Intact Heart Loose Patch Photolysis Reveals Ionic Current Kinetics During Ventricular Action Potentials

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Rationale: Assessing the underlying ionic currents during a triggered action potential (AP) in intact perfused hearts offers the opportunity to link molecular mechanisms with pathophysiological problems in cardiovascular research. The developed loose patch photolysis technique can provide striking new insights into cardiac function at the whole heart level during health and disease.

Objective: To measure transmembrane ionic currents during an AP to determine how and when surface Ca\(^{2+}\) influx that triggers Ca\(^{2+}\)-induced Ca\(^{2+}\) release occurs and how Ca\(^{2+}\)-activated conductances can contribute to the genesis of AP phase 2.

Methods and Results: Loose patch photolysis allows the measurement of transmembrane ionic currents in intact hearts. During a triggered AP, a voltage-dependent Ca\(^{2+}\) conductance was fractionally activated (dis-inhibited) by rapidly photo-degrading nifedipine, the Ca\(^{2+}\) channel blocker. The ionic currents during a mouse ventricular AP showed a fast early component and a slower late component. Pharmacological studies established that the molecular basis underlying the early component was driven by an influx of Ca\(^{2+}\) through the L-type channel, CaV 1.2. The late component was identified as an Na\(^{+}\)–Ca\(^{2+}\) exchanger current mediated by Ca\(^{2+}\) released from the sarcoplasmic reticulum.

Conclusions: The novel loose patch photolysis technique allowed the dissection of transmembrane ionic currents in the intact heart. We were able to determine that during an AP, L-type Ca\(^{2+}\) current contributes to phase 1, whereas Na\(^{+}\)–Ca\(^{2+}\) exchanger contributes to phase 2. In addition, loose patch photolysis revealed that the influx of Ca\(^{2+}\) through L-type Ca\(^{2+}\) channels terminates because of voltage-dependent deactivation and not by Ca\(^{2+}\)-dependent inactivation, as commonly believed. (Circ Res. 2016;118:203-215. DOI: 10.1161/CIRCRESAHA.115.307399.)

Key Words: action potentials ■ calcium signaling ■ excitation contraction coupling ■ ionic currents ■ nifedipine ■ sarcoplasmic reticulum

In mammalian hearts, the complex nature of electrophysiologic signals is critically defined by the timing of the action potential (AP) at the different layers within the ventricular wall. The ventricular AP has 5 distinguishable temporal phases driven by the activation of ionic currents. In particular, the duration and amplitude of AP phase 2 is considered to be critical for excitation–contraction coupling because it is thought to determine the magnitude of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels, which predominantly occurs during this phase. However, the time course of APs are highly heterogeneous within different areas and layers of the ventricles. These heterogeneities are driven by nonhomogenous distribution of ionic channels that produce currents underlying the AP morphology. Furthermore, diverse mammalian species display different AP time courses, indicating that the contribution of specific ionic conductances defining the AP in one species cannot be extrapolated to another one.

In This Issue, see p 183
Editorial, see p 184

Much of our knowledge about the ionic currents underlying the AP has been obtained from measurements on enzymatically dissociated ventricular cardiac myocytes. A standard method is to record APs in current clamp mode and then voltage clamping the myocytes using the recorded AP waveform (AP voltage clamp). The different ionic components can then be dissected using pharmacological tools. This approach has generated a great deal of knowledge, but it has several drawbacks and limitations. For example, the exact ventricular area and layer from where the tested dissociated myocytes originated is largely unknown. The electric, metabolic, and mechanical coupling within the ventricular syncytium (and its influence on the AP) is lost...
when cells are dissociated. Furthermore, ionic currents in isolated myocytes are usually recorded at room (not physiological) temperature and in the presence of exogenous intracellular Ca\(^{2+}\) buffers to help sustain cell viability. We developed loose patch photolysis (LPP) to overcome these obstacles. It allowed us for the first time to simultaneously measure transmembrane currents \(i_m\) during a triggered AP in an intact heart by activating or inhibiting ionic conductances using photosensitive compounds.

We recently reported\(^7\) that, in mouse epicardium, AP phase 2 occurs at a membrane potential \((V_m; \text{more negative than } -40 \text{ mV})\) at which L-type Ca\(^{2+}\) channels are closed.\(^8\) This finding spurred 2 questions that can be answered with our newly developed technique. These questions are: (1) if Ca\(^{2+}\) does not enter the cell during phase 2, what ionic conductance is responsible for phase 2 in mouse epicardium? and (2) when does the Ca\(^{2+}\) influx that triggers Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) occur?

Here, we used LPP to definitively show that, the Ca\(^{2+}\) influx that triggers CICR occurs during AP phase 1 in mouse epicardium and AP phase 2 is because of Na\(^{+}\)–Ca\(^{2+}\) exchanger (NCX)–mediated inward current driven by Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), not the L-type Ca\(^{2+}\) channel. The results presented here challenge the long-standing concept that, in all mammals, the AP phase 2 is responsible for triggering CICR.

Finally, our finding that Ca\(^{2+}\) influx that triggers CICR occurs during AP phase 1 opens the possibility that this scenario could also occur in the epicardium of other mammals, including humans that have a strong spike and dome AP morphology. Our state-of-the-art LPP approach is a new powerful tool that can be used to define the properties of ionic currents in the intact heart during pathophysiological situations such as ischemia or arrhythmias, where intact heart recordings are essential.

### Methods

#### Heart Preparation

Animals used in this study (male Balb/c, 3–7 weeks old) were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–23, Revised 1996) and euthanized by cervical dislocation. This protocol was approved by the Institutional Animal Care and Use Committee of the University of California Merced (No. 2011-001). Hearts were removed and cannulated onto a custom-designed recording chamber attached to a Langendorff apparatus and continuously perfused at a rate of 1 mL/min with Tyrode solution. Tyrode solution consists of (in mmol/L): 140 NaCl, 5.4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 0.33 Na2HPO\(_4\), 10 HEPES, 10 glucose, and pH=7.4. The temperature of the solution outside the heart was controlled with a Peltier unit that allowed us to maintain the heart at quasi-physiological temperatures during the entire experiment. All solutions were equilibrated with 100% O\(_2\). Hearts were paced at the apex, at 6 Hz using an optically isolated current stimulator (Isostim, WPI, Sarasota, FL) at 32°C.

### Fluorophore Loading

Rhod-2 and X-Rhod-5F AM were used to measure myoplasmic Ca\(^{2+}\) concentration and Di-8-ANEPPS to record the membrane potential. The dyes were dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) with 2.5% pluronic, the stock solution was finally diluted in Tyrode solution to reach a final dye concentration of 45 μmol/L for the Ca\(^{2+}\) indicators and 1.5 μmol/L for the potentiometric dye. Perfusion with dyes started after the spontaneous heart rate became regular. After 30 minutes of perfusion at room temperature, the solution containing the fluorescent dye was switched to Tyrode solution, and temperature was increased to 32°C within 10 minutes. In all the experiments, the hearts were perfused with 10 μmol/L blebbistatin to reduce the motion. All the recordings were performed at the epicardial layer. All the dyes were obtained from Life Technologies (Carlsbad, CA) and additional drugs and salts from Sigma (St. Louis, MO).

#### Pulsed Local Field Fluorescence Microscopy Setup

Pulsed local field fluorescence microscopy has been extensively described elsewhere.\(^{19,20}\) Briefly, the excitation light source was obtained from a green (532 nm) Yag laser. The light pulses were focused by an objective into a small (200 μm in diameter, 0.67 NA) multimode optical fiber. Emitted light was carried back through the same fiber, filtered and focused on an avalanche photodiode (Perkin Elmer, Waltham, MA). The recordings were obtained by gently placing one end of fiber on the tissue to immobilize the fiber optic relative to the heart surface. This procedure greatly attenuated motion artifacts.

In addition, intracellular membrane potential was also recorded with sharp glass microelectrodes filled with 3-mol/L KCl and connected to an electrometric amplifier (WPI, Sarasota, FL). Data acquisition was performed with a multifunction acquisition board (National Instruments, Austin, TX) controlled by a custom-designed, G-based software program (LabVIEW).

#### LPP Setup

This novel technique allowed us to record ionic currents during a triggered epicardial AP. The system is a combination of 3 major elements (Online Figure I): loose patch recordings,\(^{21,22}\) local field optical measurements,\(^{19,20}\) and fiber optic laser–driven flash photolysis.\(^{23–27}\) The first component, a dual patch clamp allowed us to voltage clamp at the same potential the interior of a macro-patch pipette and the surrounding bath. This configuration equalized both potentials to eliminate the pipette seal leak current. As illustrated in Figure 1A,

\[
V_0 = -R_i \cdot i_m = R_i (i_{Seal} + i_m)
\]

where \(V_0\) and \(R_i\) are the current-to-voltage converter output voltage and feedback resistor, respectively. \(i_m\) is the current flowing through the patch pipette; \(i_{Seal}\), the leak current through the loose seal; and \(i_m\), the membrane current. Usually, because of a poor electric seal between the pipette and the tissue

\[
i_{Seal} \gg i_m
\]

then

\[
i_m = i_m + i_{Seal} = \frac{V_0 + V_A - V_{Bath}}{r_m}
\]

where \(V_m\) is the membrane potential; \(r_m\), the membrane resistance; \(V_0\), the potential inside the pipette; \(V_{Bath}\), the extracellular potential outside the heart; and \(r_{Seal}\), the seal resistance.

If

\[
V_A = V_{Bath} \Rightarrow i_{Seal} = 0
\]

then

\[
V_A = V_{Bath} \Rightarrow i_m = 0
\]

Thus, voltage clamping the bath and the pipette at the same potential allowed us to directly evaluate the membrane current during an AP.

The second component, a local field fiber optic positioned inside the patch pipette was used to optically record the AP inside the
pipette. As seen in Figure 1B, the currents recorded during an AP after sealing the patch pipette against the tissue displayed a complex behavior. The complexity of this signal relies on the fact that the recorded membrane current is the summatory of the total ionic current plus a capacitive current.

Indeed,

\[ i_m = i_c + \sum i_i \]

where \( i_c \) is the capacitive current and \( i_i \) is each of the individual ionic currents occurring during the AP. The capacitive current will depend on the rate at which the membrane potential changes below the pipette. So,

\[ i_m = C_m \frac{dV_m}{dt} + \sum i_i \]

where \( C_m \) is the membrane capacitance. Because we are interested in the ionic currents, the previous equation can be rearranged as follows:

\[ \sum i_i = i_m - C_m \frac{dV_m}{dt} \]

Thus, to evaluate the total ionic current, the membrane current and the membrane potential need to be evaluated at the same site. This was performed by optically recording the membrane potential at the patch pipette with a local field optical fiber positioned inside the pipette (Figure 1C). The resting membrane potential and the amplitude of the optically recorded AP were calibrated with the aid of an electric signal obtained with a sharp microelectrode positioned close to the patch pipette (Figure 1C).

The third component, a flash photolysis system, allowed us, by the use of light-sensitive drugs, to fractionally change the number of ion channels or the open probability of those channels within the patch. For example, Ca\(^{2+}\) currents can be activated (ie, relieved from partial block) by the photolysis of dihydropyridines (eg, nifedipine \(^{26,27}\) inside the loose patch pipette. Nifedipine partial blockade was locally relieved by UV illumination generated by a DPSS UV laser (355 nm; DPSS Lasers Inc, Santa Clara, CA). UV light was optomechanically shuttered for 1 to 40 ms and applied through either by a quartz fiber-optic positioned inside or by an additional quartz multimode fiber positioned outside the patch pipette. The pipettes have a tip diameter of 250 μm and the optical fiber 200 μm (depth of field ≈30 μm) making it possible to record currents from 25 to 50 myocytes. Using this procedure, we were able to record membrane currents (capacitive and ionic) during the AP before and after the UV flash. The subtraction of the resulting currents before and after the UV flash allowed us to dissect specific ionic currents (see Results section of this article).

Data were recorded with an acquisition system Digidata 1440A (Molecular Devices, Sunnyvale, CA) using pClamp 10 software. Fluorescence, membrane potential, and currents were always recorded from the left ventricular epicardium.

**Statistical Analysis**

Data are expressed as means±SE; statistical significance was tested using ANOVA. The difference was considered to be significant if the value of \( P \) was <0.05.

**Results**

**Recording Transmembrane Currents During a Triggered AP**

Measuring ionic currents in multicellular preparations has been challenging both in application and in interpretation...
because of difficulties to spatially voltage clamp the tissue and the impossibility of obtaining high-resistance electric seals using giant patch pipettes.

To overcome these problems, we developed the novel LPP technique that allowed us to measure \( i_m \) during a triggered physiological AP. This was achieved by voltage clamping to the same potential the interior of a giant patch pipette and the bath where the heart was immersed. This procedure eliminates the seal current \( (i_{\text{seal}}) \) by zeroing the potential difference between the pipette and the bath. A scheme of the system is shown in Figure 1A. With the bath potential clamped to \( V_A \), \( i_{\text{seal}} \) is eliminated, and the entire remaining current flowing through the pipette will be the current flowing across the plasma membrane \( (i_m) \); see Methods section of this article). Figure 1B illustrates a membrane current induced by an AP and recorded in a loose seal before and after the patch pipette was gently pressed against the ventricular epicardial layer. The recorded signal presents a complex biphasic behavior composed of the stimulus artifact (large downward spike) and a compound current waveform that includes capacitive and ionic currents. As described in Methods section of this article to evaluate the capacitive component, the \( V_m \) during an AP needs to be recorded in the same location as \( i_m \). This was achieved by loading the heart with the potentiometric dye Di-8-ANEPPS and positioning a pulsed local field fluorescence microscopy optical fiber inside the recording glass pipette (Figure 1C). Traces of optically and electrically recorded APs in conjunction with \( i_m \) are shown in Figure 1D. The optically recorded AP allowed us to simultaneously assess the total \( i_m \) in conjunction with the \( i_e \) in an intact heart assuming that the membrane-specific capacitance is 1 \( \mu \)F/cm\(^2\) (Figure 1E).

### Dissecting Transmembrane Currents During an AP

Although the loose patch allowed us to record \( i_m \) locally during an AP, it is problematic to selectively change the activity of a specific ionic conductance (eg, L-type Ca\(^{2+}\) current) in the patch. The traditional approach of separating the component conductances using voltage-clamp approaches cannot be adopted, because neither the patch membrane nor the cell can be voltage-clamped in situ. Changing the pipette potential with respect to the bath to activate voltage-dependent currents will result in a massive increase in \( i_{\text{seal}} \) that will prevent the electrical recording of \( i_m \). To tackle this problem, we decided to activate an ionic conductance photolytically. This was achieved by perfusing the heart with nifedipine, a well-known L-type Ca\(^{2+}\) channel blocker. The macroscopic Ca\(^{2+}\) conductance was then activated by photo-relieving the dihydropyridine partial block (Figure 2A) with the aid of an additional multimode optical fiber carrying a UV beam (Figure 2B). To test this concept, we recorded \( V_m \) (electrically) and Ca\(^{2+}\) transients simultaneously. Ca\(^{2+}\) transients were used as a reporter of SR Ca\(^{2+}\) release driven by an influx of Ca\(^{2+}\) across the plasma membrane. Perfusion of the heart with 10-\( \mu \)mol/L nifedipine produced a reduction in the AP duration and an attenuation of the amplitude of the epicardial Ca\(^{2+}\) transient with respect to their control without nifedipine (Figure 2C and 2D). Figure 2C shows a surprising result. When

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**Figure 2.** Nifedipine blocking action removed by UV photolysis. **A**, Scheme of nifedipine photo-breakdown. Nifedipine, such as some other dihydropyridines, contains an O-nitrobenzyl moiety that confers their photolability. The photolytic product no longer inhibits L-type Ca\(^{2+}\) current.\(^{26,27}\) **B**, Experimental arrangement to measure Ca\(^{2+}\) or \( V_m \) using fluorescent dyes and electric \( V_m \) by means of an intracellular microelectrode while applying UV pulses through an additional quartz multimode optical fiber. **C**, Simultaneous recordings of action potentials (APs) with an intracellular microelectrode and Ca\(^{2+}\) transients with Rhod-2 AM, before and after the perfusion of 10 \( \mu \)mol/L nifedipine. Nifedipine reduced the amplitude of Ca\(^{2+}\) transients and the duration of AP. On illumination (UV flash), the breakdown of nifedipine occurs relieving the inhibition of L-type Ca\(^{2+}\) channels allowing an increase in the influx of Ca\(^{2+}\) that promotes a larger release from the sarcoplasmic reticulum as illustrated in the **bottom**. Similar results were obtained in 10 hearts. **D**, Concurrent electric and optical recordings of AP in hearts loaded with Di-8-ANEPPS. Nifedipine induced a shortening of AP duration. However, nifedipine photolysis had no significant effect on the time course of APs (n=6 hearts).
Ca\textsuperscript{2+} influx inhibition was removed locally by the photolysis of nifedipine, no statistically significant changes were observed in the AP time course (APD\textsubscript{90} before flash 45.2±2.2 ms versus APD\textsubscript{90} after flash 47.9±3.6, \(P \leq 0.05\)). This result was unexpected. In contrast, the Ca\textsuperscript{2+} transient after the flash was dramatically enhanced, demonstrating that the photolysis of nifedipine did have profound effects on EC coupling. One possible reason for the unaltered AP time course is that the \(V_m\) recorded with the microelectrode was not obtained exactly at the same location where nifedipine was photolyzed (the microelectrode was positioned 1 mm away from the photolysis optical fiber). To evaluate this explanation, we performed simultaneous electric and optical recordings of APs (Figure 2D). As illustrated in Figure 2D, the local optical recording produced the same surprising result. Local photolysis of nifedipine changed neither the electrically nor the optically recorded AP significantly. This result can be interpreted by the fact that nifedipine photolysis might not have changed the plasma membrane conductance. However, the fact that the Ca\textsuperscript{2+} transient was markedly enhanced (Figure 2C) demonstrates that nifedipine photolysis did enhance Ca\textsuperscript{2+} influx. The second interpretation is that the local current produced by Ca\textsuperscript{2+} influx through L-Type Ca\textsuperscript{2+} channels is not able to affect \(V_m\) perceptibly because of the strong isopotential coupling (electrotone) imposed by the tissue neighboring the recording area. In other words, despite the increased Ca\textsuperscript{2+} influx, \(V_m\) did not change because it was physiologically clamped by the surrounding membrane.

The latter premise was evaluated by recording APs electrically at different distances from the photolysis site. A schematic representation of the experiment is presented in Figure 3A. Figure 3B illustrates typical AP recordings obtained before and after the nifedipine photolysis at varying distances from the photolysis site. For each spatial position, the black traces represent 3 consecutive APs before the UV flash. On the application of a UV pulse, it is possible to observe a small but distinct effect on the AP (red traces) repolarization after the flash. Two clear observations can be made. First, although the photolytic pulse induced a subtle change in the AP occurring just after the UV pulse, this effect essentially disappears in the following AP. This could be the result of the rapid rebinding of nifedipine after photolysis because of its continuous perfusion. Second, the closer the microelectrode is positioned with respect to the optical fiber used for photolysis, the larger is the effect on the AP repolarization. To quantify the maximum nifedipine-induced effect on the AP repolarization, 2 consecutive AP traces (before and after the flash at the photolysis site) were subtracted. The differential records obtained at increasing photolysis fluences (density of energy) display an early and a late differential component (traces not shown). The early and late components of this difference correspond to changes in AP phase 1 and phase 2, respectively. Figure 3C shows the percentile difference \(((V_m\text{ after photolysis} - V_m\text{ before photolysis}) / V_m\text{ before photolysis}) \times 100\) induced by the nifedipine photolysis at different fluences of AP recorded at the center of the photolysis site. The percentile difference in the early differential component is significantly larger than in the

![Figure 3](http://circres.ahajournals.org/)

**Figure 3. Testing the electrotone imposed by the tissue neighboring the recording area.** A, Scheme of the experiment. The microelectrode was positioned at different distances from the photolysis fiber. B, Action potential (AP) traces recorded at the indicated distances from the photolysis site, before (black traces) and after (red traces) the UV flash. The flash intensity (fluence) used for these records was 6.5 J/cm\(^2\). C, Effect of nifedipine photolysis effect on the AP repolarization. Two consecutive AP traces (before and after the flash at the photolysis site) were subtracted. The differential records obtained at increasing photolysis fluences (density of energy) display an early and a late differential component. The early and late components of this difference correspond to changes in AP phases 1 and 2, respectively. The percentile difference between the early and the late component (\(n=5\) hearts) were calculated using the formula \(\frac{V_m\text{ after flash} - V_m\text{ before flash}}{V_m\text{ before flash}} \times 100\). D, Spatial distribution of the photolysis induced changes in the early and late differential component of the membrane potential at different distances from the photolysis site. Teal corresponds to the early differential component, whereas the orange corresponds to the late differential component. The data were fitted with the spatial component of a cable equation: \(V_m(x)/V_m(0) = e^{-\lambda x}\).
late differential component (n=5 hearts). The electrotonic effect was assessed by evaluating the space constant of the epicardial tissue after the nifedipine photolysis. Interestingly, Figure 3D shows the spatial attenuation of the early differential component was more abrupt than for the late differential component (273±25 µm versus 507±46 µm). These results suggest that under the fluence range <10 J/cm², the difference in the membrane potential change induced by photolysis is <4%. In addition, the larger spatial attenuation of the early differential component was expected because the temporal filtering introduced by the cable properties of the tissue is larger for faster differential components than for slower ones.

The increase in the ionic current that triggers SR Ca²⁺ release could be related to changes in the $V_m$ after the photolysis or because of specific effects on the macroscopic Ca²⁺ conductance. As we already showed (Figures 2 and 3), the effect of photolysis on the AP is negligible. Despite this appearance, it could be possible that the small change in the AP was the result of a large change in 2 opposing conductances. However, the most probable cause is a change in the nifedipine-sensitive current, which could result from changes in the number of available channels or an increase in the open probability as a result of the photo-degradation of nifedipine. These various possibilities were tested by recording $i_m$ and optical APs simultaneously (Figure 4A). We implemented a protocol in which APs and $i_m$ were recorded before and after a UV flash. Traces obtained using this protocol are shown in Figure 4B, followed by the subtraction of those consecutive AP and $i_m$ traces, Figure 4C. The $V_m$ subtraction before and after the UV flash indicates that $V_m$ did not change by photolysis. This result reinforces the idea that the tissue outside the patch is imposing an electrotonic. Moreover, $i = C(dV_m/dt)$ was canceled during the $i_m$ subtraction because $V_m$ did not change with photolysis. The differential ionic current ($Δi_m$) obtained after subtraction showed a biphasic morphology displaying an early fast component ($i_{early}$) and a late slower ($i_{late}$) component. Thus, $Δi_m$ displayed in Figure 4C represents a nifedipine-sensitive ionic current. This current flows locally in the patch membrane, but scarcely affects the recorded AP because of the electrotonic dominated by the $V_m$ in the larger surrounding membrane area. Hence the $Δi_m$ is recorded during a normal AP that is not significantly altered by the local removal of nifedipine.

Mouse epicardial APs exhibit a phase 2 that is partially mediated by Ca²⁺ release from the SR. Figure 5A illustrates that perfusion with ryanodine (Ry) and thapsigargin (Tg), a combination of drugs that impairs Ca²⁺ release from the SR, dramatically reduces AP phase 2. With the novel LPP approach, we are in the unique position to determine which of the 2 components of the nifedipine-sensitive ionic current is responsible for AP’s phase 2.

Properties of the Early and Late Components of the Photolytically Activated Ionic Currents
To dissect which of the ionic currents was underlying phase 2, we decided to explore if $i_{early}$ (Figure 5B) was activated by Ca²⁺ release from SR. The $i_{late}$ was significantly reduced (89.0±2.1%) in hearts treated with Ry plus Tg (Figure 5C and 5D) in comparison with the effect of the drugs on $i_{early}$ (15.4±1.3%; Figure 5D and 5E). Note that in Figure 5C, early currents recorded after the flash decrease monotonically in time. This effect is because of nifedipine rebinding to dihydropyridine receptor on the photolyzed area (Online Figure II).

Figure 4. Dissecting ionic currents during triggered action potential (AP). A, Experimental arrangement to measure optically $V_m$ and electrically $i_m$ during photolysis. B, Traces of APs and $i_m$ in the presence of 10 µmol/L nifedipine. Upper and lower records (eg, $V_m$ and $i_m$ and $V_m$ and $i_m$) were recorded under identical conditions in the same location. The arrow indicates the time when nifedipine was photolyzed (traces $V_m$ and $i_m$). C, Difference between $V_m$ and $i_m$ ($ΔV_m$) and $i_{early}$ ($Δi_m$). No changes were observed in the differential trace ($Δi_m$) after nifedipine photolysis. However, the subtraction $i_{late}$ revealed a nifedipine-sensitive current that displays an early fast component and a slower late component (n=6 hearts).
The ionic nature of $i_{\text{early}}$ was determined using a pharmacological approach. The heart was perfused with CdCl$_2$, a Ca$^{2+}$ channel blocker in the presence of Ry plus Tg to eliminate $i_{\text{late}}$. This maneuver reversibly inhibited $i_{\text{early}}$ (78.7±3.2%; Figure 6A and 6B) indicating that voltage-dependent dihydropyridine-sensitive Ca$^{2+}$ channels mediate this kinetic component.

Figure 5. Ionic currents underlying action potential phase 2. A, Effect of Ry (10 μmol/L) and Tg (2 μmol/L) perfusion on epicardial action potentials (APs). The treatment had a strong effect impairing the development of AP phase 2 (n=18 hearts). B, Differential ionic current after photolysis (violet arrow) of 10 μmol/L nifedipine (control; n=22 hearts). C, Differential ionic current in a heart perfused with Ry and Tg (n=9 hearts). D, Comparison of ionic current traces in the absence and in the presence of Ry and Tg. E, Histogram showing that the late component $i_{\text{late}}$ is highly inhibited by Ry and Tg, whereas the early $i_{\text{early}}$ is mostly unaffected (n=5, P<0.02).

Figure 6. Probing the molecular nature of the differential ionic currents. A, 100 μmol/L CdCl$_2$ was able to block the fast component ($i_{\text{early}}$) in a reversible manner (n=3 hearts). B, Comparison of the amplitude and kinetics of $i_{\text{early}}$ during CdCl$_2$ treatment. C, Effect of 10 μmol/L SEA0400 on epicardial action potentials (APs). SEA0400 is a potent blocker of Na$^+$–Ca$^{2+}$ exchanger (NCX) and had a similar effect as Ry and Tg treatment on the AP repolarization (n=6 hearts). D, Effect of SEA0400 on the amplitude of epicardial Ca$^{2+}$ transients. As expected, NCX’s block induced an increase in the Ca$^{2+}$ transient amplitude (n=3 hearts). E, Effect of SEA0400 on the photolically activated ionic currents (n=3 hearts). F, Histogram showing that SEA0400 had a selective effect on $i_{\text{late}}$ indicating that in mouse epicardium, an NCX current activated by sarcoplasmic reticulum Ca$^{2+}$ release is involved in the genesis of AP phase 2. *Populations means are significantly different at a level of 0.05 (ANOVA).
As already shown, \( i_{\text{late}} \) is an inward current mediated by SR \( \text{Ca}^{2+} \) release. Among several possible candidates, an electrogenic influx of \( \text{Na}^{+} \) through the NCX forward mode seems to be a likely candidate.\(^7\,^28\) To evaluate this option, we perfused hearts with 10-μmol/L SEA0400,\(^29\) an NCX blocker. Figure 6C shows that SEA0400 has a similar effect on AP phase 2 like Ry plus Tg. To evaluate if the effect of SEA0400 was produced by an impairment of the \( \text{Ca}^{2+} \) influx to the cells, we recorded \( \text{Ca}^{2+} \) transients. Figure 6D shows that SEA0400 does not decrease the amplitude of the myocytes’ \( \text{Ca}^{2+} \) transients but rather induced an increase.\(^30\) This effect was consistent with SEA0400 producing a block of NCX that leads to an increase in the intra SR \( \text{Ca}^{2+} \) content. To further demonstrate that \( i_{\text{late}} \) was mediated by an \( \text{Na}^{+} \) influx through NCX, we recorded currents in the presence and the absence of SEA0400. A typical trace is shown in Figure 6E where SEA0400 selectively blocked \( i_{\text{late}} \) (72±13%) without affecting (11.6±3.1%) \( i_{\text{early}} \) (Figure 6F). Together, these results demonstrate that, during an epicardial AP, there is a fast influx of \( \text{Ca}^{2+} \) through Ca\( ^{2+} \) channels responsible for \( i_{\text{early}} \). In addition, there is a late influx of Na\( ^{+} \) mediated by SR \( \text{Ca}^{2+} \) release activation of NCX forward mode, defining \( i_{\text{late}} \).

Even though the early and fast \( \text{Ca}^{2+} \) influx during the AP seems to be responsible for triggering SR \( \text{Ca}^{2+} \) release, additional evidence is needed. Assessing if incremental \( \text{Ca}^{2+} \) influxes during AP can induce incremental \( \text{Ca}^{2+} \) transients strengthened this line of evidence. Experiments testing this idea are presented in Figure 7A–7C, where \( \text{Ca}^{2+} \) currents (Figure 7A) and \( \text{Ca}^{2+} \) transients (Figure 7B) were recorded in the same membrane patch. The idea was to increase the amplitude of the \( \text{Ca}^{2+} \) current locally by photolyzing progressively greater fractions of nifedipine on the patch surface using UV laser pulses of increasing energy. The direct linear relationship between \( \text{Ca}^{2+} \) current and \( \text{Ca}^{2+} \) transient amplitudes (Figure 7C) allowed us to quantify the gain of CICR at the intact heart level, for the first time.

A different approach to evaluate the relationship between \( \text{Ca}^{2+} \) influx and \( \text{Ca}^{2+} \) release was to analyze the relationship

![Figure 7. Testing the gain of CICR at the intact heart level.](http://circres.ahajournals.org/)

A. Ionic currents during triggered action potentials (APs) illustrating the effects of varying the fraction of nifedipine broken down by applying UV pulses of different energies. Similar results were obtained in 6 hearts. B. \( \text{Ca}^{2+} \) transients, measured with X-Rhod-5F AM, recorded simultaneously at the same location where the ionic currents (A) were obtained. The colors of the fluorescent traces correspond to the loose patch photolysis currents. C. Relationship between \( \text{Ca}^{2+} \) transients amplitude and \( i_{\text{early}} \). There is a direct relationship between intracellular \( \text{Ca}^{2+} \) and the \( \text{Ca}^{2+} \) influx (n=3 hearts). D. Ionic current traces obtained with different flash energies in hearts not perfused with exogenous \( \text{Ca}^{2+} \) indicators. E. Fluence dependency of \( i_{\text{early}} \) (blue) and \( i_{\text{late}} \) (black), both current components saturate for high flucenes indicating that at these energies all nifedipine molecules were locally broken down. F. Nonlinear relationship between \( i_{\text{early}} \) and \( i_{\text{late}} \) (n=15 hearts). G. Time courses of normalized early currents recorded at different flucenes. H. Relaxation time constants of the early currents as function of the photolysis fluence. I. Relaxation time constants of the early currents as function of the peak early current amplitude (n=4 hearts).
between \(i_{\text{sec}}\) and \(i_{\text{early}}\). One advantage of this approach is that \(i_{\text{sec}}\) (NCX forward current) will be proportional to the local dyadic \(Ca^{2+}\) concentration during systole. This approach differs from previous measurements using fluorescence indicators in that they only report the mean average intracellular \(Ca^{2+}\) concentration. Another advantage is that the myocytes’ internal \(Ca^{2+}\) channel capacity is not altered by adding an exogenous buffer as occurs when using a \(Ca^{2+}\) indicator dye. Figure 7D illustrates currents recorded at different nifedipine photolysis fractions (i.e., different fluences). Figure 7E shows the peak values of \(i_{\text{sec}}\) and \(i_{\text{early}}\) as a function of the fluence. It is possible to observe that both \(i_{\text{sec}}\) and \(i_{\text{early}}\) saturate as a function of the fluence; indicating that for high fluence values (>16 J/cm²) most of the nifedipine has been photolyzed. The relationship between \(i_{\text{sec}}\) and \(i_{\text{early}}\) was plotted in Figure 7F. This curve showed a predominantly nonlinear behavior in comparison with the one illustrated in Figure 7C, which presents a more linear relationship, possibly because of the additional buffering effect introduced by the \(Ca^{2+}\) indicator. A interesting finding was that early currents with larger magnitude do not terminate faster than smaller ones (Figure 7A and 7D). This idea was specifically evaluated in Figure 7G–7I. Figure 7G illustrates traces at different photolysis fluences that were normalized to the peak current for each trace. Every trace displayed the same kinetics for different fluences, suggesting that the magnitude of the current does not affect the current termination. Furthermore, data from 5 different hearts show that the kinetics of relaxation of the early currents were not dependent on the fluence of the photolytic pulse (Figure 7H) or the amplitude of the early current (Figure 7I). This result challenges the established idea31–33 that, in mammalian hearts, the termination of \(Ca^{2+}\) influx through L-type \(Ca^{2+}\) channels is mostly mediated by \(Ca^{2+}\)-dependent inactivation.

If \(Ca^{2+}\)-dependent inactivation is not the main mechanism that terminates \(Ca^{2+}\) influx, in murine hearts during excitation–contraction coupling, then which is the mechanism? To address this question, we performed experiments to evaluate in which of the AP phases, the \(Ca^{2+}\) influx that triggers CICR occurs. Interestingly, we found that most of the \(Ca^{2+}\) current that triggers CICR occurs during phase 1 (Figure 8A). In order for this to happen, L-type \(Ca^{2+}\) channels need to be activated at an early stage of the AP (phase 0). Because in phase 0, \(V_m\) moves quickly toward the \(Ca^{2+}\) electrochemical potential, the amount of \(Ca^{2+}\) entering into the cells will be small. At the beginning of phase 1, a fraction of L-type \(Ca^{2+}\) channels are still open. Thus, throughout phase 1 repolarization, a massive influx of \(Ca^{2+}\) enters the cells during the deactivation of the \(Ca^{2+}\) current. This is driven by a rapid increase in the \(Ca^{2+}\) driving force (Figure 8A). Finally, when the AP reaches phase 2, the \(V_m\) is negative enough to completely close the L-type \(Ca^{2+}\) channels.18 Thus, this suggests that the influx of \(Ca^{2+}\) terminates because of voltage-dependent deactivation (in a tail current fashion) and not by \(Ca^{2+}\)-dependent inactivation. This phenomenon resembles \(Ca^{2+}\) entry into the presynaptic terminal during a neuronal AP repolarization to induce neurotransmitter release.34–36 This novel concept alters the currently accepted paradigm in cardiac \(Ca^{2+}\) signaling.

To further evaluate the idea that \(Ca^{2+}\) influx triggering \(Ca^{2+}\) release occurs during phase 1, we performed experiments to change the kinetics of phase 1 repolarization. Phase 1 is a phase of rapid repolarization that is largely governed/acted upon by the activation of the transient outward current, \(i_n\) (Kv. 4.2).37 Thus, to slow the repolarization kinetics of phase 1, we partially blocked \(i_n\) with 200 μmol/L of 4-aminopyridine. Figure 8B and 8C show the nifedipine-sensitive current and the AP recorded simultaneously. 4-Aminopyridine significantly modified the time course of both the current and the AP repolarization kinetics (Figure 8D and 8E). Superposition of the nifedipine-sensitive currents before and after 4-aminopyridine in Figure 8F illustrates the dramatic increase in the rapid spike of \(Ca^{2+}\) current during phase 1, as well as the greater and prolonged inward Na+ current during phase 2. A summary of several experiments (n=5) is presented in Figure 8G and 8H. Slowing down phase 1 increased significantly the time to peak and the half duration of \(i_n\) with no significant effect on the amplitude (Figure 8I). Thus, this result supports the argument that the \(Ca^{2+}\) influx across the plasma membrane occurs during phase 1.

**Discussion**

Although there have been several efforts to record ionic currents in multicellular cardiac preparations, this is the first report of ionic currents recorded in a perfused heart during a physiologically triggered AP. In the early days of the voltage-clamp technique, several groups recorded ionic currents in cardiac tissue using the sucrose gap technique.38–41 Even though these pioneering experimental contributions were important for the advancement of cardiac electrophysiology, these multicellular preparations presented a poor spatial and temporal control of the potential. This limitation was partially overcome by a modification of the cell-attached patch-clamp recording,42 the loose patch clamp,43,44 This technique has been used to measure ionic currents in multicellular cardiac preparations, such as trabeculae and papillary muscle.45–46 However, none of these reports was performed on an intact perfused heart during a physiological triggered AP.

The evaluation of ionic currents during an AP usually has been performed using whole-cell AP voltage clamp.47–50 With this approach, the AP waveform is first recorded in a particular myocyte under current clamp and then used to voltage clamp different myocytes. Although this approach made it possible to record ionic currents driven by an AP waveform, it does present several drawbacks. For example, as we already mentioned, myocytes from which AP are recorded can come from different regions than the one that will be voltage clamped. In addition, the AP in an isolated cell will be different from one recorded in an intact tissue because neighboring layers of cells can affect the waveform of the local AP. In addition, the study of ionic currents in isolated myocytes is usually done at room temperature and in the presence of a high concentration of intracellular \(Ca^{2+}\) buffers to increase the viability of the isolated myocytes. Under these conditions, it is unlikely that the time course of the T-tubule potential during an AP voltage clamp will reach the rate of depolarization–repolarization that actually occurs during a physiological AP. This last limitation is mostly defined by the cable properties of the tubular system. In 1 report, the ionic currents were recorded during a physiological AP50 in cell-attached configuration on embryonic chick heart cells. Unfortunately,
all these recordings were obtained from isolated cells that are electrically, metabolically, and mechanically uncoupled from the rest of the tissue, a situation that is highly unphysiological.

Here, we introduced a new methodological approach that overcomes most of the experimental problems described above. A unique advantage of LPP is that it made possible the recording of membrane currents during a physiologically triggered AP on an intact perfused heart (Figure 1). The L-type Ca\(^{2+}\) channels were selectively reactivated by photo-degrading an antagonist (Figure 2). The isopotential condition was imposed by the rest of the unphotolyzed area (Figure 3). The experiments were performed at a quasi-physiological body temperature (33–35°C) and heart rates (6 Hz). These factors will strongly modify the NCX currents because of the steep temperature dependence (\(Q_{10}=3.8\))\(^{48,51,52}\) and the frequency dependence of Ca\(^{2+}\) release.\(^{51}\) The studies reported here using our new approach revealed several unexpected results that alter the existing paradigm of the cardiac AP.

The ionic currents recorded with LPP at the epicardial layer of a perfused heart during an AP showed a fast early component and a slower late component (Figure 4), which we interpret as L-type Ca\(^{2+}\) current and NCX current, respectively. The early component is similar to the one reported by Sah et al\(^{49}\) using AP voltage clamp. Interestingly, the slower late component is activated by Ca\(^{2+}\) released from the SR (Figure 5D and 5E) and seems to be partially responsible for the genesis of the AP phase 2 (Figure 5A).
recorded in the mouse epicardium. Pharmacological experiments designed to evaluate the molecular basis underlying the early and late component of the nifedipine-sensitive currents determined that the early component was sensitive to Cd2+ (Figure 6A and 6B), indicating that this current is mediated by an influx of Ca2+ through the L-type channel, CaV 1.2. Although Cd2+ has also a blocking effect on the NCX, the experiments with Cd2+ were performed in hearts that were pretreated with Ry and Tg (to avoid NCX activation) thus, it is likely that the currents shown in Figure 6A and 6B were not the result of the blocking effect of Cd2+ on NCX1 but rather on CaV 1.2. Furthermore, the late component was inhibited with micromolar concentrations of the NCX blocker SEA0400 (Figure 6E and 6F). At this concentration, SEA0400 also attenuated the AP phase 2 (Figure 6C). However, because the amplitude of Ca2+ transients increased (Figure 6D), this indicated that the predominant effect of SEA0400 was to inhibit NCX and not the L-type Ca2+ channels. This result is consistent with experimental findings in transgenic animals by Pott et al28 showing that NCX was involved in the genesis of AP phase 2. Knockout of NCX alters the AP waveform and pacemaker activity in sinoatrial node cells.3 Here, we show that electrogenic Na+ influx through NCX contributes substantially to prolonging the plateau phase of the AP.

The gain of the CICR process has been defined in recordings from isolated myocytes. In this article, we showed that LPP can be used to evaluate CICR ex vivo. This is an important parameter to evaluate mechanisms involved in defining cardiac contractility. Figure 7A–7C showed not only that the early inward current was carried by Ca2+ ions, but that increasing the number of available nifedipine-sensitive Ca2+ channels produced an equivalent effect on the amplitude of Ca2+ currents and Ca2+ transients. Moreover, the fact that there is a relationship between the late current and the early current (Figure 7F) indicates that the local gain of CICR can be evaluated in a perfused heart. This last result is important because it will help to determine the strength of the coupling between Ca2+ influx and Ca2+ release in normal and pathological conditions, such as ischemia and heart failure. Although it is difficult to make a direct comparison between our data and previous published data because of NCX current-voltage dependency as well as intracellular and extracellular Ca2+ and Na+ concentrations, we found that the magnitude of both the early Ca2+ current and the late NCX current recorded using LPP (Figure 7E) are similar to the ones reported using whole-cell voltage clamp in enzymatically isolated mouse ventricular myocytes.28,54,55

Another important and unanticipated result was that the decay of Ca2+ current (early component) does not become faster as the size of the current increases (Figure 7D). This finding puts some constrains to the general idea that, in all mammalian hearts, the Ca2+ influx is terminated by Ca2+-dependent inactivation. In Figure 8A, we demonstrated that although Ca2+-dependent inactivation is not the mechanism involved in the termination of the Ca2+ influx, the fast Ca2+ influx terminates. This termination then is because of voltage-dependent deactivation during AP phase 1 repolarization. Thus, although L-type Ca2+ channels activate during phase 0, most of the Ca2+ influx occurs during phase 1. Interestingly, it is possible to observe that the early component/peak is composed by a first fast inward current and a second component/peak that is larger and longer. We think that Ca2+ is getting into the cell during both peaks. The difference in magnitude can be explained if we consider that most of the Ca2+ influx through L-type Ca2+ channels (second peak) occurs during the deactivation of the channels and during the action potential (AP) phase 1. In order for the deactivation to occur, the channels need to open before phase 1 (AP phase 0). This is possible because the AP reaches its peak value in 1 ms and at this point, the driving force for Ca2+ is highly reduced, which could explain the kinetics of the first (fast and short) of the 2 peaks, which will not contribute significantly to the activation of the excitation–contraction coupling process. The concept of Ca2+ of getting into the cell has been previously suggested in isolated cells by Sah et al50 and Clark et al52 but has not been widely accepted. This idea was further explored in Figure 8B–8H where we changed the rate of phase 1 repolarization by partially blocking iNCX with 4-aminopyridine. Here, the slowing down of phase 1 produced a significant prolongation of the time to peak and the half duration of the early Ca2+ current (Figure 8F and 8H), but not on the current amplitude (Figure 8H).

To conclude, LPP allowed the recording of ionic currents from cells electrically and metabolically coupled during their own AP at the intact heart level. Local activation of Ca2+ currents or local increase in myoplasmic Ca2+ does not have a significant effect on AP repolarization. Nifedipine-sensitive inward current has 2 components: an ifast that is driven by Ca2+ influx through L-type Ca2+ channels and an NCX-dependent ilate component activated by SR Ca2+ release. Larger L-type Ca2+ currents do not terminate faster than small ones indicating that Ca2+-dependent inactivation is not the main mechanism for current termination during an epicardial AP in mouse ventricle. Finally, the transmembrane Ca2+ influx that triggers SR Ca2+ release occurs during phase 1 repolarization in a tail current fashion.

Among several possibilities, we think that this mechanism involving an early Ca2+ influx through the L-type Ca2+ channels during phase 1 and a late Na+ influx through the NCX during phase 2 has not been previously described and is now revealed by LPP. This is because of LPP’s ability to record ionic currents during an AP in the intact heart, when all the other conductances are active. Consequently, this speeds up the T-tube depolarization and repolarization. In contrast, when AP whole-cell voltage clamp is used, only the current of interest is activated and the tubular capacitance and series resistance slow down the kinetics of several ionic currents at the T tubes. Therefore, the kinetics of Ca2+ influx to the cell change and subsequently modify the SR Ca2+ release that activates the NCX current.

In summary, by the use of light-sensitive compounds, LPP is useful for isolation and functional characterization of a particular ionic current from cells mechanically, electrically, and metabolically coupled during their own AP at the intact heart level, during health and in disease states.

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DISCLOSURES
None.

REFERENCES

**What Is Known?**

- Ventricular ionic currents are usually recorded from enzymatically dissociated cells by voltage clamping.
- Phase 2 of the action potential (AP) is driven by Ca2+ influx through L-type channels.
- During an AP, the L-type Ca2+ current is terminated by a Ca2+-dependent inactivation mechanism.

**What New Information Does This Article Contribute?**

- We measured ionic currents in intact hearts under physiological AP.
- The Ca2+ influx that triggers Ca2+ release occurs during phase 1 (not phase 2) of the AP, whereas the activation of Na+-Ca2+ exchanger forward current drives phase 2.
- Current through L-type Ca2+ channel is terminated by voltage-dependent deactivation.

Ventricular myocytes are electrically and metabolically coupled. These couplings determine the electric properties of the heart.

**Novelty and Significance**

Unfortunately, these currents are usually measured in isolated myocytes, which differ significantly in their properties from the functional syncytium. In the intact heart, the electric and metabolic status will determine the key mechanisms involved in AP generation. Here, using the intact heart, we recorded ionic currents underlying the AP. Our approach revealed that the Ca2+ influx through L-type Ca2+ channels that triggers sarcoplasmic reticulum Ca2+ release occurs during phase 1, whereas phase 2 results from Na+-Ca2+ exchanger activity. These findings suggest that the mouse epicardial AP is different from other reported ventricular APs, where the activity of L-type channels has been indicated as being responsible for phase 2 of the AP. Moreover, our findings also indicate that Ca2+-dependent inactivation is not the central mechanism for current termination during AP. This approach will help to address questions that can only be evaluated on functional hearts providing a bridge between classical electrophysiological studies and cardiac function under normal and pathological conditions.
Intact Heart Loose Patch Photolysis Reveals Ionic Current Kinetics During Ventricular Action Potentials
Josefina Ramos-Franco, Yuriana Aguilar-Sanchez and Ariel L. Escobar

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Supplemental Material

Loose Patch Photolysis setup

The system is based on three major parts. *First*, a multiple voltage clamp system that allows the clamping of the potential inside the loose patch pipette and measure the membrane current (A). A second voltage clamp to voltage clamp surrounding bath (B). And *C*, an amplifier to electrically measure the membrane potential. *Second*, a PLFFM to optically record Ca^{2+} transients and APs (D). Finally, a flash photolysis system that can photo-break different photosensitive compounds under the loose patch pipette. Photolysis was produced by UV illumination generated by a DPSS UV laser (355 nm). UV light was optomechanically shuttered for 1-50 ms and applied through an external quartz multimode fiberoptic (E).
Nifedipine rebinding.

Online Figure II

Effect of nifedipine rebinding after the UV photolytic pulse. As the heart is continuously perfused with a tyrode solution containing nifedipine, the uncaged nifedipine can rebind to DHPR that were previously blocked by nifedipine. (N= 4 hearts)