# 2 Recording of Ionic Currents Under Physiological Conditions: Action Potential-Clamp and 'Onion-Peeling' Techniques

Ye Chen-Izu, Leighton T. Izu, Bence Hegyi, and Tamás Bányász

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Y. Chen-Izu, Ph.D. (✉)
Departments of Pharmacology, Biomedical Engineering, Internal Medicine/Cardiology, University of California, Davis, Davis, CA 95616, USA
e-mail: ychenizu@ucdavis.edu

L.T. Izu, Ph.D. • B. Hegyi, M.D., Ph.D.
Department of Pharmacology, University of California, Davis, Davis, CA 95616, USA
e-mail: ltizu@ucdavis.edu; bhegyi@ucdavis.edu

T. Bányász, M.D., Ph.D.
Departments of Pharmacology, Biomedical Engineering, Internal Medicine/Cardiology, University of California, Davis, Davis, CA 95616, USA

Department of Physiology, University of Debrecen, Debrecen 4012, Hungary
e-mail: banyasz.tamas@med.unideb.hu



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## 2.1 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 time course of AP. A critically important question of the field is how the ionic currents behave individually and interact collectively during the AP cycle of 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 the 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.

## 2.2 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 [1] 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 [2] and molecular structure models [3] were also used to describe some channels. However, standard voltage-clamp experiments often used non-physiological conditions. The ion species and concentration 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 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 [2, 4].

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 later.) The experimental protocol mainly involves the following steps (Fig. 2.1) with some variations: (1) Under current-clamp ($I = 0$) mode, record the steady state AP of the cell. (2) Apply this AP waveform as the voltage command onto the same cell under voltage-clamp. After reaching steady state, the net current output should be zero as it serves as

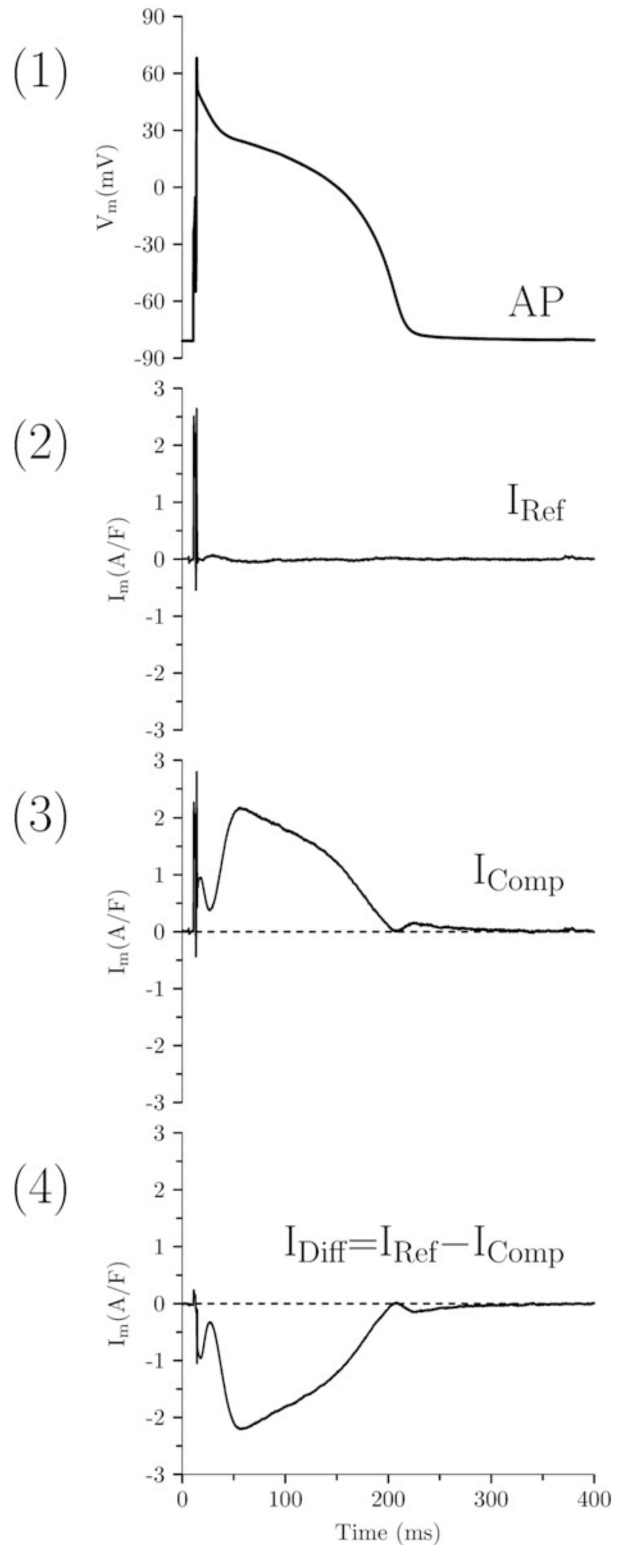


**Fig. 2.1** $^{self}$AP-clamp technique (1) record the cell's steady state AP. (2) Use this AP as the voltage-clamp command to record the total net current output, $I_{Ref}$. (3) Isolate a current by using its specific blocker to remove it from the total net current output, and record the compensation current from the amplifier, $I_{Comp}$. (4) The current of interest is obtained as the difference current: $I_{Diff} = I_{Ref} - {}_{Comp}$

the reference current, $I_{Ref}$. (3) Isolate the current of interest by using its specific blocker to remove it from the net current output, seen in the 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 [5, 6], 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 at every moment 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 blocker application.

A fundamental difference between the AP-clamp technique and the conventional voltage-clamp technique is 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).

## 2.3 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 phases of AP. The cell was stimulated by a brief current pulse and then 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 [7].

At the beginning, the idea to record the membrane current using a prerecorded AP as the voltage command was used for various applications. Bastian and Nakajima studied the T-tubule function in skeletal muscle fiber with double sucrose gap method using prerecorded AP as the voltage command [8, 9]. In other experiments, AP-clamp was used simply to test the effectiveness of the space clamp in axon [6, 10]. 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" [6].

Another technique to measure the membrane conductance during AP (without using prerecorded AP) utilizes two electrodes [11, 12]. 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 is 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 [13].

The breakthrough took place during 1990s following three important publications. Trautwein and his colleagues used digitized AP from spontaneously beating rabbit sinoatrial node cell [5] to stimulate the guinea pig ventricular myocyte [14] 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 [15]. 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 [16], neurocytes [17], as well as plant cells [18]. Combined with epifluorescent $Ca^{2+}$ measurement, AP-clamp technique became a powerful tool for studying the $Ca^{2+}$ dynamics in cardiac myocytes [19–21].

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 [22, 23]. 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' method. Previously, traditional voltage-clamp and AP-clamp technique were used to record only one current in any one cell. The Onion-Peeling method uses a series of channel blockers to sequentially dissect out the currents in a single cell under AP-clamp [24, 25]. The ability to measure many currents in a single cell enables study of the Individual Cell Electrophysiology of excitable cells.

## 2.4 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.

### 2.4.1 Using 'Typical' or 'Standardized' AP

Individual cells display distinctive APs with some degree of cell-to-cell variations. To simplify experiment, a 'typical' or 'standardized' AP can be used, instead of the cell's own AP, as the command voltage in AP-clamp [19]. 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. It is all right as long as the reference current can reach a steady state, indicating a stable seal condition is achieved. Next step, apply a specific blocker to remove the current of interest, and drug-sensitive current can be obtained by subtracting the reference current.

### 2.4.2 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 losing 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 [26]. 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 [27].

### 2.4.3 Dynamic Clamp

A special variant of the AP-clamp method is the Dynamic Clamp [22, 23]. 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. [2] 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.

A significant limitation in using the Dynamic Clamp technique to study cardiomyocytes excitation–contraction coupling is due to the fact that the ionic homeostasis (i.e., $Ca^{2+}$ and $Na^{2+}$ homeostasis) may not be maintained by injecting electric current alone. The $Ca^{2+}$ and $Na^{2+}$ homeostasis are maintained by ion fluxes through various $Ca^{2+}$ and $Na^{2+}$ channels, transporters, and Na/Ca exchanger. Hence, injecting electrons instead of ions under Dynamic Clamp might alter the ion homeostasis during AP cycle. This limitation can be overcome by the $^{self}$AP-clamp technique described later.

### 2.4.4 The $^{self}$AP-Clamp Technique

Recently, a new version of the AP-clamp method was developed, called $^{self}$AP-clamp technique [25]. The essence of this technique is to preserve the $Ca^{2+}$ cycling (and $Ca^{2+}$ homeostasis) during AP. Triad of conditions are used to achieve this: (1) the cell's own steady-state AP is used as the voltage-clamp command waveform; (2) the internal and external solutions use physiological ionic composition; and (3) the cytosolic $Ca^{2+}$ is only buffered by intrinsic buffers, without adding exogenous buffer, to preserve the intrinsic $Ca^{2+}$ homeostasis. Using these physiologically relevant conditions, the cardiomyocyte under $^{self}$AP-clamp experiences its own steady-state AP, with $Ca^{2+}$ cycling in a physiological milieu, and is contracting normally. Hence, the triad of conditions used in $^{self}$AP-clamp allows recording of the natural flow of ionic currents and also the intrinsic ion homeostasis during the AP cycle.

The purpose of $^{self}$AP-clamp is to measure the ionic current that naturally flows during the cell's own AP under physiologically relevant condition. The data can be used to tune the mathematical models of the ionic currents, which might be based on the conventional voltage-clamp data obtained under simplified conditions, to better reflect the physiological reality. The power of $^{self}$AP-clamp can be further brought out by the AP-clamp Sequential Dissection method described later.

### 2.4.5 The AP-Clamp Sequential Dissection or 'Onion-Peeling' Technique

A step forward from $^{self}$AP-clamp is the development of the AP-clamp Sequential Dissection (called "Onion-Peeling" or Onion-Peeling) technique [24, 25, 28]. This innovative technique allows, for the first time, recording of multiple ionic currents from the same cell. First, the cell is placed under $^{self}$AP-clamp; a specific blocker is used to record a particular ionic current; next, another blocker is used to dissect out its corresponding current; next, more blockers are applied sequentially to dissect out more ionic currents. Thus, 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 under $^{self}$AP-clamp. Figure 2.2 shows a recording of four different currents ($I_{Ks}$: chromanol-293B sensitive, $I_{Kr}$: E4031 sensitive, $I_{K1}$: $Ba^{2+}$ sensitive, $I_{NISO}$: nisoldipine sensitive) in a single guinea pig ventricular myocyte using the Onion-Peeling technique.

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 studying how the inward currents and the outward currents counterbalance in the same cell to shape the AP under physiological condition and give rise to arrhythmogenic activities under pathological condition. The Onion-Peeling technique enables study of the Individual Cell Electrophysiology (see later).

## 2.5 Applications of the AP-Clamp Methods

### 2.5.1 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 investigate one current from

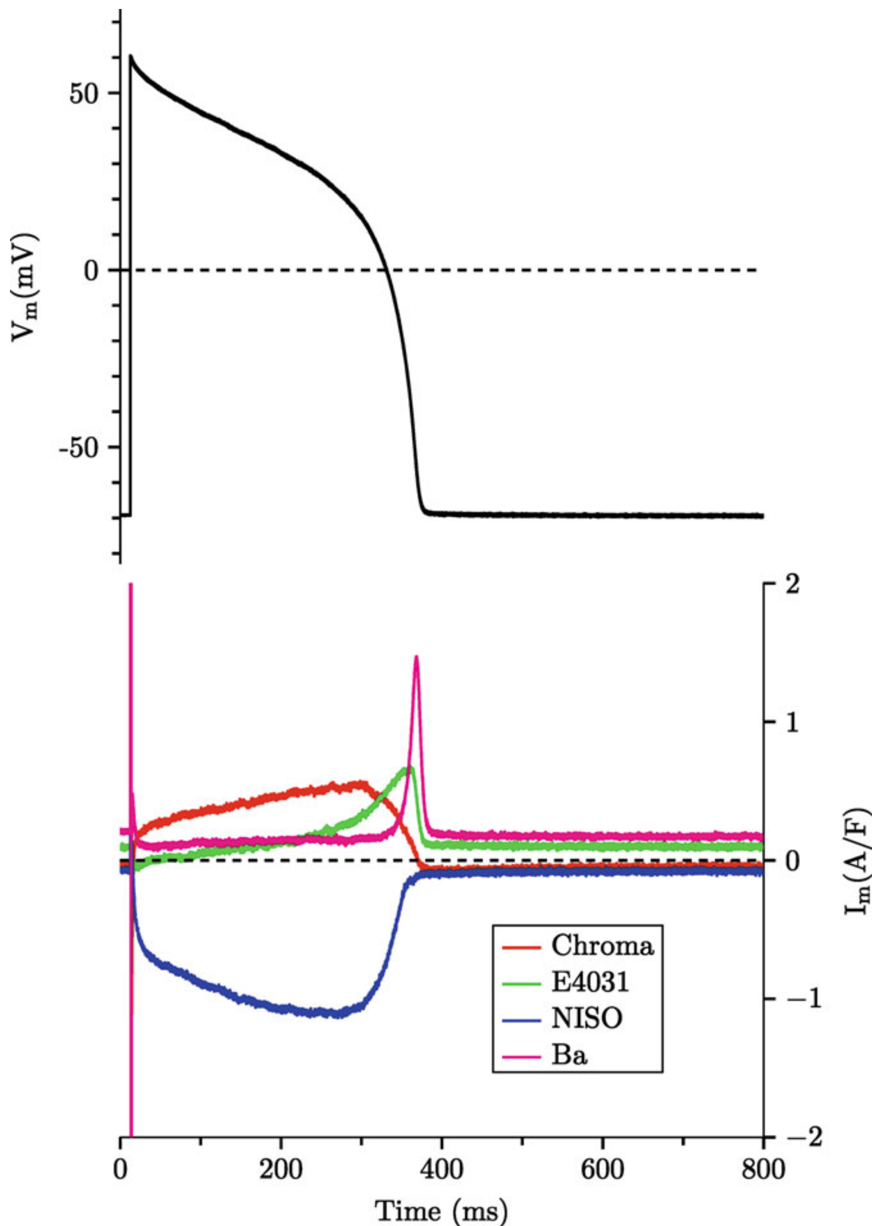


**Fig. 2.2** Onion-Peeling recording of multiple ionic currents from a single guinea pig ventricular myocyte: $I_{Ks}$: chromanol-293B sensitive, $I_{Kr}$: E4031 sensitive, $I_{K1}$: $Ba^{2+}$-sensitive, $I_{NISO}$: nisoldipine sensitive current

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 [29]. It has been evident that discrepancies exist between the model simulations and the AP-clamp measured current [24], and model simulations still fail to reproduce some of the AP dynamics (i.e., EAD, adaptation, restitution) [2, 4, 30, 31]. The significant contribution of the Onion-Peeling technique is that it can generate a new type of data: recording of the currents flowing during AP under physiological condition can help to build accurate and realistic models; recording of multiple currents in a single cell enables study of the Individual Cell Electrophysiology (ICE).

The cell's AP is determined by integration of all of the ionic currents flowing across the cell membrane via ion channels, exchangers, transporters, pump, etc. In short, the AP is a cell-level phenomenon. In order to gain an in-depth quantitative understanding of the ionic mechanisms that control the AP, we need to integrate the ionic currents at the single cell level, and therefore study of the ICE. Due to cell-to-cell variability, the current density of certain currents may vary in individual cells. However, if the inward and the outward currents can counterbalance one another, different combinations of currents can generate similar APs [24]. Nonetheless, under pathological conditions, the cell-to-cell variability may become more pronounced to widen the heterogeneity among cells. The Onion-Peeling technique enables us to study the coordination or discoordination of multiple currents in the single cell and to gain comprehensive view of how they work concertedly to shape the APs in health and diseases, and in response to aging, stress, drug, etc. Such ICE study will be critically important for understanding the heterogeneity of cells (transmural gradient, regional difference, inherent variance, etc.) as well as the impact of cellular variability on the population behavior at tissue and organ levels.

### 2.5.2 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 [32–34]. 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 [35]. Similar transmural differences were also obtained in ventricular myocytes isolated from human hearts [36]. This double-peaked morphology of $I_{Ca,L}$ in epicardial 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.

### 2.5.3 Studying the Complex Effects of Pathological Conditions

Pathological conditions wrought by disease, stress, drug, etc., usually cause complex changes in multiple currents and $Ca^{2+}$ handling molecules. For example, in a rabbit model of pressure and volume overload-induced non-ischemic heart failure, researchers have found changes in several key molecules that determine the cardiac AP and arrhythmias [37, 38]: $I_{K1}$, an important $K^+$ channel 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 β-adrenergic receptors, which profoundly influence the AP and the $Ca^{2+}$ signaling was also altered. These, and perhaps 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 [39]. The Onion-Peeling 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.

### 2.5.4 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 [40, 41]. Hence, the profile of the drug-sensitive current recorded under AP-clamp can give clues toward 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 [42] and the effects of acetylcholine and adenosine on $I_{K1}$ in ferret cardiac myocytes [43] were clarified using the AP-clamp technique.

## 2.6 Technical Aspects

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

### 2.6.1 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. 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 the membrane permeable buffers (bicarbonate) seem to improve robustness of the cells. We found that the cells worked better if stored for 1–2 h 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 noncontracting cell types). However, sometimes cells tend to stick to the bottom of the perfusion chamber. Adding a small drop of the albumin 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 Onion-Peeling technique [25] 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 was 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 Onion-Peeling 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, 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 min 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 min of pacing for the cell to reach equilibrium between the cytoplasm and the pipette solution after establishing the whole-cell ruptured seal configuration.

### 2.6.2 The Patch Pipette and the Whole-Cell Seal Configuration

The patch pipette used for the AP-clamp technique is the same as those used for traditional voltage-clamp experiments. We use borosilicate glass pipette with tip resistance of 1.5–2.5 MΩ. The technical details of pipette fabrication are given by 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 or Onion-Peeling 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 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.

### 2.6.3 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 complicated and time consuming (if we have to overwrite protocol parameters) than simply turning the control knobs on the stimulator (e.g., grass simulator). 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.

## 2.7 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 that 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

**Table 2.1** Specific inhibitors for the major ionic currents in cardiac cells

| Ion channel and transporter | Inhibitor | Dosage | Reference |
|---|---|---|---|
| $I_{Na}$ | TTX | 1–10 μM | [44, 45] |
| $I_{Na\text{-}Late}$ | Ranolazine | 10 μM | [46] |
| $I_{Na\text{-}Late}$ | GS-458967 | 0.1–1 μM | [47] |
| $I_{Ca\text{-}L}$ | Nifedipine | 1 μM | [48] |
| $I_{Ca\text{-}L}$ | Nisoldipine | 0.1 μM | [35] |
| $I_{NCX}$ | SEA0400 | 3 μM | [49, 50] |
| $I_{to}$ | 4-Aminopyridine | 1 mM | [51, 52] |
| $I_{Kr}$ | E-4031 | 1 μM | [26, 51] |
| $I_{Ks}$ | HMR-1556 | 30 nM | [53, 54] |
| $I_{Ks}$ | Chromanol-293B | 1–10 μM | [55] |
| $I_{K1}$ | $BaCl_2$ | 50 μM | [51, 56] |
| $I_{K\text{-}Ca}$ | Apamin | 100 pM, 1 nM | [57, 58] |
| $I_{Cl\text{-}Ca}$ | N-(*p*-amylcinnamoyl) anthranilic acid | 5 μM | [59] |
| $I_{Cl\text{-}Ca}$ | 9-Anthracene carboxylic acid | 0.5 mM | [60] |
| $I_{Cl\text{-}Ca}$ | Niflumic acid | 50 μM | [61, 62] |
| $I_{Cl\text{-}small}$ | Chlorotoxin | | [63] |
| $I_{Cl,ligand}$ | Picrotoxin | | [64] |
| $I_{Ca\text{-}T}$ | NNC 55-0396 | | [65] |
| $I_{Ca\text{-}T}$ | R(-)efodipine | | [66] |

effects on other channels besides their primary target. Using these nonspecific drugs in the AP-clamp experiment would yield a composite current contaminated with other current(s).

How to make sure the Onion-Peeling 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 measure 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 nonspecific 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 [24]. If the drug is found to have nonspecific 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 2.1 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 Onion-Peeling 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 elevates the intracellular $Ca^{2+}$ concentration that triggers many biochemical events and modulate numerous $Ca^{2+}$-sensitive currents. For example, to record the L-type $Ca^{2+}$ current using the Onion-Peeling 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 Onion-Peeling 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.

## 2.8 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 Onion-Peeling 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 Onion-Peeling 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.

## Problems

2.1. How is the cell's action potential shaped by the inward currents and the outward currents?
2.2. What are the advantages of using AP-clamp and 'Onion-Peeling' technique, instead of conventional voltage-clamp, to record ionic currents?
2.3. Does $Ca^{2+}$ transient during the action potential cycle affect ionic currents and the action potential? How to preserve the $Ca^{2+}$ cycling during action potential?
2.4. What are the basic requirements for performing successful AP-clamp experiments?

## Solutions

2.1 The inward currents provide the Depolarization and the outward currents provide the Repolarization; these opposing currents counterbalance to shape the cell's action.
2.2 AP-clamp recording provides a different type of data from conventional voltage clamp: (a) the ionic currents are directly recorded during AP with $Ca^{2+}$ cycling in a physiological milieu, mimicking in vivo condition; (b) the unprecedented ability to measure multiple currents in the same cell allows deciphering their relationships in the same cell, which is necessary for studying how multiple currents interact and integrate in the single cell to shape APs.
2.3 1. Not adding any exogenous $Ca^{2+}$ buffer;
2. Using the cell's own steady-state AP as the voltage-clamp command waveform;
3. Using internal and external solutions having physiological ionic composition;
4. Using physiological stimulation frequency and body temperature.

2.4 1. High-quality isolated single cells that can reach steady-state AP;
2. Using the internal and external solutions with physiological ionic composition, pH, and osmolarity;
3. Making the whole-cell seal with low access resistance ($<5$ MΩ) and seal condition kept constant throughout the entire experiment;
4. Using highly specific channel blockers to obtain drug-sensitive current.

## Further Study

- The recent advances in patch-clamp techniques and their application in cardiac electrophysiology are summarized by Bébarová [67].
- How AP-clamp can be used to test the selectivity of drugs acting on cardiac ion channels are reviewed by Szentandrássy et al. [68].
- AP-clamp studies can reveal the exact frequency-dependent properties of ionic currents under AP [69].
- Dynamic clamp can be used to assess ionic current properties during action potentials with beat-to-beat variability [70].
- Dynamic clamp can be used to study the consequences of cardiac ion channel mutations in causing inherited arrhythmias [22, 71].
- Dynamic clamp used in the field of cardiac stem cell research [72].

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