Yonsei Medical Journal
Vol. 35, No. 2, 1994

Effect of Hypothermic Cardioplegia on Cardiac Protection

-I. Effect of hypothermic cardioplegia on the cytosolic Ca^{2+} concentration in rat ventricular myoctes-

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Cytosolic Ca^{2+} concentration of rat ventricular cells was measured under varying experimental conditions by using a fluorescent Ca^{2+} indicator, Fura-2. Resting $[Ca^{2+}]_i$ of rat myocyte was 150± 30 nM (n=39), and this value was compatible with others. The Perfusion of cardioplegic solution significantly increased $[Ca^{2+}]_i$, and this effect was further augmented by hypothermia ($p<0.05$). Application of nifedipine (5×10^{-7} M) to the perfusate or pretreatment of caffeine (10 mM) had no apparent effect on this cardioplegia-induced $[Ca^{2+}]_i$ change. But Ni^{2+} (5 mM), an antagonist of Na^+/Ca^{2+} exchange mechanism, prevented the $[Ca^{2+}]_i$ change during cardioplegia ($p<0.05$). Magnitude of cardioplegia-induced $[Ca^{2+}]_i$ increase was also dependent on the Ca^{2+} concentration of cardioplegic solution. These results suggest that Na^+/Ca^{2+} exchange may play an important role in cardioplegia-induced $[Ca^{2+}]_i$ change. To rule out the possibility whether the protective effect of hypothermic cardioplegia is due to the preservation of high-energy phosphate store or decreasing the transmembrane ionic fluxes by phase transition, we exhausted a energy store of cardiac cell by application of 2,4 dinitrophenol to the bath and measured its effect on $[Ca^{2+}]_i$ change during cardioplegia. Hypothermic cardioplegia delayed the onset of $[Ca^{2+}]_i$ increase and decreased its amplitude compared to those of normothermic cardioplegia.

From the above results, hypothermic cardioplegia may protect the cardiac cells from ischemic insult by preserving a high-energy phosphate store. Application of Ni^{2+} to the cardioplegic solution or reduction of external Ca^{2+} concentration also had some protective effect, since it prevented $[Ca^{2+}]_i$ increase during cardioplegia.

Key Words: Rat myocyte, cardioplegia, Na^+/Ca^{2+} exchange, hypothermia, ischemia, fura-2

Reperfusion after cardiac operation or temporary myocardial ischemia can induce irreversible cell damages by massive Ca^{2+} influx into the myocyte (Hearse *et al.* 1978; Steenbergen *et al.* 1990). Myocardial contracture and the activation of endogenous protease by this excess Ca^{2+} destruct the membrane integrity and cause cell death (Poole-Wilson *et al.* 1984). Hypothermic cardioplegia has been known to protect the myocyte from this ischemic insult (Hearse *et al.* 1980; Rosenfeldt *et al.* 1980). It has been reported that the protective effect of hypothermia may be related to its ability to reduce cytosolic Ca^{2+} concentration by maintaining the activity of energy-dependent Ca^{2+} extrusion system during ischemia or to decrease the transmembrane ionic fluxes by phase transition (Jones *et al.* 1982; Rich & Langer, 1982; Ferrari *et al.* 1990; Tani, 1990). However, the mechanism of cytosolic Ca^{2+} ac-

Received January 25, 1994

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This work was supported by a development project grant and faculty fund of Yonsei University College of Medicine of 1991

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cumulation during myocardial ischemia and the relation between the cytosolic Ca^{2+} concentration and the protective mechanism of hypothermic cardioplegia is still not understood (Ferrari *et al.* 1990; Liu *et al.* 1990; Liu *et al.* 1991).

Recently, it has become possible to measure the $[Ca^{2+}]_i$ of single cardiac myocyte by using a fluorescent Ca^{2+} indicator, Fura-2 (Lee *et al.* 1987). Measurement of Ca^{2+} with Fura-2 has advantages compared to other Ca^{2+} measuring methods, such as ion selective method (Thomas, 1982; Chapman, 1986). It shows very rapid time response, and can accurately measure the Ca^{2+} concentration from diastolic (100 nM) to systolic (>1 μM) Ca^{2+} level (Cobbold & Rink, 1987; Lee *et al.* 1987; Tsien, 1989).

In this experiment, we investigated the mechanism of Ca^{2+} accumulation during cardioplegia by using a Fura-2 and examined protective effect of hypothermic cardioplegia on myocardial ischemia. Preliminary result of our work had been reported as an abstract form (Lee *et al.* 1992)

MATERIALS AND METHODS

Preparation of single ventricular myocytes

Adult rats (250 g~350 g) were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/Kg). The heart was removed and transferred to a beaker which contained the normal Tyrode solution. The heart was then Langendorff-perfused with the normal Tyrode solution at 37℃. The flow rate was set at 8 ml/min and the perfusion pressure was maintained at 30~40 cm H_2O. Once the heart beating appeared stable, the perfusion was switched to the nominally Ca^{2+}-free Tyrode solution for 5 minutes. The heart was then perfused with the Tyrode solution containing collagenase (type 1A, 0.04%), hyaluronidase (type II, 0.03%), 50 μM Ca^{2+}. At the end of the enzyme perfusion, the heart was cut down and the ventricles were minced. The minced ventricular tissues were incubated with the Tyrode solution containing collagenase (type 1A, 0.04%), hyaluronidase (type II, 0.03%), deoxyribonuclease (type VI, 0.002%), trypsin (type IX, 0.002%), 2% albumin, 50 μM Ca^{2+} for 5 min at 37℃. Thereafter, this mixture of minced ventricular tissues and single ventricular cells was filtered through guaze and the filtrate was centrifuged at 500 rpm for 5 min. The cell pellet was resuspended in a 0.5 mM Ca^{2+}-Tyrode solution and stored at 4℃.

Measurement of Ca^{2+} concentration

Fura-2 loading method: The single ventricular cells were incubated with normal Tyrode solution containing 5 μM Fura-2 acetoxymethyl ester (Fura-2 AM) for 20 min at 37℃. The cells were then centrifuged and the supernatant was removed. The cell pellet was resuspended in normal Tyrode solution and kept at 4℃ until they were used.

Measurement of intracellular Ca^{2+} concentration: The Ventricular myocytes settled down on the bottom of experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The Solutions were flowed at 3 ml/min by peristaltic pump, and the temperature of experimental chamber was changed rapidly by using a two input lines with different temperatures (37℃ and 20℃). Fura-2 loaded myocytes were alternately excited with ultraviolet (UV) light of 340 nm and 380 nm. The resulting Fura-2 fluorescence was transmitted to 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 (as shown in figure 1). The ratio of fluorescence intensity at 340 nm and 380 nm was determined by a computer program (DM3000CM), and the Ca^{2+} concentration was calculated by using a following equation (Grynkiewicz *et al.* 1985)

$$[Ca^{2+}] = K_b \times \beta\ [(R - R_{min}) \times (R_{max} - R)]$$

K_d: dissociation constant of Fura-2 for Ca^{2+} (37℃ =224 nM, 20℃=132 nM)

β: ratio of the 380 nm fluorescence signals of Fura-2 without Ca^{2+}/with saturated Ca^{2+}

R: fluorescence ratio at 340/380 nm

R_{min}/R_{max}: values of R for Fura-2 without Ca^{2+}/with saturated Ca^{2+}

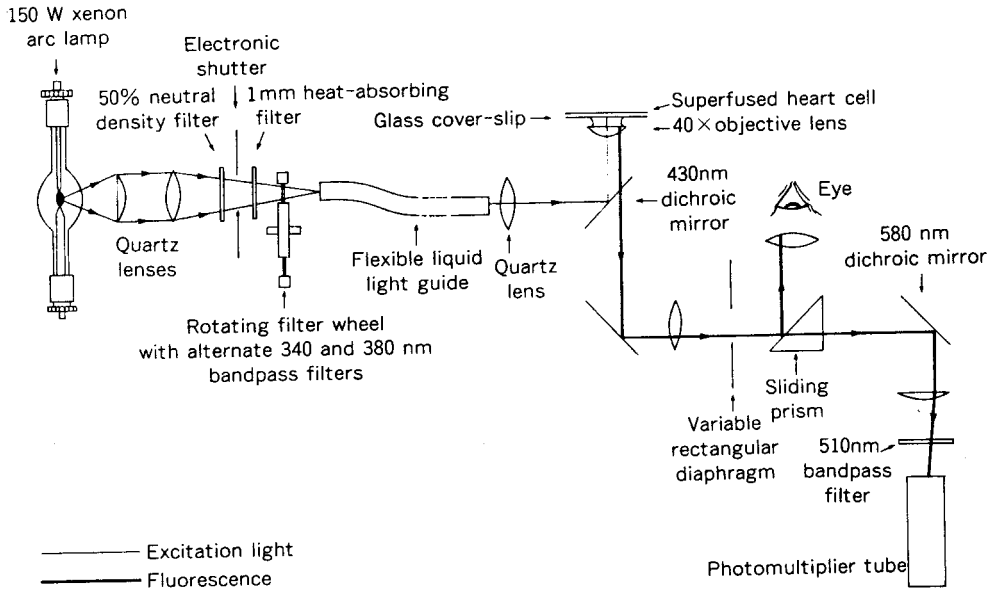

Fig. 1. *A schematic diagram of epi-fluorescence o ptical system.*
Spex cation measurement system was used for measurement of cytoplasmic Ca^{2+} concentration. UV light from xenon lamp was transmitted to rotating filter wheel through a neutral density filter and heat absorbing fliter. The rotating filter wheel composed of three 340 nm filters and three 380 nm filters, which were arranged alternately. Excitation light from filter was transmitted to microscope via a flexible optic fiber. A 430 nm dichroic mirror in the microscope reflected the UV light to the cell via a FLUOR objective lens. The resulting fura-2 fluorescence from the cell was transmitted to side port of microscope. In the side port, fluorescence light was passed through variable sized diaphragm which confine the cell under study and prevent the contamination of fluorescence from neighboring cells. The flourescence light was reflected by a 580 nm dichroic mirror to a photomultiplier tube via a 510 nm emission filter. The output of the photomultiplier tube transferred to the computer and stored in hard disk for further analysis.

Estimation of intracellular Ca^{2+} concentration and fura-2 compartmentalization.

To obtain the intracellular Ca^{2+} concentration, we used an invivo calibration technique to minimize the error due to intracellular viscosity or Ca^{2+} insensitive products of Fura-2 (Ponie, 1990; Roe *et al.* 1990). The protocol used in our experiment is briefly summarized in figure 2.

Fura-2 also may be compartmentalized to intracellular organelle (Di Virgilio *et al.* 1990), so that the recorded fluorescence may be contaminated by non-cytoplasmic source. This compartmentalizaiton may interfere the accurate estimate of cytoplasmic Ca^{2+} concentration (Frampton *et al.* 1991). The extent of compartmentalization depends on the Fura-2 loading method (Roe *et al.* 1990), therefore we first assessed the extent of Fura-2 compartmentalization as shown in figure 3. Application of digitonin (12 μM) to the bath, which is known to increase the permeability of sarcolemma only, increased $[Ca^{2+}]_i$ transiently and then application of triton X−100 (5%), which increases the permeability of intracellular organelle membrane, showed no change in fluorescence intensity. This result suggested that there was no significant compartmentalization of Fura-2 with our loading method.

Solution compositions

The composition of normal Tyrode solution was : NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1; NaH_2

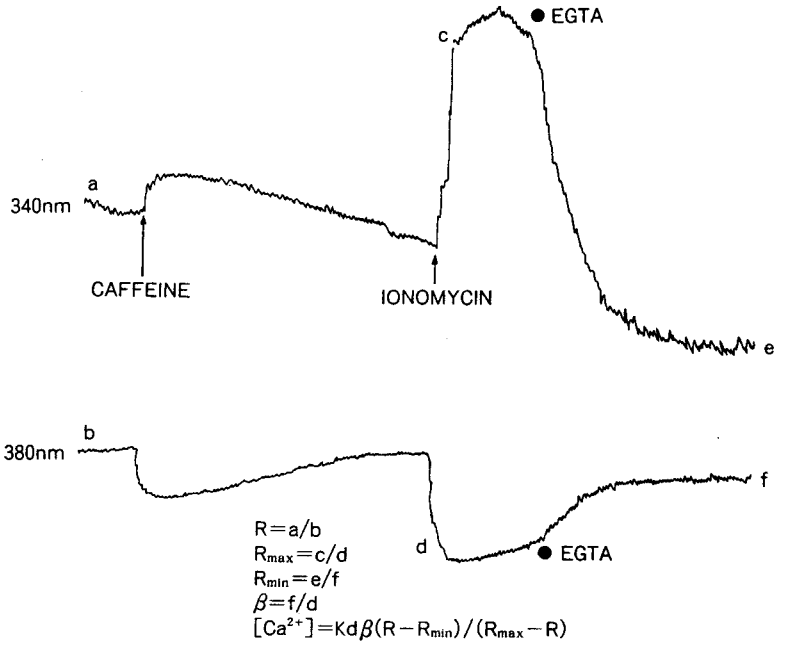

Fig. 2. *Calibration of fura-2 fluorescence.*
The fura-2 loaded ventricular cell was excited by 340 nm and 380 nm alternately during a perfusion of normal Tyrode solution (top trace; 340 nm, bottom trace; 380 nm). The cell was then perfused with 10 mM caffeine contained Tyrode solution which resulted an increase in fluorescence ratio (R). After the fluorescence intensity returned to control level, ionomycin (25 μM) was directly added to the bath. There was a marked increase in fluorescence ratio. The peak amplitude of this fluorescence change represent a full saturation of fura-2 with intracellular Ca^{2+} (R_{max}). The perfusate was then changed to zero Ca^{2+}-Tyrode solution which contained 10 mM EGTA. This produced a rapid decline of fluorescence intensity and represent a full dissociation of fura-2 with intracellular Ca^{2+} (R_{min}). The use of ionomycin caused a decrease in absolute fluorescence intensity by fura-2 leakage from the cell. To compensate this factor, we used the β for calculation of Ca^{2+} concentration. β is the ratio of maximum fluorescence during excitation at 380 nm (d) to minimum fluorescence during excitation at 380 nm (f).

PO_4 0.3; HEPES 10; Glucose 5.5 (mM); pH=7.4 with Tris. 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). All chemicals used in this experiment were purchased from Sigma Co. (St Louis, USA) except Fura-2 (Molecular probe).

Data analysis and statistics

Ca^{2+} concentration was calculated by using a computer program (DM 3000 CM, Spex Co.) and expressed as a mean S.E. The change of $[Ca^{2+}]_i$ during cardioplegia was expressed as △ $[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$-resting $[Ca^{2+}]_i$). Statistical comparisons were made using an unpaired t-test.

RESULTS

Cytosolic Ca^{2+} concentration change during cardioplegia

To expose a cell to a similar condition of cardiac operation, we perfused the ventricular cells with cardioplegic solutions (St. Thomas Hospital Solution, Ronbinson *et al.* 1991) and measured a $[Ca^{2+}]_i$ by using a Fura-2. Resting

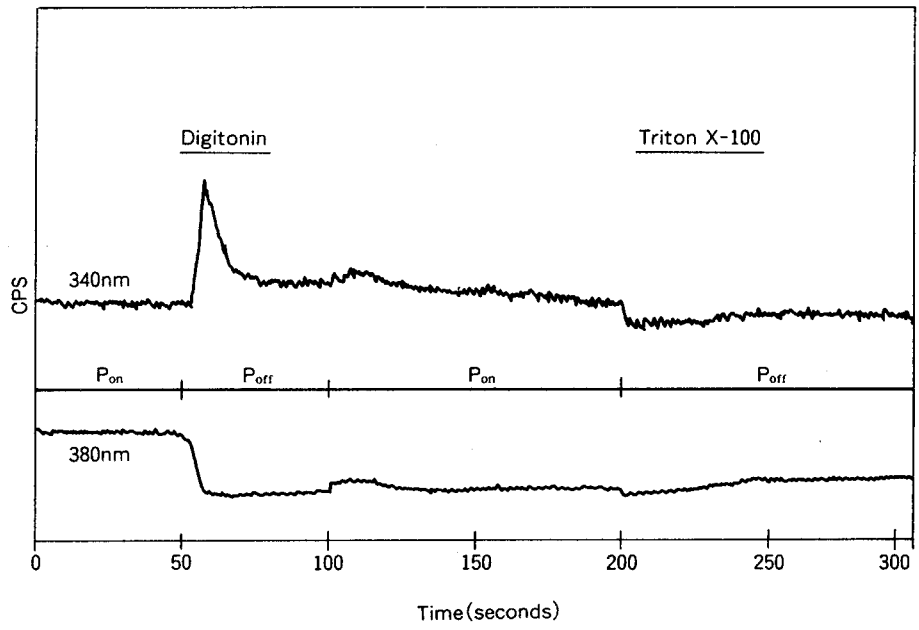

Fig. 3. *Measurement of fura-2 compartmentalization.*
The fura-2 loaded ventricular myocyte was excited with 340/380 nm UV lights and resulting fluorescence change was recorded (top trace; 340 nm, bottom trace; 380 nm). Perfusion was stopped and the digitonin was added to the chamber where indicated. The application of digitonin (12 μM) to the chamber caused a transient increase in fluorescence intensity. Perfusion was restarted to eliminate the residual cytosolic fura-2 molecule after the fluorescence change was stabilized. The perfusion was then stopped and triton X−100 (5 %) was added to the bath. There was no significant change in fluorescence intensity by appliction of triton X−100. CPS; counts per second, P_{on}; period of solution flow, P_{off}; period of solution stop.

$[Ca^{2+}]_i$ of rat ventricular cell was 150±30 nM (n=39) and this value was compatible with others (Liu *et al.* 1991). However, the resting $[Ca^{2+}]_i$ of rat myocytes obtained in our experiment might be lower than the value of end-diastolic $[Ca^{2+}]_i$ in beating myocytes (Lee and Clusin, 1987).

When the cell was perfused with the cardioplegic solution, $[Ca^{2+}]_i$ of ventricular myocyte was increased significantly from resting value. The extent of $[Ca^{2+}]_i$ elevation was more prominent during hypothermic (20℃) cardioplegia than during normothermic (37℃) cardioplegia (Fig. 4-A, B; 435 nM±48 nM, n=27 vs 297±13 nM, n=35). However, a simple reduction of temperature in normal Tyrode solution had no apparent effect on $[Ca^{2+}]_i$ compared to those of hypothermic cardioplegia (Fig. 4-C).

The elevation of $[Ca^{2+}]_i$ reached a maximum within 2~3 minutes and then gradually declined to a steady state level, despite the continuous perfusion of the cardioplegic solution, but $[Ca^{2+}]_i$ remained significantly elevated throughout the period of cardioplegia(Fig. 4D). Because the initial change of $[Ca^{2+}]_i$ during cardioplegia reached to a steady state after 6-8 minutes of perfusion, we perfused the cardiac cell for 6~8 minutes and examined the underlying mechanism of this transient $[Ca^{2+}]_i$ change during cardioplegia.

Effect of Ca^{2+} channel blocker on $[Ca^{2+}]_i$ during cardioplegia

Depolarization of membrane potential by high external K^+ induces a Ca^{2+} influx through the voltage-depenent Ca^{2+} channels and consequently increase the $[Ca^{2+}]_i$ of cardi-

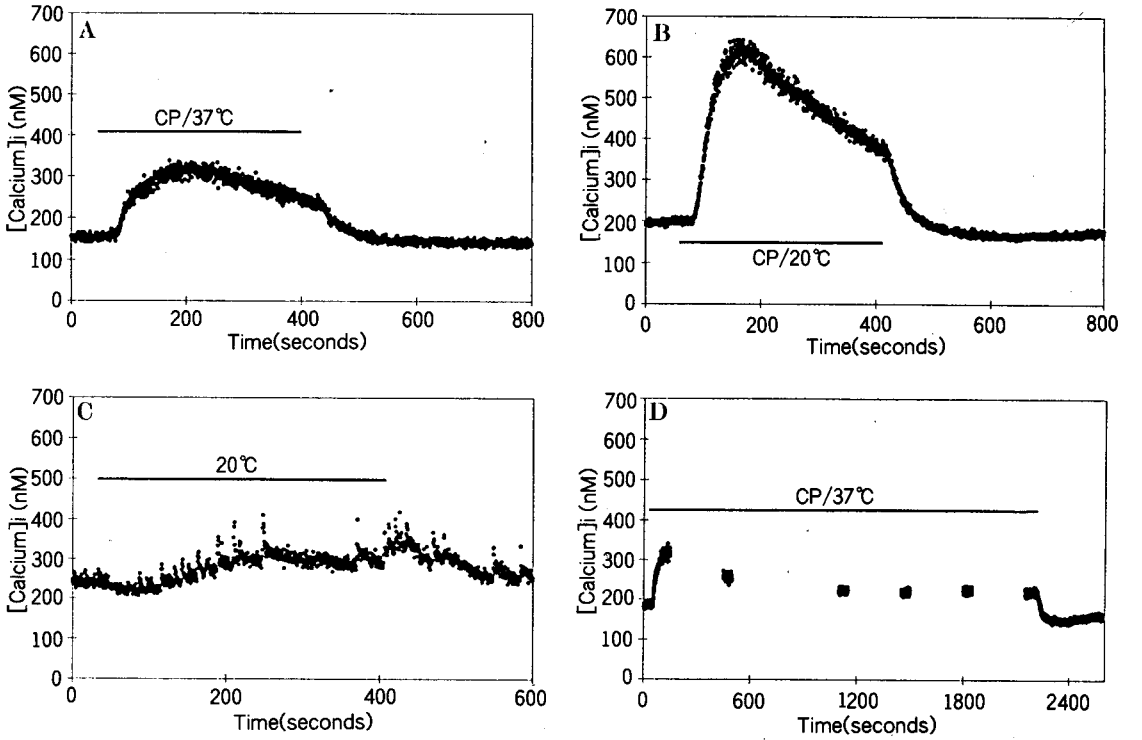

Fig. 4. *Effect of temperature on [Ca^{2+}]$_i$ changes in ventricular myocytes.*
Ventricular myocytes was perfused with normal Tyrode solution and resting [Ca^{2+}]$_i$ were measured by fura-2. The perfusing solution was then changed to normothermic cardioplegic solution (A), hypothermic cardioplegic solution (B) and hypothermic Tyrode solution (C). The time dependent changes in [Ca^{2+}]$_i$ during cardioplegia was recorded intermittently for 40 minutes (D). CP/37℃; perfusion of normothermic caridoplegia. CP/20℃; perfusion of hypothermic cardioplegia.

ac myocytes (Powell *et al.* 1984). This Ca^{2+} influx through the Ca^{2+} channels can be effectively blocked by organic Ca^{2+} channel blockers, such as nifedipine (Tsien, 1983). We measured effect of nifedipine on cardioplegia-induced [Ca^{2+}]$_i$ change and examined the role of Ca^{2+} channels in the transient increase of [Ca^{2+}]$_i$ during cardioplegia.

Application of nifedipine (5×10^{-7} M) to the cardioplegic solution had no apparent effect on the [Ca^{2+}]$_i$ change during normothermic cardioplegia compared to the control (Fig. 5-A), and its effect was statistically insignificant (control: $\triangle[Ca^{2+}]_i=119.4\pm15$ nM, nifedipine treated group: $\triangle[Ca^{2+}]_i=108.1\pm13$nM, n=8). Application of nifedipine during hypothermic cardioplegia also had no apparent effect on [Ca^{2+}]$_i$ change(Fig. 5-B), and its effect was statistically insignificant (control: $\triangle[Ca^{2+}]_i=193.6\pm37$ nM, nifedipine treated group: $\triangle[Ca^{2+}]_i=170.4\pm38$ nM, n=7).

Effect of sarcoplasmic reticulum on [Ca^{2+}]$_i$ during cardioplegia.

To rule out the possibility whether the transient 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 cardioplegia on [Ca^{2+}]$_i$ after emptying the SR by pretreatment of 10 mM caffeine. Application of caffeine (10 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 second application

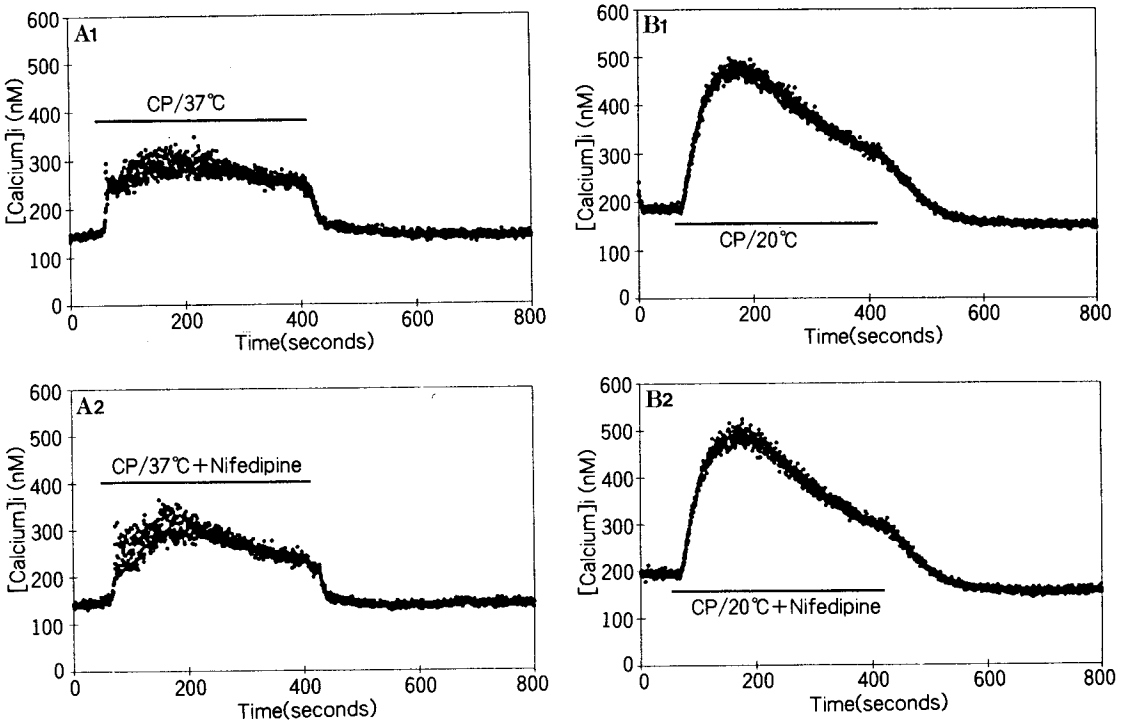

Fig. 5. *A) Effect of normothermic cardioplegia on $[Ca^{2+}]_i$ in the absence (A1) and presence (A2) of nifedipine (5×10^{-7} M) in the perfusate. B) Effect of hypothermic cardioplegia on $[Ca^{2+}]_i$ in the absence (B1) and presence (B2) of nifedipine (5×10^{-7} M) CP/37℃; perfusion of normothermic cardioplegia, CP/20℃; perfusion of hypothermic cardioplegia*

of caffeine in the same cell did not cause any change in $[Ca^{2+}]_i$, which suggests the complete emptying of SR by pretreatment of 10 mM caffeine (Fig. 6-A1).

Pretreatment of caffeine slightly decreased the $[Ca^{2+}]_i$ change during normothermic cardioplegia compared to the control (Fig. 6-A2, 3), but the effect of caffeine was not stastistically significant (control: $\triangle[Ca^{2+}]_i=136.2\pm27$ nM, caffeine treated group: $\triangle[Ca^{2+}]_i=90.6\pm15$ nM, n=8). Pretreatment of caffeine during hypothermic cardioplegia also slightly decreased the $[Ca^{2+}]_i$ change and its effect was statistically insignificant (Fig. 6-B; control: $\triangle[Ca^{2+}]_i=201.7\pm38$ nM, caffeine treated group: $\triangle[Ca^{2+}]_i=130.0\pm30$ nM, n=7).

Effect of external Ca^{2+} concentration on $[Ca^{2+}]_i$ change

To investigate the effect of external Ca^{2+} on $[Ca^{2+}]_i$ change, we perfused the cardiac cell with different Ca^{2+} containing cardioplegic solutions and measured its effect on $[Ca^{2+}]_i$ change during hypothermic cardioplegia. Because the complete removal of external Ca^{2+} can induce the massive Ca^{2+} influx during reperfusion ("Ca^{2+} paradox", Alto & Dhalla, 1979; Rich & Langer, 1982), we changed external Ca^{2+} concentration of cardioplegic solution from 0.2 mM to 2 mM. When the cardiac cell was perfused with 0.2 mM Ca^{2+}-containing cardioplegic solution, there was little change in $[Ca^{2+}]_i$ compared to the resting $[Ca^{2+}]_i$. Changing the Ca^{2+} concentration of cardioplegic solution from 0.2 mM to 2 mM resulted in the corresponding increase of $[Ca^{2+}]_i$ during perfusion of cardioplegic soultion. The $[Ca^{2+}]_i$ reached to the maximum value ($>2\mu$M) when the cardiac cell was perfused with the 2 mM Ca^{2+}-containing cardioplegic solution (FIg. 7).

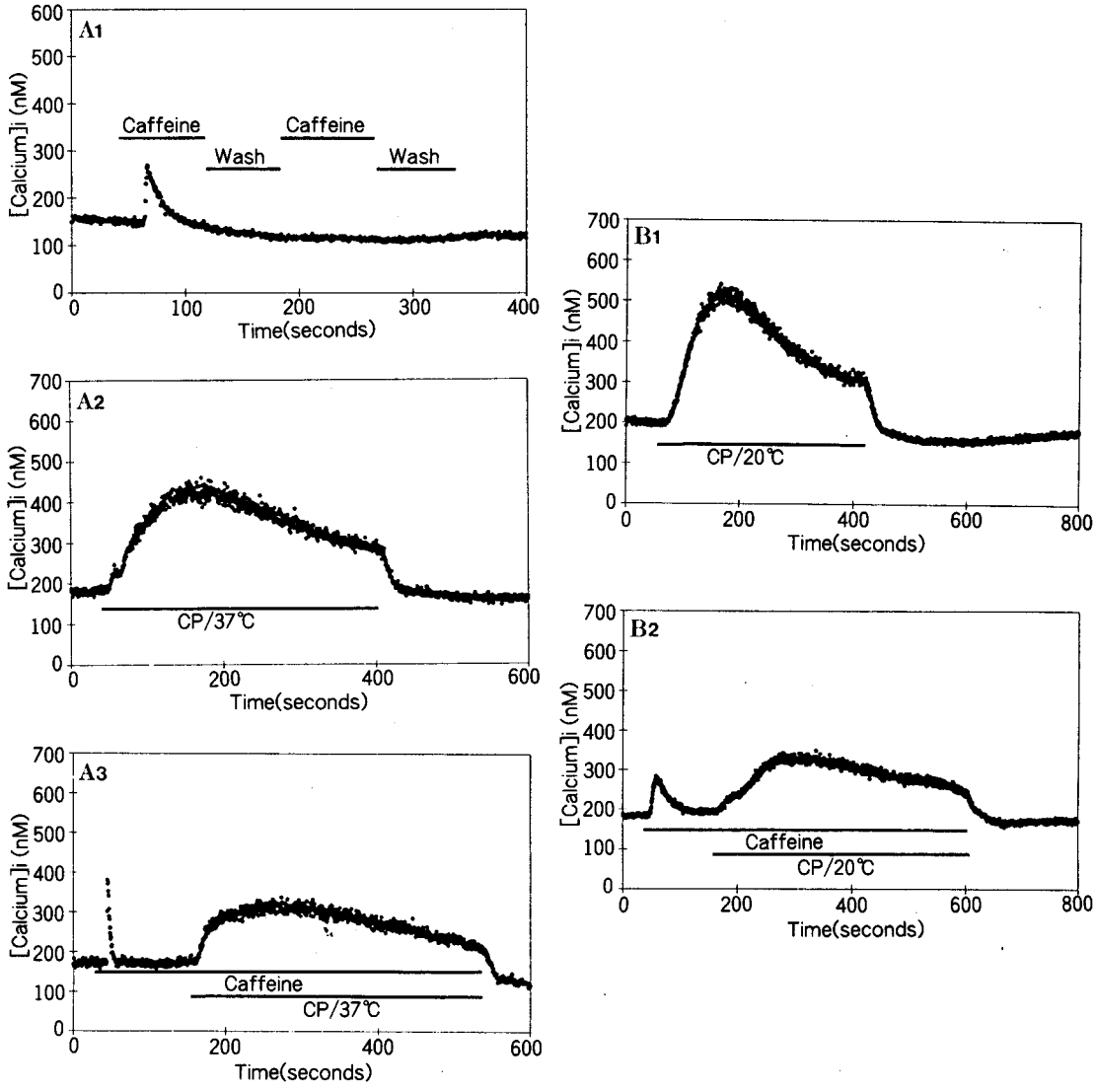

Fig. 6. *A) Treatment of 10 mM caffeine caused a complete emptying of sarcoplasmic reticulum (A1). And the effect of normothermic cardioplegia on [Ca^{2+}]$_i$ in the absence (A2) and presence (A3) of caffeine (10 mM) were measured.*
B) Effect of hypothermic cardioplegia on [Ca^{2+}]$_i$ in the absence (B1) and presence (B2) of caffeine (10 mM) in the perfusate. CP/37℃; perfusion of normothermic cardioplegia, CP/20℃; perfusion of hypothermic cardioplegia

Effect of Na^+/Ca^{2+} exchange blockers on [Ca^{2+}]$_i$ during cardioplegia

Na^+/Ca^{2+} exchange mechanism in cardiac cell membrane play an important role on the excitability and Ca^{2+} homeotasis of cardiac cells (Hilgeman, 1990). Depolarization of the membrane potential by high external K^+ or increase of [Na^+]i by myocardial ischemia can increase the Ca^{2+} influx through Na^+/Ca^{2+} exchange mechanism (Wilde & Kleber, 1986).

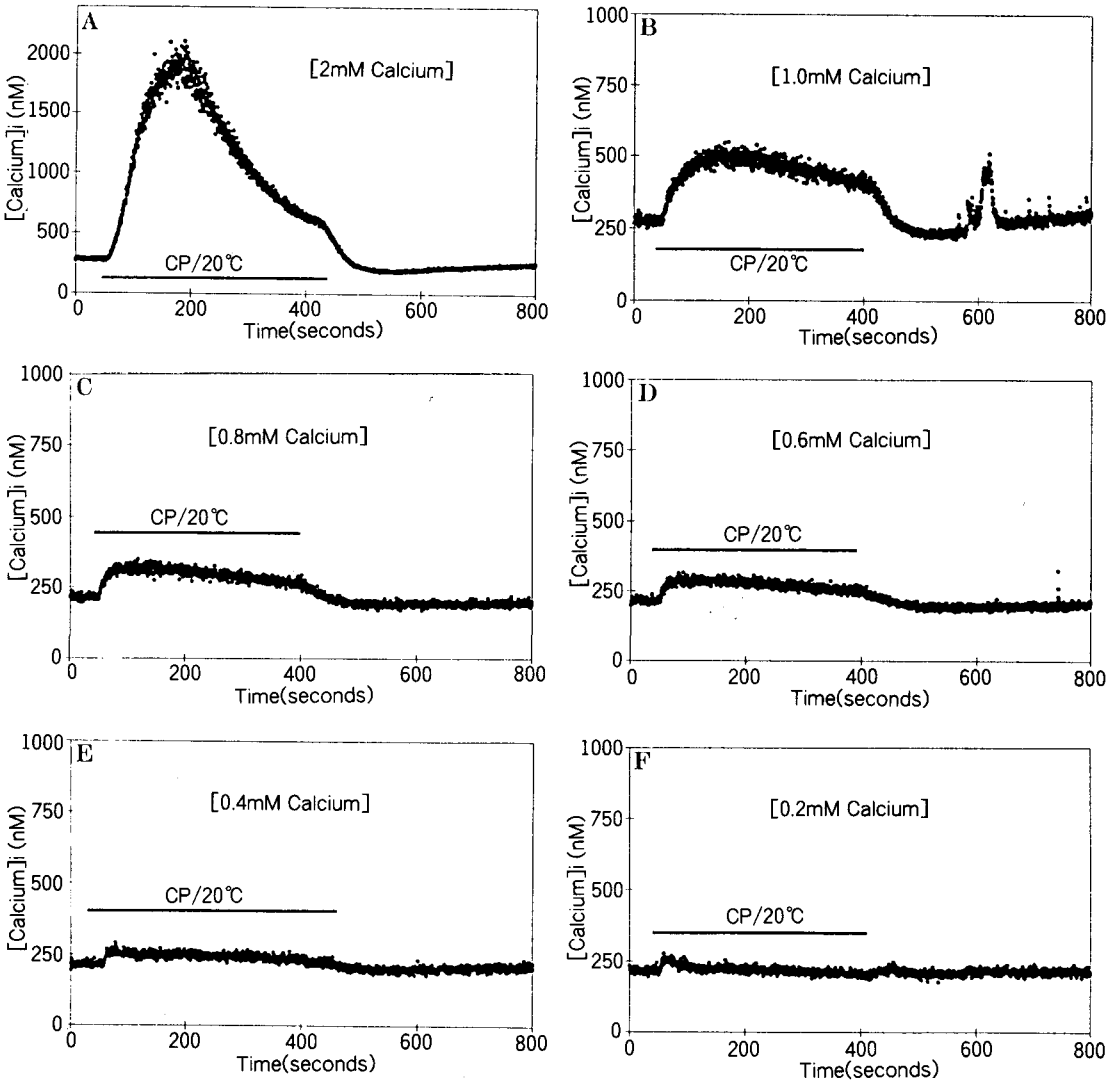

Fig. 7. *Ca^{2+} concentration of cardiopleic solution was changed from 0.2 mM to 2mM and measured its effect on cytoplasmic Ca^{2+} concentration during hypothermic cardioplegia. CP/20℃; perfusion of hypothermic cardioplegia*

The specific blocker of Na^+/Ca^{2+} exchange has not been known, but the divalent cation, Ni^{2+}, can inhibit the Na^+/Ca^{2+} exchange at high concentrations (Kimura *et al.* 1987). We applied Ni^{2+} to the cardioplegic solution and examined its effect on cardioplegia-induced transient increase of $[Ca^{2+}]_i$. The application of Ni^{2+} (5 mM) to the cardioplegic solution significantly decreased $[Ca^{2+}]_i$ change during normothermic cardioplegia compared to the control (Fig. 8-A1,2; $p<0.05$, control: $\triangle[Ca^{2+}]_i = 103.3 \pm 15$ nM, Ni^{2+} treated group: $\triangle[Ca^{2+}]_i = 27.8 \pm 9.5$ nM, $n=7$). Combined treatment of caffeine and Ni^{2+} further decreased $[Ca^{2+}]_i$ change during normothermic cardioplegia (Fig. 8-A3; Ni^{2+} & caffeine treated group: $\triangle[Ca^{2+}]_i = 19.2 \pm 8.6$ nM, $n=7$). The effect of Ni^{2+} during hy-pothermic cardioplegia was similar as those

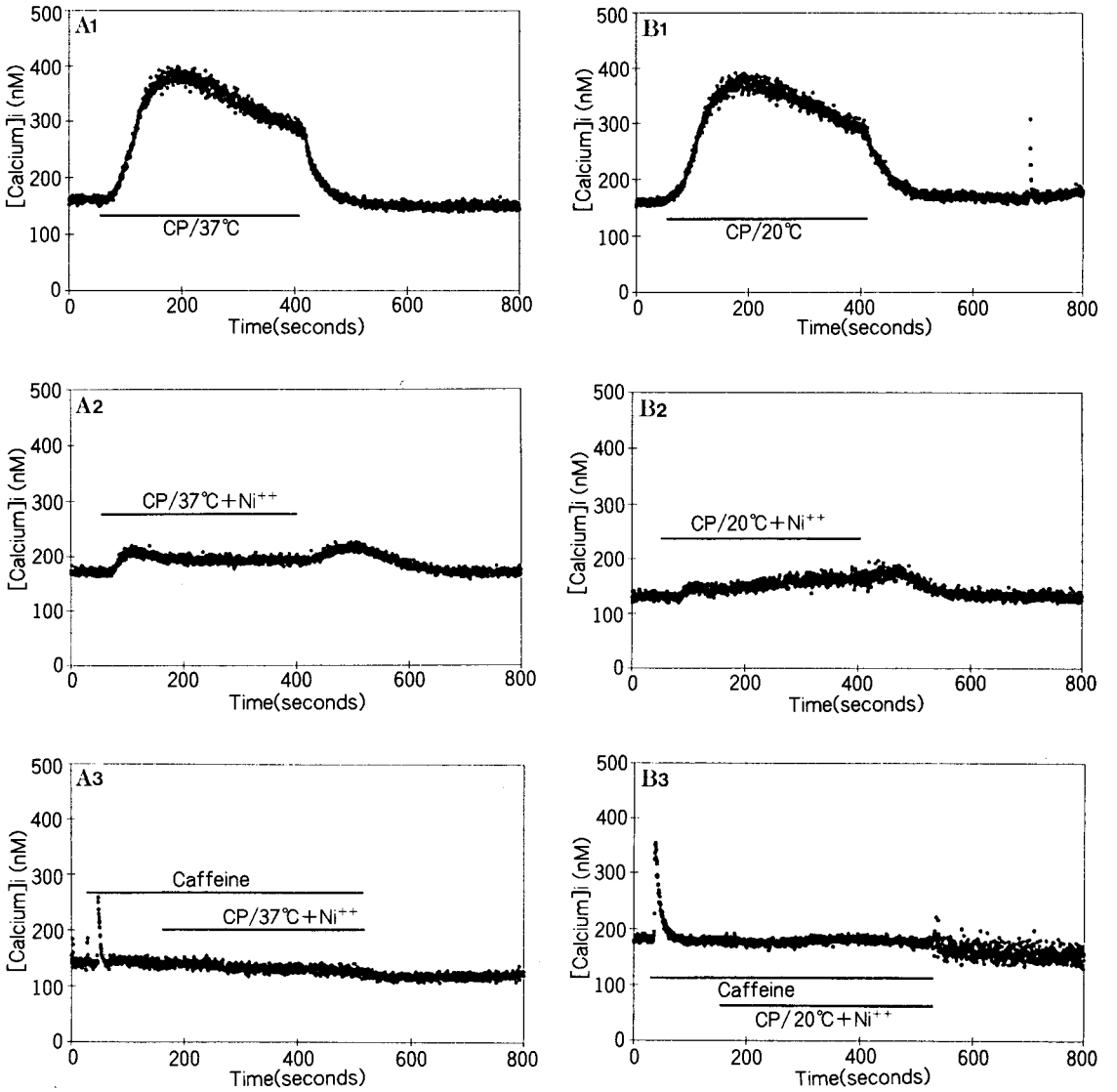

Fig. 8. *A) Effect of normothermic cardioplegia on $[Ca^{2+}]_i$ in the absence (A1) and presence (A2) of Ni^{2+} (5 mM) in the perfusate.*
B) Effect of hypothermic cardioplegia on $[Ca^{2+}]_i$ in the absence (B1) and presence (B2) of Ni^{2+} (5 mM) in the perfusate. Combined treatment of caffeine (10 mM) and Ni^{2+} (5 mM) completely prevented the $[Ca^{2+}]_i$ changes during normothermic (A3) and hypothermic cardioplegia (B3). CP/37℃; perfusion of normothermic cardioplegia, CP/20℃; perfusion of hypothermic cardioplegia

in normothermic cardioplegia (Fig. 8-B1,2; control: $\triangle[Ca^{2+}]_i = 193.6 \pm 37$ nM, Ni^{2+} treated group: $\triangle[Ca^{2+}]_i = 22.5 \pm 7.7$ nM, n=8), and combined treatment of caffeine and Ni^{2+} completely blocked $[Ca^{2+}]_i$ change during hypothermic cardioplegia (Fig. 8-B3; Ni^{2+} & caffeine treated group: $\triangle[Ca^{2+}]_i = 7.1 \pm 5.7$ nM, n=7).

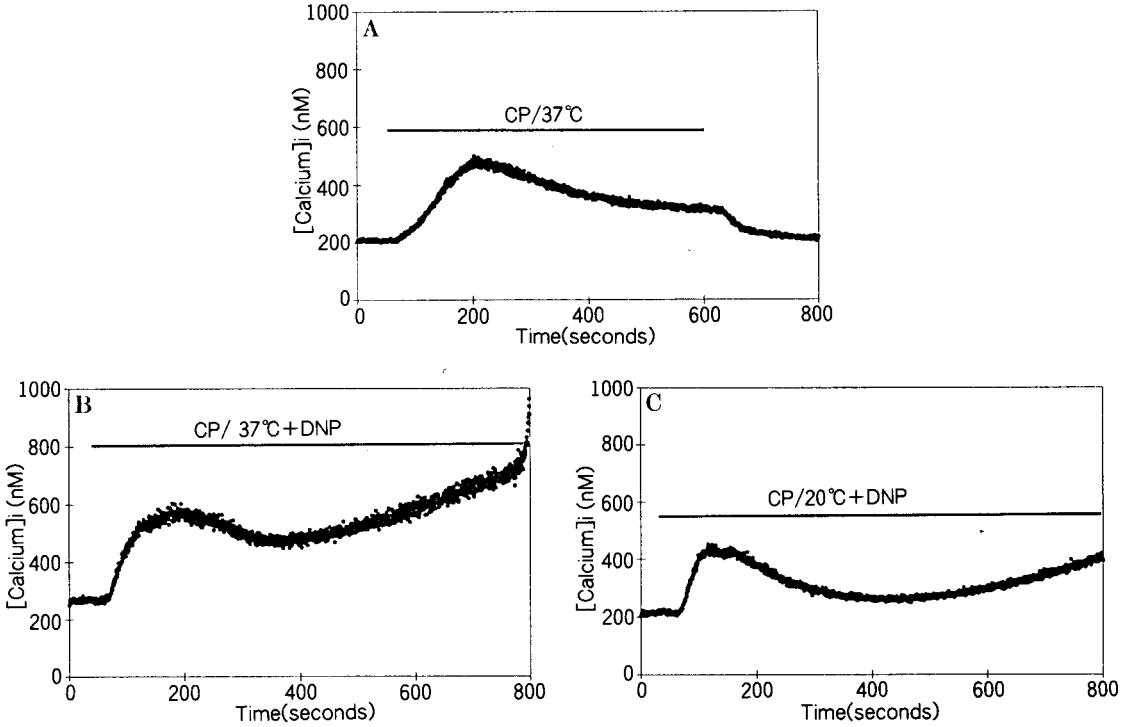

Fig. 9. *Effect of DNP (0.1 mM) on $[Ca^{2+}]_i$ during control (A), normothermic cardioplegia (B) and hypothermic cardioplegia (C). CP/37℃; normothermic cardioplegia, CP/37℃+DNP; normothermic cardioplegia with 0.1 mM DNP, CP/20℃+DNP; hypothermic cardioplegia with 0.1 mM DNP.*

Effect of ischemic insult on $[Ca^{2+}]_i$ during cardioplegia

To examine whether the protective effect of hypothermia on myocardial ischemia is due to the preservation of high energy phosphate store or not, we treated the cardiac cells with 2,4 dinitrophenol (DNP) which blocks the formation of ATP in mitochondria (Stryer, 1988). Application of DNP (0.1 mM) to the bath showed different effect on $[Ca^{2+}]_i$ with the temperature of cardioplegic solutions. When cardiac cells were perfused with DNP-contained normothermic cardioplegic solution, $[Ca^{2+}]_i$ initially changed as those of control, and then it gradually increased over 1 μM and caused a contracture of cardiac cell. In the case of hypothermic cardioplegia, the initial transient change of $[Ca^{2+}]_i$ was similar to those of normothermic cardioplegia. However, the onset and the amplitude of secondary $[Ca^{2+}]_i$ increase was diminished compared to those of normothermic cardioplegia (Fig. 9, n=3).

DISCUSSION

Myocardial hypothermia has been known to reduce the severity of ischemic damage, and it has been used to protect the myocardium from ischemic insult during cardiac operation (Ferrari *et al.* 1990; Ronbinson *et al.* 1991). However, the underlying mechanism of its protective effect is still uncertain. Here, we measured the $[Ca^{2+}]_i$ under different experimental conditions and investigated the mechanism of $[Ca^{2+}]_i$ change during cardioplegia.

It has been well known that the magnitude

of $[Ca^{2+}]_i$ during ischemia and reperfusion was closely related to the extent of cardiac cell damage. The low $[Ca^{2+}]_i$ before and during ischemia slows the rate of ATP depletion during ischemia, delays the onset of contracture and the significant rise in $[Ca^{2+}]_i$ (Murphy *et al.* 1983; Steenbergen *et al.* 1990; Huddleston *et al.* 1992). During cardiac operations, the high K^+-cardioplegic solution leads to a membrane depolarization which increase a $[Ca^{2+}]_i$ by Ca^{2+} influx through the voltage-dependent Ca^{2+} channel (Pappano, 1970; Tsien, 1983; Powell *et al.* 1984). However, in our experiment addition of Ca^{2+} channel blockers to the cardioplegic solution did not cause any significant reduction of $[Ca^{2+}]_i$ during normothermic cardioplegia (Fig. 5-A). When the cardiac cell was perfused with cardioplegic solution, there would be membrane depolarization up to -40 mV (based on the Nernst equation). At this membrane potential, the open probability of Ca^{2+} channel is very low, and maintained depolarization by continusous perfusion of cardioplegic solution can stop the Ca^{2+} influx by inactivation of Ca^{2+} channel (Nelson *et al.* 1990; Pietrobon & Hess, 1990). These reports may explain the insignificant effect of nifedipine in our experiment.

When the cardiac cells are exposed to low temperature, the membrane potential of cardiac cell can be depolarized by lowering the temperature itself (Kurihara & Sakai, 1985). This low temperature-induced depolarization during hypothermic cardioplegia increase the magnitude of membrane depolarization. This increase of membrane potential may augument the Ca^{2+} influx through Ca^{2+} channel compared to those of normothermic cardioplegia (Pelzer *et al.* 1990; Pietrobon & Hess, 1990). However, application of Ca^{2+} channel blocker to the bath did not affect $[Ca^{2+}]_i$ during hypothermic cardioplegia (Fig. 5-B). These results suggest that the amount of Ca^{2+} influx through Ca^{2+} channels may not significantly contribute to the $[Ca^{2+}]_i$ increase during normothermic and hypothermic cardioplegia.

Cytoplasmic Ca^{2+} concentration is also regulated by the Ca^{2+} release from SR, and this triggered Ca^{2+} release can be increased by membrane depolarization or low temperature exposure (Kurihara & Sakai, 1985; Bers *et al.* 1989). However, based on our experimental data the contribution of this triggered Ca^{2+} release from SR by membrane depolarization was insignificant to the $[Ca^{2+}]_i$ increase during cardioplegia. Because the pretreatment of 10 mM caffeine, which was a sufficient amount to empty out the SR (Fig. 6-A1), had no significant effect on $[Ca^{2+}]_i$ change during normothermic cardioplegia (Fig. 6-A3). The effect of low temperature-induced Ca^{2+} release on $[Ca^{2+}]_i$ was also excluded, since the pretreatment of 10 mM caffeine had no effect on the $[Ca^{2+}]_i$ increase during hypothermic cardioplegia (figure 6-B). Also the temperature (20℃) used in our experiment was above the critical temperature which induces a Ca^{2+} release from SR (Kurihara & Sakai, 1985; Bers *et al.* 1989).

Another possible system to regulate the cytosolic Ca^{2+} concentration is Na^+/Ca^{2+} exchange mechanism , which shows a bidirectional exchange of Na^+ and Ca^{2+} with regard to their electrochemical concentration gradients (Kimura *et al.* 1987; Hilgemann, 1990). Ca^{2+} influx through this mechanism can be increased in the case of membrane depolarization or increase of $[Na^+]_i$ (Bers, 1987; Kimura *et al.* 1987). When the cardiac cell was exposed to hypothermic cardioplegia, $[Ca^{2+}]_i$ might be increased by Ca^{2+} influx through Na^+/Ca^{2+} exchange mechanism (Crake & Poole-Wilson, 1986; Wilde & Kleber, 1986; Murphy *et al.* 1988). To test this possibility, we changed a external Ca^{2+} concentration from 0.2 mM to 2 mM and measured its effect on $[Ca^{2+}]_i$. As shown in figure 7, a reduction of external Ca^{2+} decreased the $[Ca^{2+}]_i$ during hypothermic 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 a beneficial effects of low external Ca^{2+} on the recovery of cardiac function after myocardial ischemia (Kinoshita *et al.* 1991; Robinson *et al.* 1991). Furthermore, application of Ni^{2+}, blocker of Na^+/Ca^{2+} exchange mechanism at high concentrations (Kimura *et al.* 1987; Beuckelmann & Wier, 1989), significantly reduced the $[Ca^{2+}]_i$ change during normothermic and hypothermic cardioplegia (Fig. 8). These results suggested

that a major part of $[Ca^{2+}]_i$ increase during cardioplegia was resulted from Ca^{2+} influx through Na^+/Ca^{2+} exchange mechanism. But a small amount of $[Ca^{2+}]_i$ increase still remained in the presence of Ni^{2+}. It may be due to the incomplete block of Na^+/Ca^{2+} exchange mechansim by 5 mM Ni^{2+} or the Ca^{2+} release from intracellular Ca^{2+} stores (Fabiato, 1986; Kimura *et al.* 1987). However, a combined treatment of Ni^{2+} and caffeine completely prevent the $[Ca^{2+}]_i$ increase during normothermic and hypothermic cardioplegia (Fig. 8-A3, B3). It suggests that a Ca^{2+} release from SR can also participate in $[Ca^{2+}]_i$ increase during cardioplegia, but its contribution may be insignificant.

The protective effect of hypothermia is attributed either to the conservation of high energy phosphate store by reducing metabolic rate or to the decrease of transmembrane ionic fluxes by phase transition (Harding & Poole-Wilson, 1980; Rosenfeldt *et al.* 1980; Rich & Langer, 1982; Ferrari *et al.* 1990; Steenbergen *et al.* 1990). In this experiment, we did not directly measure the ATP and creatinine phosphate consumption rate nor phase transition during hypothermic cardioplegia, but indirect evidences were presented.

If the protective effect of hypothermia was due to the decrease of transmembrane ionic fluxes by low temperature, the $[Ca^{2+}]_i$ increase during the perfusion of hypothermic cardioplegic solution might be smaller than the $[Ca^{2+}]_i$ increase during the perfusion of normothermic cardioplegic solution. However, the perfusion of hypothermic cardioplegic solution without ischemic insult enhanced the $[Ca^{2+}]_i$ increase compared to those of normothermic cardioplegia (figure 4).

$[Ca^{2+}]_i$ of cardiac cell depends on the balance between the amount of Ca^{2+} influx and its extrusion from the cell. The amount of released Ca^{2+} from the SR or reuptaked Ca^{2+} into the SR also contribute to the regulation of $[Ca^{2+}]_i$. The possible pathways to decrease the $[Ca^{2+}]_i$ are the reuptake or extrusion of Ca^{2+} from the cytosol, and these processes use ATP as an energy source (Nelson *et al.* 1990; Tani, 1990). As presented in Fig. 9, the change of $[Ca^{2+}]_i$ was delayed and its amplitude was decreased during the perfusion of hypothermic cardioplegic solution with ischemic insult. These result suggested that the energy-dependent $[Ca^{2+}]_i$ decreasing processes were well maintained during ischemic period by the perfusion of hypothermic cardioplegic solutions, since the perfusion of hypothermic cardioplegic solution without ischemic insult enhanced the $[Ca^{2+}]_i$ increase compared to those of normothermic cardioplegia (Fig. 4).

Above results suggest that hypothermic cardioplegia protects the myocardium from ischemic insult by preservation of high-energy phosphate store, not by decreasing the transmembrane ionic fluxes (Rich & Langer, 1982; Clark III *et al.* 1991).

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