Myocardial Na\(^+\) during Ischemia and Accumulation of Ca\(^{2+}\) after Reperfusion: A Study with Monesin and Dichlorobenzamil

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ABSTRACT—The intracellular cation contents were determined in isolated perfused rat heart using cobaltic EDTA as a marker of the extracellular space. In hearts in which Na\(^+\) accumulation was induced with monensin, a Na\(^+\) ionophore, during 20 min-ischemia which otherwise did not result in accumulation of Na\(^+\), the levels of Na\(^+\) and Ca\(^{2+}\) were significantly higher after reperfusion with a significant decrease in K\(^+\). While the recovery of the cardiac mechanical function (CMF) was complete after reperfusion in control hearts, the recovery was incomplete in monensin-hearts. Dichlorobenzamil (DCB), the most specific inhibitor of Na\(^+\)-Ca\(^{2+}\) exchanger, infused for 10 min before induction of ischemia in a dose of 10\(^{-5}\) M, which produced a definite suppression of CMF (over 80%), inhibited the accumulation of Ca\(^{2+}\) and Na\(^+\) and the loss of K\(^+\) and ATP after 40 min-ischemia and reperfusion. The same dose of DCB given for 3 min before induction of ischemia and after reperfusion, which produced a less than 20% inhibition of CMF, failed to prevent the Ca\(^{2+}\) accumulation after 40 min-ischemia and reperfusion. These findings are at variance with the idea that the accumulation of Na\(^+\) during ischemia and the consequent augmented operation of Na\(^+\)-Ca\(^{2+}\) exchange is responsible for accumulation of Ca\(^{2+}\) after reperfusion.

Keywords: Ca\(^{2+}\) accumulation, Monensin, Na\(^+\)-Ca\(^{2+}\) exchange, Intracellular cation contents, Cobaltic EDTA

The intracellular accumulation of Ca\(^{2+}\) is a crucial factor for the irreversible myocardial injury that occurs after reperfusion following a prolonged period of ischemia (1, 2). According to Bourdillon and Poole-Wilson (3), the accumulation of Ca\(^{2+}\) was not due to a decrease in efflux, but due to an increase in influx. However, attempts to demonstrate the involvement of voltage-operated Ca\(^{2+}\) channels in the accumulation of Ca\(^{2+}\) have failed; Ca\(^{2+}\) antagonists given during the period of reperfusion (4, 5) did not prevent the accumulation of Ca\(^{2+}\). Ca\(^{2+}\) antagonists can prevent the accumulation when given prior to induction of ischemia. However, the inhibition was closely associated with the suppression of the myocardial mechanical work before induction of ischemia. Thus, nonspecific protective effects may be the cause of the observed prevention. Observing the increase in myocardial Na\(^+\) content during ischemia, several researchers (6–8) implicated Na\(^+\)-Ca\(^{2+}\) exchanger as another possible pathway for Ca\(^{2+}\) entry. Weiss et al. (9) reported the prevention of reoxygenation-induced Ca\(^{2+}\) accumulation in hypoxic heart by an inhibitor of Na\(^+\)-Ca\(^{2+}\) exchanger, amiloride. However, the specificity of amiloride as an inhibitor of Na\(^+\)-Ca\(^{2+}\) exchanger does not seem to be sufficiently high to derive a reliable conclusion. In view of these circumstances, we performed the present study to reexamine the importance of Na\(^+\) accumulation during ischemia and the consequent augmented operation of Na\(^+\)-Ca\(^{2+}\) exchanger for reperfusion-induced accumulation of Ca\(^{2+}\). The importance of Na\(^+\) accumulation during ischemia was assessed by producing an accumulation of Na\(^+\) using monensin during a short period of ischemia. For assessment of the role played by the Na\(^+\)-Ca\(^{2+}\) exchange system, the most specific inhibitor of the Na\(^+\)-Ca\(^{2+}\) exchange system, dichlorobenzamil, was used. Particular attention was directed to changes in myocardial function produced by these agents.
MATERIALS AND METHODS

Experiments were performed in an isolated perfused rat heart preparation (Langendorff's method). Male rats weighing around 250–350 g were lightly anesthetized with ether. Immediately after opening the thorax, the hearts were excised and transferred to ice-chilled modified Krebs-Ringer bicarbonate solution to induce rapid cessation of the heart beat. The adherent connective tissue was removed and the ascending aorta cannulated. Retrograde perfusion with a modified Krebs-Ringer bicarbonate solution from a reservoir 75 cm above the heart was begun immediately. The perfusion fluid contained 127.2 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄ and 24.9 mM NaHCO₃. It was oxygenated with 95% O₂ + 5% CO₂ gas by means of an oxygenating device as described by Neely et al. (10) to ensure P0₂ values higher than 600 mmHg and was kept at a temperature of 38°C. Sodium pyruvate (2 mM) and glucose (5.5 mM) were added to the perfusion fluid as substrates.

The coronary inflow (CF) was measured by means of an electromagnetic flowmeter probe (Statham, 1-mm i.d.) placed in the perfusate inflow-line and an electromagnetic flowmeter (Statham 2201). The left ventricular pressure (LVP) was measured by a balloon filled with saline and connected to a pressure transducer (Statham P50) with a saline-filled polyethylene tubing, which was introduced into the left ventricular cavity from the left atrium through the mitral valve. Heart rate (HR) was counted with a cardiotachometer (Sanei N2507) triggered by pressure pulses of LVP. As a measure of the total mechanical energy required for contraction (11), the product of LVP × HR was calculated.

Protocol

After a 20 min equilibration perfusion, the perfusion fluid was changed to one containing 1 mM potassium cobaltic EDTA (Co-EDTA). After a further perfusion for 20 min with this solution, global ischemia was induced by cross-clamping the aortic inflow line for 40 min. Then the hearts were reperfused for 40 min (reperfusion group). The period of reperfusion was set at 40 min to obtain a steady level of myocardial mechanical function.

When the effects of monensin was examined, the animals (both the control and the test animals) were administered intraperitoneally with 1 mg/kg of reserpine 18–24 hours before the experiments, as it is known that monensin produces significant positive inotropic and chronotropic responses by releasing catecholamines (12, 13). Prior to induction of global ischemia for 20 min, monensin (6.13 × 10⁻⁵ mg/ml) was infused for 10 min into the perfusate inflow line near the aortic cannula at a speed of 0.113 ml/min by means of an infusion pump (Harvard 940) to achieve a concentration of around 10⁻⁶ M. Ethanol, the solvent of monensin, was infused for the control hearts.

Dichlorobenzamil (DCB) (10⁻⁵ M) was infused for 10 min before ischemia or for 3 min before ischemia and then 3 min after reperfusion. The solvent of DCB, polyethylene glycol (PEG), was infused to the control hearts.

At the end of perfusion, hearts were rapidly excised and immediately frozen with a pair of Wollenberger tongs precooled in liquid N₂. The frozen tissue fragments were crushed into a fine powder in a stainless steel percussion mortar cooled in liquid N₂ (14).

Intracellular cation contents

For the determination of the intracellular myocardial cation contents, 300–400 mg of fine powder of myocardium was dried overnight at 80°C and weighed. Na⁺, K⁺, Ca²⁺ and Co²⁺ were extracted from the dried tissue powder with a method developed by Sparrow and Johnstone (15), and the total tissue contents of the cations were determined with an atomic absorption spectrometer (Hitachi 180-30) and a flame photometer (Hitachi 180-30). The intracellular cation contents were calculated using the following equation (Hoerter et al.) (16):

\[
\text{Intracellular cation contents} = \frac{\text{Tissue content} \times (\text{ECS})}{(\text{Concentration in perfusate})} - \frac{(\text{ECS})}{(\text{Concentration in perfusate})}
\]

Adenine nucleotide contents

The extraction of adenine nucleotide from the myocardium was conducted by a method developed by Khym (17). The fine powder of myocardium was homogenized at 0°C with a Polytron homogenizer (Kinematica PT 10/35) at setting 9 in ice-cold 0.6 N perchloric acid. After centrifugation at 3000 rpm for 15 min, the supernatant was neutralized with 1,1,2-trichlorofluoroethane containing 0.5 M tri-n-octylamine. After a second centrifugation at 1,000 rpm for 2 min, the supernatant was used for the determination of adenine nucleotide contents.

Determination of ATP, ADP and AMP was performed with high performance liquid chromatography (HPLC) (Waters model 6000A Solvent Delivery System
with a 330 UV Absorbance Detector). A Radial-Pak μ Bondapack C18 column (Waters) was used as the stationary phase with 0.025% trihydroxyfuran, 0.12 M KH2PO4, 0.001 M tetrabutylammonium hydrogen sulfate and 4.5% acetonitrile (pH 6.25) as a mobile phase (18). The absorbance was monitored at 254 nm. Identification of the compounds was carried out on the basis of retention time and enzymatic transformation, and the concentration of each compound was calculated from the peak height measurements using the corresponding authentic substances as standards. As sensitive measures of the energy status of the myocardium, the ATP/ADP ratio and energy charge (EC) were used. The latter was calculated as follows:

$$EC = \frac{(ATP + ADP/2)}{(ATP + ADP + AMP)}$$

Throughout the experiments, all animals were dealt with in a humane manner in accordance with recognized guidelines on animal experiments.

**Chemicals and drugs**

The following chemicals and drugs were used: ethanol (for HPLC grade, Wako Chemicals) and tetrabutylammonium hydrogen sulfate (Aldrich Chemicals). DCB, a generous gift from Nippon Soda Co., Ltd., was dissolved in PEG and diluted by distilled water (final concentration of PEG was about 0.1%). All other chemicals were obtained from Wako Chemicals. Potassium Co-EDTA (16, 19) was prepared in pure crystalline form by the method of Dwyer et al. (20).

**Statistical analyses**

Data are presented as means ± S.E. Statistical assessment of the significant difference among groups was made by one-way analysis of variance followed by Bonferroni’s method or Student’s t-test. A difference was considered significant at a probability value of less than 0.05.

**RESULTS**

The perfusion with the Krebs-Ringer bicarbonate solution containing 1 mM Co-EDTA did not affect any parameter of the cardiac function. Tissue cobalt level reached a plateau within 10 min after the start of perfusion.

The myocardial intracellular contents of Na+, K+ and Ca2+ determined after 40 min of perfusion were 64.88 ± 12.84, 361.24 ± 7.41 and 2.84 ± 0.12 μmoles/g dry weight, respectively (Table 1); and the extracellular space, intracellular space and water content were 0.4264 ± 0.0163, 0.4204 ± 0.0143 and 0.8450 ± 0.0027 ml/g wet weight (n = 12), respectively. Myocardial contents of ATP, ADP and AMP were 28.91 ± 3.08, 5.04 ± 0.30 and 0.85 ± 0.10 μmoles/g dry weight, respectively (Table 1).

**Changes in cardiac function after ischemia and reperfusion**

Figure 1 depicts the recovery of cardiac function after reperfusion for 40 min following 20, 40 and 60 min of global ischemia. The recovery was expressed as a % of the values before induction of ischemia (determined after 40 min equilibration perfusion), which were 10.3 ± 1.4 ml/min for CF, 0 mmHg for enddiastolic pressure (EDP), 138.6 ± 7.1 mmHg for LVP and 311 ± 8 beats/min for HR. The recovery of cardiac function following 20-min ischemia was almost complete in terms of any parameters of the cardiac function examined. In contrast, the recovery of cardiac function was incomplete with the ischemia of 40 min. The recovery of the total mechanical energy required for contraction, LVP × HR, was likewise significantly decreased with ischemia of 40 min, and there occurred a significant elevation of EDP. Ischemia of 60 min caused further changes in these parameters, with the exception of HR.

**Table 1. Intracellular Na+, K+ and Ca2+ contents, tissue ATP content, ATP/ADP, and energy charge (EC) of hearts after 20, 40 and 60 min of global ischemia and those of hearts after reperfusion following ischemia**

|            | Na⁺ (μmole/g dry wt.) | K⁺ (μmole/g dry wt.) | Ca²⁺ (μmole/g dry wt.) | ATP (μmole/g dry wt.) | ATP/ADP | EC     |
|------------|------------------------|----------------------|------------------------|-----------------------|---------|--------|
| Before     |                        |                      |                        |                       |         |        |
| ischemia*a| 12                     | 64.88 ± 12.84        | 361.24 ± 7.41          | 2.84 ± 0.12           | 28.91 ± 3.08 | 5.86 ± 0.29 | 0.903 ± 0.005 |
| Ischemia 20| 8                      | 102.47 ± 6.38        | 365.32 ± 9.95          | 2.44 ± 0.34           | 7.33 ± 1.12* | 1.01 ± 0.08** | 0.591 ± 0.021** |
| 40         | 9                      | 95.44 ± 9.48         | 344.01 ± 7.22          | 2.80 ± 0.16           | 2.64 ± 0.19** | 1.13 ± 0.06** | 0.254 ± 0.004** |
| 60         | 6                      | 95.80 ± 8.24         | 314.98 ± 15.33*        | 2.26 ± 0.25           | 1.42 ± 0.10** | 0.89 ± 0.05** | 0.220 ± 0.010** |
| Reperfusion|                         |                      |                        |                       |         |        |
| ischemia 20| 7                      | 69.33 ± 14.12        | 348.83 ± 8.00          | 3.34 ± 0.54           | 12.53 ± 0.84** | 3.29 ± 0.33** | 0.850 ± 0.010** |
| 40         | 8                      | 93.50 ± 19.87        | 287.05 ± 6.73**        | 8.59 ± 1.04**         | 5.42 ± 0.45** | 1.75 ± 0.14** | 0.667 ± 0.020** |
| 60         | 8                      | 142.47 ± 11.38**     | 265.92 ± 5.66**        | 12.63 ± 1.03**        | 2.75 ± 0.26** | 1.10 ± 0.07** | 0.518 ± 0.029** |

*a: 40-min perfusion. Values are means ± S.E. *P < 0.05, **P < 0.01 vs. before ischemia.
Changes in intracellular cation contents

Table 1 shows the myocardial intracellular cation contents after global ischemia of 20, 40 and 60 min and after reperfusion following the global ischemia. With ischemia of 20 min, there was a tendency for Na\(^+\) to increase. However, further lengthening of ischemia to 40 and 60 min did not bring about further increases in this parameter. The K\(^+\) content decreased with ischemia of 40 min and longer. The decrease was significant with ischemia of 60 min. The Ca\(^{2+}\) content did not change during ischemia.

As shown in Table 1, the myocardial intracellular contents of Na\(^+\) recovered after reperfusion following 20-min ischemia. It remained high with ischemia of 40 min. With ischemia of 60 min, it increased further to reach a value significantly higher than the value before ischemia. The myocardial K\(^+\) content decreased after reperfusion, reaching values significantly lower than the values before ischemia with ischemia of 40 min and longer. The Ca\(^{2+}\) content increased. Again values significantly higher than those before ischemia was achieved with ischemia of 40 min and longer. With ischemia of 60 min, Na\(^+\) and Ca\(^{2+}\) contents after reperfusion were 2 and 4 times over the pre-ischemic values, respectively.

With ischemia of short duration, there was no change in extracellular and intracellular space, while there was a significant increase in the intracellular space from ATP/ADP ratio and EC. These parameters significantly decreased with ischemia and remained low even after reperfusion.

Figure 2 depicts the relation between the recovery of the total mechanical energy required for contraction, LVP \(\times\) HR, and the myocardial intracellular contents of cations and ATP after reperfusion. A linear relationship was observed with r values of 0.908 for Na\(^+\), 0.999 for K\(^+\), 0.988 for Ca\(^{2+}\) and 1.000 for ATP, respectively.

Effects of monensin

Infusion of 10\(^{-6}\) M of monensin increased LVP by 24.4 ± 4.1 mmHg (n = 18). However, as HR decreased by 23.2 ± 7.2 beats/min (n = 18), LVP \(\times\) HR remained at the level of 119.2 ± 6.5% of the value just before administration of the drug. The infusion of ethanol (final concentration was about 0.01%), the solvent for monensin, had no effect on LVP and HR.

Changes in myocardial cation and adenine nucleotide contents produced by monensin are shown in Figs. 3 and 4. As compared with that of the ethanol group, Na\(^+\) content was higher, but the difference was not significant. The only significant change was a decrease in EC.

Ischemia of 20 min produced a decrease in Na\(^+\) content in the ethanol-group, while Na\(^+\) content increased in the monensin group. Therefore, the
As expected, ischemia produced a decrease in myocardial ATP contents and EC in both groups. The changes were significantly greater in the monensin-group. Judging from the level of ATP and EC, the metabolic insult incurred by 20-min ischemia upon the monensin-treated heart equaled that produced by ischemia of 40 min on the hearts not treated with monensin.

After reperfusion, the myocardial intracellular cation contents recovered in the ethanol-group, with the exception of Ca^{2+} that became a little higher. In contrast, in the monensin-group, Na^+ and Ca^{2+} increased and K^+ decreased further after reperfusion.

The recovery after reperfusion of ATP, ATP/ADP ratio and EC was not complete even in the ethanol-group. The recovery of these parameters was worse in the monensin group.

Figure 5 depicts the recovery after reperfusion of the cardiac mechanical function. While the recovery was good in the ethanol group, it was poor in monensin-treated hearts. Thus, the CF, LVP, HR and LVP × HR of the latter were significantly lower than those of the former, and EDP was significantly higher.

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**Fig. 2.** Correlation between LVP × HR and intracellular cation (Na^+, K^+, Ca^{2+}) and ATP contents after reperfusion for 40 min following 20, 40 and 60 min of global ischemia. Vertical lines express recovery % of LVP × HR, which are means ± S.E. plotted as percent of the initial values. Horizontal lines express contents of Na^+ (upper left), K^+ (upper right), Ca^{2+} (lower left) and ATP (lower right) which are means ± S.E. plotted as μmole/g dry weight. □: 20-min ischemia, ○: 40-min ischemia, △: 60-min ischemia. Solid lines express the regression lines. Na^+: y = −0.73x + 140.33, r = 0.908; K^+: y = 0.697x −143.72, r = 0.999; Ca^{2+}: y = −6.37x + 117.91, r = 0.988; ATP: y = 5.94x + 24.79, r = 1.000.

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**Monensin**

**Fig. 3.** Intracellular cation contents of the isolated perfused heart treated with monensin (10^{-6} M) for 10 min prior to induction of 20-min ischemia followed by 40-min reperfusion. BI, before ischemia; I, ischemia; RP, reperfusion; open column: ethanol treatment (n = 7 - 9); cross-hatched column: monensin treatment (n = 6 - 9). Values represent means ± S.E. *P < 0.05, **P < 0.01 vs. ethanol treatment.
Fig. 4. ATP contents (μmole/g dry weight), ATP/ADP ratio and energy charge (EC) of the isolated perfused heart treated with monensin (10^{-6} M) for 10 min prior to induction of 20-min ischemia followed by 40-min reperfusion. BI, before ischemia; I, ischemia; RP, reperfusion; open column: ethanol treatment (n = 7–9); cross-hatched column: monensin treatment (n = 6–9). Values represent means ± S.E. *P < 0.05, **P < 0.01 vs. ethanol treatment.

Fig. 5. Recovery % of cardiac function of the isolated perfused heart treated with monensin (10^{-6} M) for 10 min prior to induction of 20-min ischemia followed by 40-min reperfusion. CF, coronary flow; LVP, left ventricular pressure; HR, heart rate; EDP, end-diastolic pressure. open column: ethanol treatment (n = 7–9); cross-hatched column: monensin treatment (n = 6–9). Values represent means ± S.E. *P < 0.05, **P < 0.01 vs. ethanol treatment.

Effects of DCB
The 10-min infusion of 10^{-6} or 3 \times 10^{-6} M of DCB before induction of 40-min ischemia altered neither the recovery of cardiac function nor the accumulation of Na^+ and Ca^{2+} and the loss of K^+ and ATP after reperfusion. The 10-min infusion of 10^{-5} M of DCB before ischemia markedly decreased LVP by 105.4 ± 8.6 mmHg (n = 9) and HR by 59 ± 20 beats/min, so that LVP × HR was 16.0 ± 3.1% of the value just before administration of the drugs (Fig. 6). Under this condition, the accumulation of Na^+ and Ca^{2+} and the decrease in K^+, ATP, ATP/ADP ratio and EC after re-
perfusion were significantly suppressed as compared with the group treated with PEG, the solvent of DCB (Figs. 6 and 7). Infusion of $10^{-5}$ M of DCB for 3 min before ischemia and for 3 min during reperfusion resulted in a moderate decrease in $LVP \times HR$ to $78.8 \pm 7.1\%$ of the value before induction of ischemia (Fig. 6). Under this condition, the accumulation of $Na^+$ and $Ca^{2+}$ and the reduction of $K^+$ content after reperfusion were not changed (Fig. 6), although the decline of ATP and ATP/ADP ratio was alleviated (Fig. 7). The recov-

![Dichlorobenzamil](image1)

**Fig. 6.** $LVP \times HR$ before ischemia (values are expressed as percents of the value just before administration of the drugs) and intracellular cation contents of the isolated perfused heart treated with dichlorobenzamil (DCB) ($10^{-5}$ M) for 10 min before 40-min ischemia or for 3 min before ischemia and then 3 min after reperfusion. open column: polyethylene glycol (PEG) treatment ($n = 10$), cross-hatched column: treatment with DCB for 3 min before ischemia and 3 min after reperfusion ($n = 7$), filled column: treatment with DCB for 10 min before ischemia ($n = 9$). Values represent means ± S.E. *P < 0.05, **P < 0.01 vs. PEG treatment.

![Dichlorobenzamil](image2)

**Fig. 7.** ATP contents ($\mu$ mole/g dry weight) and ATP/ADP ratio and energy charge (EC) of the isolated perfused heart treated with dichlorobenzamil (DCB) ($10^{-5}$ M) for 10 min before 40-min ischemia or for 3 min before ischemia and then 3 min after reperfusion. open column: polyethylene glycol (PEG) treatment ($n = 10$), cross-hatched column: treatment with DCB for 3 min before ischemia and 3 min after reperfusion ($n = 7$), filled column: treatment with DCB for 10 min before ischemia ($n = 9$). Values represent means ± S.E. *P < 0.05, **P < 0.01 vs. PEG treatment.
tery of cardiac function after 40-min reperfusion was incomplete in both the DCB- and PEG-groups. There was no significant difference between the two groups.

DISCUSSION

In the present study, the intracellular contents of Na⁺, K⁺ and Ca²⁺ were determined in isolated perfused rat hearts with Co-EDTA as a marker of the extracellular space. This method has an advantage in that the tissue adenine nucleotide contents can be measured in the same preparation. Furthermore, the changes in extracellular space, intracellular space and water content of hearts during ischemia and reperfusion can also be determined with this method.

There was a tendency for myocardial Na⁺ content to increase with ischemia of 20 min. However, no further increase in Na⁺ was observed with ischemia of 40 min and longer, while there was a decrease in K⁺ content; the decrease was significant with ischemia of 60 min.

Reperfusion after 20-min ischemia resulted in recovery of these parameters, with the exception of Ca²⁺, that showed a tendency to increase further. Ischemia of 40 min and longer resulted in a significant increase in Ca²⁺ and a significant decrease in K⁺. With 60-min ischemia, a significant increase in Na⁺ was observed after reperfusion. These results agree with those obtained by Pridjian et al. (21) and Humphrey et al. (22).

On the basis of the finding that the extracellular marker ⁵¹Cr-EDTA did not enter the intracellular space on reoxygenation following 30-min hypoxia, Poole-Wilson et al. (23) concluded that Ca²⁺ overload is not due to disruption of the plasma membrane. Nayler et al. (24) demonstrated in the isolated rat heart that the sarcolemma was intact in ultrastructure after ischemia of 60 min, even though this preparation exhibited uncontrolled Ca²⁺ gain upon reperfusion. Therefore a possible route of Ca²⁺ influx may be a physiological pathway.

Several researchers proposed that the increase in Ca²⁺ influx was due to an augmented operation of the Na⁺-Ca²⁺ exchange mechanism during reperfusion due to the accumulation of Na⁺ during ischemia (7, 25, 26). However, in the present study in which the determination of intracellular cations was conducted with Co-EDTA as a marker of the extracellular space, Na⁺ showed only a tendency to increase during ischemia. Furthermore, the increase did not augment with the ischemia of 40 min and longer, while the Ca²⁺ accumulation after reperfusion increased depending on the duration of ischemia, and significant increases were observed with ischemia of 40 min and longer. Thus, the Ca²⁺ accumulation after reperfusion was observed without significant accumulation of Na⁺ during ischemia, and larger accumulation of Ca²⁺ after reperfusion was not associated with the larger accumulation of Na⁺ during ischemia.

In the present study, when the heart was treated with monensin, a Na⁺ ionophore, prior to induction of ischemia, a significantly larger accumulation of Na⁺ occurred during ischemia of 20 min, and an accumulation of Ca²⁺ was observed after reperfusion. However, compared with the degree of ischemic damages as assessed by the level of ATP and EC, which were equivalent to those produced by 40-min ischemia without monensin, the increase in Ca²⁺ was small, disproportionately small if one additionally takes into consideration the fact that the Na⁺ accumulation during ischemia was greater under this condition.

Thus, the accumulation of Na⁺ during ischemia can not be the sole cause of Ca²⁺ accumulation after reperfusion. This conclusion is in harmony with that of Crake and Poole-Wilson (27). According to them, Na⁺-Ca²⁺ exchange can only be a minor mechanism since the uptake of Ca²⁺ on reoxygenation could not be inhibited by lithium substitution for sodium introduced after the onset of hypoxia.

In hearts treated with monensin, K⁺ and ATP decreased and the mechanical function deteriorated after ischemia-reperfusion despite the fact that LVP × HR before ischemia was not different from that of the control group. The significant inhibitory effects on ADP-stimulated (State 3) respiratory rates, respiratory control ratio and ADP/O ratio as reported by Schlafer and Kane (28) in isolated mitochondria from the rabbit heart with monensin (≥ 10⁻⁷ M) may be the cause of these deleterious effects.

It is true that 10⁻⁵ of DCB, a selective inhibitor of Na⁺-Ca²⁺ exchanger with a potency 100-fold over amiloride (29), when given before induction of ischemia prevented the accumulation of Na⁺ and Ca²⁺ and the loss of K⁺ and ATP, but a marked decrease of LVP × HR to 16% of the control was observed before induction of ischemia with this dose of DCB; and doses of DCB lower than 10⁻³ M, which did not produce any inhibition of LVP × HR, failed to prevent the myocardial accumulation of Ca²⁺. Kim and Smith (30) reported that the EC₅₀ of DCB for inhibition of Na⁺-Ca²⁺ exchange in sarcolemmal vesicles of guinea pig heart was 6 × 10⁻⁷ M, and a Kᵢ of 4 × 10⁻⁸ M was reported for the inhibition of Na⁺-Ca²⁺ current by Bielefeld et al. (31) in frog atrium. As was demonstrated in our previous paper (Shiga et al. (5)) with Ca²⁺ antagonists, there exists a close relation between the level of LVP × HR before induction of ischemia and the amount of Ca²⁺ accumulated after reperfusion.
Thus, the prevention by DCB may be ascribed not to the specific effects on the Na\(^+\)-Ca\(^{2+}\) exchanger but to the inhibitory effects of this compound on the myocardial mechanical function before induction of ischemia. The fact that 3-min infusion of 10\(^{-5}\) M DCB before induction of ischemia, which produced only a slight inhibition of LVP \(\times\) HR, combined with another 3-min infusion of 10\(^{-5}\) M DCB after reperfusion did not inhibit the accumulation of Na\(^+\) and Ca\(^{2+}\) and the loss of K\(^+\) provides further support for this idea. The reason why ATP, ATP/ADP ratio and EC recovered to a level much the same as the one attained in the experiment with 10\(^{-5}\) M of DCB given for 10 min before induction of ischemia is not clear at present. As a cause of inhibition of the myocardial mechanical function, inhibition of Ca\(^{2+}\) influx via slow Ca\(^{2+}\) channels as reported by Kim and Smith (30) is conceivable. The poor recovery of the mechanical function of the heart treated with DCB may also be explained on the same basis.

Then what is the mechanism of accumulation of Ca\(^{2+}\) after reperfusion? We have at present no clearcut explanation. However, the disruption of the surface membrane or sarcoplasmic reticulum by free radicals as reported by some investigators (32, 33) may be the cause of this accumulation of Ca\(^{2+}\)

In hearts treated with polyethylene glycol, the Na\(^+\) concentration was higher and ATP, lower after 40 min-ischemia and reperfusion than those in control hearts. This is in agreement with the results obtained in isolated perfused rat kidney which showed the exacerbation of the hypoxic damage by polyethylene glycol (34).

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