Intracellular Ca\textsuperscript{2+} Modulation during Short Exposure to Ischemia-Mimetic Factors in Isolated Rat Ventricular Myocytes

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Abstract

We investigated the effects of different ischemia-mimetic factors on intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]). Ventricular myocytes were isolated from adult Wistar rats, and [Ca\textsuperscript{2+}] was measured using fluorescent indicator fluo-4 AM by confocal microscopy. Intracellular pH was measured using c5-(and-6)-carboxy SNARF-1 AM, a dual emission pH-sensitive ionophore. Myocytes were exposed to hypoxia, extracellular acidosis (pHo 6.8), Na-lactate (10 mM), or to combination of those factors for 25 min. Monitoring of [Ca\textsuperscript{2+}] using fluo-4 AM fluorescent indicator revealed that [Ca\textsuperscript{2+}] accumulation increased immediately after exposing the cells to Na-lactate and extracellular acidosis, but not during cell exposure to moderate ischemia. Increase in [Ca\textsuperscript{2+}] during Na-lactate exposure decreased to control levels at the end of exposure period at extracellular pH 7.4, but not at pH 6.8. When combined, Na-lactate and acidosis had an additive effect on [Ca\textsuperscript{2+}] increase. After removal of solutions, [Ca\textsuperscript{2+}] continued to rise only when acidosis, hypoxia, and Na-lactate were applied together. Analysis of intracellular pH revealed that treatment of cells by Na-lactate and acidosis caused intracellular acidification, while short ischemia did not significantly change intracellular pH. Our experiments suggest that increase in [Ca\textsuperscript{2+}] during short hypoxia does not occur if pHi does not fall, while extracellular acidosis is required for sustained rise in [Ca\textsuperscript{2+}] induced by Na-lactate. Comparing to the effect of Na-lactate, extracellular acidosis induced slower [Ca\textsuperscript{2+}] elevation, accompanied with slower decrease in intracellular pH. These multiple effects of hypoxia, extracellular acidosis, and Na-lactate are likely to cause [Ca\textsuperscript{2+}] accumulation after the hypoxic stress.

Key words: intracellular Ca\textsuperscript{2+}, intracellular acidosis, ischemia

Introduction

Elevated intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) during ischemia and reperfusion is known to induce damage to the heart. During ischemia, interruption of the oxygen supply and substrates promotes ATP hydrolysis and glycolysis, which together induce intracellular acidosis. Intracellular acidosis during ischemia may cause elevation of [Ca\textsuperscript{2+}], by stimulation Na\textsuperscript{+}–H\textsuperscript{+} exchanger resulting in Na\textsuperscript{+} influx, which increases [Ca\textsuperscript{2+}], by activation of Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger. On the other hand, ATP depletion decreases function of Ca-ATPases of sarcoplasmic reticulum and sarcolemma further leading to elevation of [Ca\textsuperscript{2+}].\textsuperscript{1–4}

Ischemia-induced changes in intracellular environment like depletion of ATP, lactate accumulation, and intracellular acidosis are often used to simulate ischemic damage of the heart.\textsuperscript{3,5,6} Different cellular models, including single fresh cardiomyocyte preparations, require short-lasting ischemia to study the effects of ischemia and reperfusion on cellular function.\textsuperscript{3,4} Differences in ischemia-mimetic solutions can differently affect [Ca\textsuperscript{2+}] and thus be crucial for the research of simulated cardiac ischemic injury. In this study, we compared the effects of 25 min-lasting simulated hypoxia with glucose deficiency,
lactate accumulation, and extracellular acidosis on [Ca^{2+}]
  during hypoxia and reoxygenation in single ventricular cardiomyocytes. Additionally, we examined the role of intracellular pH (pHi) on changes of [Ca^{2+}], under the same conditions. The results of this study provide new insight into effects of simulated ischemia that alter [Ca^{2+}], which produce cellular damage.

**Materials and Methods**

**Animals**

Adult male Wistar rats (175–250 g) were used in this study. Animals were kept on 12-h cycles of light and dark, and received water and standard food *ad libitum*. The investigation was conducted in accordance with the Institutional Animal Use and Care Committee of the Medical College of Wisconsin (Milwaukee, Wisconsin), and in accordance with U.S. National Institutes of Health standards (NIH Publication 95–23, revised 1996).

**Cell isolation**

Ventricular myocytes were isolated by enzymatic dissociation. Animals were anesthetized with an intraperitoneal injection of thiobutabarbital (Inactin, 100–150 mg/kg, Sigma-Aldrich, St. Louis, MO) and heparin (1000 U, Abraxis, Schaumburg, IL) to prevent blood clotting. The heart was rapidly removed and mounted to Langendorff perfusion system. All perfusion solutions were continuously gassed with 95% O2 and 5% CO2, and temperature was held at 37 °C. Perfusion with a 0 mM Ca^{2+} solution for 5 min was followed by 11 min of recirculation; the heart was rapidly removed and mounted to a nylon mesh (200 μm; Spectrum Laboratories, Inc., Rancho Dominguez, CA) at room temperature for 15 min before recordings started. SNARF-1 was excited at 543 nm with a green HeNe laser. Emitted fluorescence was simultaneously collected at 590 and 650 nm via a 40×/1.4 oil-immersion objective (Nikon). The 640 nm/590 nm emission ratio from each cell was calculated and converted to pHi values using nigericin calibration technique. Nigericin, a H^+/K^+ exchanger ionophore sets [K^+]_o=[K^+]_i, and thus pH_o=pHi. Calibration curve was made by exposing the cells to different pH (pH 6.0, pH 7.5, and pH 9.0) buffers in a depolarizing high K+ buffer (140 mM KCl, 1.0 mM MgCl₂, 5 mM D-glucose, 10 mM HEPES, in the presence of 10 mM nigericin). To reduce contamination of the superfusion system with nigericin, calibration was not done after every experiment. Instead, the pH calibration data were obtained separately from individual cells and were averaged for more than 15 cells.

**Solutions**

For baseline measurements, myocytes were superfused with normoxic Tyrode solution containing (in mM): 132 NaCl, 10 HEPEs, 5 D-glucose, 5 KCl, 1 CaCl₂, and 1.2 MgCl₂ equilibrated at room air; PO₂ 199±14.1 mmHg; pH 7.4 adjusted with NaOH. For simulated hypoxia, cells were placed into a closed hypoxic chamber (RC-21B, Warner Instruments, Hamden, CT) and perfused under controlled conditions. Hypoxia was simulated by perfusing the myocytes with hypoxic Tyrode solution containing (in mM): 132 NaCl, 10 HEPEs, 0 D-glucose, 8 KCl, 1 CaCl₂, and 1.2 MgCl₂; pH 7.4; PO₂ 26.5±2.8 mmHg, bubbled with 100% N₂ for 20 min. Acidosis was simulated by perfusing the cells with Tyrode solution at pH 6.8. Lactate accumulation was induced by adding 10 mM Na-L-lactate to Tyrode solution without osmotic compensation. Hypoxia, acidosis, and lactate accumulation were simulated with HEPES-buffered glucose-free Tyrode solution containing (in mM): 118 NaCl, 10 HEPEs, 8 KCl, 1 CaCl₂, 1.2 MgCl₂, 1 Na₃HPO₄, and 10 mM Na-L-lactate, pH 6.8; gassed with 100% N₂ for 20 min; PO₂ 26.5±2.8 mmHg. To diminish contamination by room air, all solutions were placed in glass syringes and delivered to recording chamber using an air-tight tubing system. The PO₂ of superfusing solutions was measured with a gas analyzer (Radiometer ABL 505, Radiometer, Copenhagen, Denmark).
Statistical analysis

Data are presented as means±SD, with the sample size (n). To test differences between groups, we used one-way analysis of variance with Bonferroni’s post-hoc test. Analysis of variance for repeated measures with Tukey’s post-hoc test was used for temporal comparisons between groups. A p value of 0.05 or less was considered significantly different.

Results

Following equilibration in normoxic HEPES-buffered Tyrode solution for 5 min, myocytes were exposed to simulated hypoxia (PO2 = 25 mmHg) at extracellular pH of 7.4. Figure 1a shows the effect of hypoxia applied for 25 min on [Ca2+]i in ventricular myocytes. During the exposure to hypoxia there was a slow increase of [Ca2+]i. At the end of this period [Ca2+]i increased to 106±5% (p>0.05). When hypoxic solution was replaced with normoxic Tyrode solution, [Ca2+]i returned to the preischemic levels.

Figure 1b shows the effect of acidic Tyrode solution (pH 6.8) on [Ca2+]i. The rate of [Ca2+]i increase was faster compared to hypoxia-induced [Ca2+]i increase, and at the end of 25 min period it was 116±4% (p<0.05). Washout of acidic Tyrode solution slowly returned [Ca2+]i to control levels. In contrast to the effect of hypoxia, [Ca2+]i recovery was prolonged. This suggests that the [Ca2+]i regulating mechanisms are impaired by lowering extracellular pH.

Na-lactate (10 mM) was used to induce intracellular lactate accumulation and intracellular acidosis. Figure 2a shows the changes in [Ca2+]i during the application of lactate at extracellular pH 7.4. Initially, there was rapid increase in [Ca2+]i, which was similar to that induced by acidic Tyrode solution, 118±10% (p<0.05). After the initial rise in [Ca2+]i, there was a slow partial recovery of [Ca2+]i, which reached control levels after 25 min. This result suggests that [Ca2+]i regulating process is active at normal extracellular pH.

When cardiac cells were exposed to hypoxia together with acidosis (pH 6.8) and Na-lactate, there was rapid in-
increase in $[\text{Ca}^{2+}]_i$ (149±15%), which did not recover during the hypoxic period. This implies that Na-lactate induces fast increase in $[\text{Ca}^{2+}]_i$, which persist when extracellular pH is decreased, indicating that regulating processes are less active at decreased extracellular pH. During early reperfusion, $[\text{Ca}^{2+}]_i$ decreased to the preschismic levels, but then significantly increased through reoxygenation, indicating that cells were damaged during simulated hypoxia (Figure 2b). Figure 3 summarized the change in $[\text{Ca}^{2+}]_i$ induced by hypoxia, Na-lactate, and extracellular acidosis or with combination of these factors.

The changes in $\text{pH}_i$ resulting from exposure to hypoxia, Na-lactate, extracellular acidosis, and combination of these factors are shown in Figure 4. Hypoxia induced small decrease in $\text{pH}_i$ (0.07±0.02 pH units, $p>0.05$). Na-lactate induced faster $\text{pH}_i$ decrease (0.15±0.03 pH units, $p<0.05$), which remained constant. Exposure of the cells to extracellular acidosis caused the decrease in $\text{pH}_i$ that was slower than $\text{pH}_i$ decrease during the Na-lactate exposure, but it was more profound (0.22±0.05 pH units, $p<0.05$). This corresponds to an increase in $[\text{Ca}^{2+}]_i$, as seen when cells were exposed to acidic Tyrode solution. Under the conditions of hypoxia with decreased extracellular pH and Na-lactate, the decrease in $\text{pH}_i$ was the largest (0.42±0.08 pH units, $p<0.05$).

Discussion

In this study, we examined the effects of different ischemia-mimicking factors on $[\text{Ca}^{2+}]_i$ and $\text{pH}_i$ in isolated ventricular myocytes. The main objective of this study was to compare the measurements with different types of hypoxia-mimicking solutions and to determine temporal relationship between the rise in $[\text{Ca}^{2+}]_i$ and $\text{pH}_i$. Our results showed that moderate ischemia ($\text{PO}_2 = 25 \text{ mmHg}$) had no effect on the rise in $[\text{Ca}^{2+}]_i$, and decrease in $\text{pH}_i$ during the tested period. Na-lactate at 10 mM concentration rapidly increased $[\text{Ca}^{2+}]_i$, which subsequently recovered at extracellular pH 7.4. When Na-lactate was combined with extracellular acidosis (pH 6.8) this effect was prevented. Extracellular acidosis induced slower rise in $[\text{Ca}^{2+}]_i$, that was accompanied with slow intracellular acidification. Combination of those factors also induced the rise in $[\text{Ca}^{2+}]_i$, after the removal of solutions.

Accumulation of $[\text{Ca}^{2+}]_i$ is one of the main factors causing cardiac damage during ischemia, and rise in $[\text{Ca}^{2+}]_i$ causes irreversible myocardial damage. In myocardial ischemia $\text{pH}_i$ decreases due to ATP hydrolysis, increased glycolysis, and increased lactate production. In addition, protons can not be removed due to impaired circulation. The lack of ATP impairs the function of $\text{Ca}^{2+}$-pumps, while acidosis induces an additional rise in $[\text{Ca}^{2+}]_i$, by activation of Na$^+$/Ca$^{2+}$ exchanger. When myocytes are exposed longer to an acidic solution, Na$^+$/H$^+$ exchanger activity is stimulated resulting in a Na$^+$ influx, which subsequently induces Na$^+$/Ca$^{2+}$ exchanger to operate in reverse mode resulting in rise in $[\text{Ca}^{2+}]_i$. Studies performed on animal models showed that Na$^+$/H$^+$ exchanger inhibitors, such as amiloride derivatives, can reduce intracellular Na$^+$ accumulation and subsequent rise in $[\text{Ca}^{2+}]_i$, during ischemia. These inhibitors were also beneficial in reducing myocardial ischemia and reperfusion injury.

To study the effects of ischemia and reoxygenation on cardiac myocytes, different ischemia-mimicking solutions are used. Those solutions can induce hypoxia, glucose deficiency, hyperkalemia, hypercapnia, acidosis, lactate accumulation, or substrate deprivation. For ischemia and reperfusion experiments, extended cellular viability is essential to study different cellular processes at reoxygenation. The long duration of hypoxia is limiting factor to study ischemia and reperfusion injury in fresh cellular preparations, like cardiac myocytes, which have to be used within several hours of isolation. On the other
hand, to induce ischemic damage of resting cardiomyocytes by sole hypoxia, usually several hours of hypoxia are necessary. Additionally, cardioprotective strategies like ischemic and anesthetic preconditioning are maximally potent when fresh cells are used\(^3\). For that reason the use of the short ischemia and reoxygenation-mimicking factors is necessary in many experimental models.

It has been reported that rat cardiomyocyte superfused with an anoxic medium lacking glucose does not increase \([\text{Ca}^{2+}]_i\); during prolonged hypoxia (more than 1 hour)\(^1\). This is in agreement with our experiments where 25 min of hypoxia without glucose did not significantly increase \([\text{Ca}^{2+}]_i\). Our experiments suggest that increase in \([\text{Ca}^{2+}]_i\); during short hypoxia does not happen if pH\(_i\) does not fall. In an earlier study, it was found that 20 mM Na-lactate induces intracellular acidosis and rise in \([\text{Ca}^{2+}]_i\). But when it was accompanied with extracellular acidosis (pH 6.4) at used concentration, Na-lactate induced severe twitch potentiations and myocytes went into an irreversible contracture at reoxygenation\(^1\). We observed similar effect when 20 mM Na-lactate was used. The high lactate concentrations can damage the myocytes through increased osmolarity, intracellular acidosis, and increase in \([\text{Ca}^{2+}]_i\). Myocyte hypercontracture leads to rupture of plasma membrane and rapid cell death (within 10 min), which is not suitable for studying the effect of reoxygenation on cellular function. Contrary, others have reported that Na-lactate is not responsible for anoxia and reoxygenation damage in cardiac myocytes\(^1\). Intracellular lactate increases up to 5–10 mM during anoxia in isolated ferret heart. In the present study, we used 10 mM Na-lactate to mimic those conditions present during ischemia in isolated hearts. Cells exposed to Na-lactate and acidosis had more severe increase in \([\text{Ca}^{2+}]_i\), and decrease in pH\(_i\). This is in agreement with the finding that intracellular acidosis induced by CO\(_2\), and Na-lactate can increase \([\text{Ca}^{2+}]_i\) transients\(^1\). Furthermore, there was a faster increase in \([\text{Ca}^{2+}]_i\); during Na-lactate exposure, than during extracellular acidosis. Increase in \([\text{Ca}^{2+}]_i\); has an early rapid phase and slower delayed phase dependent on Na\(^{+}\)–H\(^{+}\) exchanger activity. There are several mechanisms responsible for the early increase in \([\text{Ca}^{2+}]_i\); during Na-lactate exposure. One of the factors that could induce rapid rise in \([\text{Ca}^{2+}]_i\); upon exposure to Na-lactate is acute intracellular acidosis, when intracellular buffering mechanisms become hypersaturated. Under the conditions of intracellular acidosis, binding capacity of intracellular proteins for \([\text{Ca}^{2+}]_i\); is reduced, and acidosis reduces \([\text{Ca}^{2+}]_i\); uptake by sarcoplasmic reticulum. In addition to this, protons directly inhibit Na\(^{+}\)–Ca\(^{2+}\) exchanger, which normally extrudes \([\text{Ca}^{2+}]_i\); in resting myocytes\(^2\).

The decrease in \([\text{Ca}^{2+}]_i\); observed under prolonged exposure to Na-lactate was prevented with extracellular ac-
PROMJENE UNUTARSTANIČNOG Ca²⁺ TIJEM KRTAKOTRAJNOG IZLAGANJA ČIMBENICA KOMA OPONASAJU HIPOKSIJU U VENTRIKULARNIM MIOCITIMA ŠTAKORA

Istražili smo učinak različitih čimbenika koji oponašaju ishemiju na koncentraciju unutarstaničnog Ca²⁺ ([Ca²⁺]). Ventrikularne srčane stanice smo izolirali iz srca odraslih Wistar štakora, a [Ca²⁺] smo mjerili korištenjem fluorescentnog indikatora fluo-4 AM uz uporabu konfokalnog mikroskopa. Unutarstanični pH smo mjerili koristeći c5-(and-6)-carboxy SNARF-1 AM. Srčane stanice smo izložile hipoksijskom izlaganju za 25 minuta. Mjerenje [Ca²⁺], koristeći fluo-4 AM indikator, pokazalo je da se nakupljanje [Ca²⁺] povećalo nakon izlaganja miocita na Na-laktat i izvanstaničnoj acidozi, ali ne i hipoksiji. Porast u nakupljanju [Ca²⁺] tijekom izlaganja Na-laktatima smanjen je na razinu kontrole vrijednosti na kraju izlaganja [Ca²⁺] koji je na konzervativnoj pH 7,4, ali ne i pri pH 6,8. Na-laktat i izvanstanična acidoza imali su aditivni učinak na porast [Ca²⁺]. Nakon odstranjivanja otopina, [Ca²⁺] je se povećao samo kod su acidoze i Na-laktat bili pri miješeni zajedno. Analiza unutarstaničnog pH pokazala je da su Na-laktat i izvanstanična acidoza uzrokovali smanjenje pH, dok kratkotrajna hipoksijska ishemija nije značajno promijenila unutarstanični pH. Naši rezultati upućuju na da se [Ca²⁺], ne povećava tijekom kratkotrajne hipoksijske ishemijske smanjene unutarstanični pH, i izvanstanična acidoza uzrokovala je ishemiju [Ca²⁺] i hipoksijski stres. U usporedbi S Na-laktatom, izvanstanična acidoza uzrokovala je sporije smanjenje pH, a Na-laktat bili pri mijenjeni zajedno. Analiza unutarstaničnog pH pokazala je da su Na-laktat i izvanstanična acidoza uzrokovali smanjenje unutarstaničnog pH, dok kratkotrajna hipoksijska ishemija nije značajno promijenila unutarstanični pH. Naši rezultati vrijedni zaupaju na da se [Ca²⁺], ne povećava tijekom kratkotrajne hipoksijske ishemije, a su smanjene unutarstanični pH, dok je izvanstanična acidoza potrebna za trajno povećanje [Ca²⁺], koje su uzrokovane Na-laktatom. U usporedbi s Na-laktatom, izvanstanična acidoza uzrokovala je sporije smanjenje pH, a Na-laktat bili smanjeni ne smanjeni unutarstanični pH. Udržani učinci hipoksijske ishemije, izvanstanične acidoze i Na-laktata najvjerojatnije uzrokuju nakupljanje [Ca²⁺], nakon hipoksijskog stresa.