Intracellular Ca\(^{2+}\) release underlies the development of phase 2 in mouse ventricular action potentials

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Intracellular Ca\(^{2+}\) release underlies the development of phase 2 in mouse ventricular action potentials. Am J Physiol Heart Circ Physiol 302: H1160–H1172, 2012. First published December 23, 2011; doi:10.1152/ajpheart.00524.2011.—The ventricular action potential (AP) is characterized by a fast depolarizing phase followed by a repolarization that displays a second upstroke known as phase 2. This phase is generally not present in mouse ventricular myocytes. Thus we performed colocalized electrophysiological and optical recordings of APs in Langendorff-perfused mouse hearts founding a noticeable phase 2. Ryanodine as well as nifedipine reduced phase 2. Our hypothesis is that a depolarizing current activated by Ca\(^{2+}\) released from the sarcoplasmic reticulum (SR) rather than the “electrogenicity” of the L-type Ca\(^{2+}\) current is crucial in the generation of mouse ventricular phase 2. When Na\(^{+}\) was partially replaced by Li\(^{+}\) in the extracellular perfusate or the organ was cooled down, phase 2 was reduced. These results suggest that the Na\(^{+}/Ca^{2+}\) exchanger functioning in the forward mode is driving the depolarizing current that defines phase 2. Phase 2 appears to be an intrinsic characteristic of single isolated myocytes and not an emergent property of the tissue. As in whole heart experiments, ventricular myocytes impaled with microelectrodes displayed a large phase 2 that significantly increases when temperature was raised from 22 to 37°C. We conclude that mouse ventricular APs display a phase 2; however, changes in Ca\(^{2+}\) dynamics and thermodynamic parameters also diminish phase 2, mostly by impairing the Na\(^{+}/Ca^{2+}\) exchanger. In summary, these results provide important insights about the role of Ca\(^{2+}\) release in AP ventricular repolarization under physiological and pathological conditions.

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VENTRICULAR REPOLARIZATION is a key event in understanding cardiac electrical activity, both under normal or pathophysiological conditions (51). Although ventricular action potentials (APs) usually present a late depolarizing phase (phase 2) before the final repolarization (8), electrical signals recorded from different ventricular layers (i.e., epicardium, mid-myocardium, and endocardium; Ref. 33) display different kinetic features of this repolarization process. These kinetic differences are responsible for a transmural repolarization gradient throughout the ventricular wall (34). Moreover, this functional sequence of events sheds light on a variety of clinical electrocardiographic problems related to cardiac arrhythmias (10, 20). Furthermore, epicardial APs display a complex morphology named “spike and dome” behavior (33). Changes in the time course of this process are commonly observed in several pathological conditions such as the appearance of the J wave during hypothermia (55) and the Brugada syndrome (54). In both conditions, these alterations can serve as a substrate for the genesis of arrhythmias.

Several experimental animal models, showing human-like electrophysiological characteristics (i.e., canine or feline), have been used to mimic in vitro and in vivo alterations in the electrical behavior of the epicardium (19). However, those animal models do not have the genetic advantage of mouse models where cardiac transgenesis has been well established (18). Unfortunately, mouse cardiac electrophysiological properties have been considered to be different from those of other mammals. For example, durations reported for mouse APs are on average up to 10 times shorter than human ones (23, 40). Furthermore, compelling evidence from experiments performed on isolated mouse ventricular cardiac myocytes shows that the repolarization of the AP is highly monotonic lacking of a distinguishable phase 2 (1, 9, 22, 25, 29, 30, 35, 40, 50, 52). This specific characteristic has set some limitations on the use of mice as a model for the study of syndromes where alterations of the repolarization of the ventricle are central in the genesis of the disease.

In the present study, we evaluated the controversial subject of the presence of phase 2 in mouse hearts and the mechanisms involved in the genesis of phase 2. We tested the hypothesis that a depolarizing current activated by Ca\(^{2+}\) released from the sarcoplasmic reticulum (SR) rather than the “electrogenicity” of the L-type Ca\(^{2+}\) current is crucial in the generation of mouse ventricular phase 2. This mechanism has been suggested to be involved in the development of a slow phase of repolarization in different experimental models (rat, the cardiac structure (trabeculae), and experimental conditions (temperature of 26°C) rather than the ones presented in the present study (46).

To test our hypothesis, we first performed several experimental series to resolve the controversy between patch-clamp measurements in isolated myocytes and our optically recorded epicardial APs. We also took into account the effect of the regional localization of the epicardial recordings, the experimental approach (optical vs. electrical), the temperature, the dependency on intracellular Ca\(^{2+}\) release, the role of the Ca\(^{2+}\) conductances in the plasma membrane, the transport of Na\(^{+}\) ions through the Na\(^{+}/Ca^{2+}\) exchanger, and the emergent properties of the tissue.

We conclude that APs recorded from ventricular myocytes show phase 2 both at the whole heart and at the isolated cell levels. This phenomenon is highly dependent on temperature and intracellular Ca\(^{2+}\) dynamics. Finally, we postulate that phase 2 appears as a consequence of the activation of ionic conductance/transport (i.e., Na\(^{+}/Ca^{2+}\) exchanger) driven by Ca\(^{2+}\) release from the SR.
METHODS

Whole heart preparation. Hearts were dissected from young C57BL/6 mice (3 to 7 wk old). Briefly, each animal was euthanized by cervical dislocation and the heart was rapidly removed. The heart was placed in the recording chamber, where the aorta was cannulated and placed into a horizontal Langendorff apparatus (48). The heart was constantly perfused with normal Tyrode solution containing the following (in mM): 2 CaCl₂, 140 NaCl, 5.4 KCl, 1 MgCl₂, 0.33 Na₂HPO₄, 10 HEPES, and 10 glucose, pH 7.4 at 37°C. In Li⁺ replacement experiments, NaCl was equimolarly replaced with LiCl. The temperature of the solution outside the heart was controlled with a Peltier unit. Pacemaker cells of the sinoatrial and atrioventricular nodes were electrically ablated with the aid of an ophthalmic bipolar pencil (Mentor Ophthalmics, Santa Barbara, CA) to gain external control of the heart rate. During the experiments, each heart was continuously paced at 4 or 5 Hz through a silver bipolar electrode placed at the base of the left ventricle. The stimulus electrodes were wired to an electrical stimulator ISOSTIM A320 (World Precision Instruments, Sarasota, FL) controlled by a PC. All solutions were equilibrated with 100% oxygen. This protocol was approved by the University of California Merced Institutional Animal Care and Use Committee.

Ventricular myocyte dissociation. Mouse ventricular myocytes were enzymatically isolated using the Langendorff coronary retroperfusion technique (38). Briefly, each heart was placed on a Petri dish containing a Tyrode solution at 37°C. The aorta was cannulated, and the coronary arteries were washed with Tyrode solution for 6 min, followed by 6 min with a Ca²⁺-free Tyrode solution containing the following (in mM): 140 NaCl, 5.4 KCl, 0.33 Na₂HPO₄, 10 HEPES, and 10 glucose, pH 7.4 at 32°C. The heart was then perfused for 7 min with Ca²⁺-free Tyrode solution containing 1 mg/ml type 2 collagenase (322 U/mg) (Worthington Biochemical). Subsequently, hearts were perfused with a Ca²⁺-free Tyrode solution and the cells were dissociated by the aid of a fire-polished glass Pasteur pipette in the presence of 1 mg/ml of BSA. Finally, the cells were filtered with a nylon mesh and maintained in Tyrode solutions containing 0.2 and 0.1 mg/ml of BSA. Perfusion with the dye began after the spontaneous heart rate became regular (within 10 min after cannulation). The time of loading was 25–35 min.

Membrane potential was optically measured with the photometric dye di-8-annepes (Invitrogen). The dye (10 μg) was dissolved in 45 μl of DMSO with 2.5% pluronic and added to 1 ml of normal Tyrode. Perfusion with the dye was carried out at room temperature for 30 min, then the perfusate was switched to Tyrode solution, and the temperature was increased to 37°C. Downward fluorescence signals reflecting plasma membrane depolarization were used as an indication of dye loading. After di-8-annepes was removed from the perfusate, the amount of dye that remained in the plasma membrane and the tubular system was enough to generate a detectable signal for ≥3 h. Optical recordings of the membrane potential were calibrated against intracellular microelectrode measurements for each experiment.

Optical setup. A modified version of our custom-made pulsed local field fluorescence (PLFF) microscopy setup (14, 37) was employed to measure APs from intact hearts. Solid-state neodymium-doped yttrium aluminum garnet (Nd-YAG) lasers were used as an illuminating source. Green light (532 nm) was obtained from a MGL-50B-1 CW Nd-YAG laser (Enlight Technologies, Branchburg, NJ).

The pulses of light used for excitation were focused with an aspheric lens (Thorlabs; NA 0.47) into multimode optical fibers for the transmission of the exciting light to the epicardial layer (32, 47). The light emitted by the dye was carried back through the same fiber, filtered with a 590-nm emission barrier filter to eliminate the reflected excitation component, and focused on an avalanche diode. The photocurrent was finally amplified by a resistive current-to-voltage converter controlled by a Digital Signal Processor (DSP 320; Texas Instruments). The head-stage unit and their corresponding high-voltage power supply were custom built. The electrical signals produced by the measurements of fluorescence were filtered to a bandwidth of 500 kHz and sampled at a frequency of 5 MHz. The acquisition system was controlled by a PC running a custom-designed G-based software program (LabVIEW; National Instruments).

Finally, one end of the optical fiber was gently placed a few hundreds of micrometers from the tissue surface. The motion of the heart was noticeably decreased upon addition of 10 μM blebbistatin to the perfusing Tyrode solution. A number of studies (11, 16, 17, 47) have shown that, at this concentration, blebbistatin has no significant side effects on either Ca²⁺ transients or APs.

Whole heart electrophysiological measurements. Transmembrane APs were recorded from the left atrium and epicardial layer of the right and left ventricles over the anterior wall. The signals were recorded using glass microelectrode filled with 3 M KCl (10–20 MΩ resistance) connected to a high input impedance amplifier (World Precision Instruments-Duo 773 Electrometer). The electrical signals were filtered to a bandwidth of 500 kHz, digitally sampled at a frequency of 5 MHz, and then digitally resampled to 2 kHz. The acquisition system was controlled by a PC running a custom-designed G-based software program (LabVIEW; National Instruments).

AP refractoriness was obtained by applying an additional stimulation pulse at different times with respect to the regular pacing pulses. By changing the time interval between electrical stimulations, we were able to obtain the values for the refractory period.

Single myocytes electrophysiological measurements. Ventricular myocyte APs were measured under current-clamp conditions (Axopatch 200B; Molecular Devices) using intracellular sharp microelectrodes filled with 3 M KCl (40–60 MΩ resistance). Pipette resistance and capacitance was analogically compensated to improve the recordable bandwidth. Data were filtered at 10 kHz and digitized through a Digidata 1440A acquisition system (Molecular Devices) with pClamp 10 software at a sampling rate of 100 kHz. Ventricular myocytes were placed on a chamber in the stage of an inverted microscope (Olympus IX70). Myocytes were continuously perfused with oxygenated Tyrode solution at 37°C.

Analysis of the recordings. An integration-subtraction procedure was utilized to measure the contribution of phase 2 to the total area under the AP. First, phases 0 and 1 of the AP were fitted with an exponential function of the form:

\[ V_m(t) = V_{m_0} + V_{amp} \left(1 - e^{-\frac{t-t_1}{\tau_m}}\right) e^{\frac{t-t_1}{\tau_{ap}}}, \]

where \( V_m \) is the resting membrane potential, \( V_{amp} \) is the amplitude of the AP, \( \tau_m \) is the time constant for phase 0, and \( \tau_{ap} \) is the time constant for phase 1, and \( t_1 \) is the delay time with respect to the electrical stimulus. Note that first, all the amplitudes were measured as a difference between the actual membrane potential and the resting membrane potential.

Second, the area under this function \( V_{ap} \) was calculated using a numerical integration procedure (Aap). Finally, the contribution of the phase 2 to the total AP was calculated using the following expression

\[ \%ph_{ph_1} = \frac{A_{ap} - A_{ap01}}{A_{ap}} \cdot 100 = \frac{A_{ap2} - A_{ap02}}{A_{ap}} \cdot 100 \quad \text{where } A_{ap2} = A_{ap} - A_{ap01} \]

Statistical analysis. Data are expressed as means ± SE; \( n \) indicates the number of independent experiments. The total number of animals used in this study was 52. Statistical significance was tested using
ANOVA. The difference was considered to be significant if the value of \( P \) was <0.05.

**Drugs and chemicals.** All reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) unless indicated.

**RESULTS**

**Presence of phase 2 in epicardial AP.** Mouse epicardial electrical activity has usually been measured using monophasic AP recordings (29). Although this approach is very useful to detect the presence of local APs, it only provides a qualitative description of the epicardial electrical activity. In this study, we performed PLFF microscopy measurements on mouse hearts previously loaded with the potentiometric dye di-8-anneps. Figure 1A shows both an optically and electrically recorded AP from the left ventricle using a 200-\( \mu \)m optical fiber and a intracellular sharp microelectrode, respectively. The four phases of a ventricular AP can be clearly observed. Two very distinguishable features are a very deep phase 1 and a prominent phase 2. In Fig. 1C, the contribution of phase 2 to the AP is shown in a bar graph for the electrical and optical recordings. There are no significant differences in the AP kinetic parameters (\( n = 15 \) animals) using either method. Figure 1C, inset, shows a graphical scheme that illustrates how the contribution of phase 2 to the AP was evaluated. A detailed description of the mathematical procedure has been described in METHODS. This method for evaluating the contribution of phase 2 to the AP provided us with a parameter that has less scattering than the usual report of AP durations (APDs) at different levels of repolarization. However, the contribution of phase 2 to the atrial AP was negligible (Fig. 1B). A comparison of the contribution of phase 2 to the repolarization of the AP between the left atrium and the left ventricle is shown in a bar graph presented in Fig. 1D. The observed differences (15 animals left ventricle and 5 animals left atrium) are statistically significant (\( P < 0.05 \)). Additionally, APD at 50 and 90% of the repolarization (APD\(_{50}\) and APD\(_{90}\)) are also presented in Table 1.

Potentiometric dyes not only stain the surface membrane but the T system as well (49). On the other hand, electrical recordings are self-calibrated, and although the myocytes are electrically connected between them, the electrical signal measured with this technique is spatially confined by the space constant \( \lambda \) of the tissue. In Fig. 2A, we show simultaneous electrical and optical APs recordings measured from the epicardium of the left ventricle in a mouse heart perfused with a Langendorff apparatus. Similar kinetic features as the deep phase 1 and a prominent phase 2 are evident in both optical and electrical recordings.

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**Fig. 1. Phase 2 in epicardial ventricular action potential (AP).** A: typical optical recording of a left ventricle AP. Four phases of AP can be clearly observed. B: electrical AP recordings measured from the left ventricular (LV) epicardium of a perfused mouse heart. Both types of recordings (A) show identical kinetic features (B). Optical recordings of left atrium AP, the AP traces (\( A_{opt} \)) relaxed monotonically to the resting membrane potential. C: comparison between the contribution of phase 2 to left ventricle and left atrium AP (\( n = 15 \) animals left ventricle and 5 animal left atrium) is statistically significant (\( P < 0.01 \)). Inset: graphical scheme that illustrates how the contribution of phase 2 to the AP was evaluated. D: bar graph of the percentage of phase 2 contributions to the epicardial AP. There are not significant differences between the AP kinetic measured with either of 2 techniques. Recordings were performed at 37°C and externally paced at 5 Hz. Electrical recordings were performed by impaling cells with a 20 M\( \Omega \) microelectrode and optical recordings using a 200-\( \mu \)m optical fiber.
Table 1. *Intact heart AP parameters*

<table>
<thead>
<tr>
<th>LV Epicardium (5 Hz/37°C)</th>
<th>Electrical AP (n = 15)</th>
<th>Optical AP (n = 15; 200-μm Ø fiber)</th>
<th>Electrical AP (n = 5)</th>
<th>Optical AP (n = 5; 1,000-μm Ø fiber)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential</td>
<td>mV</td>
<td>66.7 ± 1.3</td>
<td>73.2 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>mV</td>
<td>76.2 ± 1.7</td>
<td>83.8 ± 3.5</td>
<td>63.1 ± 2.9</td>
</tr>
<tr>
<td>Phase 0, mV</td>
<td>40.1 ± 0.5</td>
<td>41.2 ± 0.9</td>
<td>42.7 ± 1.8</td>
<td>39.9 ± 3.3</td>
</tr>
<tr>
<td>Phase 2, mV</td>
<td>40.5 ± 0.6</td>
<td>41.7 ± 1.0</td>
<td>45.3 ± 1.2</td>
<td>42.0 ± 3.2</td>
</tr>
<tr>
<td>Magnitude</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1, mV</td>
<td>36.1 ± 1.6</td>
<td>35.1 ± 5.1</td>
<td>40.7 ± 4.3</td>
<td>23.7 ± 4.0</td>
</tr>
<tr>
<td>APD50%, ms</td>
<td>87.2 ± 3.2</td>
<td>87.6 ± 3.2</td>
<td>102.6 ± 5.8</td>
<td>100.3 ± 7.6</td>
</tr>
<tr>
<td>APD90%, ms</td>
<td>33.3 ± 5.4</td>
<td>37.6 ± 4.6</td>
<td>51.3 ± 12.8</td>
<td>54.9 ± 14.1</td>
</tr>
<tr>
<td>Time to peak, ms</td>
<td>2.6 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>5.8 ± 0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 15 hearts. Action potential (AP) parameters of mouse ventricular epicardium recorded at 37°C and 5 Hz. APD90%, AP duration at 90% repolarization; APD50%, AP duration at 50% repolarization. *P < 0.05.

electrical measurements, when a 200-μm multimode optical fiber and a 20 MΩ microelectrode were used at the same location. Usually, an important limitation of measuring intracellular signals from multicellular preparations in three dimensions is to know where the measured signals are coming from. For example: how different myocytes, located across and along the ventricular wall of an intact beating heart, will contribute to the measured intracellular AP? We have already shown that the PLFF microscopy provides the possibility to measure fluorescence signals from different tissue levels depending on the transmural depth of our recordings and the contribution of APs from deeper layers.

Nonetheless, phase 2 was clearly present independently of the transmural depth of our recordings and the contribution of this phase to the AP was not significantly different for measurements obtained with optical fibers having different diameters (Fig. 2C). Table 1 summarizes the differences between AP kinetic parameters measured with the two types of optical fibers and the simultaneous electrical recordings.

In canine models, the distribution of the time courses of APs depends not only on the transmural dispersion but also present differences along the epicardial layer. To test whether these epicardial differences were also present in the mouse model, we performed experiments to map the epicardial AP waveforms. Regions like the base, middle, and apex of the left ventricle and the middle of the right ventricle were measured. Traces of all epicardial recordings are shown in Fig. 3A. We

Fig. 2. Phase 2 in epicardial ventricular AP: effect of the fiber optic diameter. A: comparison of typical electric and optical recordings when a 200-μm optical fiber was used; traces are mostly identical. B: electrical and optical traces measured with a 1,000-μm optical fiber show significant differences in the delay time from the stimulus and the time to peak (see Table 1). C: bar graph that shows the contribution of phase 2 to the AP for simultaneous electrical and optical recordings using a 1,000-μm optical fiber. Although the phase 2 contribution seems to be larger for the optical recording, no statically differences were found between the 2 type of measurements (n = 5 animals). All the recordings were performed at 37°C, and the electrical recordings performed with a 20-MΩ microelectrode.
can observe that the magnitude of phase 1 and the APD$_{90}$ of the repolarization are different for the various recording areas. In addition, a plot of the relative contribution of phase 2 for the different regions is shown in Fig. 3B. This plot shows that although there are no significant differences between optical and electrical recordings for the same region, the differences between regions are highly statistically significant ($P < 0.05$; $n = 4–15$ hearts).

The fact that epicardial APs recorded from different regions of the heart displayed a significant phase 2 at a heart rate of 5 Hz opened the question of what will happen with the APD at a higher heart rate. Indeed, the resting heart rate of the mouse heart is close to 600 beats/min (10 Hz). To test if phase 2 in epicardial APs was compatible with a physiological heart rate, we performed experiments pacing the heart at different rates. Figure 4 shows the results from these experiments. Figure 4A illustrates the AP waveforms at heart rates from 5 to 10 Hz. It is possible to observe that phase 2 gets shorter with no changes in phase 1 duration. Both the APD$_{90}$ (Fig. 4B) and the contribution of phase 2 to the AP (Fig. 4C) become significantly shorter and smaller as the heart rate increases. Note that the APs still show a significant contribution of phase 2.

Relationship between epicardial phase 2 and intracellular Ca$^{2+}$ dynamics. Ventricular AP phase 2 has been largely associated with an influx of Ca$^{2+}$ through the L-type voltage-activated Ca$^{2+}$ channel (dihydropyridine receptor) (8, 44). To test if in mouse models phase 2 has the same pharmacological properties as in other mammalian species, we perfused hearts with 10 $\mu$M of nifedipine (a Ca$^{2+}$ channel blocker). Figure 5A shows optical and electrical APs recordings from the left ventricle, where the application of nifedipine has a profound effect on the AP repolarization. This effect was completely removed after 15 min of washout of the drug. Figure 5B illustrates the statistical analysis of the contribution of phase 2 during the application and the washout of the drug. Nifedipine induced a significant reduction of the phase 2 ($n = 5$ animals; $P < 0.01$).

In other mammalian models, dihydropyridines not only reduce the Ca$^{2+}$ current through L-type Ca$^{2+}$ channels (26) but also reduce Ca$^{2+}$ released from the SR in an indirect way. This mostly occurs by an impairment of the Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) process due to a reduction in the size of the Ca$^{2+}$ trigger. Figure 5C exemplifies the effect of nifedipine on epicardial Ca$^{2+}$ transients measured by PLFF microscopy. Hearts loaded with the Ca$^{2+}$ indicator rhod-2 were perfused with a Tyrode solution containing 10 $\mu$M of nifedipine. The drug produced a significant decrease in the amplitude of the Ca$^{2+}$ transients ($n = 5$ animals); as shown in Fig. 5C, the decrease produced by nifedipine is statistically significant (Fig. 5D).
Although the current opinion on the role of L-type Ca\(^{2+}\) channels on the repolarization of the AP is that the Ca\(^{2+}\) current itself is mainly responsible for this long depolarizing effect during phase 2, it is possible that the Ca\(^{2+}\) released from the SR plays a key role in the genesis of the AP phase 2. Ryanodine, an alkaloid that binds to the SR Ca\(^{2+}\) release channel and locks the channels in a high open probability low subconductance state, and thapsigargin, a noncompetitive inhibitor of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase pump, were used to impair SR Ca\(^{2+}\) release. Figure 5G illustrates the effect of 10 \(\mu\)M ryanodine and 2 \(\mu\)M thapsigargin on Ca\(^{2+}\) transients measured on the left ventricle epicardial layer. A dramatic and significant decrease in the amplitude of the Ca\(^{2+}\) transients was observed even though ryanodine does not have a direct effect on the dihydropyridine receptor (Fig. 5H). Figure 5E shows the effect of 10 \(\mu\)M ryanodine and 2 \(\mu\)M thapsigargin on the time course of epicardial AP. The elimination of SR Ca\(^{2+}\) release produces a very similar effect as nifedipine. Figure 5F shows the statistical analysis for the contribution of phase 2 to the repolarization of the AP.

**On the mechanism that transduces intracellular Ca\(^{2+}\) dynamics into phase 2 of epicardial AP.** Experiments illustrated in Fig. 5 show that contribution of phase 2 to the repolarization of the AP dramatically depends on SR Ca\(^{2+}\) release. Additionally, our laboratory has already reported that the kinetics of intracellular Ca\(^{2+}\) transients recorded from the left ventricle epicardial layer highly depends on temperature (32). Therefore, it is likely to expect that temperature will have a strong influence on the phase 2. We performed experiments to test this hypothesis, and the results are shown in Fig. 6. Temperature has an important effect in all the kinetic parameters of the AP. Specifically, if we increase the temperature from 23 ± 1°C (Fig. 6B) to 37 ± 1°C (Fig. 6A), it is possible to observe a decrease in the magnitude of phase 1 and an increase in the contribution of phase 2 to the AP repolarization. A statistical analysis of the phase 2 contribution is presented in Fig. 6C. Temperature has a significant effect on phase 2 in both optically and electrically recorded APs (\(n=5\) animals; \(P<0.01\)). As temperature was increased from room temperature to 37°C the contribution of phase 2 increases by 119 ± 22%. This suggests that not only the kinetics of the Ca\(^{2+}\) transients but also that the mechanism that translates the changes in myoplasmic Ca\(^{2+}\) concentration into a depolarizing current during phase 2 are extremely temperature dependent (see DISCUSSION). The fact that an increase in temperature induces an increase in phase 2 is somehow counterintuitive. In fact, it is reasonable to expect that an increase in temperature will accelerate any kinetic process. However, the APD\(_{50}\) increased from 13.66 ± 0.3 to 43.27 ± 0.8 ms (\(n=5\) animals) when the temperature was increased from 23°C to 37°C. On the other hand, this same increment in the temperature did not produce a significant change in the APD\(_{50}\) (79.3 ± 6.8 ms to 92.7 ± 6.0 ms; \(n=5\) animals; \(P>0.05\)). This is consistent with the idea that temperature mostly changes the morphology of the AP. Additionally, there were also no significant changes in the resting membrane potential when temperature was changed (−70.76 ± 2.9 to 72.47 ± 4.5 mV; \(n=5\) animals; \(P>0.05\)).

To further explore if there were other AP kinetic properties modified by temperature in an anomalous way, we performed double-pulse experiments to evaluate the temperature dependency of the epicardial AP refractory period. Data presented in Fig. 6, D–F, show that an increase in the temperature produced a decrease in the refractory period. The reduction in the refractory period could be explained by a faster recovery from inactivation of Na\(^{+}\) channels when temperature was increased (39) from 23°C to 37°C. Additionally, the enlargement in the rate of depolarization (phase 0) for the same temperature change could also contribute to the reduction of the refractory period at higher temperatures. Indeed under this condition, the time to peak of the AP was significantly reduced from 5.37 ± 0.95 to 2.24 ± 0.55 ms (\(n=5\) animals; \(P<0.05\)).

The high temperature dependency of phase 2, in addition to the effect of intracellular Ca\(^{2+}\) dynamics on the repolarization of the AP, drove us to speculate that the depolarizing membrane current during phase 2 was carried through a Ca\(^{2+}\)
Phase 2 in Mouse Ventricular Action Potential

A

Control (LV)  
Nifedipine 10 μM (LV)  
Washout (LV)

B

Control
Nifedipine 10 μM
Washout

Nifedipine
Electrical AP  
Optical AP (200 μm fiber)

5 Hz 37°C

C

Control
Nifedipine 10 μM

0.3 F

20 ms

D

Control
Nifedipine 10 μM

*p<0.01

Ca²⁺ Transient

5 Hz 37°C

E

Control (LV)  
Ryanodine (Ry) 10 μM
Thapsigargin (Thap) 2 μM

F

Control
Ry. + Thap.

*p<0.01

Ca²⁺ Transient

5 Hz 37°C

G

Control
Ry 10 μM + Thap 2 μM

0.2 F

20 ms

H

Control
Ry. + Thap.

*p<0.01

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mediated transporter. The most likely transmembrane protein governing this kinetic process appears to be the Na+/Ca2+ exchanger. Unfortunately, known pharmacological antagonists of this transporter also interact with other membrane proteins involved in the CICR process. On the other hand, it is well known that Li+ cannot be transported through the Na+/Ca2+ exchanger. Furthermore, Li+ can permeate just as well as Na+ through the voltage-dependent Na+ channel allowing the upstroke of an AP. Thus the equimolar replacement of Na+ by Li+ in the external Tyrode solution can serve as a way to evaluate the fractional contribution of the Na+/Ca2+ exchanger to the phase 2. Experiments testing this idea are shown in Fig. 7A where the effect of replacing half of the NaCl by LiCl in optically recorded APs is presented. This fractional equimolar substitution of Na+ by Li+ has a dramatic effect on the AP repolarization. However, the effect on the repolarization of the AP shown in Fig. 7A can be explained as a direct reduction of the transport turnover number of the Na+/Ca2+ exchanger or by a direct effect of the Li+ treatment on the amplitude of the Ca2+ transient. If indeed Li+ reduces the Ca2+ released from the SR, then an acceleration of the repolarization process is also expected to be observed. To test this latter idea, we

Fig. 6. Phase 2 in epicardial ventricular AP: temperature dependency. A and B: effect at 37°C (A) and room temperature (23 ± 1°C) (B) on AP recorded electrically and optically. Decrease in temperature accelerates the late repolarizing phase of APs. Graph bar presented in C shows that temperature has a significant effect on the phase 2 contribution to the AP (n = 5 animals; P < 0.01). D and E: typical double pulse experiments design to measure the absolute refractory period (ARP) at 37°C and at 23 ± 1°C, respectively. It is possible to observe that although the AP are longer at 37°C than at 23 ± 1°C the ARP is shorter (F; n = 5 animals; P < 0.02). Experiments were obtained by externally pacing the heart at 4 Hz and AP measurements were performed by means of glass microelectrodes or by using 200-μm optical fibers.
measured the amplitude of the epicardial Ca\(^{2+}\) transients measured under the same experimental conditions than the one described for Fig. 7A but using the indicator rhod-2 to track the intracellular Ca\(^{2+}\). The results presented in Fig. 7B show that the Li\(^+\) treatment does not diminish the amplitude of the Ca\(^{2+}\) transient; instead it induces an increment of this intracellular signal most likely due to an increase in intra SR Ca\(^{2+}\) load. A summary of the results obtained in AP recordings when Na\(^+\) was equimolarly replaced by different fractions of Li\(^+\) is presented in Fig. 7C. Phase 2 contribution to the AP was significantly decreased when 50 and 75% of Na\(^+\) was replaced by Li\(^+\). These results indicate that most of the membrane current that depolarizes the AP during phase 2 is carried through the Na\(^+\)/Ca\(^{2+}\) exchanger when this transporter is activated by Ca\(^{2+}\) released from the SR.

Phase 2 at the single cell level. Most of our actual knowledge on the kinetics of ventricular mouse APs comes from isolated ventricular myocyte experiments, where membrane potentials have been recorded using the whole cell patch-clamp recording under current-clamp conditions (3, 22, 36). Although this is a very powerful approach, it has the disadvantage that the myocytes are usually dialyzed through the recording pipette. Additionally, the internal solution usually contains a high concentration of the Ca\(^{2+}\) buffer EGTA to prevent cell contraction during an AP-induced Ca\(^{2+}\) release. Moreover, usually these experiments are generally performed at room temperature (22) to maintain the myocyte viability for a longer time. Experiments published under these conditions never display a phase 2 during the repolarization of the AP. To test if phase 2 is a single cell property that has been impaired by the factors described above, we performed experiments in enzymatically isolated ventricular myocytes impaled with high resistant sharp microelectrodes at 37°C. Figure 8A illustrates a chart of APs every 200 ms elicited by current pulses applied through the

![Fig. 7. Na\(^+\)/Ca\(^{2+}\) exchanger regulates phase 2 in ventricular AP.](image1)

A: equimolar substitution of 70 mM of NaCl by 70 mM LiCl in the extracellular perfusate dramatically reduces the contribution of phase 2 to normalized optically recorded AP from the left ventricle of intact mouse hearts (n = 5 animals; P < 0.02). Traces are illustrated in arbitrary units (AU). B: Effect of replacing 70 mM Na\(^+\) by 70 mM Li\(^+\) on Ca\(^{2+}\) transients. Amplitude of the Ca\(^{2+}\) transients was increased by 20% (n = 5 animals; P = 0.004). Traces are presented as absolute fluorescence. C: bar graph demonstrating the incremental effect of Li\(^+\) replacement on the phase 2 contribution to the AP (n = 5 animals). All experiments were performed at 37°C, and the hearts were paced a 4 Hz.

![Fig. 8. Phase 2 in ventricular AP: isolated myocytes.](image2)

A: isolated myocytes APs recorded under current clamp using 50-MΩ sharp microelectrodes. Cell was hyperpolarized to −79 mV and electrically stimulated by injecting short (0.2 ms) depolarizing current pulses through the recording microelectrode. All the phases of the AP (including phase 2) are present in the recording. B: effect of temperature on the repolarization of the AP. It can be observed that the reduction of the temperature accelerate the repolarization. This effect is further illustrated in C, where the temperature dependency of the phase 2 contribution to the AP has been plotted in a bar graph. At lower temperatures, a significant reduction can be observed in phase 2 (n = 4–6; P < 0.002).
microelectrode when the membrane potential was held at −79 mV. Under these experimental conditions, it is possible to clearly observe all the phases of the AP: a fast phase 0, a very deep phase 1, and a distinguishable phase 2. To evaluate if single cell phase 2 was also temperature dependent, we performed experiments at room temperature and 37°C. A typical recording of this type of experiment in the same cell is exemplified in Fig. 8B, and it shows a very similar phase 2 temperature trend as that recorded in the intact ventricle when the temperature was increased from room temperature to 37°C. Figure 8C shows a bar graph that represents the statistics on the contribution of phase 2 to the AP at two different temperatures (n = 4 to 6 animals; P < 0.002). Illustration of the kinetic parameters of single cell APs is shown in Table 2. These results indicate that mouse ventricular AP phase 2 is a characteristic of the single myocyte and not an emergent property of ventricular tissue.

**DISCUSSION**

*Phase 2 on intact perfused mouse hearts.* The presence of phase 2 during the repolarization of mouse ventricular AP has been a very controversial subject (40, 43). Indeed, this electrophysiological characteristic has challenged the utility of the mouse as a viable alternative to other larger mammalian models for the study of electrocardiological disorders. In this study, we evaluated the molecular and cellular mechanisms that delineate the kinetics of mouse ventricular AP repolarization. Using several different experimental approaches, we found that all of them show clear evidence of a strong contribution of phase 2 during the ventricular AP repolarization (Fig. 1). The fact that the small diameter optical fiber (200 μm) recordings and electrical recordings using sharp microelectrodes measurements are not significantly different suggests that cells beneath the tip of the optical fiber are sufficiently isopotential compared with the intracellular microelectrode recording (Fig. 2A). However, significant differences were found between the optical and the electrical recordings when a larger diameter optical fiber (1,000 μm) was used to optically measure the APs (Fig. 2B). The most important differences between a recording with a large diameter optical fiber and the intracellular microelectrode electrical measurement were the slower rise phase (phase 0) and the smaller magnitude of phase 1 of the optical recorded AP (1,000 μm). In addition, an early appearance of phase 0 was observed in optical recordings when compared with the electrical measurements. We have already shown that the fiber diameter does not only define the lateral optical spatial resolution but the optical axial resolution as well (37). For example, a fiber having a larger diameter not only delivers and collects photons from a wider area but also can detect signals from deeper layers within the tissue. The fact that the depolarizing phase 0 starts sooner in the optical recording than in the electrical one indicates that there are anatomical regions within the optical recording volume that depolarize earlier than the local intracellular measurement.

AP cannot only occur at different delay times in different regions of the epicardial layer (i.e., apex, base, left ventricle, right ventricle, etc.) but also can display disparity in morphology. This issue was explored in Fig. 3 where we showed that the middle of the left ventricle has a larger contribution of phase 2 than the apex and that the right ventricle displays a larger phase 2 than the left ventricle. These results indicate that there is a dispersion of the epicardial cellular repolarization properties from different regions of this outermost ventricular layer. Moreover, phase 2 is present during the repolarization of the epicardial AP at different heart rates (Fig. 4), indicating that at the intact heart level phase 2 is a physiological manifestation and not a mere consequence of a bradycardic heart rate.

**Comparison of whole heart AP recordings with previous reports.** There has been a common consensus in the field about the absence of phase 2 in mouse ventricular APs. With exception of our previous preliminary contributions (32, 47), there have been no reports using optical fiber techniques evaluating the kinetic features of ventricular AP repolarization in mouse models. However, several articles (2, 21, 36) have been published in Langendorff-perfused mouse hearts, using optical mapping techniques to measure ventricular APs. In the articles from Baker (2) and London (36), the authors conclude that there is no a plateau phase under control conditions. Glukhov (21) reported APDs that are longer than the ones reported by Baker (2) and London (36). Nevertheless, their recordings display a smaller distinguishable difference between phase 1 and phase 2 compared with the optical recordings presented in our current work. In general, differences between our data and optical mapping recordings could be interpreted as differences in optical resolution between both methods. Although optical mapping has differences with PLFF microscopy in spatial resolution, it is unlikely that these differences could explain alone the absence of phase 2. However, summarized scattering signals from the neighbor myocardium can introduce an important effect by making slower the rate of relaxation of phase 1. This could make the optical signal recorded by optical mapping to be more triangular in shape, masking phase 2 (36).

Additionally, several authors (1, 25, 29) have reported intracellular electrical recordings using sharp microelectrodes in whole hearts or reduced mouse ventricular preparations at a mammalian physiological temperature (~37°C). The studies of Knollmann (30) and Hong (25) showed ventricular APs that relax fast and monotonically, not presenting a phase 2 plateau. Additionally, Anumonwo (1) showed a prominent phase 2 in fibers from the conduction system but recordings from endocardial and epicardial layers showed no indication of a plateau phase. The differences between our results and the ones presented by these authors could be due to the use of pharmacological agents to reduce contraction that also affect Ca2+ release from the SR (29) or the use of reduced ventricular preparations (1, 27).

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Values are means ± SE; n = 6-4 hearts. AP parameters of mouse ventricular isolated myocytes recorded using current-clamp technique (5 Hz) at 37°C and room temperature. *P < 0.05, room temperature vs. 37°C.
Finally, there are multiple published reports (9, 29, 30, 50) of electrical recordings using extracellular MAPs in mouse ventricles at ~37°C. These authors do not see any indication of a phase 2 in the repolarization of APs. However, there is one report (34) where the authors described the existence of a plateau phase in their extracellular recordings. They suggested that the differences between their recordings and the ones from others rely on the fact that Liu’s (35) recordings were obtained in vivo.

Relationship between phase 2 and intracellular Ca\(^{2+}\) dynamics. A classical fingerprint of phase 2 in the repolarization of the AP is the role of the L-type Ca\(^{2+}\) current as the primary ionic current maintaining the plateau phase (4). Figure 5 shows that 10 \(\mu\)M nifedipine, a dihydropyridine that specifically blocks the voltage-dependent L-type Ca\(^{2+}\) channel, produces a significant change in the contribution of phase 2 to the repolarization of the mouse ventricular AP. This effect was completely washed out after perfusing the coronary arteries with a drug-free Tyrode solution for 15 min. This result is consistent with the idea that the L-type Ca\(^{2+}\) channel is a key molecular entity in the pathway that controls the genesis of the phase 2 during the AP repolarization. In most mammalian species, activation of the L-type Ca\(^{2+}\) current in the ventricular tissues is not only involved in maintaining the depolarization during phase 2, but it also triggers the opening of RyR2 located on the facing junction of the terminal cistern of the SR and activates the CICR process (15). In fact, blocking L-type Ca\(^{2+}\) currents with nifedipine will halt both processes as we show in Fig. 5, A and C. Traditionally, it has been accepted that the plateau phase is sustained by the depolarizing effect of the Ca\(^{2+}\) current and not as a consequence of the Ca\(^{2+}\) release by the SR. The results shown in Fig. 5E specifically challenge this concept. Our experiments were designed to evaluate if the Ca\(^{2+}\) released by the SR itself could have an impact in the genesis of phase 2. Without a reasonable doubt, this seems to be the case in hearts treated with a combination of ryanodine and thapsigargin. Neither of the drugs have a direct detectable effect on the L-type Ca\(^{2+}\) channels (14, 27) at the concentrations that were used in these experiments. This result suggests that the Ca\(^{2+}\) released from the SR can activate a Ca\(^{2+}\)-dependent depolarizing current that largely contributes to the phase 2 in APs measured in the ventricle of mouse models.

Even though the experiments shown in Fig. 5 revealed the role of intracellular Ca\(^{2+}\) dynamics in the repolarization of the AP, they are not sufficient to completely define the molecular entity of this plasmalemma Ca\(^{2+}\)-activated current involved in the genesis of phase 2. In principle, this depolarizing current can be carried by an efflux of anions or an influx of cations. Although Ca\(^{2+}\)-activated chloride channels have been identified in cardiac ventricular myocytes (53), an influx of cations is most likely to occur due to the larger electrochemical gradients for cations compared with the lower concentration gradients for chloride. Moreover, it has been shown that in Na\(^{+}/\text{Ca}^{2+}\) exchanger knockout animals APs repolarize faster than wild types at the single cell level (24, 41).

One typical difference between a membrane ionic flux carried through a voltage-activated channel and one mediated by a transporter is the temperature dependency of both processes. The amplitude of macroscopic L-type Ca\(^{2+}\) currents has only a very moderate temperature dependency with a Q\(_{10}\) of 1.56 (28), a single channel conductance with a Q\(_{10}\) of 1.63 (28), and a higher temperature dependency for the inactivation (Q\(_{10}\) 2.3) (31). In contrast, the activation of the Na\(^{+}/\text{Ca}^{2+}\) exchanger is extremely temperature dependent with a Q\(_{10}\) between 3 and 4 (5, 45; for a review, see Ref. 7). Following the same line of arguments, it is possible to expect that if the Na\(^{+}/\text{Ca}^{2+}\) exchanger activation is involved in the genesis of phase 2, the temperature will have a profound effect on this plateau phase of the ventricular AP repolarization. The results showed in Fig. 6 completely support this idea. At room temperature, the APs display a very moderate phase 2 that is significantly increased when the temperature was raised to 37°C. However, the lengthening of the APD\(_{50}\) produced by the appearance of phase 2 at 37°C did not increase the refractory period. In fact, the refractory period was significantly shorter at 37°C (Fig. 6, D-F). This decrease in the refractoriness at a longer APD can be explained mostly by a parallel increase in the recovery from inactivation rate and the activation rate of the Na\(^{+}\) channels (Nav1.5) without a significant change in the APD\(_{50}\) at higher temperatures. This idea is supported by the fact that the time to peak of the epicardial AP is significantly shorter at 37°C than at room temperature.

The temperature dependency of phase 2 is a compelling but not a conclusive evidence to demonstrate that the Na\(^{+}/\text{Ca}^{2+}\) exchanger is the transporting protein that defines the AP plateau. The classical way to deal with this paradigm is to use a pharmacological blocker/antagonist of the transporter. However, typical blockers of the Na\(^{+}/\text{Ca}^{2+}\) exchanger as KBR-7943 or SEA-0400 also block L-type Ca\(^{2+}\) channels in canine ventricular myocytes (6). As shown in Fig. 5 A a blockade of the L-type Ca\(^{2+}\) channel will dramatically reduce the AP phase 2. On the other hand, Li\(^{+}\) has traditionally been used as a way to discriminate between the transport through the Na\(^{+}/\text{Ca}^{2+}\) exchanger and other Na\(^{+}\)-dependent pathways (12, 45). Li\(^{+}\) can permeate though the Na\(^{+}\) channel but cannot be translocated through the active transport mechanisms such as the Na\(^{+}\)-K\(^{+}\) ATPase or the Na\(^{+}/\text{Ca}^{2+}\) exchanger. Figure 7A illustrates that the partial replacement of Na\(^{+}\) by Li\(^{+}\) dramatically reduces the contribution of phase 2 to the repolarization of the AP. Moreover, the effect on the AP repolarization is not due to an attenuation of the intracellular Ca\(^{2+}\) release from the SR. In the presence of Li\(^{+}\) (Fig. 7B), the amplitude of the Ca\(^{2+}\) was even larger than in control experiments. Furthermore, the larger the fraction of Na\(^{+}\) that was replaced by Li\(^{+}\) the larger the effect on phase 2 as seen in Fig. 7C.

To summarize, we propose that an influx of Na\(^{+}\) through the forward mode of the Na\(^{+}/\text{Ca}^{2+}\) exchanger is at least partially responsible for phase 2. Consequently, the temperature dependency of phase 2 is mostly defined by the thermodynamics of the Na\(^{+}\) influx driven through this transporter.

Phase 2 at the single cell level. The electrophysiological profile of isolated cells and cells forming part of an intact tissue can be dramatically different. Some of the factors that can contribute to these differences include electrical connectivity between cells, accumulation of ions in the extracellular space, and exosion to complex vectorial mechanical forces present in the tissue. These factors disappear when the cells are isolated in a dish and proteins of the extracellular matrix have been digested during the dissociation procedure. In particular, the question if phase 2 is a single cell characteristic or an emergent property of the tissue is central to our working hypothesis. Figure 8, A and B, shows that not only isolated...
ventricular cardiac myocytes from mouse hearts present a phase 2 but also demonstrates that the kinetics of the AP repolarization displays a similar behavior as the one observed in the intact organ.

Therefore, why do our results show a predominant phase 2 that was not observed by other authors? Most of the published data dealing with the repolarization of single ventricular myocytes were done at room temperature and using the whole cell patch-clamp technique under current-clamp configuration (3, 22, 51). Under this experimental condition, it is very likely that the absence of phase 2 in those recordings is mostly due to the temperature effect on the genesis of the plateau. However, there is also an extensive set of published experimental work where experiments were performed under whole cell configuration but at temperatures between 32 and 37°C. Under these conditions, the repolarization of APs does not display phase 2 either (13, 36). One possible explanation is that the presence of high concentration of Ca\(^{2+}\) buffers like EGTA and/or the dialysis effect produced by the whole cell membrane rupture will impair the intracellular Ca\(^{2+}\) dynamics. Indeed, these factors can be critical in defining the amplitude and rate of Ca\(^{2+}\) release from the SR and the degree of activation of the Ca\(^{2+}\)-dependent plasmalemma transport system. Interestingly, Remme et al. (42, 43) performed experiments using the perforated patch methodology at 37°C, a situation that highly resembles our sharp microelectrode recordings. Although in their work these authors did not comment on the detailed characteristics of the AP repolarization, it is possible to observe a phase 2 very similar to the one illustrated in our single cell experiments (Fig. 8A). This observation supports our hypothesis that phase 2 is an intrinsic property of mouse ventricular myocytes AP.

To conclude, we demonstrated for the first time the presence of the mouse ventricular APs phase 2. This phase 2 occurs at a more negative membrane potential compared with other mammalian species and is mediated by the activation of the Na\(^{+}/Ca^{2+}\) exchanger. The contribution of phase 2 to the repolarization of the APs is different for different epicardial regions and is also present at the isolated single myocyte level. Temperature and intracellular Ca\(^{2+}\) release from the SR regulate the repolarization process by modifying the ionic transport through the Na\(^{+}/Ca^{2+}\) exchanger. This mechanism present in the mouse model could be extrapolated to other mammalian species (46). Finally, we think that this work further validates the use of mouse models to study normal and pathophysiologic phenomena related to cardiac arrhythmias.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.F. and A.L.E. conception and design of research; M.F., A.D.P., and A.L.E. interpreted results of experiments; M.F., A.D.P., and A.L.E. prepared figures; M.F. and A.L.E. drafted manuscript; M.F., A.D.P., and A.L.E. edited and revised manuscript; M.F., A.D.P., and A.L.E. approved final version of manuscript.

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