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Changes in Intracellular Ca^{2+} Concentration of Rabbit Coronary Artery Smooth Muscle Cell during Ischemic Cardioplegic Period

Young Ho Lee, Gyu Bog Choi*, Duck Sun Ahn, and Bok Soon Kang

To elucidate the possibility whether an elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in rabbit coronary artery myocytes during ischemic cardioplegic period may serve as one of the mechanisms of the 'no-reflow' phenomenon or not, the changes in $[Ca^{2+}]_i$ were measured under ischemic cardioplegia conditions using a fluorescent Ca^{2+} indicator, fura 2/AM. When single cells were perfused with cardioplegic or ischemic cardioplegic solutions, $[Ca^{2+}]_i$ was significantly increased and the degree of $[Ca^{2+}]_i$ elevation was further augmented by the ischemic cardioplegic solution. Pretreatment of a sarcoplasmic reticulum emptying agent, 20 mM caffeine, had no effect on ischemic cardioplegia-induced $[Ca^{2+}]_i$ changes, but application of a Ca^{2+} channel blocker, 5×10^{-7}M nifedipine, or an antagonist of Na^+/Ca^{2+} exchange, 5 mM Ni^{2+}, significantly inhibited the $[Ca^{2+}]_i$ elevation, respectively. The magnitude of ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation was dependent on the Ca^{2+} concentration of perfusate in the range of 0 and 2.5 mM. When Ni^{2+} was added to the reperfusion solution, recovery of ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation was very rapid compared with the controls. It is concluded that ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation may serve as one of the mechanisms of the 'no-reflow' phenomenon in rabbit coronary artery smooth muscle cells. We propose that Na^+/Ca^{2+} exchange may serve as a key function in ischemic cardiopelgia-induced $[Ca^{2+}]_i$ elevation.

Key Words: Ischemic cardioplegia, 'no-reflow' phenomenon, $[Ca^{2+}]_i$, coronary artery

Reperfusion with normal blood after cardiac operation or temporary myocardial ischemia can induce reperfusion injuries such as myocardial 'stunning' (Bolli, 1990), accumulation of Ca^{2+} in the myocardial cell (Shen and Jennings, 1972; Bourdillon and Poole-Wilson, 1981), ventricular arrhythmia (Hearse and Tosaki, 1987), vascular injury and 'no-reflow' (Kloner *et al.* 1974). This reperfusion injury occurs only when there has been inadequate myocardial protection during the preceding ischemic period. Many recent studies are, therefore, focused on the minimization of ischemic injury as well as reperfusion injury.

Studies to identify the cause and to minimize the effect of ischemic and reperfusion injuries have been mainly focused on injuries in the myocardial cell. However, not only injuries in myocardial cell during reperfusion but also irreversible ischemic-reperfusion injuries such as 'no-reflow' in the coronary artery during ischemia or after reperfusion were observed (Kloner *et al.* 1974; Opie, 1989).

'No-reflow' is a specific type of vascular

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Department of Physiology, College of Medicine, Yonsei University, Seoul, Korea
*Department of Internal Medicine, College of Medicine, Ehwa Womans University, Seoul, Korea
Address reprint request to Dr. Y.H. Lee, Department of Physiology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea

damage occurring when removal of a coronary occlusion does not lead to the restoration of coronary flow. There are three major explanations for the 'no-reflow' phenomenon such as endothelial cell edema (Kloner *et al.* 1974), microvascular plugging by platelets or thrombi (Kloner and Alker, 1984; Kloner *et al.* 1989) and coronary occlusion by ischemic contracture of the myocardium (Kloner *et al.* 1974).

However detailed mechanisms of the 'no-reflow' phenomenon are not known. More study is needed to understand the relationship between the 'no-reflow' and contractility of the smooth muscle cell in the coronary artery that is closely related to the control of coronary blood flow. Moreover, it is not clearly known why the reversible injuries occur such as vascular injury during reperfusion after ischemia, change in cell membrane permeability (Kloner *et al.* 1977) and low-reflow phenomenon (Heyndricks *et al.* 1978; Bolli *et al.* 1990). However, it is predictable that, as in the case of myocardial cell, Ca^{2+} accumulation in the smooth muscle cell of the coronary artery is probable cause of the injuries.

In this study, to elucidate the possibility whether elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) during an ischemic cardioplegic period is one of the mechanisms of the 'no-reflow' phenomenon or not, changes in $[Ca^{2+}]_i$ were measured in the single smooth muscle cell of the coronary artery perfused with ischemic cardioplegic solution in order to expose a cell to similar condition under cardiac operation.

MATERIALS AND METHODS

Preparation of single coronary artery smooth muscle cells

Rabbits (2.5 kg) of either sex were anesthetized by injecting sodium pentobarbital (60 mg/kg) and heparin (2,000 IU/kg) into the ear vein, and they were exsanguinated by cutting a carotid artery. The left anterior descending coronary artery was dissected with the neighboring cardiac muscle under a surgical microscope, and then moved to a preparation chamber. The surrounding connective and fat tissue was carefully removed with fine scissors and a pincette under a surgical microscope.

The coronary artery strip was incubated in a plastic bottle containing Tyrode solution without Ca^{2+} and Mg^{2+}, at 37℃ for 5 min. The strip was then transferred to another glass bottle containing collagenase solution (Ca^{2+} and Mg^{2+}-free Tyrode solution+0.1% collagenase), and incubated for 20~30 min. After completion of enzymatic digestion, the coronary artery strip was gently agitated using a blunt tipped glass pipette until it was dispersed to the single cells. The single cells were resuspended in normal Tyrode solution containing albumin (0.1%) and stored at 4℃ for further experiment.

Measurement of $[Ca^{2+}]_i$

Fura 2/AM loading method: Fura 2 was incorporated into the cells as the acetoxymethyl (AM) ester. A 1 mM-stock solution in dimethylsulphoxide (DMSO) was added to a suspension of cells (3×10^5/ml) to give a final concentation of 5 μM and incubated at 37℃ for 60 min. Pluronic F-127 (Molecular Probe, Eugene, Oregon, U.S.A., 0.02%), a noncytotoxic detergent, was added to increase the solubility of fura 2/AM. The cells were then centrifuged and the supernatant was removed. The cell pellet was resuspended in a normal Tyrode solution and kept at 4℃ until they were used.

Measurement of $[Ca^{2+}]_i$: The single cells settled down on the bottom of experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The solutions flowed at 3 ml/min by peristaltic pump, and the temperature of experimental chamber was 37℃. Fura 2/AM loaded cells were alternately excited with ultraviolet (UV) light of 340 nm and 380 nm. The resulting fura 2 fluorescence was transmitted to a photomultiplier tube via a 510 nm emission filter. The output from the photomultiplier tube was transfered to a computer (IBM AT compatible) and stored for further analysis. The ratio of fluorescence intensity at 340 nm and 380 nm was determined by a computer program (DM 3000 CM).

Absolute Ca^{2+} concentration was not calcu-

lated in theses experiments because a large part of the intracellular fura 2 binds to soluble proteins and the dissociation constant of fura 2 for Ca^{2+} and fluorescent characteristics of fura 2 may be changed from that obtained in vitro (Karaki, 1990). Instead, the ratio of these two fluorescence (F_{340}/F_{380}) was used as an indicator of relative $[Ca^{2+}]_i$.

Solution compositions and drugs

The composition of normal Tyrode solution for the cell isolation was: NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1; NaH_2PO_4 0.3; HEPES 10; glucose 5.5 (mM); pH=7.4 with Tris. The Krebs-Henseleit solution used in the measurement of Ca^{2+} concentration was composed of NaCl 110; KCl 4.7; $MgSO_4$ 1.2; $CaCl_2$ 1; KH_2PO_4 1.2; $NaHCO_3$ 10; glucose 5.5 (mM); pH 7.4 aerated with 95% O_2+5% CO_2 gas. The cardioplegic solution used in this experiment contained: NaCl 110; KCl 20; $MgCl_2$ 16; $CaCl_2$ 1; $NaHCO_3$ 10; glucose 5.5 (mM) (St. Thomas' Hospital Solution, Robinson *et al.* 1991). The ischemic cardioplegic solution was made by subtracting glucose from the cardioplegic solution and was aerated with 95% N_2+5% CO_2 gas instead of 95% O_2+5% CO_2 gas.

Drugs used were: caffeine, nifedipine, collagenase (type 1A), nickel chloride, bovine serum albumin (all from Sigma, Kansas, Missouri, U.S.A), fura 2/AM, dimethylsulphoxide (DMSO), pluronic F-127, ethyleneglycol-bis-(β-aminoethylester)-N,N'-tetraacetic acid (EGTA), (Molecular Probe, Eugene, Oregon, U.S.A.).

Data analysis and statistics

Ratio (F_{340}/F_{380}) was expressed as a relative values of the maximum fluorescence ratio by

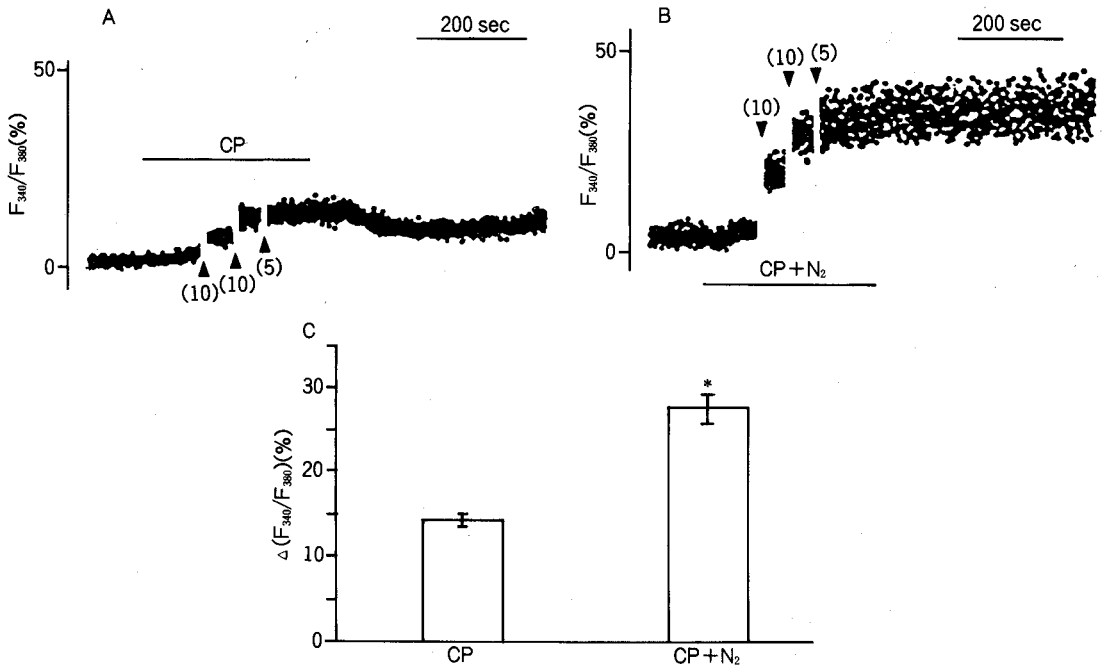

Fig. 1. *Changes in $[Ca^{2+}]_i$ during cardioplegia and ischemic cardioplegia.*
***A** and **B**: representative traces illustrating effects of cardioplegia (**A**, CP: cardiplegic solution) and ischemic cardioplegia (**B**, CP+N_2: cardioplegic solution gassed with 100% N_2) on the $[Ca^{2+}]_i$ of a single rabbit coronary artery smooth muscle cell.*
***C**: effects of cardiplegia (CP) and ischemic cardiplegia (CP+N_2) on $[Ca^{2+}]_i$. Values are means±s.e.means (n=7; *p<0.05 when compared with values at cardioplegia). ▲(or ▼) indicates time of block off UV light in order to protect the cell from excessive UV light exposure.*

high-K^+. The change of F_{340}/F_{380} during cardioplegia or ischemic cardiopegia was expressed as $\triangle$ (F_{340}/F_{380})={peak (F_{340}/F_{380})-resting (F_{340}/F_{380})}. The student paired t-test was used to evaluate the statistical significance of differences between means. Values of $P<0.05$ were considered to be significant.

RESULTS

Changes in $[Ca^{2+}]_i$ during cardioplegia and ischemic cardioplegia

Fig. 1 shows change in $[Ca^{2+}]_i$ while the cell was perfusated with the cardioplegic and the ischemic cardioplegic solutions. When the smooth muscle cell was perfusated by a normal KH solution, the resting $[Ca^{2+}]_i$ was 134.2 ±34.0 nM (n=43). This value was compatible with other findings (Katsuyama *et al.* 1991). When the perfusate was subsequently replaced with the cardioplegic solution for 30 min, the $[Ca^{2+}]_i$ was noticeably increased compared to that of the control as shown in Fig. 1A. In this experiment $[Ca^{2+}]_i$ did not change during the initial 10 min but started to gradually increase after 10 min and reached a steady state at a significantly higher concentration. When the cell was rewashed with a normal KH solution, $[Ca^{2+}]_i$ returned back to the resting level in 2 to 3 min. When the cell was perfused subsequently with the ischemic cardioplegic solution, $[Ca^{2+}]_i$, as shown in Fig. 1B, it reached a plateau within 30 min at a level significantly higher than that in the case of the perfusion with the cardioplegic solution {Fig. 1C; $\triangle(F_{340}/F_{380})$ during the cardioplegia= 14.2±0.8%, $\triangle(F_{340}/F_{380})$ during the ischemic cardioplegia=28.5±1.6%, n=7} Fig. 2 shows change in $[Ca^{2+}]_i$ in detail while the smooth muscle cell was perfused with the ischemic cardioplegic solutions. As shown in Fig. 2A, $[Ca^{2+}]_i$ was not changed during the initial 10 min {$\triangle(F_{340}/F_{380})$=2.1±0.6%, n=5}. However, it reached a significantly higher level in 30 min {$\triangle(F_{340}/F_{380})$=28.3 1.4%, n=5} and recovered to a resting concentration after approximately a 10 min washing with a normal KH solution (Fig. 2B). When the cell was perfused about

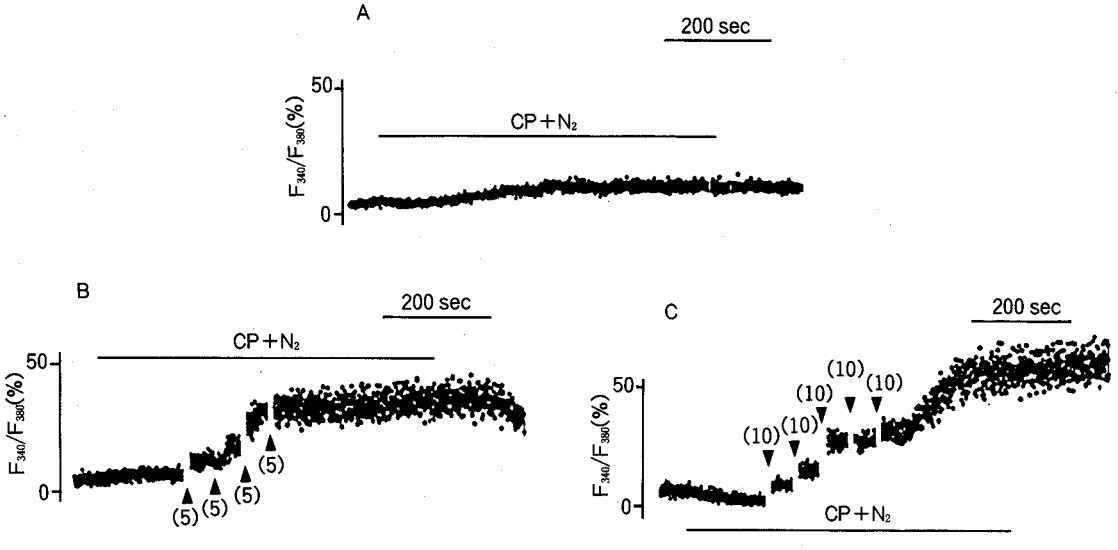

Fig. 2. *Time dependency of change in $[Ca^{2+}]_i$ during ischemic cardioplegia.*
A, B *and* ***C:*** *representative trace illustrating change in $[Ca^{2+}]_i$ when the cell was perfused with ischemic cardioplegic solution (CP+N_2: cardioplegic solution gassed with 100% N_2) for 10* ***(A)****, 30* ***(B)*** *and 60 min* ***(C)****, respectively. ▲(or ▼) indicates time of block off UV light in order to protect the cell from excessive UV light exposure.*

60 min, the $[Ca^{2+}]_i$ was increased to a significantly higher level and did not recover to the level of resting concentration by washing with a normal KH solution (Fig. 2C).

The effect of caffeine, nifedipine and nickel on changes in $[Ca^{2+}]_i$ during ischemic cardioplegia

To elucidate the mechanism(s) of $[Ca^{2+}]_i$ elevation when the smooth muscle cell is perfused with a ischemic cardioplegic solution, we studied the effect of caffeine, nifedipine and nickel on the change in $[Ca^{2+}]_i$.

To elucidate the role of Ca^{2+} channels in the increase of $[Ca^{2+}]_i$ during ischemic cardioplegia we measured the effect of nifedipine on ischemic cardioplegia-induced $[Ca^{2+}]_i$ change. As shown in Fig. 3A and 3B. when the smooth muscle cell was perfused with the ischemic cardioplegic solution containing 5×10^{-7}M nifedipine, a Ca^{2+} channel blocker, the increase of $[Ca^{2+}]_i$ during ischemic cardioplegic period was significantly reduced compared to that of the control, but not totally suppressed (Fig. 3C; control=29.9±2.2%, n=7, nifedipine=20.2±1.3%, n=6).

To identify the possibility whether the increase of $[Ca^{2+}]_i$ during cardioplegia is due to the Ca^{2+} release from sarcoplasmic reticulum (SR) or not, we measured the effect of ischemic cardioplegia on $[Ca^{2+}]_i$ after emptying the SR by pretreatment of 20 mM caffeine. As shown in Fig. 4A and 4B, application of caffeine (20 mM) to the bath caused a transient increase of $[Ca^{2+}]_i$, and it returned to the resting level in the presence of caffeine. The

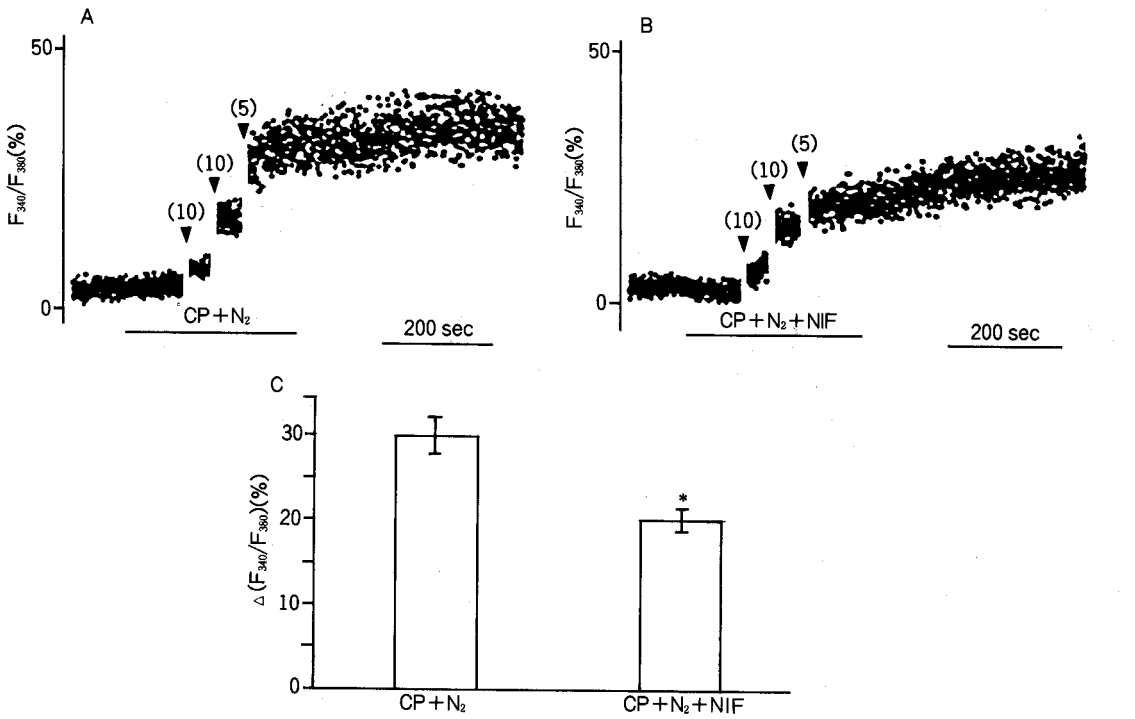

Fig. 3. *Effects of nifedipine on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation.*
***A** and **B:** representative trace illustrating effects of ischemic cardioplegia on $[Ca^{2+}]_i$ in the absence (**A,** CP+N_2: cardioplegic solution gassed with 100% N_2) and presence (**B,** CP+N_2+NIF: cardiople-gic solution containing nifedipine gassed with 100% N_2) of nifedipine (5×10^{-7}M) in the perfusate.*
***C:** effects of nifedipine on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation. Values are means±s.e.means (n=6; *$p<0.05$ when compared with values in the absence of nifedipine). ▲(or ▼) indicates time of block off UV light in order to protect the cell from excessive UV light exposure.*

second application of caffeine in the same cell did not cause any change in $[Ca^{2+}]_i$. When the solution was subsequently substituted with the ischemic cardioplegic solution containing 20 mM caffeine, $[Ca^{2+}]_i$ elevation was similar to that of the control (Fig. 4C; control=28.5±1.7%, caffeine=27.9±1.8%, n=6).

To investigate the possibility of whether the increase of $[Ca^{2+}]_i$ during ischemic cardioplegia is due to the Na^+-Ca^{2+} exchange or not, we measured the effect of cardioplegia containing 5 mM nickel. As shown in Fig. 5A and 5B, when the smooth muscle cell was perfused with the ischemic cardioplegic solution containing 5 mM of nickel, a Na^+-Ca^{2+} exchange blocker, the increase of $[Ca^{2+}]_i$ during ischemic cardioplegic period was also significantly reduced {Fig. 5C; $\triangle(F_{340}/F_{380})$=10.5±1.0%, n=9} compared to that of the control {Fig. 5C; $\triangle(F_{340}/F_{380})$=28.5±2.0%, n=9}, but not completely suppressed.

The effect of external Ca^{2+} concentration on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation

To investigate the effect of external Ca^{2+} on $[Ca^{2+}]_i$ change during ischemic cardioplegia, we perfused the smooth muscle cells with different Ca^{2+} containing ischemic cardioplegic solutions. As shown in Fig. 6, changing the $[Ca^{2+}]$ of ischemic cardioplegic solution from 0 mM to 2.5 mM resulted in the corresponding increase of $[Ca^{2+}]_i$ during perfusion of ischemic cardioplegic soultion. $\triangle(F_{340}/F_{380})$ at the 0, 0.5, 1.0, and 2.5 mM Ca^{2+} was 0.3±0.1% (Fig. 6A), 1.2±0.4% (Fig. 6B), 24.3±1.1% (Fig. 6C), and 25.1±1.5% (Fig. 6D, n=6), respectively.

The effect of nifedipine and nickel on changes in $[Ca^{2+}]_i$ during reperfusion after ischemic cardioplegia

We tested whether nifedipine and nickel

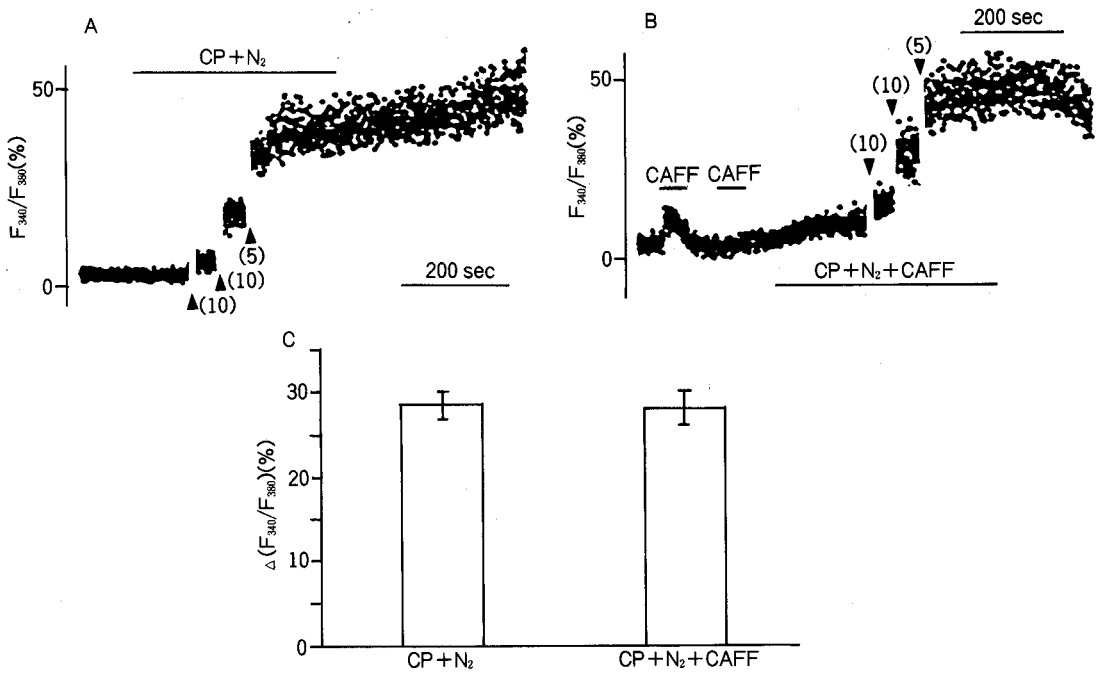

Fig. 4. *Effects of caffeine on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation.*
***A** and **B**: representative trace illustrating effects of ischemic cardioplegia on $[Ca^{2+}]_i$ in the absence (**A**, CP+N_2: cardioplegic solution gassed with 100% N_2) and presence (**B**, CP+N_2+CAFF: cardioplegic solution containing caffeine gassed with 100% N_2) of caffeine (20 mM) in the perfusate.*
***C**: effects of caffeine on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation. Values are means±s.e.means (n=6). ▲ (or ▼) indicates time of block off UV light in order to protect the cell from excessive UV light exposure.*

contribute to the recovery of $[Ca^{2+}]_i$ during the reperfusion period or not. Fig. 7 was a represented by a typical record of 7 experimental results. After $[Ca^{2+}]_i$ was significantly elevated by perfusating the smooth muscle cell with the ischemic cardioplegic solution, the perfusate was replaced by the normal KH solution (Fig. 7A) or normal KH solution containing 5×10^{-7}M nifedipine (Fig. 7B). The concentration change pattern was similar to that of the control. However, as shown in Fig 7C and 7D, when the perfusate was replaced by the normal KH solution containing 5 mM of nickel, the recovery of Ca^{2+} to the resting concentration was much faster than that of the control.

DISCUSSION

The 'no-reflow' phenomenon is a specific type of vascular damage occurring when removal of a coronary occlusion does not lead to restoration of coronary flow. But the detailed mechanisms of the 'no-reflow' phenomenon are not known. More study is needed to understand the relationship between 'no-reflow' and contractility of the smooth muscle cell in the coronary artery that is closely related to the control of coronary blood flow. However, it is predictable that, as in the case of myocardial cell, Ca^{2+} accumulation in the smooth muscle cell of coronary artery is probable cause of injuries in the coronary artery. To test this possibility, we perfused the single smooth muscle cell of a coronary artery in a

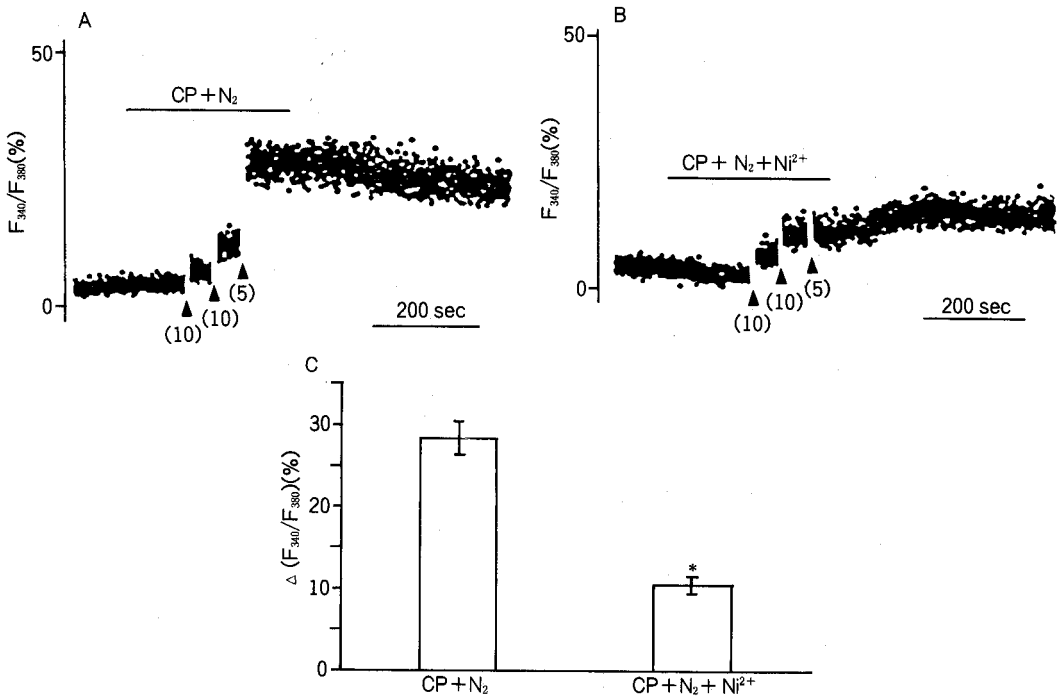

Fig. 5. *Effects of nickel on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation.*
A *and* ***B:*** *representative trace illustrating effects of ischemic cardioplegia on $[Ca^{2+}]_i$ in the absence (**A,** CP+N_2: cardioplegic solution gassed with 100% N_2) and presence (**B,** CP+N_2+Ni^{2+}: cardioplegic solution containing nickel gassed with 100% N_2) of nickel (5 mM) in the perfusate.*
C: *effects of nickel on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation. Values are means±s.e.means ($n=9$; *$p<0.05$ when compared with values in the absence of nickel). ▲(or ▼) indicates time of block off UV light in order to protect the cell from excessive UV light exposure.*

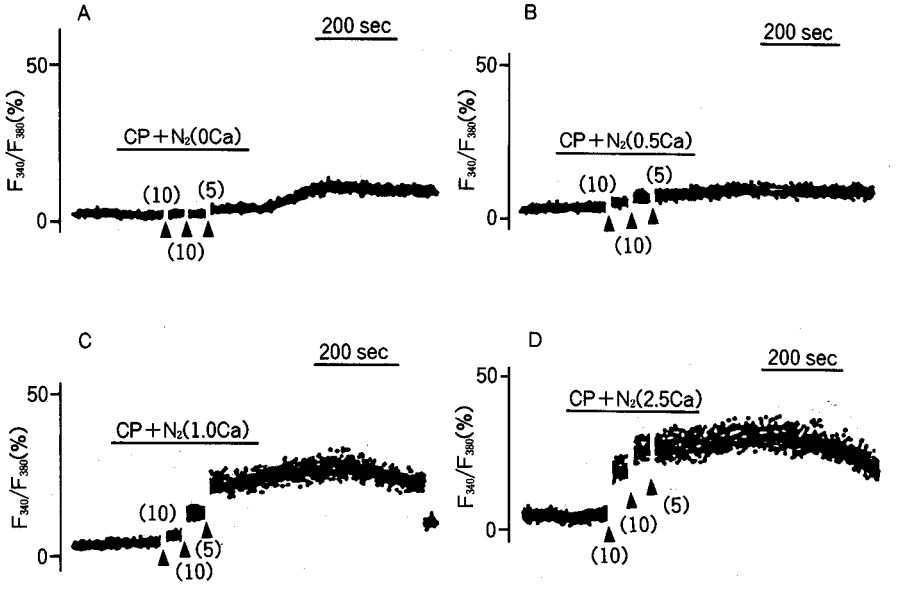

Fig. 6. *Effects of external Ca^{2+} concentration on ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation.*
A, B, C *and* ***D:*** *$[Ca^{2+}]_i$ traces illustrating when the cell was perfused with ischemic cardioplegic solution (CP +N_2: cardioplegic solution gassed with 100% N_2) containing different concentration of Ca^{2+} such as 0* ***(A),*** *0.5* ***(B),*** *1.0* ***(C)*** *and 2.5 mM (D). ▲(or ▼) indicates time of block off UV light in order to protect the cell from excessive UV light exposure.*

rabbit with ischemic cardioplegic solution in order to expose a cell to similar conditions under cardiac operation and measured a $[Ca^{2+}]_i$ by using a fura 2/AM.

When the perfusate was replaced with the cardioplegic solution, the $[Ca^{2+}]_i$ was noticeably increased and the magnitude of increase was significantly higher when the cell was perfused with the ischemic cardioplegic solution (Fig. 1). Kloner *et al.* (1974) reported that some vascular injury and 'no-reflow' phenomenon was observed when the smooth muscle cell was perfused for 90 min with an ischemic cardioplegic solution while no such damages were seen in 40 min the perfusion. Similarly, we found no change in $[Ca^{2+}]_i$ in 10 min of the perfusion, but increase of $[Ca^{2+}]_i$ with contraction was observed when the smooth muscle cell was perfused for 30 min. In the case of 60 min, further $[Ca^{2+}]_i$ elevation as well as contracture were observed and $[Ca^{2+}]_i$ elevation during ischemic cardioplegia did not recover to the resting level with reperfusion (Fig. 2).

When the cell was perfused with ischemic cardioplegic solution, the high K^+-cardioplegic solution led to a membrane depolarization which increased a $[Ca^{2+}]_i$ by Ca^{2+} influx through the voltage-dependent Ca^{2+} channel (Powell and Tathan, 1984). From the experiments used in ischemic cardioplegic solutions containing nifedipine (Fig. 3) we found that the $[Ca^{2+}]_i$ elevation was reduced. This suggests that Ca^{2+} influx via the Ca^{2+} channel is one of the causes of the $[Ca^{2+}]_i$ elevation during ischemic cardioplegia. This result is also supported by the clinical report of a cardiac muscle where a fast recovery of myocardial function was observed when the cell was perfused with cardioplegic solution containing a Ca^{2+} channel blocker (Huddleston *et al.* 1992).

$[Ca^{2+}]_i$ is also regulated by the Ca^{2+} release from SR, and this triggered Ca^{2+} release can be increased by membrane depolarization (Kurihara and Sakai, 1985). However, based on our experimental results the contribution of this triggered Ca^{2+} release from SR by membrane depolarization was insignificant to the

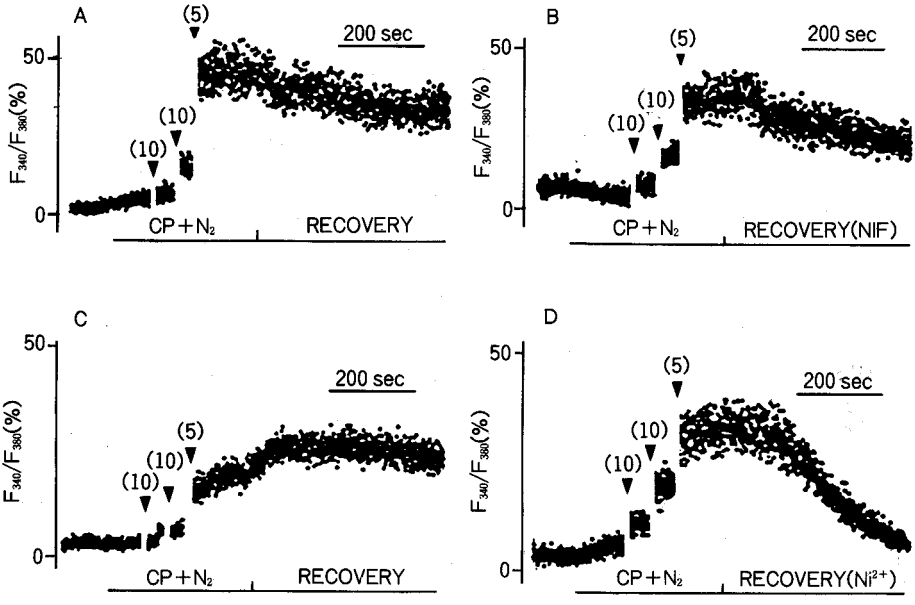

Fig. 7. *Effects of nifedipine and nickel on change in $[Ca^{2+}]_i$ during reperfusion period. After the cell was perfused with ischemic cardioplegic solution (CP+N_2: cardioplegic solution gassed with 100% N_2), the perfusate was replaced by the normal KH solution (RECOVERY).*
***A** and **B**: representative traces illustrating change in $[Ca^{2+}]_i$ during reperfusion period in the absence **(A)** and presence **(B)** of nifedipine (NIF: 5×10^{-7}M).*
***C** and **D**: representative traces illustrating change in $[Ca^{2+}]_i$ during reperfusion period in the absence **(C)** and presence **(D)** of nickel (Ni^{2+}: 5 mM). ▲(or ▼) indicates time of block off UV light in order to protect the cell from excessive UV light exposure.*

$[Ca^{2+}]_i$ elevation during ischemic cardioplegia, because the pretreatment of 20 mM caffeine, which was a sufficient amount to empty out the SR (Fig. 4), had no effect on $[Ca^{2+}]_i$ change during ischemic cardioplegia.

Another possible system to regulate the $[Ca^{2+}]_i$ is the Na^+/Ca^{2+} exchange mechanism. Depolarization of the membrane potentiated by high external K^+ of cardioplegic solution or increase of $[Na^+]_i$ by ischemia can increase the Ca^{2+} influx through Na^+/Ca^{2+} exchange mechanism (Wilde and Kleber, 1986). To elucidate this possibility, application of Ni^{2+}, blocker of Na^+/Ca^{2+} exchange mechanism at high concentration (Kimura *et al.* 1987; Beuckelmann and Wier, 1989), significantly reduced the $[Ca^{2+}]_i$ change during ischemic cardioplegia (Fig. 5). These results suggested that a major part of $[Ca^{2+}]_i$ increase during ischemic cardioplegia resulted from Ca^{2+} influx through the Na^+/Ca^{2+} exchange mechanism. Nickel, even though used as a Na^+-Ca^{2+} exchange blocker in this present study (Kimura *et al.* 1987), is also known to be a Ca^{2+} channel blocker. Therefore, the effects of nickel found in this study might be the Ca^{2+} channel blocking effect. However, the magnitude of suppression by the nickel at high concentration for ischemic cardioplegia was higher than that by nifedipine. Furthermore, we changed an external Ca^{2+} concentration from 0 to 2.5 mM and measured its effect on $[Ca^{2+}]_i$. As shown in Fig. 6, a reduction of external Ca^{2+} decreased the $[Ca^{2+}]_i$ during ischemic cardioplegia, and it may be due to the decreased Ca^{2+} influx through Na^+/Ca^{2+} exchange mechanism by low external Ca^{2+} concentration (Kimura *et al.* 1987). This effect of low external Ca^{2+} on $[Ca^{2+}]_i$ was compatible with the beneficial effects of low external Ca^{2+} upon the recovery of cardiac function after myocardial ischemia (Kinoshita *et al.* 1991; Robinson *et al.* 1991).

When the smooth muscle cell was reperfused using a normal KH solution after perfusion with an ischemic cardioplegic solution, it was found that the recovery of $[Ca^{2+}]_i$ to the resting took more than 10 min. The mechanism for the $[Ca^{2+}]_i$ elevation in cell during reperfusion is not well known. However, it has been proposed that possible causes are Ca^{2+} influx through Ca^{2+} channel (Przyklenk and Kloner, 1988) and Na^+-Ca^{2+} exchange (Renlund *et al.* 1985). Therefore, we used reperfusates containing nifedipine and nickel at high concentrations to examine this proposal. A faster recovery was found with nickel, while no improvement in the recovery was observed with nifedipine used (Fig. 7). This is most consistent with other studies. The Ca^{2+} influx is not suppressed with nifedipine lower than 1 uM (Nayler *et al.* 1980) and with the pharmacological concentration of Ca^{2+} blockers (Bourdillon and Poole-Wilson, 1981), but it is suppressed with nickel of high concentration or cyanide (Hess *et al.* 1981; Poole-Wilson *et al.* 1984).

Since 'no-reflow' is the blocking of blood flow through the coronary artery during reperfusion after a coronary occlusion (Kloner *et al.* 1974), the $[Ca^{2+}]_i$ elevation during ischemic cardioplegia should be accelerated during reperfusion in order to explain the cause of the 'no-reflow' phenomenon. But reperfusion injury takes place together with ischemic injury (Follette *et al.* 1981) and in our results, the contraction of the smooth muscle cell due to $[Ca^{2+}]_i$ elevation during ischemic cardioplegia continues for 10 minutes after reperfusion. Furthermore, when the cell was perfused about 60 min, the $[Ca^{2+}]_i$ was increased to a significantly higher level and did not recover to the level of resting concentration by washing with a normal KH solution. Therefore, it is concluded that ischemic cardioplegia-induced $[Ca^{2+}]_i$ elevation may serve as one of the mechanisms of the 'no-reflow' phenomenon in the rabbit coronary artery and the Na^+/Ca^{2+} exchange mechanism may serve as a key function in ischemic cardiopelgia-induced $[Ca^{2+}]_i$ elevation.

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