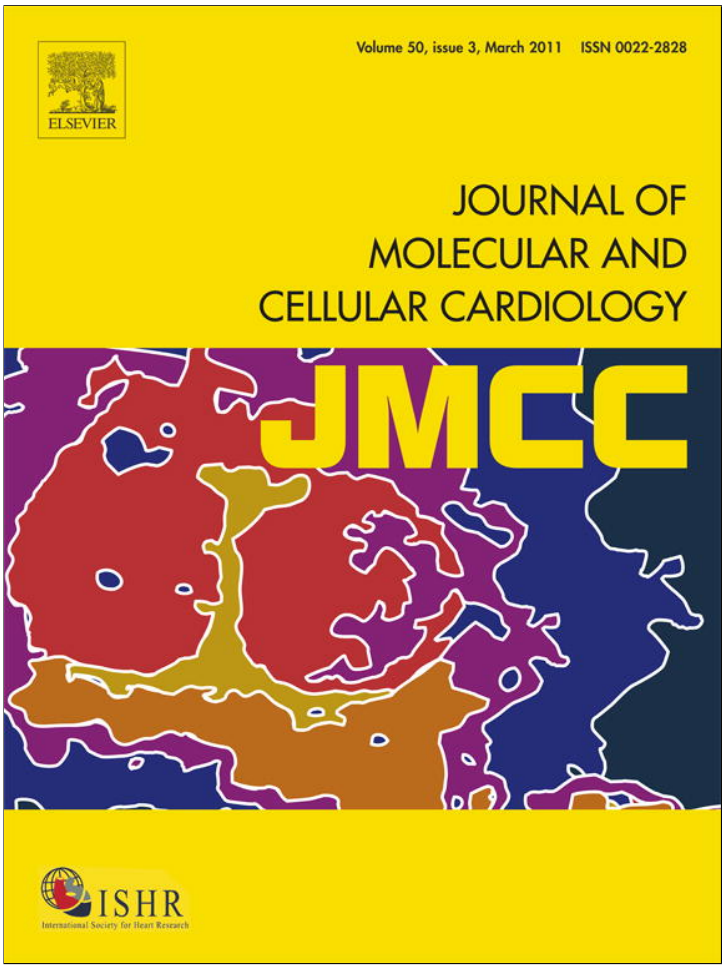

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Rapid communication

Sequential dissection of multiple ionic currents in single cardiac myocytes under action potential-clamp

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ABSTRACT

The cardiac action potential (AP) is shaped by myriad ionic currents. In this study, we develop an innovative AP-clamp Sequential Dissection technique to enable the recording of multiple ionic currents in the single cell under AP-clamp. This new technique presents a significant step beyond the traditional way of recording only one current in any one cell. The ability to measure many currents in a single cell has revealed two hitherto unknown characteristics of the ionic currents in cardiac cells: coordination of currents within a cell and large variation of currents between cells. Hence, the AP-clamp Sequential Dissection method provides a unique and powerful tool for studying individual cell electrophysiology.

1. Introduction

The cardiac action potential (AP) is shaped by a large number of ionic currents. These myriad currents work in concert to affect both dramatic and subtle changes to the AP under physiological and pathological conditions. Traditionally, there are two approaches for measuring the timing and magnitudes of ionic currents during an AP cycle. One approach involves solving the mathematical model of the cardiac AP. [1,2] The model equations are largely based on traditional voltage-clamp experiments (carried out with rectangular voltage pulses and non-physiological ionic solutions designed to study biophysical properties of the currents). Most of our current understanding of cardiac electrophysiology is born from this marriage of mathematical modeling and classical cellular electrophysiology. The other approach is to directly measure the ionic currents during an AP using the AP-clamp technique. [3–7] In this method, an AP waveform replaces the rectangular voltage pulse and physiological ionic solutions can be used.

In this study, we extend the AP-clamp technique by combining it with innovative sequential dissection method which enables us to visualize and measure multiple ionic currents from a single cell during AP. Previously, traditional AP-clamp (and voltage-clamp) technique records only one current in any one cell. Now our new method uses a series of channel blockers to sequentially dissect out the currents in a *single* cell under AP-clamp. This sequential recording of currents reminds us of peeling off layers of an onion, hence the appellation, the 'onion-peeling' method. The ability to measure many currents in a single cell enables us to study the individual cell electrophysiology, and has revealed two hitherto unknown characteristics of the ionic currents in cardiac cells: coordination of currents within a cell and large variation of currents between cells.

2. Methods

2.1. Cell isolation

Adult male Hartley guinea pigs were purchased from Charles River Laboratories (USA). A standard enzymatic technique was used to isolate ventricular myocytes [8]. 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.

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2.2. Electrophysiology

The cells were superfused with modified Tyrode solution containing (in mmol/L): NaCl 120, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, $NaHCO_3$ 25, Glucose 10, pH 7.3. The internal solution contained (in mmol/L): K-Aspartate 115, KCl 45, Mg-ATP 3, HEPES 5, cAMP 0.1, EGTA 10, pH 7.25 adjusted with KOH. After compensating for the junction potential, the pipette was placed on the cell to make a GΩ seal; the membrane patch was then ruptured to establish the whole-cell configuration. The access resistance (<5 MΩ) and the leak current (<50 pA) were kept low throughout the recording. Experiments were recorded using Axopatch 200B amplifier, DigiData 1440A analog/digital converter, and pClamp10 (Molecular Device Co.), at 21 ± 1 °C.

2.2.1. AP-clamp

The cell was stimulated at 1 Hz frequency with 2 ms long suprathreshold depolarizing pulse. After reaching a steady state, the AP was recorded under I-clamp mode (I = 0) at a sampling period of 100 μs (Fig. 1A, upper panel). After switching to V-clamp mode, this AP waveform was applied as the command voltage onto the same cell. The total net current should be zero under the cell's own AP, and recorded as the baseline current (gray trace). The cells displaying non-zero baseline were discarded. Then a selective channel blocker was

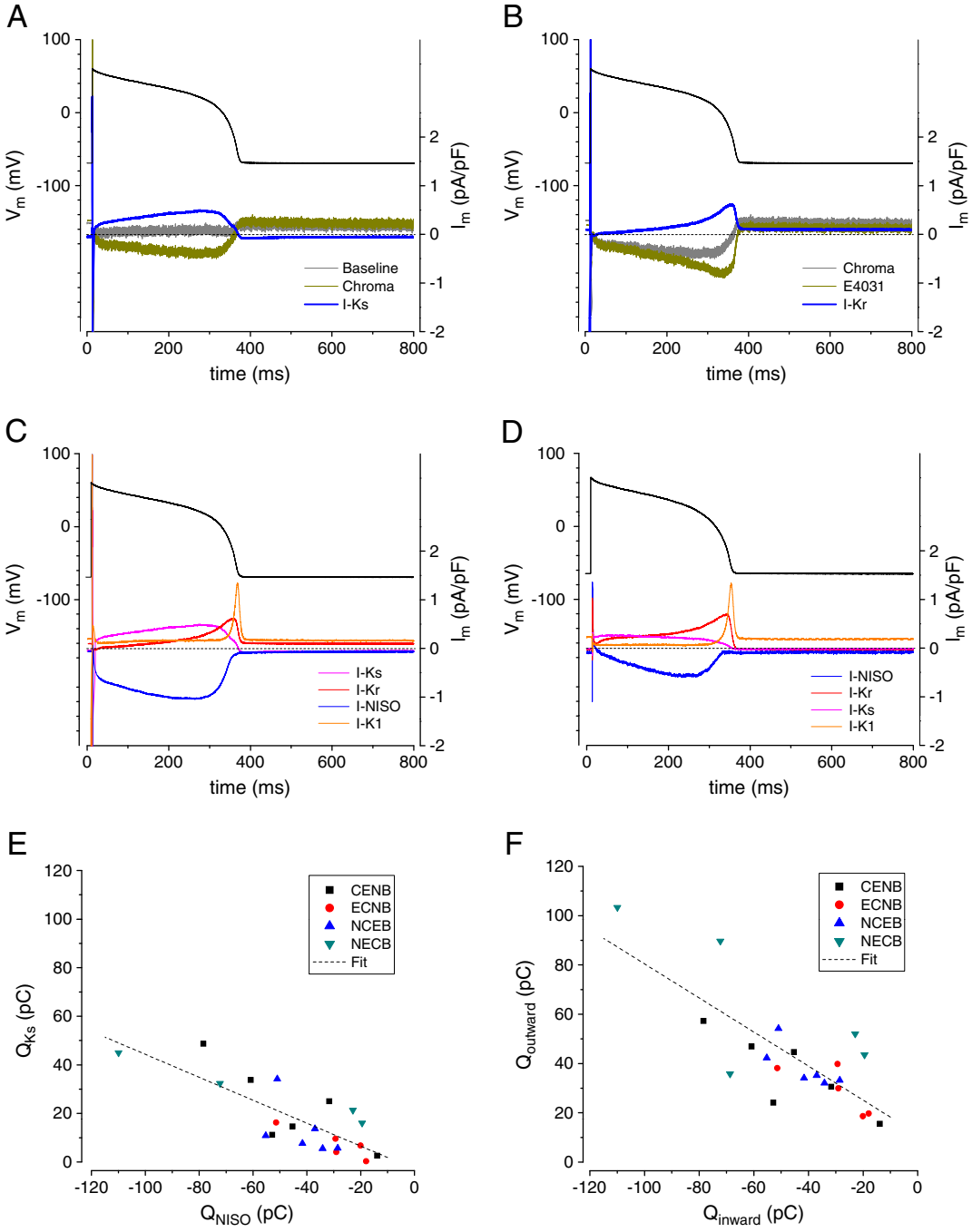

Fig. 1. A. The first current of interest, I_{Ks} is obtained by subtracting the compensation current recorded with chromanol-293B from the baseline current. B. The second current, I_{Kr} is obtained by subtracting the compensation current recorded with E4031 + chromanol from the first compensation current recorded with chromanol. C&D. Representative traces of Chromanol-293B, E4031, Nisoldipine and Ba^{2+} sensitive currents recorded in guinea pig ventricular myocytes. The sequence of channel blocker represents the order of drug application. E. Correlation between Q_{NISO} and Q_{Ks}. F. Correlation between the inward going charges carried by I_{NISO} and the sum of outward going charges carried by I_{Ks}, I_{Kr}, I_{K1}.

added into the bath to block the current of interest, and the compensation current was recorded (green trace). The current of interest (blue trace) is obtained by subtracting the compensation current from the baseline current.

2.2.2. Sequential Dissection

Following the above recording, the second blocker was added. Now, the compensation current (Fig. 1B, green trace) contained two currents blocked by the first (gray trace) and the second blockers. The second current is obtained by subtracting the second compensation current from the first compensation current (blue trace). Following the same procedure, the third, the fourth, and more blockers can be added sequentially to dissect out more currents in the same cell.

3. Results

3.1. AP-clamp sequential dissection of multiple ionic currents in single cell

A typical example of using the AP-clamp sequential dissection method to record multiple ionic currents from a single cell is shown in Figure 1C. The upper panel shows the steady state AP in guinea pig ventricular myocyte. The bottom panel shows the current density of four ionic currents recorded under AP-clamp by adding channel blockers consecutively: I_{NISO} was recorded using 10 μM nisoldipine; I_{Ks} recorded with 10 μM chromanol-293B; I_{Kr} recorded with 1 μM E4031, and I_{K1} recorded with 50 μM Ba^{2+}. These currents are present during AP in the same cell, and the dynamic profile of each current reveals its time and voltage-dependence.

To ensure that the currents recorded are independent of the blocker sequence, it is critical to use highly selective blockers at right concentrations to specifically block the target channel without affecting other channels. To test this, we scrambled the sequence of blocker application to four different groups: CENB, NCEB, ECNB, and NECB (each letter denotes the first letter in the drug name, and the order presents the sequence of drug application). Ba^{2+} was always applied at the last because it was known to be less specific whereas the other blockers were known to be specific. Figure 1C and D show sample recordings obtained with different blocker sequences. All four groups with different blocker sequences displayed similar current profiles. Statistical comparison in Table 1 also shows no significant difference between groups in AP parameters and current parameters.

3.2. Correlation between ionic currents in the same cell and large cell-to-cell variations

The parameters characterizing the currents (peak, time to peak, and total charge) varied widely from cell to cell regardless of the sequence of blocker application, as is evident from the standard deviation of each group in Table 1. The large variation may reflect an intrinsic property of cardiomyocytes or random factors such as measurement errors or cell isolation artifacts. However, the data show no systematic difference between the cells isolated from different animals, ruling out cell isolation artifacts. The currents recorded from the same cell show no systematic drift, ruling out seal-related error.

We observed that some cells had large current density for all four currents measured, but some cells had small current densities for a subset of currents. We plotted, in Figure 1E, the charge carried by I_{NISO} (Q_{NISO}) against the charge carried by I_{Ks} (Q_{Ks}) from all four drug sequence groups. Note that the Q_{NISO} and Q_{Ks} values span a wide range. Yet, there is a tight correlation between Q_{NISO} and Q_{Ks}. We also noticed that cells exhibiting large I_{NISO} usually also have large K^+ currents in general. We therefore plotted, in Figure 1F, the inward going charges (Q_{inward}) carried by I_{NISO} and the total outward going charges ($Q_{outward}$) carried by all three K^+ currents (I_{Ks}, I_{Kr}, and I_{K1}). Again, a linear relationship exists between Q_{inward} and $Q_{outward}$; the slope of the best-fit line is non-zero. Had the variations been due to random factors, the slope of the line would be zero.

Table 1
Cell, AP, and currents parameters (mean ± standard deviation).

Drug Sequence	CENB	ECNB	NCEB	NECB
Number of cells/animals	6/3	5/3	5/3	5/3
Cell capacitance (pF)	162.6 ± 58.2	193.8 ± 46.3	238.6 ± 60.8	216.6 ± 65.6
AP	Tyrode solution			
Resting potential (mV)	−68.4 ± 2.0	−69.8 ± 3.4	−70.9 ± 1.9	−68.2 ± 5.8
AP amplitude (mV)	127.4 ± 3.8	129.4 ± 4.4	129.8 ± 1.2	129.2 ± 2.3
AP plateau50 (mV)	30.2 ± 2.7	29.1 ± 4.5	32.6 ± 2.4	33.9 ± 6.1
APD50 (ms)	289.7 ± 51.7	272.4 ± 51.8	312.3 ± 44.4	300.0 ± 27.9
APD90 (ms)	329.9 ± 56.9	329.0 ± 43.7	352.3 ± 54.4	332.9 ± 30.1
I_{NISO}	Nisoldipine 10 μmol/L			
Peak (pA/pF)	−1.25 ± 0.61	−0.72 ± 0.30	−0.84 ± 0.23	−1.33 ± 0.75
ttP (ms)	189.2 ± 41.3	182.0 ± 51.8	230.9 ± 39.4	196.4 ± 50.9
Q (pC)	−43.6 ± 22.6	−29.3 ± 11.9	−42.2 ± 9.7	−52.1 ± 25.6
I_{Ks}	Chromanol-293B 10 μmol/L			
Peak (pA/pF)	0.55 ± 0.43	0.31 ± 0.25	0.27 ± 0.22	0.39 ± 0.20
ttP (ms)	173.9 ± 119.4	149.3 ± 105.0	261.1 ± 60.6	193.3 ± 90.7
Q (pC)	22.6 ± 16.8	9.2 ± 5.2	12.9 ± 10.9	23.2 ± 8.3
I_{Kr}	E4031 1 μmol/L			
Peak (pA/pF)	0.58 ± 0.26	0.65 ± 0.18	0.71 ± 0.21	0.79 ± 0.13
ttP (ms)	266.0 ± 128.2	311.7 ± 44.7	352.7 ± 48.1	322.0 ± 33.1
Q (pC)	10.4 ± 8.3	13.5 ± 6.3	20.7 ± 3.9	30.1 ± 17.4
I_{K1}	Ba^{2+} 50 μmol/L			
Peak (pA/pF)	1.42 ± 0.44	1.66 ± 0.59	1.44 ± 0.64	1.34 ± 0.18
ttP (ms)	335.5 ± 54.5	325.3 ± 45.6	365.9 ± 49.6	336.7 ± 31.5
Q (pC)	3.9 ± 1.0	6.1 ± 3.5	5.4 ± 2.5	4.3 ± 1.6

The linear regression slopes of Q_{Ks}/Q_{NISO} and $Q_{outward}/Q_{inward}$ are −0.47 and −0.69 respectively. This means that I_{Ks} carries 47% of the charges needed to counterbalance I_{NISO}, and the charge carried by the sum of I_{Ks}, I_{Kr} and I_{K1} represents 69% of the charges counterbalancing I_{NISO}. It is outside the scope of this study to identify the other missing currents. However, this analysis demonstrates how the onion-peeling method can be used to determine the relative contribution of a given current amongst the other currents in a single cell.

3.3. Relationship between the ionic currents and AP

To examine whether the cell-to-cell variation in currents could result from differences in AP, we analyzed correlations between characteristic parameters. Knowing the large variations in I_{NISO} and I_{Ks} in individual cells, we segregated the cells into two groups: 5 cells with the largest currents in one group versus 5 cells with the smallest currents in the other group. The values of Q_{NISO} and Q_{Ks} are -64.7 ± 10.1 pC (mean ± standard deviation) and 36.3 ± 13.5 pC for the large-current group, and -18.8 ± 3.2 pC and 6.1 ± 2.4 pC for the small-current group; the former is significantly larger than the latter (*t*-test $p<0.01$) for both Q_{NISO} and Q_{Ks}. The AP parameters are not significantly different between the two groups. AP duration determined at 90% repolarization (APD90) is 338.4 ± 59.3 versus 323.2 ± 42.9 ms and the plateau height is 101.8 ± 2.9 versus 99.5 ± 3.2 mV. These data provide the first experimental evidence to demonstrate that either large or small currents can generate the same AP as long as the inward and outward currents can counterbalance each other in a single cell.

4. Discussion

We have combined AP-clamp with an innovative sequential dissection technique to enable recording of multiple currents in the same cell during cardiac AP. This method has three important advantages. First, we can directly visualize and measure the dynamic behavior of the currents under an AP waveform instead of rectangular voltage pulses. Second, the internal and external solutions can be made to resemble the physiological ionic milieu, whereas traditional voltage-clamp technique requires non-physiological ionic composition to isolate a particular current. Third, it enables us to measure not one, but a multitude of currents flowing during the cell's own AP. Measuring multiple currents from a single cell enables us to see the relationships between currents within a cell. Hence, AP-clamp onion-peeling provides a unique and powerful tool for studying individual cell electrophysiology.

The sequential dissection technique, the standard voltage-clamp, and mathematic modeling are complementary ways of studying the electrical behavior of cells. Standard voltage-clamp experiments are needed to get the biophysical properties of channels and transporters, which are then used to construct mathematical models. The models, in turn, allow us to see how currents and membrane voltage shape each other. However, the traditional voltage-clamp experiments often use unphysiological solutions and Ca^{2+} buffer; therefore, the data may contain possible distortions. Direct measurements under physiological conditions with Ca^{2+} cycling are needed to assess the impact of the simplifications used in standard voltage-clamp experiments. The AP-clamp sequential dissection technique provides these independent measurements. In some cases, modeling may be necessary to analyze the AP-clamp data. For example, I_{NISO} comprises the L-type Ca^{2+} current plus some fraction of the Na^{+}/Ca^{2+} exchange current and other Ca^{2+}-dependent currents. These currents can be separated if specific blockers are available. In the absence of specific blockers, recourse to models is needed to effect the separation.

The ability to measure multiple currents from the same cell has revealed hitherto unknown characteristics of ionic currents in cardiac cells. First, within a single cell, large outward current/charge movement is matched with large inward current/charge movement and vice versa, hence the magnitudes of the depolarizing and repolarizing currents seem to be coordinated in such a way as to produce canonical AP, as seen in healthy guinea pig ventricular myocytes in this study. Second, there exists large variation in the current/charge movements between cells. These two findings suggest a more pluralistic view of cardiac cells. The magnitude of ionic currents can vary widely between cells, but the relative strength of the depolarizing and repolarizing currents are somehow constrained within a cell to produce similar APs. Variation and coordination of ionic currents in cardiac myocytes should have significant impact on the AP and play important roles in arrhythmias.

Conflict of Interest

None.

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