Published in final edited form as:

Heart Rhythm. 2013 January ; 10(1): 80–87. doi:10.1016/j.hrthm.2012.10.002.

The Ionic Bases of the Action Potential in Isolated Mouse Cardiac Purkinje Cell

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Abstract

Aims—Collecting electrophysiological and molecular data from the murine conduction system presents technical challenges. We have developed an approach for the isolation of murine Purkinje cells (PCs), characterized the major ionic currents and use the ionic data to simulate action potentials (APs) recorded from the isolated PCs.

Methods and Results—Light microscopy was used to isolate and identify PCs from apical and septal cells. Current and voltage clamp techniques were used to record APs and whole cell currents. We simulated a PC action potential, based on our experimental data. APs recorded from PCs were significantly longer than those recorded from ventricular cells. The prominent plateau phase of the PC AP was very negative (~ -40 mV). Spontaneous activity was observed only in PCs. The inward rectifier current, I_{K1} , demonstrated no significant differences compared to ventricular myocytes (VMs). However, sodium current density was larger, and the voltage-gated potassium current (I_{to}) density was significantly less in PCs compared to myocytes. T-Type Ca^{2+} currents (I_{Ca-T}) were present in PCs but not VMs. Computer simulations suggest that I_{Ca-T} and cytosolic calcium diffusion significantly modulate AP profile recorded in PCs, as compared to VMs.

Conclusions—Our study provides the first comprehensive ionic profile of murine PCs. The data show unique features of PC ionic mechanisms that govern its excitation process. Experimental data and numerical modeling results suggest that a smaller I_{to} and the presence of the I_{Ca-T} are important determinants of the longer and relatively negative plateau phase of the APs.

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Conflict of Interest: None declared.

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Keywords

Purkinje cell; murine specialized conduction system; action potential duration; ventricular myocyte; cardiac ion channels; mathematical model

Introduction

The specialized conduction system (SCS) of the myocardium is important for coordinating cardiac excitation. Components of the SCS in the ventricular myocardium include the atrio-ventricular (AV) node, the His bundle and associated left and right branches, and the cardiac Purkinje fiber network¹. There is an absolute requirement for precision in the pathway of the conduction of cardiac impulse through the SCS, and deviations therein can be arrhythmogenic and have been associated with various fatal cardiac arrhythmias². Cells within the Purkinje fiber network (Purkinje cells; PCs) distribute the cardiac electrical impulse rapidly and efficiently throughout the ventricular myocardium thereby ensuring effective synchronization of ventricular contraction. Given the role of PCs in the cardiac conduction system, a thorough understanding of excitation in PCs is very important.

The ionic bases of excitation have been investigated by taking advantage of available genetically engineered murine models³. Such studies have provided valuable information on the molecular bases of excitation in the working myocardium of the mouse heart, notwithstanding the inherent limitations^{3,4}. Compared to the working myocardium, the size of the Purkinje fiber network is very small; consequently, available data on the electrophysiology of the mouse SCS is limited, and most of our current knowledge is based on data obtained from immunohistochemistry and from optical mapping of excitation⁵⁻⁷. In a previous study, action potential (AP) properties in the right bundle branch and Purkinje fiber network were characterized in the mouse using the microelectrode technique¹. Results of that study were subsequently confirmed in current-clamp experiments in cells isolated from wild type (WT) and Cx40GFP⁺ mice⁵⁻⁷. To our knowledge, there is presently no data in literature on the major ionic currents that underlie excitation in mouse cardiac PCs.

In this study we report, for the first time, basic properties of the major ionic currents in PCs isolated from mouse hearts. Comparisons were made between currents recorded from PCs and from myocytes isolated from septal and apical regions of the ventricle. Using the ionic current data, we developed a mouse PC model by modifying an existing ventricular myocyte model⁸. The PC model was used to investigate the key ionic determinants of the unique AP morphology of the mouse PC.

Methods

Mouse cardiac Purkinje cells and ventricular myocytes were dissociated using Langendorff method as previously described⁵. All procedures were carried out in accordance with the University of Michigan guidelines for animal use and care conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 58-23, revised 1996). Experimental details are given in the Online Supplement.

Results

Morphology of murine Purkinje cells and ventricular myocytes

We studied, comparatively, morphological features of isolated cardiac PCs and ventricular myocytes (VMs). PCs were readily distinguishable from myocytes (Figure 1); although PCs had striations similar to myocytes (Figure 1A; a & b), they were spindle-shaped, longer, and have a higher length-to-width ratio than myocytes (Table S5). Lateral membranes of PCs had no obvious cell-cell contact points or “steps” (arrow heads, Figure 1A, b). These features were also evident in PCs isolated from Cx40EGFP^{+/+} mouse⁹ (Figure 1A, c & d),

During cell isolation from Cx40GFP^{+/+} mice, a small percentage of cells positive for GFP had morphological features atypical for PCs (Figure 1A, e & f). On the average, the cells were wider than PCs and had “steps” on lateral membranes (arrows in Figure 1A, e), features reminiscent of myocytes. We further investigated these cells using confocal imaging of the ventricular endocardium of Cx40GFP^{+/+} mice (Figure 1B). GFP fluorescence of Purkinje fibers was easily identifiable in the endocardial surfaces of the right ventricle. Regions of interest in Figure 1B (rectangles marked a, b and c) have been enlarged in Figure 1C. Again, whereas Purkinje fibers (marked P) are positive for GFP, myocytes (marked M) are not (Figure 1C, a), even with further digital enhancement (Figure 1C, a*). Note that the shape of cells within Purkinje fibers (Figure 1C, b & c) is consistent with the shape of isolated PCs. Importantly, note that as fibers make contact with the myocardium, cells within the fibers have “steps” on lateral membranes (Figure 1C, c) reminiscent of myocytes. We will refer to these as transitional cells. As would be expected, the yield of transitional cells was quite low. In the present study, we focused our electrophysiological investigations only on PCs, with the morphology depicted in Figure 1A,a.

Passive and active properties of PCs

All electrophysiology experiments were conducted using cells isolated from wild type mice. Compared to the ventricular myocytes, PCs had smaller membrane capacitance, consistent with the smaller cell size (Table S5). The resting membrane potential was not significantly different between PCs and myocytes (Table S5). A characterization of action potential properties (in PCs, septal and apical myocytes (Figure 2A)) is a necessary first step in the study the ionic mechanisms underlying excitation. Stimulated action potentials in PCs (n=7) (1 Hz) were significantly longer (action potential duration at 70% and 90% repolarization, APD_{70,90}; p<0.05), and maximum upstroke velocity (dV/dt_{max}) was higher (p<0.05) than in septal (n=7) and apical (n=7) myocytes (Table S5). At 10 Hz PC APDs were abbreviated, whereas apical APDs were prolonged, APD₉₀ was not significantly different between the two cell types. Isolated PCs demonstrating pacemaker activity were also observed (n=6, Figure 2B). In addition, paced PCs exhibited early-afterdepolarizations (EADs, 4 out of 7, Figure 2C), but EADs were not observed in septal or apical myocytes.

Ionic currents in isolated murine PCs and ventricular myocytes

PCs utilized for electrophysiology were identified by their distinct cell morphology and, in some instances, by the presence of spontaneous electrical oscillations. In these experiments, we focused our investigations on the Hyperpolarization-activated pacemaker current (I_f), the

inward rectifier current (I_{K1}), components of depolarization activated potassium (K_v) currents, the L-type ($I_{Ca,L}$) and the T-type ($I_{Ca,T}$) calcium currents and the sodium current (I_{Na}).

Hyperpolarization-activated and inward rectifier currents

Properties of I_f recorded from mouse PCs are shown in Figure 3 (A, B). Hyperpolarizing pulses (inset) resulted in slowly activating, Cs (5 mM)-sensitive currents (Figure 3A), with properties consistent with I_f ¹⁰. An average I_f density/voltage plot in PCs is shown in Figure 3B (n=3). I_{K1} was studied as ramp-generated (1.5 mV/sec; -100 to 0 mV), Ba^{2+} (1mM)-sensitive currents^{11,12} (Figure 3C and D). Representative currents in control and in the presence of Ba^{2+} are shown in Figure 3C. For comparison, average Ba^{2+} -sensitive currents in PCs, septal and apical cells are shown in Figure 3D. Peak inward current density at -100 mV was -2.80 ± 0.5 pA/pF (n=6) for PCs, and -2.09 ± 0.35 pA/pF (n=7) and -2.06 ± 0.29 pA/pF (n=7) respectively for septal and apical myocytes; I_{K1} peak inward and outward current density was not significantly different between the three cell types.

Depolarization activated potassium currents

Depolarization-activated potassium currents were characterized as previously described¹³. 5-second depolarizing voltage-clamp pulses were applied from a holding potential of -70 mV, in the presence of TTX (30 μ M) and nifedipine (5 μ M) to inhibit, respectively, the fast voltage-gated sodium (I_{Na}) and the $I_{Ca,L}$. Representative, normalized current traces and average peak current density/voltage plots are shown in Figure 4A. Kinetic analyses of currents (at +40 mV) were used to determine individual components of the activated currents; $I_{to,fast}$ ($I_{to,f}$), $I_{to,slow}$ ($I_{to,s}$), $I_{K,slow}$ and $I_{steady\ state}$ (I_{ss})¹³. Figure 4B (left panel) are representative traces recorded in the three cell types accompanied by their respective superimposed fits of current inactivation. Traces were fitted with two (PCs and apical cells) and three (septal cells) exponential decay functions. Figure 4B (right panel) is the voltage-dependence of the time constants (τ) of current inactivation. Details of the exponential fits are shown in Table S6. Values of τ suggest that PCs and apical myocytes have $I_{to,f}$ and $I_{K,slow}$, respectively, τ_1 , τ_2 , and that septal myocytes have an additional component, presumably $I_{to,s}$. The magnitude of steady state current (I_{ss}) was similar in all cell types.

Voltage-gated calcium and sodium currents

$I_{Ca,L}$ and $I_{Ca,T}$ currents in PCs are illustrated in Figure 5A. The two currents were separated by using two holding potentials (-50 mV and -90 mV). Representative traces of peak $I_{Ca,T}$ (at -50 mV) and $I_{Ca,L}$ (-10 mV) densities are shown as an inset. Figure 5A (right) is the voltage-dependence of $I_{Ca,L}$ and $I_{Ca,T}$ in PCs. Peak $I_{Ca,T}$ and $I_{Ca,L}$ densities in PCs averaged -0.61 ± 0.16 pA/pF, n=6, N=3 and -3.75 ± 1.32 pA/pF, n=5, N=3, respectively. The L-type calcium currents in septal and apical myocytes are shown in Figure 5B. We could not record any $I_{Ca,T}$ in apical or septal myocytes, consistent with previous reports^{14,15}. $I_{Ca,L}$ density was largest in apical cells and smallest in PCs (p<0.05). Peak $I_{Ca,L}$ density values were -3.75 ± 1.32 pA/pF, -5.3 ± 0.67 pA/pF, -7.52 ± 0.76 pA/pF, respectively for PCs, septal and apical cells. We also examined I_{Na} density in PCs and in apical myocytes (Figure 5C). From a holding potential of -160 mV, 300 msec pulses were applied from -100 mV to 0 mV in 5 mV steps. Figure 5C shows mean current density of I_{Na} PCs (n=7) and apical myocytes

($n=4$). Representative traces are shown in the inset. I_{Na} density was significantly larger at several experimental voltages in PCs when compared to apical myocytes ($p < 0.005$, from -50 to -20 mV).

Numerical modeling results

Numerical simulations were conducted to gain further insight into the ionic mechanisms of the mouse PC action potential. Figure 6A is a schematic of the newly developed PC numerical model with the sub-cellular compartments and ionic currents. The model consists of a radial spatiotemporal Ca^{2+} diffusion process between sub-sarcolemmal (subSL) and sub-sarcoplasmic reticulum (subSR) compartments (two-way arrows). PCs are devoid of t-tubules, which leads to a rather distinct calcium activation process in which calcium ions have to diffuse through the cytoplasm to reach the SR before they trigger calcium-induced-calcium-release (CICR). Therefore, we have implemented a simplistic cytosolic calcium diffusion process to obtain more realistic calcium dynamics in our model (see details in the online supplement). Figure 6B shows APs elicited in PCs by 1Hz stimulus in experiments and in the model. In the PCs, the AP was characterized by a higher maximum upstroke velocity (dV/dt_{max}) and greater AP amplitude. Similar to apical and septal myocytes, PCs have a low voltage plateau (Figure 2A, see also Online Figure S8). The ventricular myocyte model (VM) by Li et al.⁸ was also adjusted based on the experimental data as shown in online Figure S9 and table S8. Next, we attempted to delineate the contributions of I_{CaT} and cytosolic calcium diffusion, in order to account for the AP morphology differences between apical myocyte versus the PC. When I_{CaT} was blocked completely in the PC model, the low-voltage plateau in the action potential disappeared (Figure 6C) and the AP was significantly shortened (APD₉₀ reduced by 25%). Partial blockade of I_{CaT} caused a proportional shortening of APD plateau phase (data not shown).

Several factors control the rise time and decay time of calcium transients in our PC model. These included the extrusion of $[Ca^{2+}]_{subSL}$ by NCX, uptake of $[Ca^{2+}]_{subSL}$ by SERCA, and Ca^{2+} cytosolic diffusion velocity. We kept the first two parameters untouched and altered only the diffusion velocity by varying the spatial diffusion coefficient, D_{Ca} . This way, we could vary the time to peak (T_p) without significantly altering the total time of calcium transient. As shown in Figure 7A (inset), the APD₈₀ was prolonged monotonically with the increase in T_p . The slower calcium transient produced more pronounced plateau in the Purkinje AP (red trace in Fig. 7A). To study the contribution of calcium diffusion on the AP morphology, we compared the performance of the model with and without Ca^{2+} diffusion. The amplitude of $[Ca^{2+}]_i$ transients was maintained at the same level in both models (Figure 7B top panel). The spatial diffusion caused slower Ca^{2+} transients, resulting in AP prolongation with the characteristic low-voltage hump (Figure 7B). In the model without Ca^{2+} diffusion, $[Ca^{2+}]_i$ transients were faster and the AP lacked a prominent plateau.

Discussion

We have carried out comprehensive current- and voltage-clamp experiments as well as numerical modeling of excitation of mouse PCs. To our knowledge, this is the first report of such data in the literature. The main findings from the experiments and numerical modeling

suggest that PCs have a smaller I_{to} density which may, in part, be responsible for the longer PC APD. Furthermore, our simulations suggest that the presence of I_{CaT} give is important in defining the PC action potential morphology.

Morphology of isolated murine Purkinje cells and ventricular myocytes

Based on light and electron microscopy morphological analyses of fibers/cells from a variety of species, three types of PCs have been described^{16,17}; ungulates (group I), carnivores and primates (group II) and rodents (group III). In group III, PCs are smaller in size than myocytes, and are cylindrical in shape, consistent with our findings. The light and confocal microscopy data demonstrate that “transitional” PCs can make side-to-side contacts, presumably at the Purkinje-Muscle junctions.

Ionic bases of excitation in mouse Purkinje cells – experimental and numerical insights

Heterogeneity in AP morphology and ionic currents are established characteristics of myocardial cells^{13,18}. In mouse PCs, APD₉₀ values (1 Hz) are ~34% greater compared to in septal or apical cells. The plateau phase of APs in the canine, ovine as well as the rabbit PCs is relatively more negative in voltage range (~ 0 to -20 mV) compared to ventricular myocytes^{19–21}. Repolarization in phase 2 of PCs is more negative (~ -40 mV; see Figure 2A) in mouse PCs when compared to other species (see Verkerk et al, 1999²²). It is noteworthy that phase 2 repolarization in murine ventricular myocytes are relatively more negative than what has been demonstrated in other species. It is possible that the specific densities of murine I_{to} and calcium currents in the PCs and VMs might explain this low plateau phase²².

The voltage-dependent activation properties of I_f imply that the current should play a role in the spontaneously generated action potentials in PCs. Nevertheless, we cannot rule out contributions from other current mechanisms, including IK_{dd} ²³ or calcium waves.^{21,24} The molecular correlates of I_f in the rabbit have been identified as HCN2 and HCN4²⁵. Further investigations will be required to ascertain the molecular nature of the proteins that underlie I_f in mouse PCs. I_{K1} density in the rabbit²⁶, sheep²² and canine²⁷ PCs are smaller than in myocytes. There were no significant differences in I_{K1} density between mouse PCs and myocytes also consistent with what has been reported previously in human Purkinje versus ventricular cells^{28,29,30}. It is noteworthy that no significant differences were found in I_{K1} density between mouse atrial and ventricular myocytes³¹, and may suggest a peculiarity of this species.

Mouse ventricular myocytes have negligible delayed rectifier potassium currents, I_{Kr} and I_{Ks} (~0.2 pA/pF)³² and were thus not investigated in the present study. Compared to myocytes, PCs had significantly smaller transient outward currents. Analyses of current properties show that PCs and apical myocytes have similar kinetics, in which current decay could be fitted with two exponentials, presumably reflecting $I_{to,f}$ and $I_{K,slow}$. In contrast, kinetics of current decay in septal myocytes required three exponentials to be adequately fitted. This would suggest that $I_{to,s}$ (tau of ~400 msec) is a component of the activated currents^{13,33}. It might be tempting to speculate that the molecular bases of the transient outward currents in

PCs and apical myocytes are similar; however molecular identification of the channel proteins will be required to establish this.

L- and T- type calcium currents are present in mouse PCs. We propose that the T- type channel plays a role in the negative voltage range of phase 2 repolarization. In numerical simulations, only the T-type calcium current was capable of prolonging the APD in PCs. The data suggests that action potential characteristics are attributable to intracellular calcium dynamics and the T-type calcium current. Both components may contribute to the prolonged AP with the low-voltage plateau in the Purkinje cells. Our findings are consistent with changes in action potential morphology following expression in myocytes of $\alpha 1G$, which encodes T-type calcium channel³⁴. We show that I_{Na} density in PCs is significantly larger ventricular myocytes. Recently Vassalle et al³⁵ reported that in the canine cardiac PCs, a slowly inactivating sodium current (I_{Na2}) plays a role in prolonging the plateau of the action potential. We presently cannot rule out this possibility in the murine PC.

Study limitations

Our main objective in this study was to demonstrate the feasibility of recording membrane ionic currents in PCs isolated from wild type mice, without cross breeding with Cx40GFP⁺ mice. Although we have characterized the major ionic currents, these technically challenging experiments precluded the identification of molecular correlate of the ion channels. Our model assumes a symmetric morphology of a PC with the SR located at the center and consists of a simplistic cytosolic calcium diffusion process. Despite this, it enabled us to study the effects of cytosolic calcium diffusion on the AP morphology of Purkinje cells. We did not consider possible effects of alteration in Ca^{2+} -dependent inactivation of I_{CaL} , which could also further affect the shape of AP plateau. Nonetheless, we were able to speculate that cytosolic spatiotemporal diffusion and the presence of T-type calcium channels are responsible for the peculiar AP morphology. Further characterization of cytosolic calcium diffusion via experiments, and a detailed model development for the same is needed to investigate its putative role in the cell-wide calcium waves and the increased propensity of afterdepolarizations in the Purkinje cells.

Conclusion

We have demonstrated the feasibility of using available mouse models to investigate the molecular underpinnings of excitation in the specialized conduction system. Because this approach does not require cross breeding with the Cx40EGFP⁺ mouse, less time and resources are needed for such future investigations. Our experimental and numerical modeling data suggest that cytosolic calcium diffusion and T-type calcium current, alone or in combination, contribute to the prolonged AP with the low-voltage plateau in PCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Nulang Wang for her technical assistance.

Funding

This work was supported by grants RO1-GM076608 (JA), PO1-HL087226 (JJ), and the Robert and Eileen Hutton predoctoral fellowship from AHA (RV).

Abbreviations

AP	action potential
APD	action potential duration
SCS	specialized conduction system
AV node	atrio-ventricular node
PCs	Purkinje cells
GFP	green florescent protein
WT	wild type
TTX	tetrodotoxin
VM	Ventricular myocyte

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Figure 1. Morphology of a Purkinje cell (PC) and ventricular myocytes isolated from the murine myocardium

Panel A: Photomicrographs of an isolated Purkinje cell (PC; a) and ventricular myocyte (VM; b) from a wild type mouse heart. For comparison, the panel shows an isolated PC (c) from Cx40 GFP mouse (PC Cx40), where PCs were identifiable by the expression of GFP (d). Phase contrast images of cells expressing GFP (e & f) with “steps” in the lateral membrane (arrows), similar to ventricular myocytes. These were identified as transitional cells (trans Cx40). Scale bar = 20 μ M **Panel B:** GFP-positive Purkinje fibers on right ventricular endocardium. (a), (b) and (c) have been enlarged in panel C. **Panel C:** (a) Purkinje fibers (P), but not myocytes (M), are positive for GFP, (M), even with further digital enhancement show below (a*). Note regions of contact in b and c; arrowheads in subpanel b and arrows in subpanel c. Transitional cells in subpanel c have steps in lateral membranes.

Figure 2. Action potential characteristics of murine PCs and of ventricular septal and apical myocytes

Panel A (from left to right): Representative traces of action potential morphologies of PCs (n=7), septal (n=7) myocytes and apical (n=7) myocytes. **Panel B:** Pacemaker activity demonstrated in isolated Purkinje cells (n=6). **Panel C:** A representative EAD recording in a PC.

Figure 3. Hyperpolarization-activated (I_f) and inward rectifier (I_{K1}) currents in PCs
 Panel A: Representative currents recorded from all three cell types. Voltage protocol for activation of I_f (top). Currents recorded in the absence (middle) and presence of 5 mM cesium (bottom). **Panel B:** I_f peak current density-voltage relationship in PCs (n=3). **Panel C:** Representative recording of I_{K1} in a PC in the absence (red) and presence of 1 mM Ba²⁺ (black). Voltage protocol is shown as inset. **Panel D:** Current density-voltage (I-V) relationships of Ba²⁺ sensitive currents (I_{K1}) in PCs (red, n=6), septal myocytes (blue, n=7) and apical myocytes (black, n=7).

Figure 4. Depolarization activated potassium currents in isolated cells

Panel A: Current traces following membrane depolarizations from -40 mV to $+60$ mV (holding potential = -70 mV). Top panel: PCs. Middle panel: Septal Cell. Bottom panel: Apical Cell. Right: I-V relationships in PCs (red, $n=6$), septal cells (blue, $n=6$) and apical cells (black, $n=7$). Inset is voltage protocol. **Panel B:** Exponential fits to currents in isolated cells. Left panel: PCs, apical cells and septal cells (inset: voltage protocol). Right panel: Time constants of current inactivation plotted as a function of voltage (open circles for apical τ_1 are nested with other symbols, and indicated by an arrow at $+20$ mV). PCs and apical cells were fit with a second order exponential decay function while septal myocytes were fit with a third order exponential decay function.

Figure 5. Calcium and Sodium Currents in PCs and myocytes

Panel A: Voltage protocol and representative traces demonstrating the subtraction to isolate T type currents for PCs (inset). I-V relationship of T and L type Calcium currents in PCs.

Panel B: Representative traces for apical and septal myocytes (inset). I-V relationship of I_{Ca-L} for septal (black, n=6) and apical (blue, n=6) myocytes. **Panel C:** (inset) voltage protocol and (middle) representative traces of I_{Na} recorded in a PC and an apical myocyte. (Right) I-V relationship of I_{Na} in PCs (n=7) and apical myocytes (n=4).

Figure 6. Morphologically realistic numerical model of a PC

Panel A: Schematic of the PC numerical model showing subcellular compartments (sub-sarcolemmal (subSL) and sub-sarcoplasmic reticulum (subSR)) and the ionic currents in the cell. The model consists of radial spatiotemporal Ca^{2+} diffusion between subSL and subSR compartments as shown by the two-way arrows. **Panel B:** Action potentials (at 1 Hz) in PCs elicited in experiments and in the model. Horizontal and vertical scale bars represent 50 ms and 20 mV, respectively. **Panel C:** I_{CaT} blockade in the PC model resulted in a significant reduction of plateau phase (red) and abbreviation of APD.

Figure 7. Role of cytosolic Ca diffusion in AP morphology of mouse PC

Panel A: Slowing of Ca transients by decreasing diffusion coefficient (D_{Ca}) produced more pronounced plateau in the AP. Inset: APD80 increased monotonically with increase in $[Ca]_i$ time to peak (T_p). **Panel B:** Comparison between the PC model AP with and without calcium diffusion. Spatiotemporal $[Ca]_i$ diffusion resulted in slower calcium transients which prolonged the AP. PC model without $[Ca]_i$ diffusion exhibited faster transients and no plateau in the AP. Vertical scale bars represent $0.4 \mu M$ (top panels) and $40 mV$ (bottom panels).