μM (n=2), 5) NaCN 2 mM (n=3), 6) ouabain 30 μM (n=2), 7) ouabain 40 μM (n=2), 8) ouabain 50 μM (n=2) and 9) KB-R7943 10 μM (n=2). KB-R7943 is a selective inhibitor of the reverse mode reaction of Na^+-Ca^{2+} exchanger (NCX)[8]. In the control group, global ischemia was induced immediately after the control perfusion. In the other groups, hearts were perfused with Krebs-Henseleit buffer containing the respective reagents for 3 to 10 min following the control perfusion, and then global ischemia was induced. In the 2-DG 5 mM + Na pyruvate 6 mM group, to adjust the sodium concentration of the perfusion medium, the NaCl concentration was decreased to 110 mM.

Measurement of intracellular pH, and ATP, creatine phosphate and Pi contents by ^{31}P-NMR spectroscopy

^{31}P-NMR spectra were obtained at 109.25 MHz with a JEOL GX 270 spectrometer (JEOL, Tokyo, Japan). The diameter of the probe was 15 mm, and spectra were collected from transients following 45° pulses delivered at 1 sec intervals for 1 min according to the method previously reported[14]. Perfused hearts were put into a NMR tube which was placed inside the probe. Coronary effluent was evacuated from the NMR tube through an overflow outlet placed above the heart. The heart temperature was maintained at 37°C using a variable temperature unit attached to the spectrometer and a water-jacketed perfusion line. One-min spectra were collected during control perfusion, as well as from 10 to 70 sec, 120 to 180 sec, and 270 to 330 sec of ischemia for each of six hearts (Experiment 1). In another 6 hearts, 1-min spectra were collected for the control, as well as from 60 to 120 sec, and 270 to 330 sec of ischemia (Experiment 2). The mean values of the spectra obtained during ischemia were considered to be at the center of each collection period (40, 90, 150, and 300 sec), since creatine phosphate (PCr) and Pi tissue amounts are assumed to change linearly during each collection period of ischemia. At the end of the experiments, all hearts were dried at 80°C for 48 hrs and then their dry weights were measured.

To determine the intracellular pH, the equation below was used[6]:

$$pH = 6.90 - \log[(\delta - 5.805)/(3.290 - \delta)]$$

where δ is the chemical shift (in parts per million) of the resonance of intracellular Pi relative to PCr.

Absolute intracellular concentrations of ATP, PCr, and Pi were determined from the ^{31}P-NMR spectra according to the method of Pike et al.[16] using KH_2PO_4 solution as an external standard.

Chemical analysis of lactate and pyridine dinucleotides

(1) Samples

Thirty-six isolated hearts were perfused as described above for the control perfusion and global ischemia was induced afterwards. Six hearts each were freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen at the following times: during control perfusion; after 20, 40, 90, 150 and 300 sec of ischemia. The freeze-clamped samples were powdered in liquid nitrogen.

(2) Extraction of lactate and NAD

Lactate and NAD were extracted by the method of Katz et al.[10] The powdered heart tissue samples (15-25 mg wet wt) prepared as described above were homogenized with 1 ml of ice-cold 0.6 M perchloric acid solution. The homogenates were centrifuged at 1,970×g for 10 min (0°C) and then the supernatants were mixed with an equal volume of ice-cold 0.6 M $KHCO_3$ solution for neutralization. After $KClO_4$ crystals had sunk, the supernatant was removed and stored at -80°C until the lactate and NAD assays were performed. Precipitates obtained by centrifugation as described above were dissolved in a 0.1 M NaOH solution and then protein contents were measured by the Lowry method[13].

(3) Extraction of NADH

NADH was extracted by the Klingenberg method[3]. Powdered heart tissue samples (15-25 mg wet wt) were homogenized with 1 ml of a 0.5 M KOH/50% alcohol solution. The homogenates were then heated at 90°C for 5 min in a water bath and then cooled at 0°C for 5 min. An amount of 0.1 ml of each of these extracts was set aside for protein measurement and the remaining 0.9 ml was neutralized with 0.5 ml of a mixture of 0.5 M triethanolamine, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4. The resulting mixture was centrifuged as described above and the supernatant was stored at -80°C until the assay for NADH. The protein content of the alcohol extracts was measured by the Lowry method after dilution with 0.1 M NaOH solution.

(4) Chemical analyses

The lactate contents of the perchloric acid extracts were measured spectrophotometrically by the lactate oxidase-peroxidase method[19]. NAD and NADH contents were measured by the modified Karp method[9] using bacterial luciferase and alcohol dehydrogenase. The NADH assay was performed within 12 h of extraction. The bioluminescence was measured with a 101C luminescence reader (Aloka Co., Ltd.).

Calculation and statistics

From the data obtained by ^{31}P-NMR, the ATP, PCr, and Pi contents were calculated as μmol/g-dry-wt. In Experiment 1 and 2, the mean values of pH, and ATP, PCr, and Pi contents for the control perfusion and 300 sec of ischemia were nearly the same. Therefore, the results used were those from the control perfusion, and 40, 150, and 300 sec of ischemia in the case of Experiment 1 and those for 90 sec of ischemia in the case of Experiment 2.

In the chemical analyses, the lactate and pyridine dinucleotide contents were calculated as nmol/mg protein and NAD/NADH ratios were calculated for each sample. All the results were expressed as mean ± SD for each chemical analysis and ^{31}P-NMR determination time point. The uniformity of variance of the data with time was analyzed by the Bartlett test. Statistical differences between the control data and the ischemia data were checked by one-way analysis of variance and then Dunnett's multiple comparison test using a software package (SAS 6.03, SAS Institute Japan Ltd.). $P<0.05$ was considered significant. Regressions between lactate contents and NADH contents and NAD/NADH ratios were calculated according to the simple regression line calculation method.

Results

Transient increase in contraction (TIC)

In the control perfusion, the left ventricular developed pressure (LVDP) and the heart rate ranged from 80 to 120 mmHg and 160 to 210 beats/min (n=22), respectively. In the control group, there was no remarkable change in EDP during the first 5 min of global ischemia. A typical TIC is shown in Fig. 1A. This TIC occurred 20 to 40 sec after the start of global ischemia and its pressure did not go above the half of the preceding LVDP. The typical TIC was observed in 17 hearts except for 3 hearts in the 2-DG 11 mM group and each one heart in the IAA 150μM and NaCN 2 mM groups. Therefore the appearance rate of typical TIC was 77% (17/22). Under constant-flow perfusion, the TIC was also observed, with electrical pacing and without it (data not shown). Furthermore, when hearts were perfused repetitively in a short-term ischemia (5 min) and reperfusion (10 min) cycle, the TIC was observed at a similar time from the initiation of ischemia (data not shown). When hearts were perfused for 3 min before ischemia initiation with 11 mM 2-DG, which inhibits glycolytic flux, the TIC was completely absent and there was no change in EDP or LVDP during perfusion (Fig. 1B). When the 2-DG concentration was reduced to 5 mM (2-DG 5 mM + pyruvate 6 mM group), these effects of

2-DG were still achieved by increasing the perfusion period to 10 min (data not shown). However, the effect of IAA, another glycolytic inhibitor, was not as constant as that of 2-DG in this respect. The TIC was not diminished when hearts were perfused with 100 μM IAA for 5 min before ischemia induction, but in the case of perfusion with IAA at 150 μM, the TIC was completely absent in one of the two hearts tested (Fig. 1C), but in the other one, the TIC

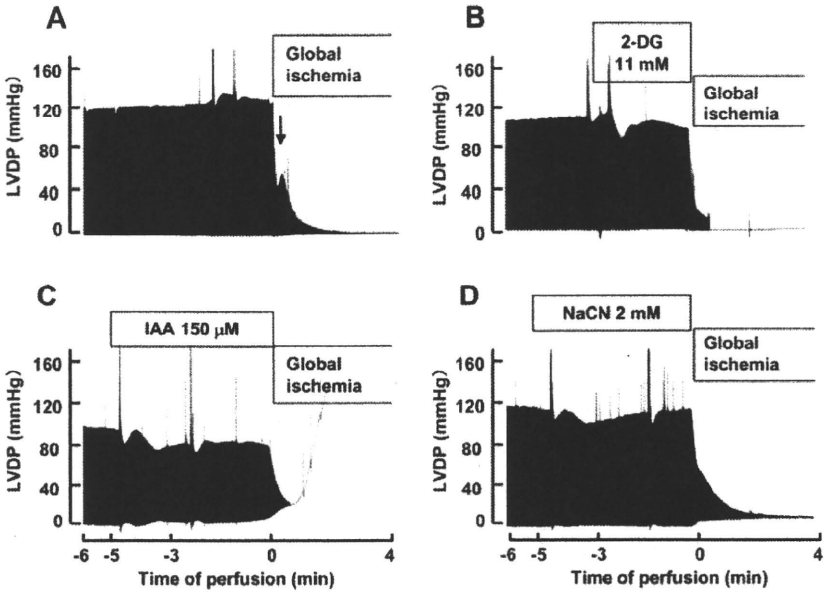

Fig. 1. Continuous recording of heart function during the control perfusion and early stage of ischemia. A: control perfusion and global ischemia - arrow shows a typical TIC, B: 2-DG 11 mM perfusion and global ischemia, C: IAA 150 μM perfusion and global ischemia, D: NaCN 2 mM perfusion and global ischemia.

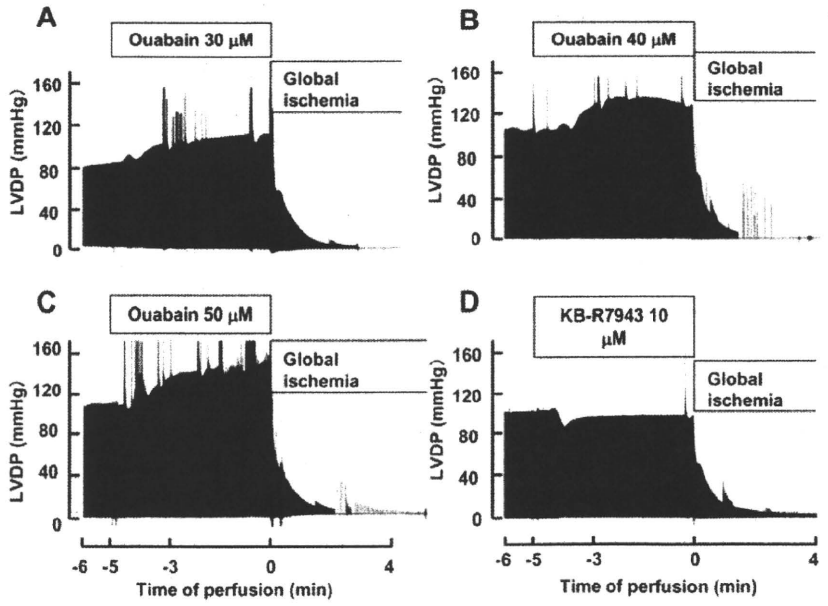

Fig. 2. Continuous recording of heart function during the control perfusion and early stage of ischemia. A: ouabain 30 μM perfusion and global ischemia, B: ouabain 40 μM perfusion and global ischemia, C: ouabain 50 μM perfusion and global ischemia, and D: KB-R7943 10 μM perfusion and global ischemia.

partially remained. With IAA perfusion, there was a more rapid rise in the EDP just after the heart stopped beating in ischemia than there was for 2-DG perfusion (Fig. 1C). Compared with these glycolytic inhibitors, it was more difficult to evaluate the effect of NaCN, a mitochondrial respiratory inhibitor. When hearts were perfused with 2 mM NaCN for 5 min, in many cases, the LVDP rapidly decreased to less than 2/3 of the preceding level and no TIC appeared after ischemia initiation. However, in three other hearts used to evaluate the effect of NaCN under the same perfusion conditions, there was only a slight change in the LVDP or there was no change at all, and the TIC was attenuated, though it did not fully disappear (Fig. 1D). When hearts were perfused with ouabain, a Na^+, K^+-ATPase inhibitor, for 5 min before ischemia initiation, a positive inotropic effect began at a concentration of 30 μM, which progressively increased up to 100 μM. In the case of initiating ischemia after perfusion with 30 μM ouabain, the TIC clearly persisted (Fig. 2A), but when the ouabain concentration was increased to 40 μM, the TIC seemed to be buried in the ischemic function declining curve (Fig. 2B), and then at 50 μM, the TIC was completely absent (Fig. 2C). Thus, the concentration of ouabain that caused the TIC to completely disappear was higher than the concentration that induced a positive inotropic effect. A TIC was clearly evident when hearts were perfused with 10 μM of KB-R7943 for 5 min before ischemia initiation (Fig. 2D). In the substances tested in this study, only 2-DG completely blocked the occurrence of TIC in all hearts used without affecting on the cardiac function.

Changes with time of pyridine dinucleotide contents, NAD/NADH ratio, and lactate content

Changes with time of pyridine dinucleotide contents, NAD/NADH ratio, and lactate content under the experimental conditions of our study are shown in Table 1. For the control perfusion, the NADH and NAD contents were 0.3 ± 0.1 nmol/mg protein and 5.8 ± 0.6 nmol/mg protein, respectively, and the NAD/NADH ratio was 24.6 ± 5.3 (n=6). The mean NADH content rapidly increased initially, being about twice the control value at 20 sec of ischemia and 5 times the control level at 40 sec of ischemia ($p<0.05$ vs control), and continued to increase until 300 sec of ischemia. There was no significant change in the NAD content during ischemia. As a result, the total pyridine dinucleotide content (NAD + NADH) steadily increased in pace with the NADH content increase up to 150 sec of ischemia, and the mean values at 150 and 300 sec of ischemia were significantly higher ($p<0.05$) than that of the control perfusion. Initially the NAD/NADH ratio decreased sharply - to about two-thirds of the control ($p<0.05$) at 20 sec of ischemia and one-fifth of the control after 40 sec of ischemia. It continued

Table 1. NAD, NADH, and Lactate Contents in the Perfused Rat Heart, NAD/NADH Ratios, and Changes in these Parameters after Global Ischemia

	Time (sec) after the onset of global ischemia					
	Control	20 sec	40 sec	90 sec	150 sec	300 sec
NAD	5.8 ± 0.6	6.2 ± 0.8	5.9 ± 0.7	5.6 ± 0.8	5.6 ± 0.7	5.1 ± 0.8
NADH	0.3 ± 0.1	0.5 ± 0.2	1.3 ± 0.4*	1.6 ± 0.2*	1.9 ± 0.2*	2.2 ± 0.1*
NAD+NADH	6.1 ± 0.7	6.6 ± 0.9	7.2 ± 0.8	7.2 ± 0.9	7.5 ± 0.8*	7.4 ± 0.7*
NAD/NADH	24.6 ± 5.3	16.4 ± 9.4*	5.0 ± 2.1*	3.4 ± 0.3*	2.9 ± 0.4*	2.3 ± 0.4*
Lactate	4.6 ± 1.9	5.2 ± 2.8	6.4 ± 2.9	26.6 ± 6.8*	50.8 ± 10.3*	98.8 ± 12.0*

Numerical values are shown as nmol/mg protein (mean ± SD, n=6)
*$p<0.05$ vs control values by Dunnett multiple comparison test

to gradually decrease until 300 sec of ischemia.

The lactate content was 4.6 ± 1.9 nmol/mg protein (n=6) in the control perfusion, and hardly changed up to 40 sec of ischemia. Thereafter, there was a sharp increase and it was about 5 times the control value after 90 sec of ischemia ($p<0.05$). It continued to increase up to 300 sec of ischemia.

Correlations between lactate content and NADH content, and NAD/NADH ratio

The correlations between lactate content and NADH content, and NAD/NADH ratio obtained under these experimental conditions, are shown in Figs. 3A and 3B. From both graphs, it is clear that the regressions between lactate content and NADH content, and NAD/NADH ratio switch in a non-continuous manner during ischemia progression. There was a critical switching point from aerobic oxidation (low NADH content) to anaerobic glycolysis (lactate production) at 40 to 90 sec of ischemia.

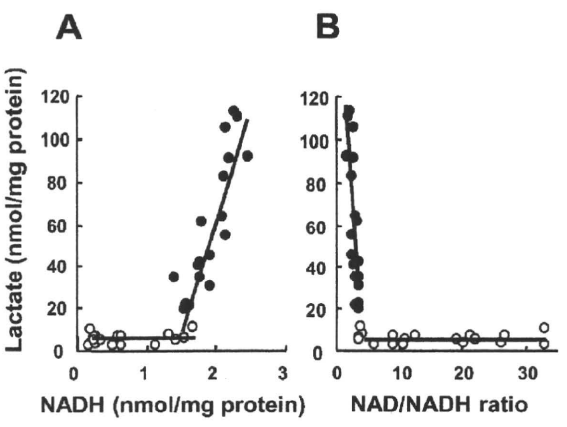

Fig. 3. Regressions between lactate content and NADH content (A) and NAD/NADH ratio (B) in the early stage of ischemia. Data were obtained from six isolated heart tissue samples during control perfusion, and after 20, 40, 90, 150 and 300 sec of ischemia (○ : control perfusion to 40 sec of ischemia, ● : 90 to 300 sec of ischemia). In Fig. 3A, regression line for control perfusion to 40 sec of ischemia: $Y = 1.44X + 4.42$, $r = 0.30$, and regression line for 90 to 300 sec of ischemia: $Y = 93.8X - 122.4$, $r = 0.86$ ($p<0.01$). Regression line for control perfusion to 40 sec of ischemia: $Y = -0.02X + 5.62$, $r = 0.06$, and regression line for 90 to 300 sec of ischemia: $Y = -39.7X + 172.9$, $r = 0.73$ ($p<0.01$) in Fig. 3B.

Changes in intracellular pH and ATP, PCr, and Pi contents with time

The mean intracellular pH was 7.36 ± 0.07 (n=6) during the control perfusion (Fig. 4). After the start of ischemia, the pH dropped to 7.18 ± 0.03 in 40 sec. Thereafter, the pH dropped continuously, registering 6.60 ± 0.05 at 300 sec of ischemia.

The mean PCr, ATP, and Pi contents during the control perfusion were 46.8 ± 8.9, 23.1 ± 3.8, and 16.0 ± 4.4 μmol/g-dry wt, respectively. Following the start of global ischemia, the PCr content rapidly decreased and at 40 and 90 sec of ischemia, the contents were approximately half and one-sixth of the control level, respectively (net decrease at 40 sec of ischemia was about 22 μmol/g-dry wt). On the other hand, there was hardly any change in ATP content up to 300 sec of ischemia. The Pi content rapidly increased with global ischemia, and at 40 sec of ischemia it was approximately 2.5 times the control level (net increase was about 23 μmol/g-dry wt).

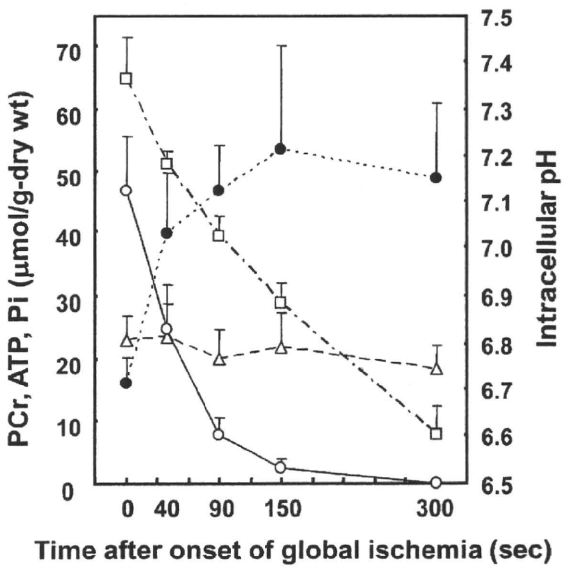

Fig. 4. Changes in intracellular pH, and ATP, creatine phosphate and Pi contents during the early stage of ischemia. □ : Intracellular pH, ○ : creatine phosphate, △ : ATP, ● : Pi

Discussion

A similar TIC has been observed in other studies in which the functions of isolated and perfused rat heart were continuously recorded[15, 26] but little mention was made of it. TIC is different from ischemic contracture and it is interesting to note that it occurs in the early stage of global ischemia when both metabolic and mechanical functions rapidly change.

We first noted that the occurrence of TIC is related to glycolytic activity. As mentioned in the results section, the TIC was completely absent with pre-perfusion of 2-DG, and this result was reproducible in all cases. However, in the case of pre-perfusion with IAA, the TIC did not always disappear and the results were not very reliable. The TIC did not disappear in the case of perfusion with 100 μM IAA, a concentration of IAA widely considered to be effective for inhibiting glycolysis[5]. On increasing the IAA concentration, the TIC was first inhibited at 150 μM (Fig. 1C). The difference in the inhibitory effect of these two reagents on TIC may be attributed to their modes of action and delivery. 2-DG is phosphorylated by hexokinase like glucose but cannot be further metabolized later on and blocks glycolysis, whereas IAA blocks glycolytic flux by inhibiting glyceraldehyde-3-phosphate dehydrogenase[21]. 2-DG is actively taken into the cardiomyocytes through the glucose transport pathway but IAA can only enter cells by passive diffusion.

ATP generated by glycolysis is thought to be intimately connected with the functions of the channel and pumps in the sarcolemma (SL) and sarcoplasmic reticulum (SR) membranes of cardiac myocytes. Glycolytic enzymes are compartmentalized (localized) in or near these membranes: in the SL membrane, the local ATP drives the Na^+, K^+-pump (ATPase)[7] and controls the opening of the K_{ATP}-channel[22], and in the SR membrane, it drives the Ca^{2+}-pump (ATPase)[25]. In global ischemia, the functions of the channel and pumps mentioned above are thought to be maintained until the very last moment when the supply of ATP from the corresponding glycolytic pathway ends. But the fact is that the glycolytic activity or flux localized in the membrane systems may be possibly affected even in the early period of ischemia, since the intracellular NADH and Pi concentrations increase rapidly while the intracellular pH has not yet decreased so much (Fig. 4). However, it is unclear whether these metabolic changes induced by global ischemia equally affect the glycolytic activity or flux localized by the two membrane systems. It is also unclear how the change of local glycolytic activity or flux affects the functions of the channel and pumps under consideration in the early stage of ischemia. However, the fact that TIC could be diminished by inhibiting glycolytic flux, suggests that it might be related to changes in activity in the channel and/or pumps since they are regulated by glycolysis.

Our second important finding was that TIC seemed to be diminished by inhibiting Na^+, K^+-pump activity with ouabain. However, the ouabain concentration that diminished the TIC was different from the concentration that induced a positive inotropic effect, so the results in this case should be carefully considered. It has been shown that when Na^+, K^+-pump activity is inhibited by cardiac glycosides, the intracellular Na^+ concentration ($[Na^+]i$) increases, and positive inotropy and Ca^{2+} overload occur[20]. Another study has reported that the positive inotropic effect is induced by intracellular Ca^{2+} ($[Ca^{2+}]i$) accumulation after the forward mode reaction of NCX, which is linked to Na^+, K^+-pump activity, is inhibited[18]. In this study, there was an obvious inotropic effect after starting the 30 μM ouabain perfusion, though a distinct TIC was evident after the start of global ischemia (Fig. 2A). As the ouabain concentration was increased, the TIC was buried in the ischemic function declining curve (Fig. 2B) to a greater

extent, and finally completely disappeared at a concentration of 50 μM of ouabain, for which there had been a strong positive inotropy effect (Fig. 2C). These results suggest that the TIC occurs independently from the $[Ca^{2+}]i$ increase induced by forward mode NCX inhibition and that at higher ouabain concentrations, the large increase in $[Ca^{2+}]i$ induced by the forward mode NCX inhibition might have buried the small rise in $[Ca^{2+}]i$ thought to trigger the TIC.

The TIC was not diminished by preperfusion with 10 μM KB-R7943. This suggests that the reverse mode reaction of NCX is not responsible for the TIC, since this concentration of KB-R7943 is thought to be sufficient to inhibit the reverse mode reaction of NCX even under these experimental conditions[8]. Thus, it seems unlikely that the TIC is induced by the increase in $[Ca^{2+}]i$ from the inhibition of NCX's forward mode reaction, which follows Na^+, K^+ pump activity inhibition by ouabain perfusion, or the stimulation of NCX's reverse mode reaction.

It is interesting that the onset of TIC seems to precede metabolic switching from aerobic oxidation to anaerobic glycolysis. As is well known, intracellular oxygen concentration rapidly decreases after the onset of global ischemia and mitochondrial respiration ceases thereafter as a consequence. At the same time, the uptake and oxidation of pyruvate by mitochondria also ceases, with the result that the cytosolic pyruvate concentration increases and lactic dehydrogenase (LDH) is stimulated towards lactate production. Similarly, the increased intracellular NADH concentration stimulates the LDH reaction in which NADH is oxidized to NAD. Thus, metabolic switching from aerobic oxidation to anaerobic glycolysis is thought to be triggered via feedback stimulation of LDH reactions induced by rapid increases in intracellular pyruvate and NADH concentrations that occur in the early stage of ischemia. Our study showed that in Langendorff perfused rat heart tissue, this switching occurs at a time between 40 and 90 sec of ischemia. While it is hard to believe that the TIC is directly induced by the accumulation of intracellular pyruvate and NADH that occurs before metabolic switching, the increases in the concentrations of these substances may contribute to the changes in activity of the glycolytic pathway after the start of global ischemia. Also, Ca^{2+} sensitivity of cardiac muscle fibers is affected by drops in intracellular pH and to a greater extent by an intracellular Pi increase. In this study, up to 40 sec of ischemia, pH dropped slightly and there was an increase in intracellular Pi in pace with a decrease in PCr. However, during this short period of ischemia, it was very unlikely that muscle fibers had become hypersensitive to Ca^{2+} ions in the above manner and induced the TIC as a result.

In conclusion, we were able to demonstrate that in the Langendorff perfused rat heart, TIC occurs during a short period preceding metabolic switching from oxidative phosphorylation to anaerobic glycolysis, and that it is intimately related to glycolytic activity in the early phase of global ischemia. The mechanisms connecting glycolysis and TIC should be clarified in future studies.

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ラットの単離心臓をランゲンドルフ潅流し、虚血を惹起した時の初期に起こる一過性の収縮は、解糖系の活性に依存している

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要旨：ランゲンドルフ潅流したラットの心臓に虚血を惹起すると，その直後の20～40秒の間に一過性の収縮（ITC）が起こる。この収縮は，虚血を惹起する直前に11 mM の2-デオキシグルコースで3分間潅流するか，5 mM 2-デオキシグルコース＋6 mM ピルビン酸の混合液で潅流すると，起こらなかった。また，150μM のヨード酢酸を潅流した時もこの収縮は見られなかったが，2 mM のシアン化ナトリウムを潅流した直後には，わずかな収縮が観察された。さらに，ウワバインは30μM ではこの収縮に影響を与えなかったが，50μM を虚血直前に潅流すると，この収縮が消失した。Na^+，Ca^{2+}交換体（NCX）の選択的抑制剤である KB-R7943は10μM ではこの収縮に影響を与えなかった。好気的代謝のマーカーである NAD/NADH 比は，虚血開始直後から急速に低下し，虚血40秒後には虚血前値の1/4になった。一方，嫌気的代謝のマーカーである乳酸生成は虚血40秒後まではほとんど変化しなかったが，それ以降に急激に増加した。^{31}P-NMR 法で心臓の代謝を観察したところ，虚血40秒後までにクレアチン燐酸が急激に減少し，それの裏返しとして無機リンが増加したが，ATP 含有量は虚血90秒までほとんど変化しなかった。また，細胞内 pH は虚血40秒後までにわずかに低下した。これらの実験結果から，ランゲンドルフ潅流したラットの心臓で虚血直後に見られる一過性の収縮（ITC）は，虚血によって引き起こされる好気的代謝から嫌気的代謝への切り換え（スイッチング）に先立つわずかな時間に起こり，しかも解糖系の代謝に関係しているらしい，ことが示唆された。

索引用語：　NAD/NADH 比　／　乳酸　／　Na^+, K^+－ATPase　／　エネルギー代謝　／　代謝スイッチング

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