero. (3) Isolate the current of interest by using its specific blocker to remove it from the net current output, seen as a compensation current from the amplifier, I_{Comp}. (4) The current of interest is then obtained as the difference current: $I_{Diff} = I_{Ref} - I_{Comp}$. What happens behind the scene is that the cell's AP, generated by all the membrane currents working in concert, is recorded under current-clamp with I=0. When this AP is applied as the command voltage onto the same cell under voltage-clamp, the net current output (seen as I_{Ref}) should be zero (Doerr et al., 1989; M.E. Starzak & R. J. Starzak, 1978), and the amplifier does not need to inject any compensation current to maintain the AP as long as the cell condition remains stable. When a particular current is blocked by its specific inhibitor, then the amplifier has to inject a compensation current (seen as I_{Comp}) in place of the blocked current in order to maintain the AP under voltage-clamp. This compensation current is the mirror image (negative) of the particular current that had been blocked. Hence, by subtracting I_{Comp} from I_{Ref} we can obtain the current that was originally flowing during the AP prior to the specific inhibitor application.

A fundamental difference between the AP-clamp technique and the conventional voltage-clamp technique is in the way of separating the current of interest from the other currents. In conventional voltage-clamp experiments, the current of interest is recorded under conditions that suppress all other currents by using custom-made voltage protocols, simplified ionic solutions, and sometimes blocking other 'contaminating' currents. In contrast, the AP-clamp experiments take an opposite approach to record the 'absence of the current' by blocking the current of interest using its specific inhibitor while allowing all other currents to flow during AP. By subtracting the net current output before and after the blocker application, all other currents (unaltered by the blocker) are cancelled out, and the current of interest is thereby obtained. The major advantage of the AP-clamp technique is

that it enables us to record the ionic currents under *in situ* conditions (i.e. during AP, with Ca^{2+} cycling, in a physiological milieu, and undergoing contraction).

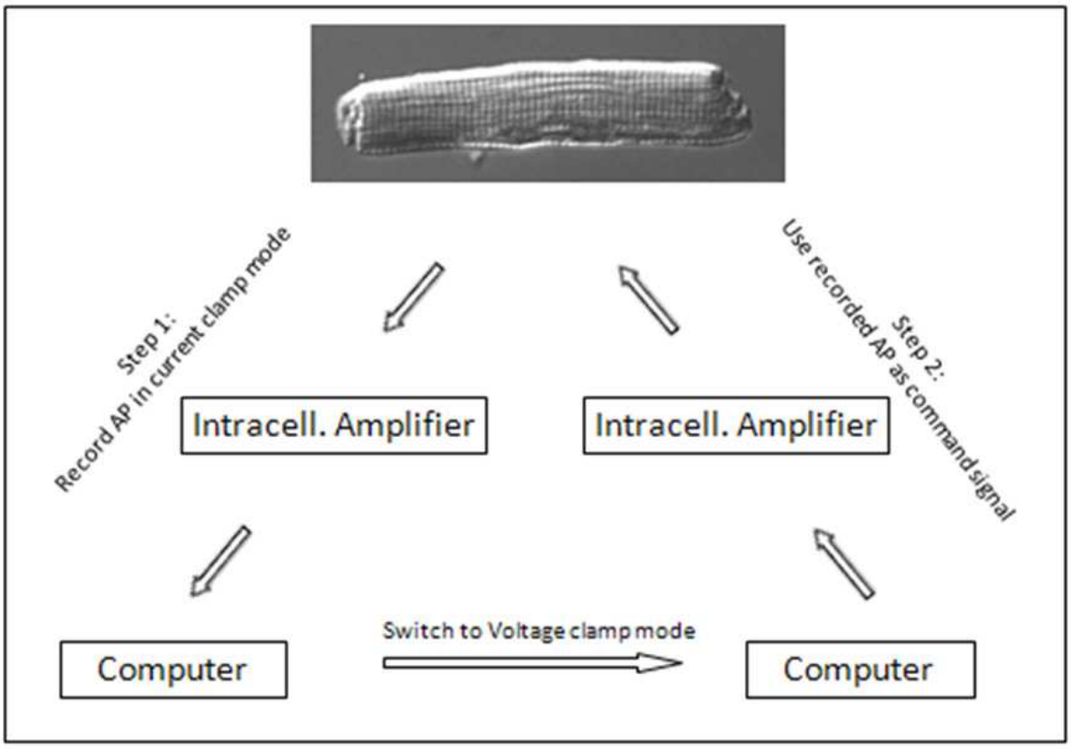


Fig. 1. The Flow chart of the Action Potential-clamp technique.

3. A short historical review

Attempts to determine the transmembrane currents during AP-clamp can be dated back to 1970s. One approach used in those pioneering experiments was to switch the amplifier from current-clamp mode to voltage-clamp mode and record the instantaneous current at different phase of AP. The cell was stimulated by a brief current pulse and the action potential was allowed to develop in a free running mode until the amplifier was switched to voltage-clamp mode and the voltage was frozen to the value at that moment for measuring the instantaneous current. (Bezanilla et al., 1970)

At the beginning, the idea to record the membrane current using a pre-recorded AP as the voltage command was used for various applications. Bastian & Nakajima studied the T-tubule function in skeletal muscle fiber with double sucrose gap method using pre-recorded AP as the voltage command. (Bastian & Nakajima, 1974; Nakajima & Bastian, 1974). In other experiments AP-clamp was used simply to test the effectiveness of the space clamp in axon (M.E. Starzak & R. J. Starzak, 1978; M.E. Starzak & Needle, 1983). Though the goals of those early studies were very different from the later studies in which AP-clamp was used to study the contributions of the membrane currents to shaping the AP, we can identify the essential elements of the AP-clamp technique in those experiments. The AP was recorded under current-clamp mode and then stored and used as the command voltage waveform in voltage-clamp mode. Most importantly the concept of 'zero current' is already established in

those publications: "To produce the most accurate reproduction of this action potential, the voltage clamp currents must include no contributions due to ineffective space clamp" (M.E. Starzak & R. J. Starzak, 1978).

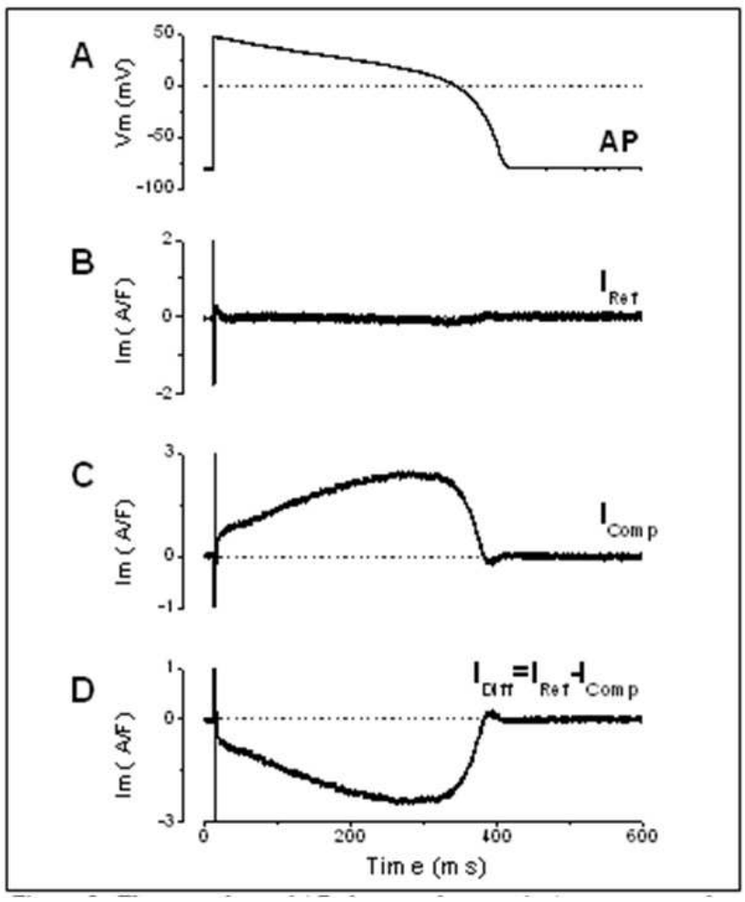


Fig. 2. The procedure of AP-clamp technique. **A**: Action potential is recorded in current clamp mode. **B**: Zero current or Reference current (I_{ref}) is recorded in voltage clamp mode. **C**: Using a specific channel inhibitor compensation current (I_{comp}) is recorded. **D**: The inhibitor sensitive current is determined as the difference current

Another technique to measure the membrane conductance during AP (without using pre-recorded AP) utilizes two electrodes (Fischmeister et al., 1984; Mazzanti & DeFelice, 1987). The first electrode had access to the cell's interior for recording the membrane potential under current-clamp mode; the second electrode was sealed to a patch of membrane in cell-attached configuration and clamped to the desired voltage (under voltage-clamp mode. The fundamental difference between this technique and the AP-clamp method are twofold. First, the two electrode technique recording of the current is limited only to the membrane patch inside the second pipette; hence only the current of limited number of channels and sometimes only single channel recording were performed. Second, the AP was not controlled with this method; hence variability of the APs would result in variability of the currents and sometimes this method was used to record membrane currents during spontaneous AP (Jackson et al., 1982).

The breakthrough took place during 1990s following three important publications. Trautwein and his colleagues used digitized AP from spontaneously beating rabbit sinoatrial node cell (Doerr et al., 1989) to stimulate the guinea pig ventricular myocyte (Doerr et al., 1990) and to record membrane currents during AP. In both cases, specific blockers (D-600, Ni^{2+}) and current subtraction were used to dissect the ionic currents during AP. These two papers were the first to describe the profile of individual ionic currents (L and T-type calcium currents) directly recorded during AP. By using specific blockers and current subtraction, they established the basic principles of the AP-clamp method. The third paper published AP clamp data obtained in nerve fiber also using the specific blocker and the current subtraction method to visualize sodium and potassium currents during AP (de Haas & Vogel, 1989). AP-clamp technique became a popular tool during 1990s and was used for mapping the key membrane currents that shape AP in several cell types including cardiac myocytes (Bouchard et al., 1995), neurocytes (Barra, 1996), as well as plant cells (Thiel, 1994). Combined with epifluorescent Ca^{2+} measurement, AP-clamp technique became a powerful tool for studying the Ca^{2+} dynamics in cardiac myocytes (Arreola et al., 1991; Grantham & Cannell, 1996; Puglisi et al., 1999).

During 2000s, a unique variation of the AP-clamp, called Dynamic Clamp technique, was developed in which an isolated cell (or a mathematical model of the cell) is coupled electrically to another cell. The first cell or the model provides the AP that is used as the voltage command onto the second cell (Bereczki et al., 2005; Wilders, 2006). The greatest advantage of the Dynamic Clamp is that the AP obtained from the first cell or model can be manipulated by changing the conditions (ionic milieu, stimulation parameters…etc.) or model parameters. This allows the experimenter to study the effects of changing AP on the dynamics of the currents.

A further extension of AP clamp technique is the sequential dissection of membrane currents or the "Onion-Peeling" (O-P) method. Previously, traditional voltage-clamp and AP-clamp technique was used to record only one current in any one cell. The O-P method uses a series of channel blockers to sequentially dissect out the currents in a single cell under AP-clamp. (Banyasz et al., 2011; Banyasz et al., 2012) The ability to measure many currents in a single cell enables study of the Individual Cell Electrophysiology of excitable cells.

4. Variations of the AP-clamp technique

Since the AP-clamp technique was introduced, several variations of the technique have been implemented. These variants use different modifications to circumvent technical limitations and provide new information on the properties of ionic currents. It is not our intension to discuss all possible modifications; here we list the characteristic features of several most frequently used variants.

4.1 Using 'standardized' or 'typical' AP

Individual cells display distinctive APs with some degree of cell-to-cell variations. To eliminate the individual variations of AP, a "characteristic or standardized" AP can be used instead of the cell's own AP as the command voltage in AP-clamp (Arreola et al., 1991). This AP could be obtained from a 'typical' cell, tissue or generated by a mathematical model. The

consequence of using such standardized AP instead of the cell's own AP is that the reference current is no longer flat ($I_{Ref} \neq 0$). Development of any kind of deflection from the zero level or appearance of a hump on the reference (zero) current may be an indicator of rundown or some other type of instability. This advantage, i.e. the possibility of monitoring the stability of our preparations is lost when a foreign AP is used as a voltage command. The pharmacological subtraction is also a crucial and necessary step here; the difference current gives the drug-sensitive current.

4.2 Using 'modified' or 'reconstructed' AP

Modified or reconstructed AP was used in some AP-clamp experiments for various reasons. Some were designed to tease out certain properties of the currents; some were to circumvent technical difficulties. For example, such a situation is generated when we need to record a delayed potassium current with small amplitude in the range of 0.1-0.5 nA. This current can be recorded with good resolution if the amplifier is set to ±1-2 nA input range. Nevertheless, this setting cannot reliably hold the voltage clamp during the upstroke of the AP where the voltage dependent Na^+ channels generate a large current with 100-150 nA peak amplitude. We have two equally poor options here. If we keep the amplifier Gain high to maintain the high resolution, the voltage clamp at the beginning of the AP would be lost. If we lower the Gain, the fidelity of the voltage clamp would be kept but the resolution of the current recording would be poor. To prevent loosing the voltage clamp, we can modify the AP by adding a short depolarizing step (i.e. 10 ms, to -30 mV) prior to the upstroke of the AP. (Varro et al., 2000) This depolarizing step can inactivate the voltage dependent Na^+ channels to allow the amplifier to hold the voltage clamp, and thereby circumvent this technical problem. Modification of other parameters of the AP (duration, plateau height, diastolic interval etc.) can also be used in AP-clamp experiments to study the ionic mechanisms that shape the AP. (Rocchetti et al., 2001)

4.3 Dynamic Clamp

A special variant of the AP-clamp method is the Dynamic Clamp (Bereczki et al., 2005; Wilders, 2006). In this case the AP voltage command comes from a current-clamped cell (Cell -1) or, alternatively, from a mathematical model. This AP waveform is used as the command voltage to voltage-clamp another cell (Cell-2). The current recorded from the Cell-2 is then fed back to the Cell-1 or to the mathematical model so it can modify the morphology of the AP accordingly. In this configuration, the two systems are in dynamic connection and in real time coupling.

Originally developed to study interactions between neural cells, the Dynamic Clamp technique provides a powerful tool for studying the dynamic interaction of currents and AP. For example, Weiss et al (Mahajan et al., 2008) used Dynamic Clamp method to investigate the Ca^{2+} modulation of ionic currents during AP in the cardiac myocyte. They first eliminated the intracellular Ca^{2+} cycling (by depleting the SR load), and then used mathematical model ('Cell-1') to generate the Ca^{2+} transient and feed the data into an AP-clamped cell (Cell-2) to record the L-type Ca^{2+} current. The data provide valuable information on how the L-type Ca^{2+} channel are modulated by the Ca^{2+} transient during AP cycle.

4.4 The "Onion-Peeling" (O-P) technique

Recently, a new version of the AP-clamp method was developed, called "Onion-Peeling" (O-P) technique, by making two significant modifications (Banyasz et al., 2011; Banyasz et al., 2012): (1) a triad of conditions is used to directly record ionic currents during AP, with Ca^{2+} cycling, in a physiological milieu; (2) multiple ionic currents are recorded from the same cell by sequentially applying the channel blockers one-by-one to dissect out each of the ionic currents. Figuer-2 shows the recording of four different currents (I_{Ks}: chromanol, I_{Kr}: E4031, I_{K1}: Ba^{2+}, and I_{NISO}: nisoldipine sensitive current) in a single guinea pig ventricular myocyte using the O-P technique. All currents are recorded after reaching steady state. To directly record the ionic currents during AP with Ca^{2+} cycling in a physiological milieu allows us to construct accurate and realistic models. The unprecedented ability to measure multiple currents in the same cell enables us to study the Individual Cell Electrophysiology (see below).

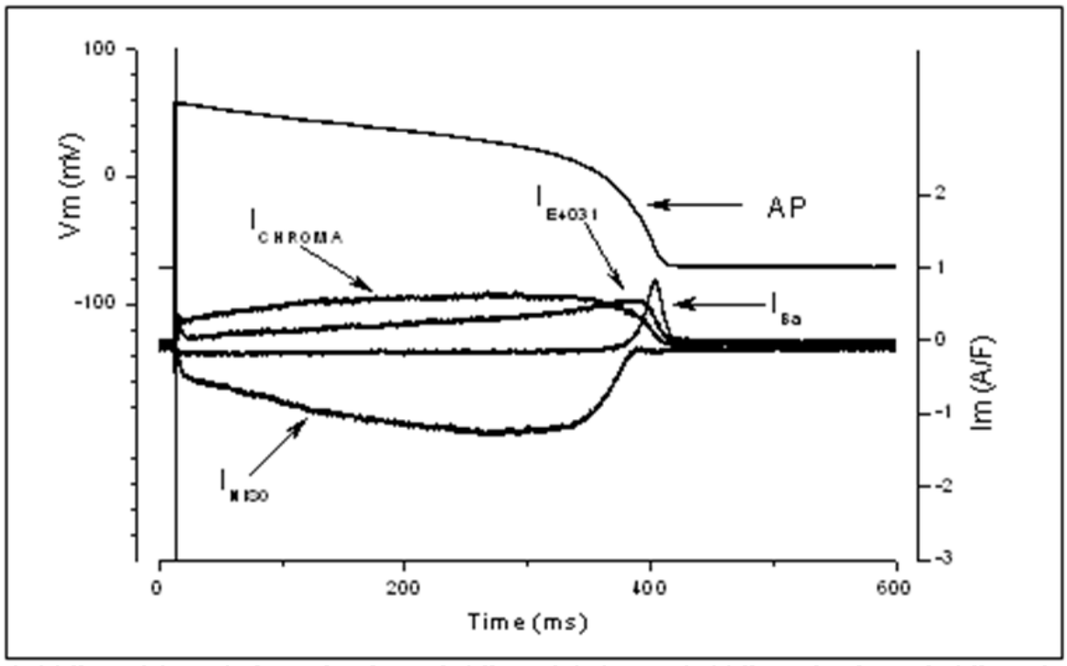


Fig. 3. Onion Peeling recording of multiple currents in guinea pig ventricular myocytate. AP: Action Potential.
I_{CHROMA}, I_{Ba}, I_{NISO}, I_{E4031} detones Chromanol-293B, Ba^{2+}, Nisoldipine and E4031 sensitive currents respectively.

5. Applications of the AP-clamp and Onion-Peeling (O-P) methods

5.1 Study of the Individual Cell Electrophysiology (ICE)

The cell's AP is a finely choreographed dance involving many ion channels and transporters interacting with each other via the membrane potential and intracellular Ca^{2+}. How do we determine the role of individual ion channel/transporter in shaping the AP and which channels/transporters are altered by disease, stress, drug etc.? Traditional voltage-clamp studies record one current from any one cell, and different currents from different cells. The

data averaged from many cells are then used to construct a canonical AP model. However, the averaged canonical AP model may not reflect the behavior of single cells due to cell-to-cell variability. (Marder & Taylor, 2011) It has been known that discrepancies exist between the model simulations and the AP-clamp measured current (Banyasz et al., 2011), and model simulations still fail to reproduce some important AP dynamic behaviors (i.e. EAD, adaptation, restitution) (Decker et al., 2009; Faber et al., 2007; Mahajanaw et al., 2008; Pasek et al., 2008).

The significant contribution of the O-P technique is that it provides a new and different type of data from all previous voltage-clamp experiments; the O-P technique allows recording of *multiple* ionic currents flowing through different channels/transporters during AP in the same single cell (see Figure 3). The ability to measure multiple ionic currents in the same cell provide experimental data to directly study how inward and outward currents work concertedly to shape the AP in individual cells, and hence the Individual Cell Electrophysiology (ICE).

The cell's AP is shaped by all of the currents flowing across the cell membrane via ion channels, exchangers, transporters etc. In order to gain in-depth quantitative understanding of the ionic mechanisms that control the AP and arrhythmias, we need to study how different currents interact and integrate at the single cell level. Due to cell-to-cell variability in the protein expression and modulation, the density of certain ionic currents may vary in different cells. Nevertheless if the inward and the outward currents can counterbalance one another, different combinations of the currents can generate similar APs. (Banyasz et al., 2011) Under pathological conditions, disproportional changes in some currents may upset the balance to cause long or short AP duration, early or delayed afterdepolarizations that lead to arrhythmias. Using O-P to measure multiple currents in the same cell provides necessary data for determining how the proportion of the currents is altered to upset the balance and cause arrhythmogenic APs. Furthermore, disease conditions may widen the heterogeneity among cells to cause more pronounced cell-to-cell variability. Using O-P to conduct the ICE study will be critically important for understanding the impact of cellular variability (inherent variance, transmural gradient, regional difference, disease associated variance, etc.) on the population behavior at tissue and organ levels.

5.2 Mapping the regional and transmural differences

It has long been known that the AP morphology has characteristic epicardial versus endocardial differences: a spike-and-dome AP profile was seen in the epi-, but not in the endocardial ventricular myocytes from canine and human hearts. Early studies show that different K^+ current density in different layers of the myocardium may contribute to this heterogeneity (Litovsky & Antzelevitch, 1988; Liu et al., 1993; Liu & Antzelevitch, 1995). When L-type Ca^{2+} currents ($I_{Ca,L}$) of epi- and endocardial ventricular myocytes were compared using traditional voltage-clamp technique, the voltage dependence, activation and inactivation kinetics of the currents were found to be identical. Interestingly, when the profile of $I_{Ca,L}$ was studied as the nisoldipine-sensitive current using the AP-clamp technique, marked differences were observed between epi- and endocardial cells. Although $I_{Ca,L}$ showed a sharp spike followed by a rapid decay in both endo- and epicardial cells following the upstroke of the AP, a hump developed in the $I_{Ca,L}$ record in the epi- but not in the endocardial cells. (Banyasz et al., 2003) Similar transmural differences were also

obtained in ventricular myocytes isolated from human hearts (Fulop et al., 2004). This double-peaked morphology of $I_{Ca,L}$ in epi-cardial cells and the relationship between the profile of AP and $I_{Ca,L}$ was revealed only under AP-clamp but not with traditional voltage-clamp method. The AP-clamp technique provides a direct method for studying the relationship between currents and membrane potential during AP.

5.3 Studying the complex effects of pathological conditions

Cardiac arrhythmias are a leading cause of morbidity and mortality in the developed world. Yet, pharmacological approaches to treat arrhythmias have met with only limited success, in large part due to incomplete understanding of the cellular and molecular mechanisms of arrhythmias. Pathological conditions wrought by disease, stress, drug etc. usually cause complex changes in multiple ion channels/transporters and Ca^{2+} handling molecules. The changes in proteins involved in determining electrical currents of the cardiac myocytes can cause abnormalities in the profile and the time course of APs, leading to arrhythmias. To elucidate the pathologic mechanisms behind abnormal APs one needs to understand the changes in the kinetics of ionic currents. For example, in a rabbit model of pressure & volume overload induced non-ischemic heart failure, researchers have found changes in several key channel/transporter that determine the cardiac AP and arrhythmias (Pogwizd, 1995; Pogwizd et al., 2001): I_{K1}, an important K^+ current that controls the repolarization of AP, was reduced; the Na^+/Ca^{2+} exchanger, a key regulator of the Ca^{2+} signaling and AP profile, was up-regulated; the expression of ß-adrenergic receptors, which profoundly influence the AP and the Ca^{2+} signaling was also altered. The reduced repolarization power (weakened I_{K1}) combined with augmented depolarizing drive (up-regulated Na^+/Ca^{2+} exchanger current), and probably more, changes at molecular level manifest at cellular level to cause delayed afterdepolarizations which can trigger premature APs. The abnormal APs at the cellular level further manifest at the tissue and organ level to cause cardiac arrhythmias. These changes in the rabbit heart failure model resemble some of the changes found in human congestive heart failure (Nattel et al., 2007).

Traditionally, the dynamic behavior of ionic currents during AP was studied using voltage-clamp experiments in conjunction with mathematical modeling. The standard voltage-clamp technique uses custom-made voltage protocols to determine the voltage dependency and the kinetic properties of each individual current. As a basic rule, only one single current can be measured from any one cell because different experimental conditions (ionic milieu, voltage protocol, inhibitors to block contaminating currents etc.) are required to record different current. For example, it is impossible to record I_{K1} and Na^+/Ca^{2+} exchanger current from the same cell using standard voltage-clamp technique. The novelty of the O-P technique is that it enables recording of multiple currents from the same single cell, by sequentially applying specific inhibitors of different currents under AP-clamp. The O-P technique gives us unprecedented ability to conduct the ICE study of the complex effects of pathological conditions on altering multiple currents and to study their collective effects on shaping APs in the single cell. The O-P recording of multiple currents enables us to study how the proportions of different currents are altered in diseased myocardium compared to healthy hearts (see the previous chapter on individual cell electrophysiology).

5.4 Characterization of drug effects

The AP-clamp technique can be used economically for studying drugs with multiple actions. As shown previously, each ionic current has its own characteristic profile during AP, called fingerprint. Knowing the fingerprint of each individual current, we can identify the suspected drug-sensitive currents (perhaps several of them) in a single AP-clamp experiment (Szabo et al., 2007; Szabo et al., 2008). Hence, the profile of the drug-sensitive current recorded under AP-clamp can give clues towards the identity of currents that might be affected by the drug. This initial screening with AP-clamp prior to the systematic pharmacological study can save significant time and resource in studying the drug effects.

Effects of receptor agonists or antagonists (hormones, receptor modulators or drugs) can also be readily studied by AP-clamp. Even in the case when agonist/antagonist affects only one population of receptors, signal transduction pathways often couple to more than one ionic current; AP-clamp is uniquely suited for studying the drug concentration-dependent effects on multiple currents. As example, the frequency-dependent effects of isoproterenol on $I_{Ca,L}$, I_{Ks}, and I_{Cl} in guinea pig ventricular cells (Rocchetti et al., 2006) and the effects of acetylcholine and adenosine on I_{K1} in ferret cardiac myocytes (Dobrzynski et al., 2002) were clarified using the AP-clamp technique.

6. Technical aspects

To successfully perform AP-clamp experiments requires high quality cells and instrumentation. In this chapter we will review the essential technical requirements for the AP-clamp technique.

6.1 Cell quality and the solutions

Robust cells with physiological resting membrane potential and stable APs are necessary requirements for using the AP-clamp technique. The experiments are performed in isolated single cells. Preparation of the cells is basically the same as in other voltage-clamp experiments. The only difference is that the composition of the bath solution should mimic the physiologic extracellular milieu during experiments. If the cell preparation requires using non-physiological media, try to minimize the time the cells spend in that media. The pH and the osmolarity of the solutions must be tightly controlled, and membrane permeable buffers (bicarbonate) seem to improve robustness of the cells. We found that the cells worked better if stored for 1-2 hours in the same extracellular medium as used in the AP-clamp experiments. Continuous perfusion of the bath solution is needed to maintain the cell quality throughout the experiments.

To work with a contracting muscle cell, the whole-cell seal usually last longer if we lift the cell up from the bottom of perfusion chamber after establishing the seal (no need to do this for other non-contracting cell types). However, sometimes cells tend to stick to the bottom of the perfusion chamber. Adding a small drop of the albumen solution into the chamber before the cells are placed can reduce the stickiness of the cells without reducing the success rate for seal formation.

The pipette solution used for the AP-clamp experiment should mimic the intracellular ionic milieu of the cell. Again, tight control of the pH and the osmolarity of the solutions

are crucial. Traditionally, 2-10 mmol/L EGTA is added to the pipette solution in voltage-clamp experiments to buffer cytosolic Ca^{2+} and eliminate cell contraction when working with muscle cells. Buffering the cytosolic Ca^{2+} can lengthen the lifetime of the seal significantly, and the previous voltage-clamp and AP-clamp experiments used Ca^{2+} buffers in most cases. However, our recent studies using the O-P technique (Banyasz et al., 2012) demonstrate that the current profiles during AP with Ca^{2+} transient (Ca^{2+} cycling preserved by not using exogenous Ca^{2+} buffer in the pipette solution) can be significantly different from the currents recorded with buffered Ca^{2+} (2-10 mM EGTA or BAPTA were used). Therefore, in order to record the ionic currents under physiological condition in situ, we suggest to minimize the use of exogenous Ca^{2+} buffer in the pipette solution and to measure the Ca^{2+} transient simultaneously with the O-P recording of the currents during AP.

Before recording the steady state AP to use as the voltage command for AP-clamp, we need to first test stability of the AP parameters. Keep in mind that the AP duration is never strictly constant, as a living system usually has some fluctuation in parameters. If the beat-to-beat fluctuation in the APs is large, the cell cannot be used for the AP-clamp experiment. We usually use the cells with no more than 4-5% fluctuation in the AP duration. At the same time, we use very stringent requirements for the voltage parameters. The cell must be discarded if any of the voltage parameters (resting membrane potential, peak amplitude, plateau height… etc.) displays more than 1 mV instability. Depending on the input impedance of the cell, ≤1 mV of instability in the voltage could translate into several hundred pA of fluctuation in the current measurements. We found it useful to stimulate the cell for 15-20 minutes at a constant pacing rate until it truly reaches a steady state, and then record the AP under current-clamp mode. Also, depending on the geometry of the cell it might take 15-20 minutes of pacing for the cell to reach equilibrium between the cytoplasm and the pipette solution after establishing the whole-cell ruptured seal configuration.

6.2 The patch pipette and the whole-cell seal configuration

The requirements for patch pipette used for the AP-clamp technique are the same as for those used in traditional voltage-clamp experiments. We use borosilicate glass pipette with tip resistance of 1.5-2.5 MΩ. The technical details of pipette fabrication follow the direction from the pipette puller manufacturer.

After compensating for the junction potential between the pipette solution and the bath solution, the pipette tip is placed on the cell membrane and a gentle suction is used to make a GΩ seal. Then the membrane patch inside the pipette is ruptured by suction to establish the whole-cell seal configuration. As a critical requirement for the AP-clamp technique, the access resistance must be kept low (< 5 MΩ) and constant throughout the entire experiment, because a change in the access resistance exerts significant impact on the magnitude and the dynamics of the compensation current. We suggest recording the access resistance at the beginning and at the end of the AP-clamp experiment and checking it several times during the experiment. It is important that the access resistance should remain close to the initial value throughout; if it changes significantly, the cell should be discarded from data collection.

The cell capacitance cancellation and the series resistance compensation are necessary for speeding up the capacitive transient and maintaining high fidelity of the actual voltage command across the cell membrane. When both are engaged, it is somewhat difficult to adjust the degrees of compensation without provoking 'ringing' (oscillations in the compensation circuitry), especially when dealing with large size cardiac myocytes (150-300 pF). Hence it is practically impossible to achieve 100% compensation. In our experience, about 80% compensation with 20 μs Lag is feasible for a typical cardiac myocyte without risking the 'ringing'. Higher percentage of compensation should be achievable for other cell types with smaller whole cell capacitance.

After breaking the membrane patch to establish the whole-cell seal, a junction potential would build up between the pipette solution and the cytosol. The magnitude of this junction potential can be as high as 10-15 mV depending on the composition of the pipette solution, and seen as an apparent depolarization of the membrane. It is essential not to compensate this junction potential to avoid imposing a voltage shift into the measurement. It can be somewhat deceptive when the reading of the resting membrane potential appears 10-15 mV higher than the known physiologic value and the temptation might be very strong to eliminate this apparent voltage shift. But doing so would destroy the AP-clamp measurements. In the design of the AP-clamp technique, this junction potential is automatically taken care of (dropped out from the equation) during the current subtraction. Nevertheless, if we want to analyze the voltage dependence of the currents, this junction potential must be corrected in the voltage values and taken into consideration during the analysis.

6.3 Instrumentation

The instruments (amplifier, A/D converter, computer etc.) used in AP-clamp experiments are basically the same as for voltage-clamp experiments. The specifications are determined by the cell type and current parameters we want to measure. However, there are some special considerations in deciding what particular instruments to use for the AP-clamp experiment.

- External stimulator: it is very useful (albeit not essential) to have an external stimulator to generate the electric pulses for evoking the AP. Although most electrophysiology software offers the option to program square pulse protocol to stimulate the cell in current-clamp mode, modification/adjustment of stimulation parameters (changing the amplitude or pacing rate) is more complicated and time consuming (if we have to overwrite protocol parameters) than simply turning the control knobs on the stimulator (analogue stimulator, for example). If we decide to use a square pulse generator to stimulate the cell it must be a DC stabile device, and the zero level should be truly zero with extremely low noise. Note that the manufacturer specification usually uses the maximum output voltage as reference to calculate the noise level, but we use no more than 5-10 V voltage range for the AP-clamp experiment, so the noise of the instrument needs to be extremely low.
- Amplifier: If we decide to use an external stimulator, the amplifier should have at least one command potential input compatible with the stimulator. It is also necessary to calibrate the amplifier frequently, because the AP-clamp experiment is more sensitive to voltage drift than traditional voltage-clamp experiments.

6.4 Channel inhibitors

The quality of AP-clamp data is determined primarily by the selectivity of the channel blocker used in pharmacological dissection of the current. In ideal situation, we have a highly specific drug which can selectively block the current under study without affecting any other currents. In such cases, using maximal concentration of the drug is suggested. Unfortunately, some drugs are known to have side effects on other channels besides their primary target. Using these non-specific drugs in the AP-clamp experiment would yield a composite current contaminated with other current(s).

Ion Channel & Transporter	Inhibitor	Dosage	Reference
$\mathbf{I_{Na}}$	TTX	1-10 μm	Chorvatova et al 2004; Yuill et al 2000
$\mathbf{I_{Na\text{-}Late}}$	Ranolazine	10 μm	Rajamani et al 2009
$\mathbf{I_{Ca\text{-}L}}$	Nifedipine	1 μm	Horiba et al 2008
$\mathbf{I_{Ca\text{-}L}}$	Nisoldipine	0.1 μm	Banyasz et al 2003
$\mathbf{I_{NCX}}$	SEA0400	3 μm	Birinyi et al 2005; Ozdemir et al 2008
$\mathbf{I_{Cl\text{-}Ca}}$	N-(p-amylcinnamoyl) anthranilic acid	5 μm	Gwanyanya et al 2010
$\mathbf{I_{to}}$	4-aminopyridine	1 mM	Banyasz et al 2007; Patel & Campbell 2005
$\mathbf{I_{kr}}$	E4031	1 μM	Banyasz et al 2007; Varro et al 2000
$\mathbf{I_{ks}}$	HMR-1556	30 nM	Gogelein et al 2000; Thomas et al 2003
$\mathbf{I_{ks}}$	Chromanol-293B	1-10 μM	Yamada et al 2008
$\mathbf{I_{k1}}$	$BaCl_2$	50 μM	Banyasz et al 2008; Banyasz et al 2007
$\mathbf{I_{K\text{-}Ca}}$	Apmin	100 pM, 1 nM	Xu et al 2003; Özgen et al 2007
$\mathbf{I_{Cl\text{-}Ca}}$	Niflumic acid	50 μM	Greenwood & Leblanc 2007; Saleh et al 2007
$I_{Cl\text{-}small}$	Chlorotoxin		Borg et al 2007
$I_{Cl\text{-}vol}$	Tamoxifen		Borg et al 2007
$I_{Cl,ligand}$	Picrotoxin		Etter et al 1999
$\mathbf{I_{Ca\text{-}T}}$	NNC 55-0396		Huang 2004
$\mathbf{I_{Ca\text{-}T}}$	R(-)efodipine		Tanaka 2004

Table 1. Specific inhibitors for the major ionic currents in cardiac cells.

How to make sure the O-P current dissected out by the specific channel blocker is pure?

First and foremost, we should use the most specific channel blocker known in literature. Several things can be done to check the specificity of drugs. (***A***) We do drug dose-response to check that the profile of current scales proportionally. (***B***) We use low dosage to dissect out a proportion of the target current and minimize non-specific effects. The full magnitude of the current can be recovered using the drug dose-response curve determined by using the traditional voltage-clamp experiments where other (contaminating) currents are eliminated. (***C)*** We scramble the sequence of drug application to make sure there is no crosstalk (Banyasz et al., 2011). If the drug is found to have non-specific effects even at very low dosage, we will search for a more specific blocker (i.e. experimental drugs, venoms, toxins, peptide inhibitors, antibody-targeted inhibitors, etc.) The drug list in Table-1 is our first pass, but can and will be modified as more specific drugs become available.

Special consideration is needed for recording Ca^{2+}-sensitive currents using the O-P technique. As a charge carrier, Ca^{2+} current influences the membrane potential. As a ubiquitous second messenger, Ca^{2+} also influences a number of Ca^{2+}-sensitive currents. The intracellular Ca^{2+} concentration is kept very low (100 nM) in cells at resting state (diastole in cardiac myocytes). Upon excitation, the Ca^{2+} inflow during AP drastically elevate the intracellular Ca^{2+} concentration which triggers many biochemical events and modulate numerous Ca^{2+}-sensitive currents. For example, to record the L-type Ca^{2+} current using the O-P technique, we use nifedipine to block the L-type Ca^{2+} channel. However, blocking the L-type Ca^{2+} current during AP also abolishes the Ca^{2+} transient, which, in turn, affects other Ca^{2+}-sensitive currents. Thus the nifedipine-sensitive current recorded using O-P technique would consist not only the L-type Ca^{2+} current but also other Ca^{2+}-sensitive currents including the Na^+/Ca^{2+} exchanger, Ca^{2+}-activated K^+ currents, Ca^{2+}-sensitive Cl^- currents and so on. One way to avoid this problem is to first record Ca^{2+}-sensitive currents while Ca^{2+} is normally cycling, and subsequently to add nifedipine to block the L-type Ca^{2+} current.

7. Conclusion

The AP-clamp methods provide powerful tools for studying the ionic currents under physiological conditions, and the complex effects of disease, stress, drug, mutation etc. on the ion channels and transporters. The O-P technique is uniquely suited for studying the Individual Cell Electrophysiology which is necessary for investigating the cell-to-cell variability. The AP-clamp and the O-P techniques can be used in muscle cells, neural cells, and any excitable cells where ionic currents and membrane potential play important roles in cell function.

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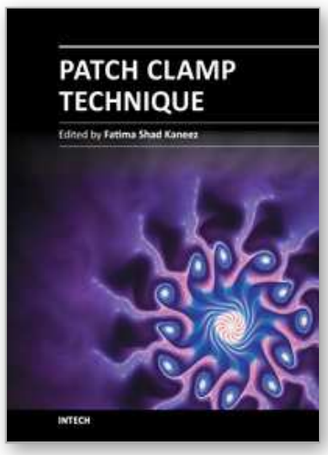
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Patch Clamp Technique
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This book is a stimulating and interesting addition to the collected works on Patch clamp technique. Patch Clamping is an electrophysiological technique, which measures the electric current generated by a living cell, due to the movement of ions through the protein channels present in the cell membrane. The technique was developed by two German scientists, Erwin Neher and Bert Sakmann, who received the Nobel Prize in 1991 in Physiology for this innovative work. Patch clamp technique is used for measuring drug effect against a series of diseases and to find out the mechanism of diseases in animals and plants. It is also most useful in finding out the structure function activities of compounds and drugs, and most leading pharmaceutical companies used this technique for their drugs before bringing them for clinical trial. This book deals with the understanding of endogenous mechanisms of cells and their receptors as well as advantages of using this technique. It covers the basic principles and preparation types and also deals with the latest developments in the traditional patch clamp technique. Some chapters in this book take the technique to a next level of modulation and novel approach. This book will be of good value for students of physiology, neuroscience, cell biology and biophysics.

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