Multiple levels of the single L-type Ca^{2+} channel conductance in adult mammalian ventricular myocytes

Ye Chen-Izu *

Department of Pharmacology, University of California, Davis, CA, USA
Department of Biomedical Engineering, University of California, Davis, CA, USA
Department of Internal Medicine, University of California, Davis, CA, USA

ABSTRACT

In cardiac muscle, Ca^{2+} entry through the L-type Ca^{2+} channel during excitation triggers Ca^{2+} release from sarcoplasmic reticulum (SR) via the ryanodine receptor, causing muscle contraction. This Ca^{2+} induced Ca^{2+} release (CICR) is locally controlled by openings of the single L-type Ca^{2+} channel that triggers the opening of ryanodine receptors located within the dyadic junction. The unitary current through the single Ca^{2+} channel determines the open probability of the ryanodine receptors, and hence the efficacy of CICR. Here, we report findings of multiple conductance levels of L-type Ca^{2+} channels in freshly isolated rat ventricular myocytes. When 10 mM Ca^{2+} was used in the pipette solution as the charge carrier in the cell-attached configuration, the most frequently occurring conductance levels are 6.9 pS and 2.9 pS. Three distinct conductance levels were also observed, although infrequently, in the same cell, corresponding to unitary currents of 0.33 pA, 0.24 pA, and 0.17 pA upon depolarization to −10 mV. In conclusion, our data demonstrate the existence of multiple single L-type Ca^{2+} channel conductance levels in the adult mammalian ventricular myocytes with Ca^{2+} as the charge carrier. The multiple conductance levels present heterogeneity in the Ca^{2+} trigger signal strength at local dyadic junctions. The physiological significance of this heterogeneity on affecting the efficacy of CICR in the cardiac muscle is discussed.

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Introduction

In cardiac muscle, Ca^{2+} entry through L-type Ca^{2+} channels during action potential triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptors, leading to elevation of the intracellular Ca^{2+} concentration that causes muscle contraction. This Ca^{2+} induced Ca^{2+} release (CICR) is *locally controlled* by the opening of single L-type Ca^{2+} channel that triggers the opening of ryanodine receptors located within 20 nm distance in the dyadic junction. The unitary current through the single L-type Ca^{2+} channel determines the open probability of the ryanodine receptors. Hence, the single channel conductance of the L-type Ca^{2+} channel significantly affects the efficacy of CICR in cardiac muscle.

Single L-type Ca^{2+} channel conductance has been measured in various preparations. Historically, most single channel studies used Ba^{2+} as the charge carrier, because channel conductance is much larger with Ba^{2+} than Ca^{2+}, making it easier to resolve the

single channel events from the background noise. For example, a pioneering study by Hess et al. [1] reported that the L-type Ca^{2+} channel in the ventricular myocyte had a single channel conductance of 25 pS with 50 mM Ba^{2+}, but only of 8 pS with 50 mM Ca^{2+} as the charge carrier. Intriguingly, multiple conductance levels were reported when Ba^{2+} was used as the charge carrier. For example, Gondo et al. [2] found that cloned rabbit cardiac L-type Ca^{2+} channel expressed in Chinese hamster fibroblast displayed four sub-conductance levels of 23 pS, 14 pS, 6 pS, and 3 pS with 110 mM Ba^{2+}. Moreover, the expression of α_1 subunit alone was sufficient to exhibit these sub-conductance levels, and coexpression of β_{2a} subunit significantly increased the number of openings in all four levels without changing the conductance values. Multiple unitary current amplitude with Ba^{2+} was also observed in the cloned channels (cardiac α_1 with skeletal $\beta\alpha_2\delta$) expressed in baby hamster kidney cells [3]. Lacerda and Brown [4] further noted that the L-type Ca^{2+} channel in neonatal rat and guinea pig also displayed at least three unitary current levels in 100 mM Ba^{2+}. However, two questions remained to be answered. One was whether the multiple conductance levels do exist in the adult mammalian cardiac myocytes rather than recombinant channels expressed in cell lines or neonatal cells. Another was whether the multiple conductance levels exist also when Ca^{2+}, instead of Ba^{2+}, is the charge carrier.

* Address: Dept. of Pharmacology, University of California, Davis, Tupper Hall, Rm. 2221, 451 Health Science Dr, Davis, CA 95616, USA. Fax: +1 530 231 5659.
E-mail address: ychenizu@ucdavis.edu.

Given the physiological importance of the single channel conductance in affecting the Ca^{2+} trigger signal strength and the efficacy of CICR in cardiac muscle, we focused this project on investigating the sub-conductance levels of L-type Ca^{2+} channels in adult mammalian ventricular myocyte using Ca^{2+} as the charge carrier. Here we report that multiple conductance levels do exist when Ca^{2+} enters the L-type Ca^{2+} channel in freshly isolated adult rat ventricular myocytes, and we characterize two most frequently observed conductance levels.

Methods and materials

Cell preparation. Ventricular myocytes were isolated from 2- to 4-month-old Wistar rats using a standard enzymatic technique. [5] Briefly, the rat was anesthetized with pentobarbital (100 mg/kg injected IP). After testing for the suppression of reflexes, the heart was explanted via midline thoracotomy, mounted on a Langendorff system and perfused with a modified Tyrode solution. Collagenase B ($\sim$1 mg/ml, F. Hoffmann-La Roche Ltd., Switzerland),
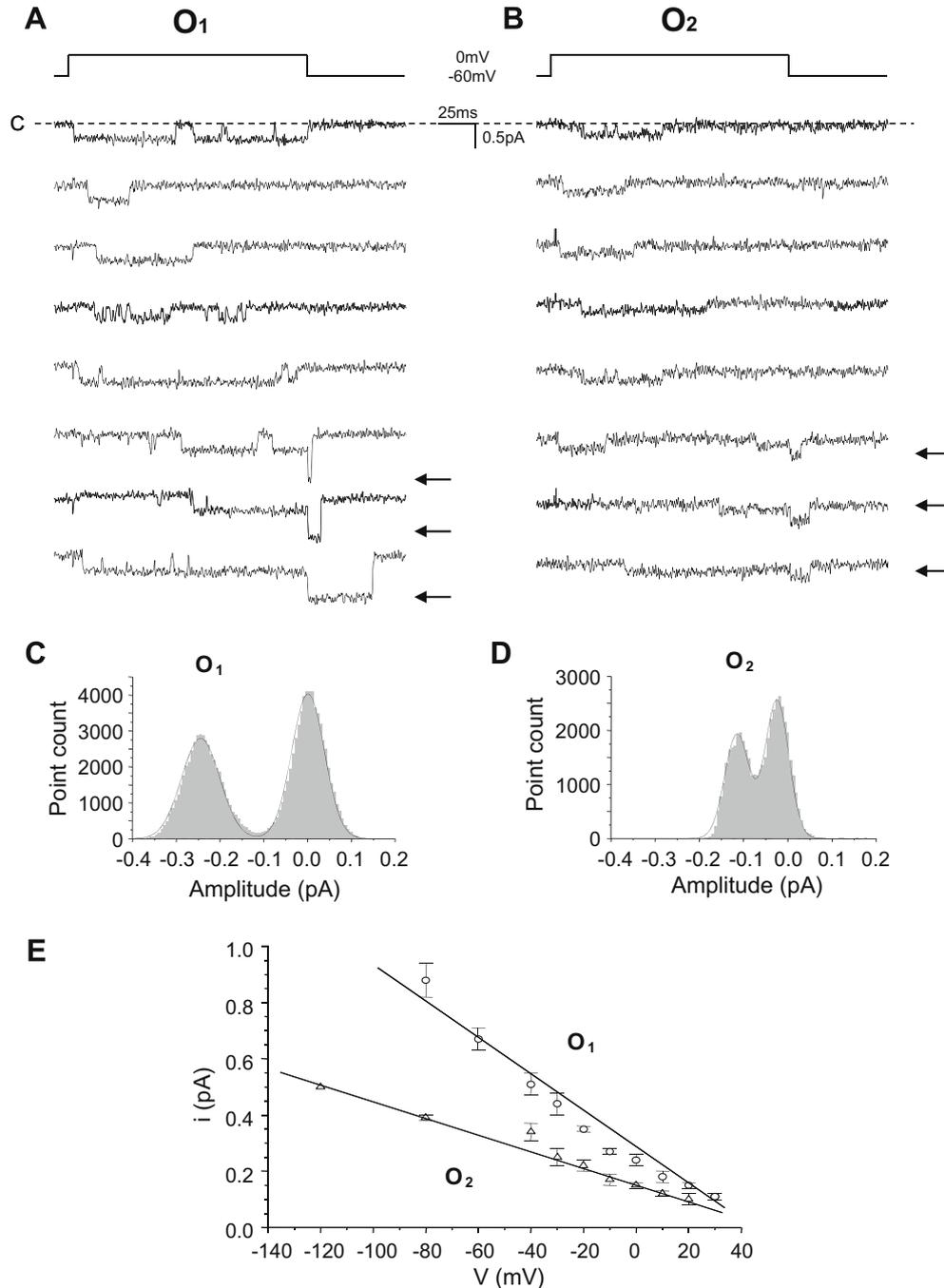

Fig. 1. Two frequently occurring single L-type Ca^{2+} channel conductance levels. The single channel activity was recorded using the cell-attached configuration under voltage-clamp. The membrane potential was held at −60 mV to inactivate the T-type Ca^{2+} channels, and then depolarized to various voltages for 200 ms to record the L-type Ca^{2+} channel activity. Representative traces of single L-type Ca^{2+} channel recordings show the unitary current flowing through the single channel opening at 0 mV. Two distinct levels of the unitary currents are shown in A and B, denoted as O_1 and O_2, respectively. The corresponding all-points amplitude histograms and the fitting results using the Gaussian function are shown in C and D. E shows the *I–V* relationship of the unitary currents (plotted are mean ± SEM, n = 13 for O_1 group, n = 11 for O_2 group). The unitary current was obtained from the Gaussian fitting results for the voltages between −30 mV and +30 mV. For voltages lower than −30 mV, the unitary current data was obtained from tail openings. The *I–V* curves were fitted with a linear function to obtain the single channel conductance levels of 6.9 pS (R^2 = 0.970) and 2.9 pS (R^2 = 0.910).

protease type XIV (~0.1 mg/ml), 0.1% BSA and 20 μM Ca^{2+} were added into the perfusion to digest the tissue. The ventricular tissue was then cut down, minced and further incubated in the enzyme solution. The dissociated cells were collected and stored in the Tyrode solution containing 1 mM Ca^{2+} at room temperature, and were used for experiments within 10 h after isolation. All the chemicals were purchased from Sigma–Aldrich if not noted otherwise.

Animal handling and all procedures were performed strictly in accordance to the National Institutes of Health (NIH) guidelines and our Institutional Animal Care and Use Committee approved protocols.

Single channel recording. We used cell-attached patch-clamp technique to record single L-type Ca^{2+} channel activity in the ventricular myocyte. The bath solution contained (in mM) K-aspartate 110, KCl 30, $MgCl_2$ 3.8, Hepes 5, EGTA 5, glucose 10, Mg-ATP 2, and $CaCl_2$ 1.2, pH 7.3. High K^+ concentration was used in bath to collapse the resting membrane potential to assist the determination of transmembrane voltage. The pipette solution contained (in mM) $CaCl_2$ 10, TEA 120, Hepes 10, pH 7.4. FPL64176 10 μM was added into the pipette solution to prolong the channel open duration [6] to aid determination of the unitary current. The patch pipette was fabricated from borosilicate glass capillary and the tip diameter was made sub-μm to facilitate capturing a single channel in the patch. After compensating for the junction potential, a tight seal (>10 GM) was made between the pipette tip and the cell membrane to form the cell-attached configuration. Voltage-clamp protocols were generated and currents recorded using an Axopatch 200B amplifier and a DigiData 1200B A/D converter (Molecular Dynamics Inc.) controlled by a computer. The current signal was filtered through a 4-pole lowpass Bessel filter at a cut off frequency of 1 kHz, digitized at a sampling rate of 10 kHz. The membrane potential was held at −60 mV to inactivate the T-type Ca^{2+} channels, and then depolarized to various potentials for 200 ms to record the L-type Ca^{2+} channel activity.

Results

We recorded the single L-type Ca^{2+} channel activity in the freshly isolated rat ventricular myocytes with Ca^{2+} as the charge carrier. The voltage was held at −60 mV for 800 ms to inactivate the T-type Ca^{2+} channel, and then depolarized to various test potentials for recording of the unitary current through L-type Ca^{2+} channel. In order to better resolve the unitary current (which ranged from 0.1 to 1.0 pA with 10 mM Ca^{2+}) from background

noise (~0.2 pA peak-to-peak) we used a known Ca^{2+} channel agonist, FPL64176, to promote the mode-2 opening of the L-type Ca^{2+} channel. Fig. 1 demonstrates two sets of most frequently recorded single channel activities. Panel A shows one set of representative traces of the channel activity at 0 mV depolarization, denoted O_1. Notice that at the end of the test voltage pulse, some traces show the tail opening (pointed to by arrows) which is produced by continued channel opening under a sudden increase of the driving force upon repolarization. Panel B shows another set of representative traces of the channel activity, denoted O_2. Importantly, the current amplitudes in the two sets of recordings are distinctively different. We plotted the amplitude histograms and best-fitted the data using the Gaussian function (Panels C and D). We then constructed the current–voltage (*I–V*) relationship using unitary currents calculated from the amplitude histograms (Panel E). To expand the range for *I–V*, we also included measurements from the tail openings obtained by stepping the membrane potential to −60 mV, −80 mV, and −120 mV. We used the *I–V* curve to calculate the single channel conductance, and obtained two clearly distinct conductance levels in the ventricular myocytes: 6.9 pS for O_1 and 2.9 pS for O_2.

The O_1 group with 6.9 pS conductance was recorded in 13 out of 25 cells; the O_2 group with 2.9 pS conductance was recorded in 11 out of 25 cells, demonstrate their frequent occurrences. The reversal potential for both conductance levels occurs near +50 mV, consistent to that reported by Hess et al. [1]. We did not see transition between the two conductance levels within a single open event, nor in any of the patches for recordings as long as 20–30 min.

We also used a ramp protocol to verify the above conductance measurements. Fig. 2A shows representative traces of the single channel opening during the voltage ramp. The slope of the open events is a direct measure of the single channel conductance. This measurement is facilitated by using FPL 64176 to promote the mode-2 events with long open durations. Notice the difference in the slopes of the currents. Panel B shows the *I–V* relations of the single channel openings. These openings can be clearly grouped into two clusters: O_1 group and O_2 group. The average conductance value of the O_1 group is 6.5 ± 0.5 pS and of the O_2 group is 2.9 ± 1.0 pS. These two conductance levels are in close agreement with those measured in Fig. 1.

On rare occasions we also observed three different conductance levels in a single patch. Fig. 3 depicts sample traces and the amplitude histogram of such a recording. The histogram can be best-fitted with the sum of four Gaussian functions: one for the closed
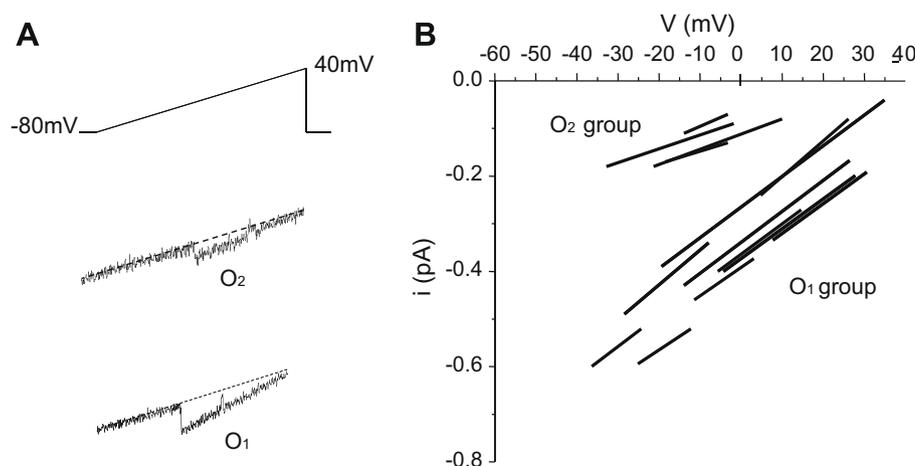

Fig. 2. Conductance levels confirmed using the ramp protocol. We used a ramp protocol to measure channel conductance during a single open event (A). The *I–V* relationship shows two distinctive groups of conductance (B). The open events in the O_1 group have a conductance of 6.5 ± 0.49 pS (mean ± SD, $n = 10$), and those in the O_2 group have a conductance of 2.9 ± 1.00 pS (mean ± SD, $n = 10$).
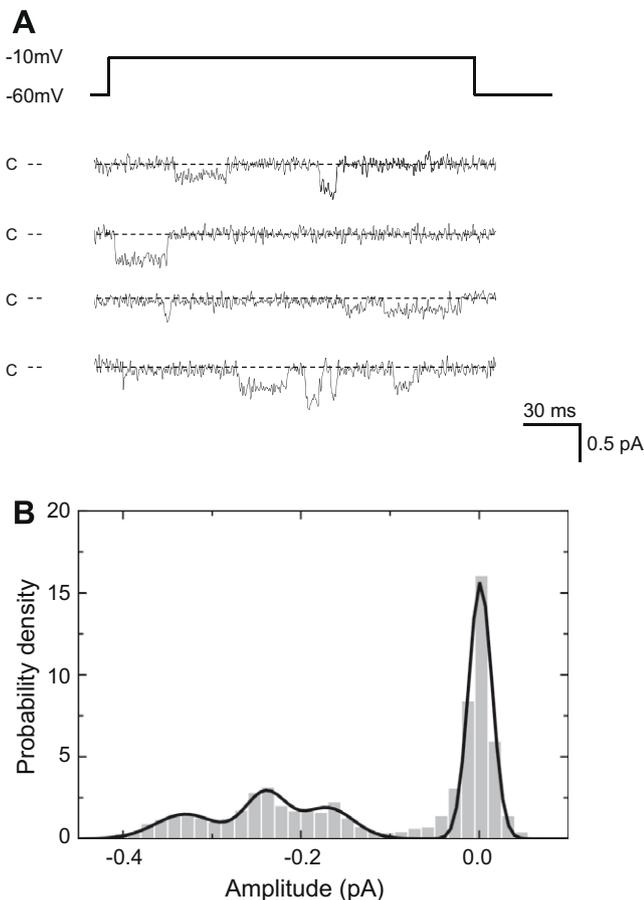
A

B

Fig. 3. Three conductance levels observed in the same cell. A shows a recording with three different unitary current levels in a single patch. B shows the all points histogram of this recording which is best-fitted with the sum of four Gaussian functions (one is for the closed state), indicating three different conductance levels.

state and three for different conductance levels of the open states. The unitary current for each conductance state at -10 mV is 0.17 pA, 0.24 pA, and 0.33 pA. The two larger values correspond to the previously characterized conductance state of 2.9 pS and 0.69 pS, respectively (also see Fig. 1E). However, the 0.17 pA value, although barely above the noise level, indicates the existence of an even smaller conductance state that is much less frequently observed (1 out of 25 cells). In an effort to catch the smaller conductance state, we used 100 mM Ba^{2+} as the charge carrier to repeat the experiments. Again, we recorded two frequently occurring conductance levels of 25 pS (5 cells) and 12 pS (5 cells), but did not catch the less frequently occurring third conductance level by chance.

Discussion

Our data clearly demonstrate that multiple conductance levels of the L-type Ca^{2+} channel do exist in mammalian adult ventricular myocyte with Ca^{2+} as the charge carrier. We have observed two larger conductance levels and had a glimpse of a third, smaller conductance level. The two larger conductance states of 6.9 pS and 2.9 pS in 10 mM Ca^{2+} correspond to the 25 pS and 12 pS states in 100 mM Ba^{2+} that we recorded in the same cell preparation. In comparison to the four conductance levels reported by Gondo et al. [2] in cloned rabbit cardiac L-type Ca^{2+} channel expressed in Chinese hamster fibroblast, the two conductance states we observed most likely correspond to the two largest conductance states in their recording (23 pS and 14 pS in 110 mM Ba^{2+}). The

third state we observed with Ca^{2+}, although rare, might correspond to the third state in their recording (6.2 pS in 110 mM Ba^{2+}). Nevertheless, the fourth state (3.2 pS in 110 mM Ba^{2+}), if it also occurs with Ca^{2+} as the charge carrier, would be submerged under noise level (0.2 pA peak-to-peak) and would have eluded detection. In fact, when mode-1 open events are dominant under physiological condition, the smaller conductance levels would be even harder to detect owning to the much shorter open duration (τ-open $\sim$0.42 ms for mode-1 in comparison to $\sim$10 ms for mode-2) [7].

We have observed three different conductance levels in the mode-2 opening of L-type Ca^{2+} channels with Ca^{2+} as the charge carrier. This finding should be distinguished from an earlier finding by Josephson et al. [8] suggesting different conductance levels in mode-1 versus mode-2. With 5 mM Ca^{2+} as the charge carrier, they found a conductance of 6.1 pS for mode-2 opening, but an average of 3.1 pS for all openings (including the predominant mode-1 and much less frequent mode-2). They did not observe different conductance levels in mode-2 alone. It is intriguing that the second conductance level of 2.9 pS we found in mode-2 is close to the 3.1 pS value they found in mode-1 (including infrequent mode-2). We surmise whether FPL64176 (which was used in our study, but not in theirs) could prolong the lower conductance state at mode-1 to mode-2. This idea is in line with a notion proposed by McDonough et al. [9] when they found that FPL64176 increased the ionic current without affecting the gating current in the recombinant L-type Ca^{2+} channel (α_{1C}). They hypothesized that FPL64176 might cause an inactivate state to become conducting without affecting gating current. Our finding provides new data for further deciphering the conductance levels of different kinetic states in the L-type Ca^{2+} channel. Furthermore, we did not see any transition between the two conductance levels in middle of an open event, nor shifting of the conductance levels within the same patch when only a single channel was present (24 out of 25 patches, the 1 patch showing three conductance states most likely contains three channels). This suggests that the different conductance levels most likely arise from different kinetic state, rather than from sub-conductances within the same state.

The existence of multiple levels of the single L-type Ca^{2+} channel conductance in ventricular myocytes has significant physiological implications to the cardiac excitation–contraction coupling. During the action potential, L-type Ca^{2+} channels open to allow Ca^{2+} entry into the cell. The Ca^{2+} entering through a single channel open event can quickly reach the ryanodine receptor clusters located about 20 nm away in the dyadic junction, and trigger the opening of the ryanodine receptors to release larger amount of Ca^{2+} from the intracellular Ca^{2+} stores in SR; the resulting intracellular Ca^{2+} transient then causes the muscle contraction. Importantly, the open probability of ryanodine receptors is dependent on the amount of Ca^{2+} entry through a single L-type Ca^{2+} channel opening. Hence, different single channel conductance levels give rise to heterogeneity of the Ca^{2+} trigger signals; for example, the different unitary currents of 0.33 pA, 0.24 pA, and 0.17 pA in our observation would give rise to different amounts of Ca^{2+} entry during a single channel open event. The different Ca^{2+} trigger signal strength is expected to affect the efficacy of CICR at local dyadic junctions and thereby affecting the excitation–contraction coupling in cardiac muscle.

Acknowledgments

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