Na\(^+\)-induced Ca\(^{2+}\) influx through reverse mode of Na\(^+\)-Ca\(^{2+}\) exchanger in mouse ventricular cardiomyocyte

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Keywords: Pathology Section, reverse mode of Na\(^+\)-Ca\(^{2+}\) exchanger, dobutamine, action potential, voltage-gated ion channel, ventricular cardiomyocyte

Abbreviations: NCX, Na\(^+\)/Ca\(^{2+}\) exchanger.

Received: June 15, 2015 Accepted: July 24, 2015 Published: August 06, 2015

ABSTRACT

Background: Dobutamine is commonly used for clinical management of heart failure and its pharmacological effects have long been investigated as inotropics via β-receptor activation. However, there is no electrophysiological evidence if dobutamine contributes inotropic action due at least partially to the reverse mode of Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) activation.

Methods: Action potential (AP), voltage-gated Na\(^+\) (\(I_{Na}\)), Ca\(^{2+}\) (\(I_{Ca}\)), and K\(^+\) (\(I_{to}\) and \(I_{K1}\)) currents were observed using whole-cell patch technique before and after dobutamine in ventricular cardiomyocytes isolated from adult mouse hearts. Another sets of observation were also performed with Kb-r7943 or in the solution without [Ca\(^{2+}\)]\(_o\).

Results: Dobutamine (0.1–1.0 μM) significantly enhanced the AP depolarization with prolongation of AP duration (APD) in a concentration-dependent fashion. The density of \(I_{Na}\) was also increased concentration-dependently without alternation of voltage-dependent steady-status of activation and inactivation, reactivation as well. Whereas, the activities for \(I_{Ca}\), \(I_{to}\), and \(I_{K1}\) were not changed by dobutamine. Intriguingly, the dobutamine-mediated changes in AP repolarization were abolished by 3 μM Kb-r7943 pretreatment or by simply removing [Ca\(^{2+}\)]\(_o\) without affecting accelerated depolarization. Additionally, the ratio of APD\(_{50}/\APD_{90}\) was not significantly altered in the presence of dobutamine, implying that effective refractory period was remain unchanged.

Conclusion: This novel finding provides evidence that dobutamine upregulates of voltage-gated Na\(^+\) channel function and Na\(^+\) influx-induced activation of the reverse mode of NCX, suggesting that dobutamine may not only accelerate ventricular contraction via fast depolarization but also cause Ca\(^{2+}\) influx, which contributes its positive inotropic effect synergistically with β-receptor activation without increasing the arrhythmogenetic risk.
INTRODUCTION

Dobutamine is a sympathomimetic drug that has long been used in the treatment of heart failure [1] and cardiogenic shock [2] on the basis of its positive inotropic action. Its primary mechanism is direct stimulation of β-receptors of the sympathetic nervous system coupled with intracellular Ca\(^{2+}\) mobilization through G-protein-couple receptor and cyclic adenosine-monophosphate (cAMP) pathway. However, if other potential mechanism except for β-receptor stimulation involved in dobutamine-mediated intracellular Ca\(^{2+}\) mobilization is not fully understand so far. Recent finding has shown that Na\(^{+}\) influx during the early phase of action potential (AP) induces an intracellular Ca\(^{2+}\) increase through activation of the reverse mode of Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX) [3–5] and there is no such report in the literatures if dobutamine share the similar mechanisms. Therefore, this study was designed to answer the following questions regarding the electrophysiological profiles of dobutamine: [1] if voltage-gated Na\(^{+}\) channel is activated; [2] if Na\(^{+}\) influx induces the Ca\(^{2+}\) entry via the reverse mode of NCX in an extracellular-dependent manner; and [3] the relation between [1] and [2]. To experimentally verify these questions, both AP and voltage-gated ion channel currents (I\(_{Na}\), I\(_{Ca}\), I\(_{K1}\), and I\(_{g}\)) were investigated on ventricular cardiomyocytes isolated from adult mouse heart, respectively, using whole-cell patch technique [6, 7] before and after administration of dobutamine. Additionally, separate sets of current-clamp observations were also performed by pretreatment of cardiomyocytes with Kb-r7943, selective NCX blocker, with normal Tyrode’s solution or calcium free Tyrode’s solution to verify the involvement of NCX and Ca\(^{2+}\)-dependency. This observation provides a solid evidence to suggest that dobutamine mediates Na\(^{+}\) influx through voltage-gated Na\(^{+}\) channel during AP depolarization and consequently induces an extracellular Ca\(^{2+}\)-dependent Ca\(^{2+}\) influx through the reverse mode of NCX in ventricular cardiomyocyte of adult mouse heart.

MATERIALS AND METHODS

Animals

For consistency, only 8 week-old young adult male mice were used for experiments unless otherwise indicated. All animal protocols were approved by Harbin Medical University or Indiana University School of Medicine Institutional Animal Care and Research Advisory Committee.

Chemical agents

Dobutamine and Kb-r7943 [8], a selective blocker for the reverse mode of Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX), were ordered directly from Sigma-Aldaich. Chemical agents for cell culture, enzymatic isolation of cardiomyocyte, and recording solutions were purchased from the regular commercial sources.

Ventricular cardiomyocyte isolation

Adult mouse single ventricular cardiomyocytes were isolated using Langendorff-perfused mouse hearts and standard enzymatic techniques as previously reported with minor modifications [9]. Briefly, mice were heparinized and sacrificed by cervical dislocation. The hearts were rapidly removed and retrogradely perfused through the aorta using a modified Langendorff apparatus. The preparation was perfused with calcium free Tyrode’s solution (in mM: NaCl 126, KCl 5.4, HEPES 10, NaHPO\(_4\) 0.33, MgCl\(_2\) 1.0, taurine 10, glucose 10, and pH adjusted to 7.4 with 1.0 N NaOH) for 5 min, and then switched to digestive solution (calcium free Tyrode’s solution containing Collagenase type-II 0.4 mg/ml, protease 0.02 mg/ml and BSA 1.0 mg/ml). Left atria and ventricular tissue were collected and titrated gently in calcium free Tyrode’s solution containing 0.5% BSA to obtain single cells. All solutions were gassed with 95% oxygen and 5% carbon dioxide. Single rod-shaped and Ca\(^{2+}\) tolerant cells with clear cross-striations (Figure 1A) were used for electrophysiological investigation.

Electrophysiology

Both current- (AP) and voltage-clamp recordings (I\(_{Na}\), I\(_{Ca}\), I\(_{K1}\), and I\(_{g}\)) were conducted using standard whole-cell patch-clamp techniques [10, 11] with an Axopatch 700B amplifier (Axon Instruments). Briefly, The recording electrodes (Borosilicate glass, Sutter) were pulled (P-97, Sutter Instruments) and polished (F-83, Narishige) down to 1.2 – 1.8 MΩ when filled with pipette solution. After formation of the gigahm-seal, the capacitance was electronically compensated and the cell membrane under the pipette tip was then ruptured by a brief increase in suction, forming the whole-cell recording configuration. After 2–5 min period for intracellular dialysis, the Tyrode solution was changed by bath perfusion of extracellular recording solution designed for I\(_{Na}\), I\(_{Ca}\), I\(_{K1}\), and I\(_{g}\) recordings, respectively. All cells were recorded at room temperature (22 – 23°C). Current amplitude data of each cell was normalized to its cell capacitance (current density, pA/pF). Current-voltage relationship (I-V curve) was presented by the currents normalized by the peak currents. Voltage-dependent activation and steady-state inactivation profiles were calculated by Boltzmann fitting function.

Recording solution

For AP, normal Tyrode’s solution was used (in mM): NaCl 125, KCl 4.5, NaH\(_2\)PO\(_4\) 1.8, NaHCO\(_3\) 24, CaCl\(_2\) 1.8, MgCl\(_2\) 0.5, and Glucose 5.5 and with pH adjusted to 7.40 with 1.0 N NaOH; and the pipette solution contained (in mM): K-glutamate 130; KCl 15; NaCl 5.0; Mg-ATP 5;
MgCl\textsubscript{2} 1.0; EGTA 5.0; CaCl\textsubscript{2} 1.0; HEPES 10, and pH adjusted to 7.2 with KOH.

For \(I_{\text{Na}}\), pipette solution was (in mM): CsOH 125; Aspartic acid 35; tetraethylammonium chloride 30; HEPES 11; Mg-ATP 5.0; EGTA 10; phosphocreatine 3.6, pH adjusted to 7.30 with 1.0 N CsOH, and recording solution contained (in mM): NaCl 50; MgCl\textsubscript{2}-6H\textsubscript{2}O 1.2; CaCl\textsubscript{2} 1.8; Tetraethylammonium chloride 125; CsCl 5.0; HEPES 20; Glucose 11; 4-AP 3.0; MnCl\textsubscript{2} 2.0; and pH adjusted to 7.30 with 1.0 N CsOH.

For \(I_{\text{Ca}}\), pipette solution was prepared (in mM): CsCl 20, MgCl\textsubscript{2}-6H\textsubscript{2}O 1, Mg-ATP 5, EGTA 10, CSOH 110, aspartate 110, HEPES 10, and pH adjusted to 7.2 using CsOH, and recording solution contained (in mM): Tris-Cl 136, CsCl 5.4, CaCl\textsubscript{2} 2.0, MgCl\textsubscript{2}-6H\textsubscript{2}O 1.0, HEPES 10, glucose 5.0, and pH adjusted to 7.4 using Tris.

For \(I_{\text{K}}\), pipette solution contained (in mM): K-glutamate 130; KCl 15; NaCl 5.0; Mg-ATP 5.0; MgCl\textsubscript{2} 1.0; EGTA 5.0; CaCl\textsubscript{2} 1.0; HEPES 10, and pH adjusted to 7.2 with 1.0 N KOH, and recording solution contained (in mM): NaCl 138, KCl 5.4, CaCl\textsubscript{2} 1.8, MgCl\textsubscript{2} 1.0, CdCl\textsubscript{2} 0.3, Nifedipine 0.02, HEPES 10, 10 glucose, and pH adjusted to 7.4 with 1.0 N NaOH.

**Statistical analysis**

Data were collected using Clampfit and analyzed using Origin and Excel. The EC\textsubscript{50} was estimated using sigmoidal fitting function from the dose-response curve. Continuous variables are presented as mean ± SD. Student’s t-tests were used to compare the means between groups. \(P \leq 0.05\) was considered statistically significant.

**RESULTS**

\(I_{\text{Na}}, I_{\text{K}}, I_{\text{to}},\) and \(I_{\text{Ca}}\) before and after treatments with dobutamine were investigated, respectively, in ventricular cardiomyocytes and only those completely recordings with control and test were included in the pooled data for further analysis. The number of observation in each group was collected from at least 3 mouse heart.

**Changes in AP discharge profiles in the presence of dobutamine**

In isolated ventricular cardiomyocyte (Figure 1A, \(n = 12\), AP discharge parameters were altered by
Table 1: Effects of dobutamine (0.1, 0.3, and 1 μM) on discharge profiles of AP recorded from ventricular cardiomyocytes isolated from adult mouse heart. Averaged data were presented as mean ± SD, n = 12 myocytes from at least 4 mice. *P < 0.05 and **P < 0.01 vs control

| Parameter | Control | 0.1 μM Dob | 0.3 μM Dob | 1 μM Dob | Washout |
|-----------|---------|------------|------------|----------|---------|
| RMP       | -79.4 ± 3.88 | -81.2 ± 3.47 | -83.6 ± 3.12* | -84.8 ± 3.44** | -80.6 ± 3.73 |
| APFT      | -57.1 ± 4.13 | -58.8 ± 3.42 | -61.3 ± 4.05* | -63.6 ± 4.66** | -58.8 ± 3.99 |
| Peak<sub>AP</sub> | 56.1 ± 5.11 | 56.9 ± 4.23 | 57.8 ± 3.41 | 58.8 ± 3.18 | 58.2 ± 4.15 |
| APD<sub>50</sub> | 3.87 ± 0.67 | 4.45 ± 0.81 | 5.51 ± 0.76* | 6.14 ± 0.64** | 4.33 ± 0.59 |
| % of APD<sub>50</sub> | 100% | 114.98% | 142.38% | 158.66% | 111.89 |
| APD<sub>90</sub> | 11.1 ± 2.04 | 13.0 ± 2.67 | 15.9 ± 3.82* | 17.7 ± 4.37** | 12.6 ± 4.02 |
| % of APD<sub>90</sub> | 100% | 117.11 | 143.24% | 159.46% | 113.51% |
| UV<sub>MAX</sub> | 193 ± 20 | 237 ± 14* | 260 ± 17** | 266 ± 27** | 208 ± 15 |
| DV<sub>MAX</sub> | -28.3 ± 4.24 | -31.3 ± 4.36 | -37.9 ± 3.24* | -46.4 ± 3.88** | -33.6 ± 3.78 |

Note: RMP: resting membrane potential (mV); APFT: AP firing threshold (mV); Peak<sub>AP</sub>: the peak of AP (mV); APD<sub>50</sub>: AP duration measured at 50% of the height (ms); APD<sub>90</sub>: AP duration measured at 90% of the height (ms); UV<sub>MAX</sub>: the maximal upstroke velocity of depolarization (mV/ms); DV<sub>MAX</sub>: the maximal downstroke velocity of repolarization (mV/ms).
Figure 2: Dobutamine-mediated increase in the current density of voltage-gated Na$^+$ channel ($I_{Na}$) without changing the voltage-dependent properties of activation and inactivation in ventricular cardiomyocyte isolated from adult mouse heart. For activation of $I_{Na}$ recording in voltage-clamp mode, the cell was held at −100 mV and 400 ms depolarization current pulse was stepped from −90 mV to +30 mV with 5 mV increments and 1-s interval between steps, for inactivation protocol, the cell was hold at −100 mV with double pulse protocol, before the test pulse 1-s conditioning pre-pulse was stepped from −120 mV to −30 mV 5 mV increment and followed by 20 ms test pulse at 2 ms immediately after the prepulse and stepped to −30 mV with 3-s step interval, for reactivation protocol, . The current density (pA) was normalized by whole-cell capacitance (pF) and voltage-dependent activation and inactivation curve were fitted by Boltzmann function (normalized conductance). All representative traces shown in this figure are from the same patch recordings. A. and B. voltage-dependent activation of $I_{Na}$ before and after 1.0 μM dobutamine. inset: the current-voltage relationship (I-V curve). Scale bars in B also apply for A; C. and D. voltage dependent inactivation and reactivation, respectively; E. voltage-dependent activation and inactivation curves; and F. voltage-dependent reactivation curves. Voltage-dependent property was fitted using Boltzmann equation and averaged data were presented as mean ± SD, n = 8 recordings from at least 3 mouse heart.
Figure 3: Effects of dobutamine on voltage-gated Ca\(^{2+}\) channel \((I_{\text{Ca}})\) recorded in ventricular cardiomyocyte isolated from adult mouse heart. For \(I_{\text{Ca}}\) recording, the cell was held at -80 mV and 400 ms depolarization current pulse was stepped from -60 mV to +60 mV with 5 mV increment and 1-s step interval. A. and B. representative recordings of \(I_{\text{Ca}}\) before and after 1 μM dobutamine, respectively; C. current-voltage relationship (I-V curve). averaged data were presented as mean ± SD, \(n = 6\) recordings from 3 mouse heart.

Figure 4: Effects of dobutamine on voltage-gated K\(^{+}\) channel \((I_{\text{K}})\) recorded in ventricular cardiomyocyte isolated from adult mouse heart. For \(I_{\text{K}}\), the cell was held at -40 mV and 600 ms depolarization current pulse was stepped from -40 mV to +50 mV with 10 mV increment and 1-s step interval. A. and B. representative recordings of \(I_{\text{K}}\) before and after 1 μM dobutamine, respectively; C. current-voltage relationship (I-V curve). averaged data were presented as mean ± SD.

Figure 5: Effects of dobutamine on voltage-gated K\(^{+}\) channel \((I_{\text{K1}})\) recorded in ventricular cardiomyocyte isolated from adult mouse heart. For \(I_{\text{K1}}\), the cell was held at -40 mV and 300 ms depolarization current pulse was stepped from -120 mV to +50 mV with 10 mV increment and 1-s step interval. A. and B. representative recordings of \(I_{\text{K1}}\) before and after 1 μM dobutamine, respectively; C. current-voltage relationship (I-V curve). averaged data were presented as mean ± SD.
and $I_w$ were not modified by the highest concentration (1 μM) of dobutamine.

**Extracellular-dependency of dobutamine-mediated changes in AP repolarization**

Even though, $I_{K1}$ and $I_{Ks}$, and $I_{Ca}$ as well were not involved in dobutamine-mediated AP repolarization changes, intracellular Ca$^{2+}$ mobilization through other transmembrane mechanism rather than voltage-gated Ca$^{2+}$ is then expected. To verify if the extracellular Ca$^{2+}$ influx occurs during the AP repolarization, removing extracellular Ca$^{2+}$ instead of using Mg$^{2+}$ would be the easiest way. In another set of experiment, the effect of dobutamine on AP was repeated in the normal recording solution with 2 mM Ca$^{2+}$. Interestingly, the similar results induced by 1 μM dobutamine were disappeared at ~2 min after complete bath perfusion (~1 ml/min) with 0 mM Ca$^{2+}$ recording solution (Figure 6A & 6B), strongly indicating the Ca$^{2+}$-dependency of dobutamine-mediated changes in AP repolarization.

**Effect of Kb-r7943 on dobutamine-mediated changes in AP repolarization**

Recent observation provides an evidence showing the potential connection between Nav1.5 and Na$^+$-Ca$^{2+}$ exchanger (NCX) [3, 4], which is a possible clue for an explanation regarding the effects of dobutamine AP discharge profiles. To test this hypothesis in our experimental condition, the APs were elicited in current-clamp configuration in ventricular cardiomyocytes. The results showed that the AP repolarization changes induced by 1.0 μM dobutamine were completely blocked by the pretreatment of cardiomyocyte with 3 μM Kb-r7943 (Figure 6C & 6D), a selective blocker for NCX, without affecting increased AP depolarization ($UV_{MAX}$).

Figure 6: Reverse mode of Na$^+$-Ca$^{2+}$ exchanger (NCX)- and extracellular Ca$^{2+}$ ([Ca$^{2+}]_{o}$)-dependent effects of dobutamine on AP discharge profiles. A. and B. APs and derivatives before and after 1 μM dobutamine and dobutamine plus 3 μM Kb-r7943; C. and D. APs and derivatives before and after 1 μM dobutamine with 2 mM [Ca$^{2+}]_{o}$ and dobutamine with 0 mM [Ca$^{2+}]_{o}$. Scale bars shown in C and D are also apply for A and B, respectively.
DISCUSSION

Dobutamine is a sympathomimetics and activates β-adrenergic receptors (β₁ and β₂ mediating cardiac and vascular effects, respectively) as an inodilator [12] and can be used in cases of congestive heart failure to increase cardiac output and positive inotropic support in the short-term treatment of patients with cardiac decompensation due to depressed contractility. Even though the clear beneficial effect of dobutamine the appropriate role of intravenous inodilator therapy in the management of congestive heart failure has long been a subject of controversy and limitation, mainly because of the side effects such as increased heart rate and O₂ consumption via β-receptor activation [13], and direct vascular effect and baroreflex feed-back regulation [14]. So, further investigation is definitely necessary to elucidate the exist electrophysiological mechanism underlying the therapeutic effect of dobutamine, which may in turn benefit for the future clinical application and pharmacological convention of dobutamine.

The major finding of this observation has demonstrated, for the first time by our knowledge, that the extracellular Ca²⁺-dependent Na⁺-induced Ca²⁺ influx is confirmed in ventricular cardiomyocyte in the presence of dobutamine in a concentration-dependent manner through voltage-gated Na⁺ channel (Nav1.5) activation during AP depolarization and consequently activation of the reverse mode of NCX and the intracellular Ca²⁺ mobilization during prolonged AP repolarization, which may be a novel mechanism of positive inotropic action of dobutamine except for the known β₁-receptor activation and G-protein coupled cAMP pathway.

Clearly, dobutamine accelerates the AP depolarization with shifting of RMP and AP firing threshold toward the hyperpolarized direction and this observation is supported by the notion of increased Iₖs density presumably due to the enhanced availability of voltage-gated Na⁺ channel at given relatively lower potential upon the inactivation profiles. In this particular case, a relatively less energy would be required to charge the membrane and therefore causing the reduction of AP firing threshold without alteration of voltage-dependent activation and inactivation properties. However, dobutamine-induced RMP hyperpolarization might be explained by the dobutamine-induced myocardial K⁺ uptake by β₁-adreneroreceptor and adenyate cyclase activation [13] and the Nernst Equation.

Not surprisingly, the outward K⁺ currents are the first to respond to a depolarizing event on account of the hyperpolarized activation profile and fast activation time constant for these currents. Moreover, as the membrane potential enters into a phase of rapid depolarization there is a marked recruitment of transient and Ca²⁺-activated K⁺ currents, which are primary outward K⁺ currents responsible for the terminating the AP upstroke and initiating a reversal in the trajectory of the membrane potential upon a Hodgkin-Huxley model [15–17]. In other words, the more faster depolarization occurs, the more K⁺ channels would be recruited during the repolarization and sequentially causing a shorter AP duration. Interestingly, although dobutamine caused a fast depolarization the larger K⁺ currents recruitment, especially the transient (Iₗᵣ), were not observed from the voltage-clamp records, suggesting other ion channel mechanism being involved in this event and the most suspicious one is Iᵢₗᵣ, unfortunately, this hypothesis was also not confirmed under the current experimental condition.

Recently, several reports indicate the potential role of the reverse mode of NCX in an intracellular Ca²⁺ mobilization [3, 4], which could be cytoplasmic Na⁺-dependent [5] and activated during the AP depolarization [18] in mouse and human. In this regard, by preincubation of ventricular cardiomyocytes with Kb-r7943, a selective NCX blocker, dobutamine-mediated changes in repolarization were disappeared without altering the depolarization, which was also confirmed by simply removing the extracellular Ca²⁺. Even though the extracellular Ca²⁺ was not added in our recording solution, the concentration for extracellular Ca²⁺ came from the distilled water and chemicals would be close to 1 μM [19], which may be enough to maintain the cell function and signaling cascades. These data strongly suggest that dobutamine causes extracellular Ca²⁺-dependently Na⁺-induced Ca²⁺ influx through the reverse mode of NCX.

Collectively, in the presence of dobutamine, unchanged ratio of APD₉⁰/APD₉⁰ may not increases the risk for arrhythmogenesis because the effective refractory period remains no change along with the similar extend prolongation of both APD₉⁰ and APD₉⁰. Meanwhile, the prolonged AP duration would limit heart rate increased by β₁-receptor stimulation, fast depolarization would produce a fast ventricular contraction and offer a relative longer period of time for cardiac diastolation. KB-R7943 is a potent, selective inhibitor of the reverse mode of the Na⁺/Ca²⁺ exchanger, but its modulatory effects on other receptor systems, such as the inhibition of Kb-r7943 on nicotinic receptor [20] and N-methyl-D-aspartate receptor [8] in nervous system, can not be excluded in cardiomyocytes under the current investigation. It would be necessary to verify the effect of Kb-r7943 on β-adrenoreceptor in the future experiment although it has not been documented so far.

ACKNOWLEDGMENTS

This project was supported by the research grants from the National Natural Science Foundation of China (81173051, 81202509, 31400983 and 31171122).

CONFLICTS OF INTEREST

These authors declare no conflict of interests.
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