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Relationship between myocardial cation content and injury in reperfused rat hearts treated with cation channel blockers

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Abstract

A role for K^+ and Ca^{2+} channel blockers in cardiac contractile dysfunction and myocardial ionic imbalance was examined in isolated rat hearts with 35-min ischemia and 60-min reperfusion. The K^+ channel blockers glibenclamide (1–30 μM) and sematilide (1–30 μM), Ca^{2+} channel blockers diltiazem (0.1–3 μM) and nicardipine (0.03–1 μM) and fast Na^+ channel blocker tetrodotoxin (0.01–0.3 μM) were delivered for the last 3-min pre-ischemia. Ischemia-induced increase in Na^+ content was attenuated by diltiazem and tetrodotoxin at all concentrations employed and by nicardipine at 0.3 μM, whereas the ischemia-induced loss of K^+ was suppressed partially by glibenclamide and sematilide and almost completely by the two drugs in combination. Left ventricular developed pressure of untreated hearts did not recover upon reperfusion, which was associated with increases in myocardial Na^+ and Ca^{2+} contents and decreases in K^+ and Mg^{2+} contents. Glibenclamide and sematilide neither enhanced the post-ischemic recovery of left ventricular developed pressure nor affected cation changes during reperfusion. Diltiazem enhanced the recovery of left ventricular developed pressure and attenuated imbalance of the myocardial Na^+ during ischemia and of all myocardial cations examined during reperfusion. The effects of nicardipine on these parameters were small. Tetrodotoxin enhanced the recovery of left ventricular developed pressure and reversed the imbalance of all myocardial cations examined during reperfusion in a concentration-dependent manner. The results suggest that blockade of transmembrane flux of K^+ during ischemia plays a minor role in the improvement of post-ischemic contractile recovery, rather blockade of transmembrane flux of Na^+ attenuates the ischemia and reperfusion injury.

Keywords: Ca^{2+} channel; Ca^{2+} overload; Contractile dysfunction; Diltiazem; Ischemia; K^+ channel; Reperfusion; Na^+ channel; Na^+ overload; Tetrodotoxin

1. Introduction

Ischemia and reperfusion induce metabolic and functional impairments of the heart mostly depending upon the severity and period of ischemia. Several possible mechanisms underlying ischemia and reperfusion-induced impairments have been proposed including disturbance in ionic homeostasis (Tani and Neely, 1989), a failure in energy production (Shen and Jennings, 1972), no reflow in the reperfused heart (Kloner et al., 1974) and disruption of cell membrane and/or subcellular organelles due to free radical attack (Zweier et al., 1987). Some investigators have emphasized that ionic disturbance in cardiac cells such as sodium overload, potassium loss and calcium overload is one of the most critical alterations responsible for ischemia and reperfusion injury (Nayler et al., 1988; Tani and Neely, 1989; Meng et al., 1991; Vandenberg et al., 1993). Upon ischemia, the heart is gradually loaded with sodium partly through Na^+/H^+ exchange, which is activated to reduce ischemia-caused increase in intracellular H^+ concentration (Poole-Wilson, 1978). Intracellular free Ca^{2+} concentration was increased during ischemia when determined by the NMR study (Steenbergen et al., 1987; Koretsune and Marban, 1990). However, cellular calcium levels, when determined by the atomic absorption method or radio-labeled Ca^{2+} method, remain relatively unchanged during a considerably long period of ischemia, particularly in perfused rat hearts (Tani and Neely, 1989; Meng and Pierce, 1991; Takeo et al., 1995). When hearts are reperfused after ischemia, sodium overload is enhanced by Na^+/H^+ exchange during the early phase of reperfusion (Vandenberg et al., 1993), and subsequent calcium overload in the cell occurs through

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Na^+/Ca^{2+} exchange (Tani and Neely, 1989). Recently, a substantial body of evidence has suggested a major involvement of sodium influx during ischemia via the Na^+ channel in ischemia and reperfusion injury (Ver Donck et al., 1993; Haigney et al., 1994; Silverman and Stern, 1994; Takeo et al., 1995; Ju et al., 1996). For example, Ju et al. (1996) have demonstrated electrophysiologically that hypoxia increases the open probability of inactivation-resistant Na^+ channels and that this channel was blocked by tetrodotoxin.

An abnormal loss of myocardial K^+ has been shown to occur during ischemia and reperfusion (Hill and Gettes, 1979, Hirche et al., 1980). The elevated loss of potassium from myocardial cells results in a shortening of the action potential plateau phase (Carmeliet, 1978; Cole et al., 1991), which is considered to play a role in the ischemia and reperfusion injury (Weiss and Shine, 1981, 1982; Coronel et al., 1988). In contrast, opening of ATP-sensitive K^+ channels (K_{ATP} channels) plays an important role in attenuation of ischemia and reperfusion injury (Cole et al., 1991; Challiner-Rogers and McPharson, 1994). Thus, the exact role for the loss of K^+ in ischemic/reperfused hearts should be further clarified.

The above consequences of ionic movement, particularly myocardial cation disturbance during ischemia and/or an early period of reperfusion, may trigger irreversible cardiac dysfunction and eventually lead to cardiac cell death (Pierce and Czubryt, 1995). Thus, blockade of transmembrane flux of Na^+, K^+ and Ca^{2+} by pharmacological agents may explore a role of individual cation flux in ischemia and reperfusion injury. In the present study, we attempted to inhibit transmembrane flux of Na^+, K^+ and Ca^{2+} by well-established ion channel blockers during ischemia and reperfusion and to determine contribution of the blocking action to recovery of cardiac function during reperfusion. Potassium channel blockade was carried out by selective or relatively selective ion channel blockers, a K_{ATP} channel blocker glibenclamide (Sturgess et al., 1985), and an inward and delayed rectifier current blocker sematilide (Lumma, 1989; Argentieri et al., 1991). Calcium channel blockade was achieved with voltage-gated Ca^{2+} channel blockers diltiazem and nicardipine (Kuga et al., 1990). A selective fast Na^+ channel blocker tetrodotoxin (Kao, 1972) was also tested in ischemic/reperfused hearts for the purpose of support to our previous hypothesis that Na^+ channel blockade plays an important role in the protection of myocardium from ischemia and reperfusion injury (Takeo et al., 1995).

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 230–280 g, were used in the present study. The experimental protocol was designed according to the *Guide for the Care and Use of Laboratory Animals* as promulgated by the National Research Council (National Academy Press, Washington D.C., 1996) and approved by the University Committee of Animal Care and Welfare.

2.2. Perfusion of heart

Perfusion of the heart was carried out by the method described previously (Takeo et al., 1995). Briefly, rats were anesthetized with diethylether and the heart was excised. Isolated hearts were placed in a glass organ bath of the Langendorff apparatus and perfused at 37°C with a constant flow rate of 9.0 ml/min with Krebs-Henseleit solution of the following composition (in mM): NaCl 120, KCl 4.8, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11. The perfusion buffer was equilibrated with a gas mixture of 95% O_2 and 5% CO_2 ($pO_2 > 600$ mm Hg). A latex balloon, connected to a pressure transducer (model TP-200T, Nihonkohden, Tokyo), was inserted into the left ventricular cavity through the mitral valve opening and secured with a ligature that included the left atrial remnants. Hearts were preloaded with 5 mm Hg of left ventricular end-diastolic pressure by inflation of the balloon. Left ventricular developed pressure, a measure of cardiac contractile force, and left ventricular end-diastolic pressure were monitored by an electronic manometer and recorded on a thermal pen recorder (model WT-645G, Nihonkohden) throughout the experiment. Coronary perfusion pressure was monitored through a branch of the aortic cannula by means of an another electronic manometer (model TP-400T, Nihonkohden) connected to a carrier amplifier (model AP-621G, Nihonkohden). At a 15-min equilibration period, the heart was paced at 300 beats/min with an electronic stimulator (model SEN 3301, Nihonkohden) via bipolar silver electrodes attached to the atrium and left ventricle and then was equilibrated for another 15 min. After ensuring equilibration, the perfusion was stopped and the heart was submerged at 37°C into an organ bath that was filled with the Krebs-Henseleit solution described above, except for the replacement of 11 mM glucose with 11 mM Tris/HCl, to avoid hypothermia-induced cardioprotection. This solution had earlier been equilibrated with a gas mixture of 95% N_2 and 5% CO_2 ($pO_2 < 20$ mm Hg), pH 7.4, and maintained at 37°C. After 35 min of ischemia, the buffer in the organ bath was drained, and the heart was reperfused for 60 min at 37°C with normal Krebs-Henseleit solution equilibrated with a gas mixture of 95% O_2 and 5% CO_2. The heart was paced throughout the experiment, except for the first 20 min of reperfusion, to avoid contractile irregularities that often occur during this period. For the purpose of comparison, rat hearts were perfused for 95 min under normoxic conditions (normoxic group).

Treatment with different concentrations of agents was carried out by infusing their solutions into the perfusing

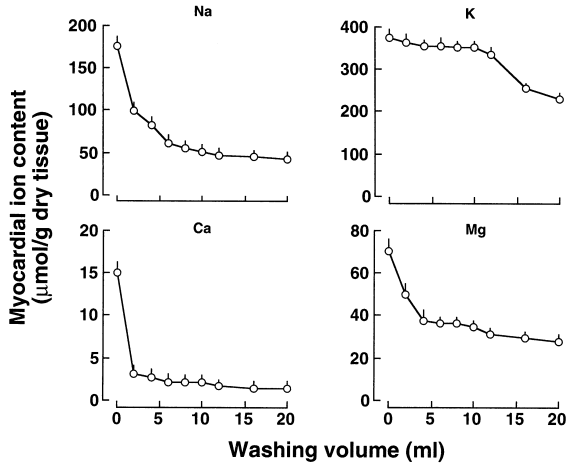

Fig. 1. Effects of different volumes of washing solution on myocardial cation content of hearts. The hearts were perfused for 30 min under normoxic conditions and then washed with different volumes (0 to 20 ml) of cold 320 mM sucrose–10 mM Tris/HCl, pH 7.4. Then, myocardial cation content was determined according to the atomic absorption method as described in Section 2. By washing with 12 ml of the buffer, an abrupt decline in K^+, Na^+ and Mg^{2+} was detected and by washing with 16 ml, Ca^{2+} content was decreased appreciably. Values represent the mean ± S.E.M. of four experiments.

buffer for the last 3 min of pre-ischemia. The agents were not included in the reperfusion buffer. Agents were dissolved in the Krebs-Henseleit solution and infused through the aortic cannula just anterior to the heart at a flow rate of 0.1 ml/min by an infusion pump (STC-523, Terumo, Tokyo, Japan). The concentrations of the agents were adjusted to deliver desired concentrations into the perfusing solution. The concentrations of K^+ channel blockers and Ca^{2+} channel blockers are reported to be enough to inhibit each channel as assessed by electrophysiological methods (Kao, 1972; Kuga et al., 1990; Argentieri et al., 1991; Tokube et al., 1996).

2.3. Measurement of creatine kinase activity in effluent

The perfusate eluted from the reperfused heart was collected to determine creatine kinase activity by the method of Bergmeyer et al. (1970) using a creatine kinase kit commercially available (CK-NAC, Boehringer Mannheim, Mannheim, Germany). The release of the enzyme was estimated as the total creatine kinase activity in the effluent.

2.4. Determination of myocardial ion content

Myocardial Na^+, K^+, Ca^{2+} and Mg^{2+} contents, were determined according to the method described previously (Tanonaka et al., 1996). Briefly, at the end of perfusion, an 8-ml of cold 320 mM sucrose–20 mM Tris/HCl, pH 7.4, was infused via the aortic cannula to eliminate ions from vascular and readily exchangeable spaces. Approximately 100 mg of the left ventricle was sampled for determination of ion content of the myocardium. The tissue was cut into pieces, weighed and dried at 120°C for 24 h. After estimation of the dry weight, the myocardium was digested at 180°C with 60% HNO_3, and then the mixture was evaporated to dryness at 180°C. The residue was reconstituted with 0.75 N HNO_3. The ion concentrations of the supernatant fluid were determined using an atomic absorption spectrometer (AA-680, Shimazu, Kyoto, Japan).

We performed two preliminary studies for measurement of tissue ion content. At first, we determined how much volume of the washing solution is practically required to obtain equilibrated values of myocardial ion content. Isolated rat hearts were perfused for 30 min with Krebs-Henseleit solution and then perfused with different volumes (0 to 20 ml) of cold 320 mM sucrose–20 mM Tris/HCl, pH 7.4 (washing solution). Myocardial ion content was determined according to the method described as above. The results shown in Fig. 1 indicate that 8–10 ml of washing was appropriate to obtain equilibrated values of tissue cation content of perfused rat hearts. Secondly, we examined how much ions of the extracellular space including vascular space can be washed out with 8-ml of the washing solution by the present procedure using the cobalt–EDTA method. Cobalt–EDTA is known to permeate into extracellular, but not intracellular, space (Sparrow and Johnstone, 1964; Kawada et al., 1992). Thus, perfusion of hearts with the buffer containing 1 mM Co^{2+} can estimate the extracellular space and thereafter extracellular and intracellular ion contents of the heart. Perfusion of the heart for 15–20 min with this solution resulted in an equilibrated value of Co^{2+} in the tissue (left panel in Fig. 2). Approximately 99% of readily exchangeable extracellular space was washed out by the 8-ml of the buffer (right panel in Fig. 2). Table 1 shows the intracellular ion contents of the heart estimated by the cobalt–EDTA method, those determined in the current method, and their ratios. There were no differences in the tissue ion content between these values, indicating that myocardial ion content determined in the present study consisted mostly of intracellular origin although values do not wholly represent intracellular free ion concentrations.

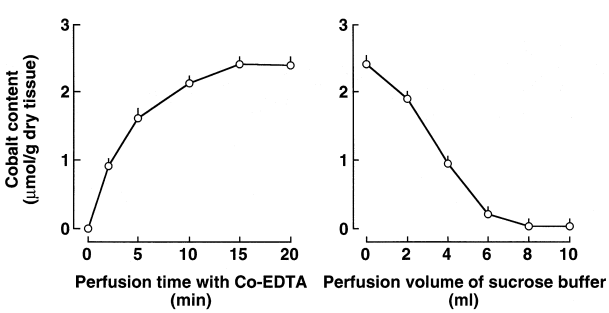

Fig. 2. The left panel shows the time course of changes in cobalt content of the heart perfused with the buffer containing 1 mM cobalt/EDTA. The right panel shows the effects of washing with different volumes (0 to 10 ml) of cold 320 mM sucrose–20 mM Tris/HCl, pH 7.4 on cobalt content of the heart preloaded with 1 mM cobalt–EDTA for 20 min. Values represent the mean ± S.E.M. of four experiments.

Table 1
Myocardial cation content determined by cobalt-EDTA method or after washing the extracellular space with 8 ml of 320 mM sucrose–20 mM Tris/HCl

	Na^+	K^+	Ca^{2+}	Mg^{2+}
(a) Washing with 8 ml buffer	55.52 ± 1.49	358.47 ± 3.52	1.77 ± 0.08	36.95 ± 0.64
(b) Estimation by cobalt–EDTA	57.05 ± 0.76	353.39 ± 2.91	1.83 ± 0.05	37.59 ± 0.84
Ratio of a/b (%)	97.3	101.4	96.7	98.3

Values (μmol/g dry tissue) represent the means ± S.E.M. of four experiments. Hearts were perfused with the Krebs-Henseleit solution for 20 min, and then washed with 8 ml of cold 320 mM sucrose–20 mM Tris/HCl, pH 7.4 (a). In contrast, hearts were perfused with the Krebs-Henseleit solution containing 1 mM cobalt–EDTA for 20 min, and then the tissue ion content was determined without washing (b). Myocardial ion content was determined by the atomic absorption method. Extracellular space necessary for determination of estimated ion content by the cobalt–EDTA method was calculated according to the method described by others (Sparrow and Johnstone, 1964; Kawada et al., 1992). There was no significant difference in cation contents between a and b groups as assessed by Student's *t*-test ($p > 0.05$).

2.5. Agents

The following agents were purchased; diltiazem (Sigma, St. Louis, MO), glibenclamide (Sigma), nicardipine (Sigma), and tetrodotoxin (Wako Pure Chemicals, Osaka). Sematilide was a kind gift from Japan Roussel (Tokyo).

2.6. Statistics

The results are expressed as the means ± S.E.M. Statistical significance was evaluated using one-way or two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's multiple comparison if necessary. Student's *t*-test was used when two groups were compared (Table 1). The relationship between two parameters was calculated by the least square method. Differences with a probability of 5% or less were considered to be significant ($p < 0.05$).

3. Results

3.1. Effects on cardiac performance

Baseline values for hemodynamic parameters were assessed after 30-min equilibration of perfused hearts. Base-

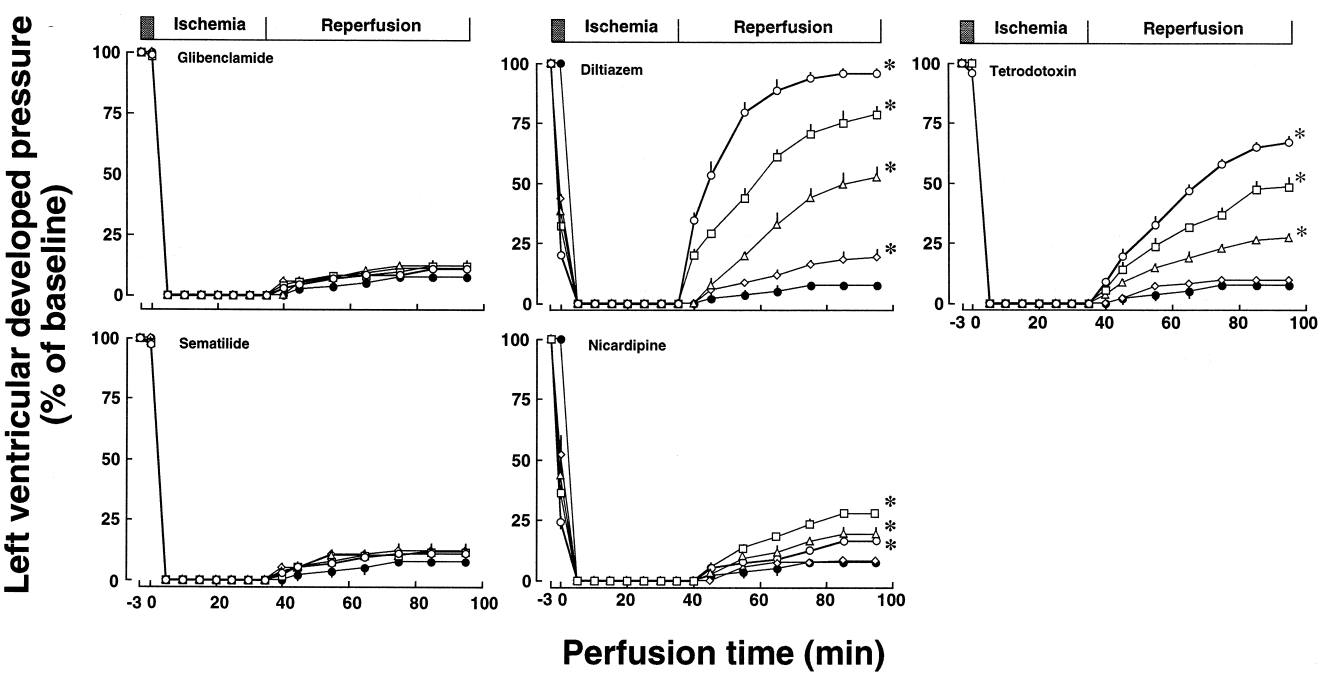

Fig. 3. The time course of changes in left ventricular developed pressure of the ischemic/reperfused heart untreated (●, $n = 6$) and pretreated with 1 (◇, $n = 4$), 3 (△, $n = 4$), 10 (□, $n = 5$) and 30 μM (○, $n = 5$) of glibenclamide (upper panel of the left column), 1 (◇, $n = 4$), 3 (△, $n = 4$), 10 (□, $n = 4$) and 30 μM (○, $n = 5$) of sematilide (lower panel of the left column), 0.1 (◇, $n = 4$), 0.3 (△, $n = 4$), 1 (□, $n = 6$) and 3 μM (○, $n = 6$) of diltiazem (upper panel of the middle column), 0.03 (◇, $n = 4$), 0.1 (△, $n = 4$), 0.3 (□, $n = 6$) and 1 μM (○, $n = 4$) of nicardipine (lower panel of the middle column) and 0.01 (◇, $n = 4$), 0.03 (△, $n = 4$), 0.1 (□, $n = 5$) and 0.3 μM (○, $n = 5$) of tetrodotoxin (upper panel of the right column). Each value represents the mean ± S.E.M. Standard errors of the symbols without any bar represent those within 3%. Statistical significance of the difference in left ventricular developed pressure between untreated and drug-treated hearts was calculated using only the values at the end of reperfusion by two-way ANOVA followed by Dunnett's *t*-test. * Significantly different from the untreated group ($p < 0.05$).

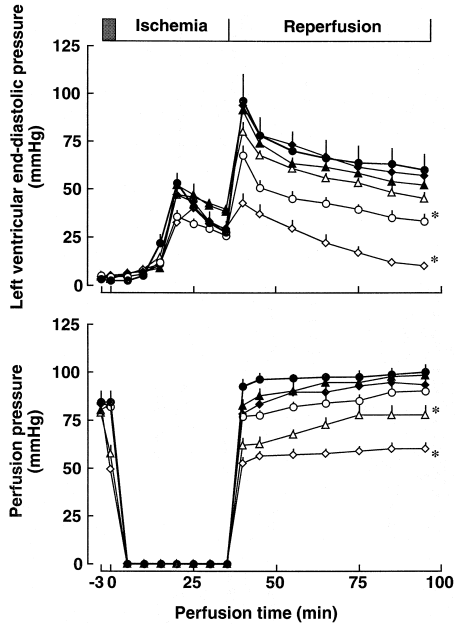

Fig. 4. The upper panel indicates the time course of changes in left ventricular end-diastolic pressure (upper panel) of the ischemic/reperfused heart untreated (●, $n = 6$) and pretreated with 10 μM glibenclamide (▲, $n = 5$), 30 μM sematilide (◆, $n = 5$), 3 μM diltiazem (◇, $n = 6$), 0,3 μM nicardipine (△, $n = 6$) and 0.3 μM tetrodotoxin (○, $n = 5$). The lower panel indicates the time course of changes in coronary perfusion pressure of the ischemic/reperfused heart untreated (●, $n = 6$) and pretreated with 10 μM glibenclamide (▲, $n = 5$), 30 μM sematilide (◆, $n = 5$), 3 μM diltiazem (◇, $n = 6$), 0,3 μM nicardipine (△, $n = 6$) and 0.3 μM tetrodotoxin (○, $n = 5$). Each value represents the mean ± S.E.M. Standard errors of the symbols without any bar are those within 3%. Statistical significance of the difference in left ventricular end-diastolic pressure between untreated and drug-treated hearts was calculated using only the values at the end of reperfusion by two-way ANOVA followed by Dunnett's *t*-test. * Significantly different from the untreated group ($p < 0.05$).

line values for left ventricular developed pressure ranged from 73 ± 2 to 81 ± 3 mm Hg ($n = 4$–6 for each group). Changes in left ventricular developed pressure are expressed as percents of the baseline values before the start of drug administration (Fig. 3). Glibenclamide and sematilide did not affect left ventricular developed pressure of pre-ischemic, normoxic hearts at all. In contrast, diltiazem and nicardipine decreased left ventricular developed pressure of pre-ischemic, normoxic heart at the end of pre-ischemia; 20 ± 1 (3 μM diltiazem) and 24 ± 1% (1 μM nicardipine) of each baseline value, respectively. Tetrodotoxin did not affect left ventricular developed pressure of pre-ischemic, normoxic hearts (Fig. 3). Ischemia reduced left ventricular developed pressure to zero, and reperfusion restored left ventricular developed pressure to less than 10% of baseline in controls. Neither glibenclamide nor sematilide (1 to 30 μM, respectively) enhanced the recovery of left ventricular developed pressure during reperfusion (Fig. 3). Combined treatment with 10 μM glibenclamide and 30 μM sematilide did not enhance the recovery of left ventricular developed pressure (12.7 ± 2.1%, $n = 5$). Diltiazem (0.1 to 3 μM) improved recovery of left ventricular developed pressure in a concentration-dependent manner (Fig. 3). Nicardipine (0.1 to 1 μM) had a similar action, although 1 μM was less effective than 0.3 μM. Diltiazem was much more effective than nicardipine, providing up to 100% recovery after 60 min of reperfusion versus only 25% with nicardipine. Tetrodotoxin (0.03 to 0.3 μM) also enhanced the recovery of left ventricular developed pressure in a concentration-dependent manner (Fig. 3), providing up to 65% recovery after 60 min of reperfusion.

Left ventricular end-diastolic pressure and coronary perfusion pressure were altered by the agents. For simplicity, only data for agents at their most effective dose for improving recovery of left ventricular developed pressure are shown (Fig. 4). In untreated hearts, left ventricular end-diastolic pressure was increased temporarily during ischemia, and markedly increased upon reperfusion. Diltiazem (3 μM) and tetrodotoxin (0.3 μM) suppressed the increase in left ventricular end-diastolic pressure during reperfusion. Reperfusion resulted in a rapid recovery of coronary perfusion pressure (Fig. 4). Diltiazem (3 μM) or nicardipine (0.3 μM) suppressed the rise in coronary perfusion pressure to an appreciable degree.

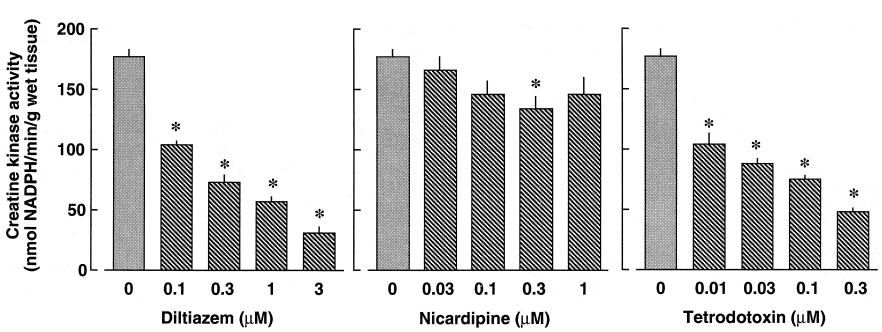

Fig. 5. Creatine kinase activities in the perfusate eluted from reperfused hearts. The hearts were untreated and pretreated with different concentrations of diltiazem (0.1, 0.3, 1 and 3 μM, $n = 4$, 4, 6 and 6, respectively), nicardipine (0.03, 0.1, 0.3 and 1 μM, $n = 4$, 4, 5 and 4, respectively) and tetrodotoxin (0.01, 0.03, 0.1 and 0.3 μM, $n = 4$, 4, 4 and 5, respectively). Each value represents the mean ± S.E.M. Statistical significance was calculated using the values for drug-treated and untreated groups by one-way ANOVA followed by Dunnett's *t*-test. * Significantly different from the untreated group ($p < 0.05$).

Table 2
Na^+, K^+, Ca^{2+} and Mg^{2+} contents at the end of ischemia in 35-min ischemic hearts untreated and pretreated with different concentrations of Na^+, K^+ and Ca^{2+} channel blockers

Groups	*n*	Na^+	K^+	Ca^{2+}	Mg^{2+}
Normoxia	6	55.75 ± 1.28	356.10 ± 3.98	2.01 ± 0.09	35.64 ± 0.47
Ischemia / reperfusion					
Untreated	6	116.37 ± 2.20	175.70 ± 2.61	2.15 ± 0.09	33.72 ± 0.09
Glibenclamide					
1 μM	4	114.61 ± 5.21	171.05 ± 7.97	2.23 ± 0.11	35.33 ± 0.78
3 μM	4	131.80 ± 10.27	190.08 ± 5.36[a]	2.30 ± 0.22	35.15 ± 0.44
10 μM	5	121.11 ± 5.50	228.54 ± 4.61[a]	2.28 ± 0.23	34.74 ± 0.95
30 μM	5	126.66 ± 4.13	234.66 ± 5.20[a]	2.28 ± 0.23	36.11 ± 1.00
Sematilide					
1 μM	5	122.08 ± 2.34	180.57 ± 6.42	2.12 ± 0.15	31.36 ± 1.31
3 μM	5	124.81 ± 6.83	217.37 ± 3.32[a]	2.12 ± 0.14	31.90 ± 0.68
10 μM	5	126.70 ± 5.59	252.69 ± 5.85[a]	2.05 ± 0.07	32.86 ± 1.95
30 μM	6	124.97 ± 1.27	290.09 ± 12.50[a]	2.00 ± 0.13	33.86 ± 1.56
Glibenclamide 10 μM + Sematilide 30 μM					
	5	116.51 ± 2.71	335.55 ± 4.60[a]	2.58 ± 0.10	33.18 ± 0.30
Diltiazem					
0.1 μM	4	103.24 ± 2.34[a]	182.77 ± 4.14	2.04 ± 0.09	32.94 ± 0.38
0.3 μM	4	88.08 ± 2.69[a]	206.45 ± 5.00[a]	2.21 ± 0.12	34.15 ± 0.65
1 μM	5	78.26 ± 1.43[a]	212.83 ± 6.26[a]	1.98 ± 0.07	33.45 ± 0.18
3 μM	6	70.81 ± 1.22[a]	206.94 ± 4.93[a]	2.11 ± 0.07	32.19 ± 0.54
Nicardipine					
0.03 μM	4	124.92 ± 3.62	175.92 ± 6.57	2.03 ± 0.08	34.61 ± 0.52
0.1 μM	4	126.85 ± 2.37	166.70 ± 2.88	2.03 ± 0.07	34.20 ± 0.90
0.3 μM	6	99.72 ± 2.48[a]	178.02 ± 2.75	2.10 ± 0.08	34.48 ± 0.59
1 μM	4	109.60 ± 4.06	175.35 ± 3.56	2.20 ± 0.04	33.51 ± 0.51
Tetrodotoxin					
0.01 μM	4	113.45 ± 2.11	167.34 ± 1.72	2.11 ± 0.05	33.54 ± 1.20
0.03 μM	4	102.90 ± 2.58[a]	169.23 ± 6.98	2.22 ± 0.07	34.57 ± 0.59
0.1 μM	4	87.65 ± 4.01[a]	189.02 ± 10.51	2.21 ± 0.07	35.16 ± 0.85
0.3 μM	5	81.08 ± 3.63[a]	160.92 ± 3.98	2.25 ± 0.07	35.48 ± 0.45

Values (μmol/g dry tissue) represent the means ± S.E.M. The hearts were subjected to 35 min of global ischemia, and their cation contents were determined according to the method described in the text. The hearts were treated with these agents for the last 3 min of pre-ischemia.
[a]Significantly different from the untreated, ischemic group as assessed by one-way ANOVA followed by Dunnett's *t*-test ($p < 0.05$).

3.2. Effects on creatine kinase release

Creatine kinase was released from normoxic hearts to a minimum degree (4.3 ± 1.0 nmol NADPH/min/g wet tissue, $n = 6$). In contrast, creatine kinase was released during reperfusion following ischemia (176.6 ± 6.3 nmol NADPH/min/g wet tissue, $n = 6$ controls). This was not altered by 1 to 30 μM glibenclamide or 1 to 30 μM sematilide (data not shown). Moreover, the combination of 10 μM glibenclamide and 30 μM sematilide also did not alter the release of creatine kinase (165.6 ± 2.7 nmol NADPH/min/g wet tissue, $n = 5$). In contrast, diltiazem and tetrodotoxin suppressed the release of creatine kinase in a concentration-dependent manner (Fig. 5). Nicardipine attenuated the release of creative kinase only at 0.3 μM to a minor degree (Fig. 5). The rank order of maximum effectiveness and the optimum concentration for effects of diltiazem, nicardipine and tetrodotoxin on creatine kinase release were the same as for actions on the recovery of left ventricular developed pressure.

3.3. Effects on myocardial ion content

Ischemia (35 min) induced a marked increase in myocardial Na^+ and a significant decrease in K^+, but it did not alter myocardial Ca^{2+} and Mg^{2+} (Table 2), compared to normoxic or pre-ischemic values (Tables 1 and 2). Glibenclamide (1 to 30 μM) and sematilide (1 to 30 μM) attenuated the decrease in myocardial K^+ of the ischemic heart in a concentration-dependent manner. The magnitude of the effect was greatest with sematilide. Combined treatment with 10 μM glibenclamide and 30 μM sematilide was even more effective (approximately 90% prevention of K^+ loss). In contrast, neither agent affected Na^+ Ca^{2+} or Mg^{2+} content. Diltiazem attenuated the ischemia-induced increase in Na^+ and decrease in K^+ in a concentration-dependent manner, but did not affect Ca^{2+}. Nicardipine suppressed the ischemia-induced increase in myocardial Na^+ to a minor degree, and only at 0.3 μM without affecting other ions (Table 2). Tetrodotoxin attenuated the

Table 3
Effects of treatment with various agents on Na^+, K^+, Ca^{2+} and Mg^{2+} contents of the pre-ischemic (normoxic) heart

Group	Na^+	K^+	Ca^{2+}	Mg^{2+}
Untreated	52.76 ± 2.03	358.15 ± 3.17	2.03 ± 0.07	35.63 ± 0.32
Glibenclamide	53.86 ± 2.45	363.35 ± 6.67	2.01 ± 0.10	37.28 ± 0.81
Sematilide	51.49 ± 1.43	352.97 ± 6.02	1.96 ± 0.11	35.47 ± 0.50
Glibenclamide + Sematilide	55.43 ± 2.20	361.19 ± 7.12	2.11 ± 0.05	36.57 ± 0.90
Diltiazem	52.39 ± 2.09	352.73 ± 3.19	1.96 ± 0.07	35.91 ± 0.49
Nicardipine	52.66 ± 1.22	357.75 ± 5.16	2.07 ± 0.10	36.07 ± 0.70
Tetrodotoxin	52.03 ± 2.23	355.46 ± 4.72	1.95 ± 0.07	35.39 ± 0.73

Values (μmol/g dry tissue) represent the means ± S.E.M. of four experiments. The pre-ischemic (normoxic) hearts were untreated and treated with either 10 μM glibenclamide, 30 μM sematilide, 3 μM diltiazem, 0.3 μM nicardipine or 0.3 μM tetrodotoxin for the last 3 min of pre-ischemia. The hearts were stopped at the end of 3-min administration of the agents, and myocardial ion content of the hearts was determined. There were no significant differences in ion contents between untreated and drug-treated groups as assessed by one-way ANOVA ($p > 0.05$).

ischemia-induced increase in myocardial Na^+ in a concentration-dependent manner without affecting other ions.

To confirm the lack of effect of the agents used on myocardial cation content of normoxic hearts, we examined the effects of the maximally effective concentrations of each agent administered during the last 3 min before ischemia on myocardial ion content. There were no significant effects with any drug (Table 3).

Reperfusion elevated myocardial Na^+ and Ca^{2+} and decreased K^+ and Mg^{2+} (Table 4), compared to normoxic or pre-ischemic values (Tables 1 and 4). Glibenclamide and sematilide did not affect these changes (Table 4). Diltiazem and tetrodotoxin suppressed changes in myocardial Na^+, K^+, Ca^{2+} and Mg^{2+} in a concentration-dependent manner, whereas nicardipine had slight but significant effects only at the higher concentrations studied (0.3 and 1 μM).

Table 4
Na^+, K^+, Ca^{2+} and Mg^{2+} contents at the end of reperfusion in hearts untreated and pretreated with different concentrations of Na^+, K^+ and Ca^{2+} channel blockers

Group	*n*	Na^+	K^+	Ca^{2+}	Mg^{2+}
Normoxia	6	55.75±1.28	356.1±4.0	2.01±0.09	35.64±0.47
Ischemia / reperfusion					
Untreated	6	144.28±2.88	97.50±3.28	11.70±0.41	18.48±0.54
Glibenclamide					
1 μM	4	135.58±3.12	98.34±7.04	13.42±0.62	19.46±0.84
3 μM	4	138.91±3.32	96.21±4.08	13.51±0.48	19.33±0.46
10 μM	5	137.78±2.55	99.22±3.48	13.41±0.29	19.44±0.59
30 μM	5	144.55±2.16	95.30±3.06	12.88±0.77	18.29±0.55
Sematilide					
1 μM	4	142.37±4.37	101.67±2.45	13.78±0.58	20.90±0.63
3 μM	4	141.89±4.46	100.04±3.61	13.59±0.68	19.84±1.51
10 μM	4	147.92±3.78	102.72±3.29	12.20±0.67	19.66±0.60
30 μM	5	140.61±0.51	104.16±5.74	11.61±0.51	19.43±0.51
Glibenclamide 10 μM + Sematilide 30 μM					
	5	138.69±4.66	99.32±5.22	13.58±0.49	18.49±0.77
Diltiazem					
0.1 μM	4	100.66±2.94[a]	150.21±5.74[a]	9.30±0.60[a]	22.64±0.96[a]
0.3 μM	4	86.20±2.27[a]	197.37±2.75[a]	6.77±0.47[a]	24.26±0.33[a]
1 μM	6	73.02±1.63[a]	245.84±4.37[a]	4.24±0.06[a]	28.31±0.56[a]
3 μM	6	68.37±1.35[a]	296.75±5.55[a]	3.23±0.14[a]	32.46±0.72[a]
Nicardipine					
0.03 μM	4	128.39±4.80	97.91±2.53	11.75±0.86	20.90±0.34
0.1 μM	4	127.50±3.02	105.57±6.32	11.52±0.52	21.66±0.52
0.3 μM	6	95.50±2.62[a]	129.59±10.27[a]	8.66±0.84[a]	22.84±0.92[a]
1 μM	4	118.26±5.21[a]	126.86±1.39[a]	6.61±0.25[a]	27.04±0.74[a]
Tetrodotoxin					
0.01 μM	4	139.41±3.63	115.74±3.89[a]	10.08±0.52	19.91±0.61
0.03 μM	4	97.54±2.48[a]	148.83±5.76[a]	7.53±0.29[a]	21.18±0.75[a]
0.1 μM	5	89.96±1.49[a]	197.84±9.06[a]	5.32±0.18[a]	25.46±0.82[a]
0.3 μM	5	78.52±2.19[a]	237.36±8.49[a]	3.69±0.23[a]	29.58±0.97[a]

Values (μmol/g dry tissue) represent the means±S.E.M. The hearts were subjected to 35 min of global ischemia followed by 60 min of reperfusion, and their ion contents were determined according to the method described in the text. The hearts were treated with these agents for the last 3 min of pre-ischemia. [a]Significantly different from the untreated group as assessed by one-way ANOVA followed by Dunnett's *t*-test ($p < 0.05$).

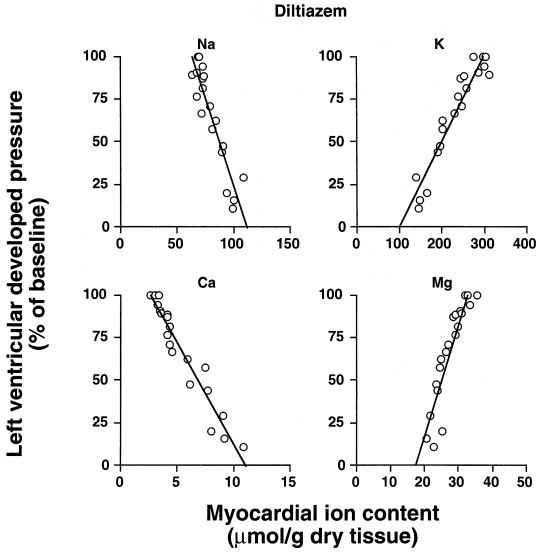

Fig. 6. The relationship between post-ischemic recovery of left ventricular developed pressure and myocardial Na^+, K^+, Ca^{2+} and Mg^{2+} of the reperfused heart treated with different concentrations (0.1 to 3 μM) of diltiazem. Significant relationships between the post-ischemic recovery of left ventricular developed pressure and myocardial Na^+, K^+, Ca^{2+} and Mg^{2+}, as assessed by the least square method, were seen ($r = -0.923$, 0.952, -0.964 and 0.910, $n = 20$; $p < 0.05$).

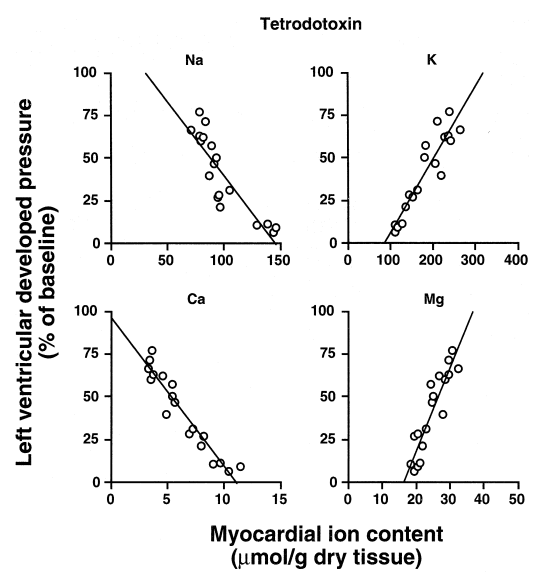

Fig. 7. The relationship between post-ischemic recovery of left ventricular developed pressure and myocardial Na^+, K^+, Ca^{2+} and Mg^{2+} of the reperfused heart treated with different concentrations (0.03 to 0.3 μM) of tetrodotoxin. Significant relationships between the post-ischemic recovery of left ventricular developed pressure and myocardial Na^+, K^+, Ca^{2+} and Mg^{2+} were seen ($r = -0.881$, 0.931, -0.955 and 0.907, $n = 18$; $p < 0.05$), as assessed by the least square method.

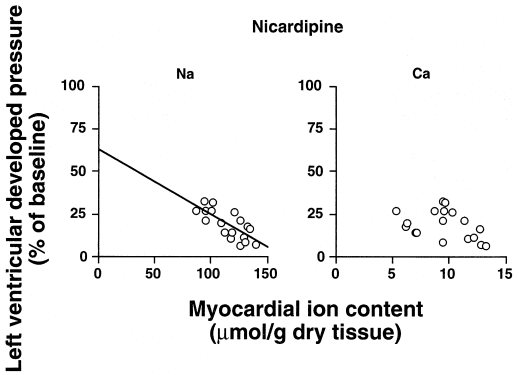

Fig. 8. The relationship between post-ischemic recovery of left ventricular developed pressure and myocardial Na^+ and Ca^{2+} of the reperfused heart treated with different concentrations (0.03 to 1 μM) of nicardipine. A significant relationship between the post-ischemic recovery of left ventricular developed pressure and myocardial Na^+ content was seen ($r = -0.748$, $n = 18$; $p < 0.05$). There was no significant relationship between post-ischemic recovery of left ventricular developed pressure and myocardial Ca^{2+} of the reperfused heart treated with different concentrations (0.03 to 1 μM) of nicardipine ($r = -0.379$) as assessed by the least square method.

3.4. Relationship between recovery of left ventricular developed pressure and myocardial ion content at the end of reperfusion at different concentrations of ion channel blockers

Recovery of left ventricular developed pressure at the end of reperfusion in each heart was plotted against myocardial Na^+, K^+, Ca^{2+} and Mg^{2+} at the same perfusion time (Figs. 6–8). None of the regressions were significant in glibenclamide- or sematilide-treated hearts; the regression coefficients in glibenclamide- and sematilide-treated groups were -0.185 and -0.415 for Na^+, 0.227 and 0.253 for K^+, 0.124 and -0.180 for Ca^{2+}, and 0.349 and -0.033 for Mg^{2+}, respectively ($p > 0.05$). In contrast, the recovery of left ventricular developed pressure was inversely related to myocardial Na^+ and Ca^{2+} and positively related to myocardial K^+ and Mg^{2+} in the diltiazem-treated hearts (Fig. 6) and tetrodotoxin-treated hearts (Fig. 7). The recovery of left ventricular developed pressure was related only to Na^+ in nicardipine-treated hearts (Fig. 8). In contrast, there were no significant relationships between recovery of post-ischemic left ventricular developed pressure and myocardial K^+, Ca^{2+} and Mg^{2+} ($r = -0.470$, -0.379 and 0.179, respectively, $n = 18$).

4. Discussion

The primary purpose of the present study was to relate the cardioprotective effects of drugs with well-established cellular targets to effects on cardiac cation content before and after reperfusion. Particularly, we focused on the role of inhibition of transmembrane flux of K^+ and Ca^{2+} during ischemia and reperfusion.

We determined myocardial ion content of ischemic and reperfused hearts with and without various ion channel blocker treatments by the atomic absorption method. This method provided myocardial cation content constituted mostly of intracellular origins, as assessed by the cobalt–EDTA method. Recently, determination of myocardial free cations, mainly Na^+, K^+ and Ca^{2+}, has been carried out by nuclear magnetic resonance (NMR) method (Murphy et al., 1991; Pike et al., 1993; Jelicks and Siri, 1995; Kupriyanov et al., 1995). The NMR method can measure real time course of minute changes in intracellular Na^+, K^+ or Ca^{2+} concentration, but cannot detect several cations in one preparation. Our method may not measure minute changes in intracellular free cations, but can measure relatively large changes in various intracellular cations in one preparation. Thus, the atomic absorption method may be relevant to determination of changes in several cation contents under pathophysiological conditions in which large changes in cation content can be induced. Interestingly, an increase in Na^+ at the end of ischemia determined by the NMR method was approximately twofold (Pike et al., 1993), similar to that obtained in our study. This suggests that qualitative change in myocardial cation content under pathophysiological conditions may be evaluated by the present method.

No changes in cardiac performance and myocardial cation content were detected in normoxic hearts by treatment with either sematilide or glibenclamide. Sematilide is believed to block K^+-delayed rectifier current (I_k) selectively (Argentieri et al., 1991), whereas several reports have shown the absence of I_k in rat hearts (Gwilt et al., 1986; Tande et al., 1990). Glibenclamide is known to inhibit K_{ATP} channel present in cell membranes (Sturgess et al., 1985) and in mitochondria (Inoue et al., 1991). Furthermore, the K_{ATP} channel can operate when cellular ATP is reduced (Noma, 1983). Thus, no change in myocardial K^+ under normal conditions (prior to drug-administration) in hearts with the K^+-channel blockers would be plausible. In contrast, K^+ was lost from the heart during ischemia and this loss was partially suppressed by glibenclamide and sematilide in a concentration-dependent manner, and almost completely blocked by the combined treatment with both agents. This inhibition was associated neither with suppression of ischemia-induced sodium accumulation nor with reperfusion-induced changes in any of the cations determined. These results suggest that the loss of K^+ may occur in ischemic, but not normoxic, hearts possibly through K_{ATP} channel and I_k channel and that only both K^+ channels can operate for the release of potassium in ischemic hearts. This indicates the difference in transmembrane flux of K^+ between normal and pathophysiological conditions. As described above, it is reported that I_k channels are absent in rat hearts (Gwilt et al., 1986; Tande et al., 1990). Therefore, the partial blockade of K^+

loss from ischemic hearts by sematilide is considered to occur by an unknown mechanism that the electrophysiological study in normoxic hearts has not elucidated yet. Despite appreciable blockade of transmembrane flux of K^+ during ischemia by glibenclamide, sematilide or a combination of the two, no enhancement of recovery of post-ischemic contractile function was seen in hearts treated with the K^+ channel blockers. This suggests that loss of K^+ induced during ischemia under the present experimental conditions is not causally related to irreversible cardiac function. In agreement with the ineffectiveness of K^+ channel blockers, several reports have shown that K^+ channel openers elicited anti-ischemic effects of hearts (Opie, 1993; Challiner-Rogers and McPharson, 1994; Cavero et al., 1995) and that mitochondrial K_{ATP} channel opener enhanced the recovery of post-ischemic contractile function (Garlid et al., 1997).

In contrast to K^+ channel modulators, Ca^{2+} channel blockers elicited diverse effects on the recovery of post-ischemic contractile function. That is, diltiazem enhanced recovery of the post-ischemic left ventricular developed pressure, attenuated changes in myocardial Na^+, K^+, Ca^{2+} and Mg^{2+} during ischemia and reperfusion and suppressed reperfusion-induced release of creatine kinase, a typical marker of cardiac cell necrosis (Vatner et al., 1978; Ganote and Kaltenbach, 1979). On the other hand, nicardipine improved, to a small degree, recovery of the post-ischemic left ventricular developed pressure and creatine kinase release and ionic imbalance of ischemic/reperfused hearts. It is well established that blockade of calcium influx during ischemia as well as reperfusion elicited potent cardioprotective effects against ischemia/reperfusion injury. Thus, the difference in the effectiveness between diltiazem and nicardipine must be a matter of debate. It is generally recognized that diltiazem is much more potent than nicardipine with respect to their cardioprotective effects. The differences in properties between 1,4-dihydropyridine derivatives and diltiazem have been documented (Ferrari et al., 1995). Dihydropyridine derivatives gain access to the external binding site of Ca^{2+} channel and the drugs with low doses act preferentially on vasculature. In contrast, diltiazem gains access to the internal site of Ca^{2+} channel lumen only when the channel is open and acts on both vascular and cardiac muscles. It is reported that Ki value for nicardipine is extremely low as compared with that of diltiazem (Ishii and Toyama, 1993) and that diltiazem is easily washed out from tissues (Nishimura et al., 1990). Although it is unknown whether nicardipine is easily washed out from the myocardium, the above observations suggest that nicardipine is liable to bind cardiac tissue during reperfusion to a greater degree than diltiazem. Such strong binding properties of nicardipine might delay recovery of cardiac function during reperfusion. These observations may account for the smaller protection of nicardipine against ischemia/reperfusion injury as compared with that of diltiazem.

To examine a role for Na^+ channel blockade in the protection of the myocardium against ischemia and reperfusion injury, a typical and selective Na^+ channel blocker tetrodotoxin (Kao, 1972) was applied to the ischemic/reperfused hearts. Although tetrodotoxin has recently been shown to enhance recovery of post-ischemic contractile function and to attenuate release of creatine kinase and lactate dehydrogenase in isolated guinea pig hearts (Le Grand et al., 1995), ionic profiles of the ischemic as well as reperfused hearts have not as yet been elucidated. We found that tetrodotoxin exerted concentration-dependent effects, and improved ischemia/reperfusion-induced contractile dysfunction and myocardial ion disturbance, thus supporting a key role of Na^+ channel blockade in the reduction of ischemia/reperfusion injury. The findings are in good agreement with our previous observations that class I type antiarrhythmic agents that have Na^+ channel blocking action, enhanced recovery of post-ischemic contractile function associated with suppression in sodium overload and calcium overload during reperfusion (Takeo et al., 1995). This improvement is probably attributable to the mechanism by which calcium overload following sodium overload in the myocardium is prevented (Lazdunski et al., 1982; Tani and Neely, 1989; Meng et al., 1991; Murphy et al., 1991).

At the end of ischemia, myocardial Na^+ was increased and K^+ was decreased, but no changes in myocardial Ca^{2+} and Mg^{2+} were seen. This suggests that non-specific transmembrane flux of ions does not occur or occurs to a minimum degree, rather a massive transmembrane flux of Na^+ and K^+ is enhanced during ischemia. Several pathways through which Na^+ accumulates in cardiac cells are possible, such as Na^+/H^+ exchanger, Na^+/K^+ pump, Na^+/Ca^{2+} exchanger, voltage-gated Na^+ channel and non-specific flux of ions across cardiac cell membrane under pathophysiological conditions. In ischemic cardiac cells, Na^+/H^+ exchange would be accelerated by increased production of intracellular H^+ (Poole-Wilson, 1978) whereas Na^+/K^+ pump is inhibited due to depletion of available myocardial energy store (Daly et al., 1984). Recently, an increasing evidence has emerged that inactivation-resistant Na^+ channels, although fast Na^+ channel is assumed to be closed during ischemia as a result of prolonged membrane depolarization, play a major role in sodium influx during ischemia (Silverman and Stern, 1994; Ju et al., 1996). That is, Ju et al. (1996) suggested that the gating behavior of the Na^+ channel dramatically changed during hypoxia, thereby generating a 'persistent Na^+-window current'. Thus, drug effects on such a 'dysfunctional' Na^+ channel may be entirely different from its effects on the physiological fast Na^+-current. This Na^+ channel is blocked by tetrodotoxin and lidocaine (Ju et al., 1996) and is activated by ischemic metabolites such as lysophospholipids (Undrovinas et al., 1992) and long chain acylcarnitine (Wu and Corr, 1995). Our findings are in good agreement with these observations, supporting the

hypothesis of major contribution of inactivation-resistant Na^+ channels to ischemia-induced increase in sodium influx.

Enhanced post-ischemic contractile recovery was seen in hearts treated with diltiazem and tetrodotoxin, or to some extent in hearts treated with nicardipine, but not in hearts treated with the K^+ channel blockers. Taken together, the critical observation that we can detect in this series of experiments is whether ion channel blockers can inhibit the excessive influx of Na^+ during ischemia: inhibition of Na^+ accumulation in the myocardium by ion channel blockers is always accompanied by a better restoration of post-ischemic contractile function. This was further supported by the close relationship between recovery of post-ischemic left ventricular developed pressure and myocardial Na^+ in diltiazem-, nicardipine- and tetrodotoxin-treated hearts (Figs. 6–8). The results support the significance of blockade of sodium overload during ischemia by pharmacological agents, which was hypothesized by us (Takeo et al., 1995) as well as others (Tani and Neely, 1989; Meng et al., 1991; Pierce and Czubryt, 1995).

Ischemia and reperfusion resulted in a marked release of creatine kinase from the heart, indicating a significant disruption of cardiac cell membrane integrity. Thus, nonspecific leakage of intracellular ions must be considered. This membrane disruption must have occurred during reperfusion because myocardial cations were altered in a selective manner at the end of ischemia. Thus, disruption of cardiac cell membrane integrity during reperfusion may be an associated event, rather than a trigger for the genesis of ischemia and reperfusion injury.

As described above, we suggested that suppression of sodium overload during ischemia may play an important role in the prevention of ischemia and reperfusion injury in diltiazem-treated hearts. If sodium overload during ischemia is a primary initiator of ischemia and reperfusion injury, then diltiazem should have the ability to block Na^+ channels. In accord with this, in vitro electrophysiological studies have shown that diltiazem inhibits fast Na^+ channels at micromolar range of the agent (Ando, 1990; Guc et al., 1993). Furthermore, as described above, the gating behavior of the Na^+ channel dramatically changed during hypoxia, thereby generating inactivation-resistant Na^+-current (Ju et al., 1996). Thus, the effects of diltiazem on Na^+ channel could be expected under ischemic conditions. It is generally admitted that 1,4-dihydropyridine derivatives have been shown not to affect Na^+ channels even at high concentrations (Kimura et al., 1982; Jurevicius et al., 1993). In contrast, Hano et al. (1991) speculated that nicardipine, but not nifedipine, despite no effects under normoxic conditions, is capable of interfering with Na^+ channels under hypoxic conditions. This speculation might account for the smaller recovery of cardiac contraction by 0.3 μM nicardipine. Taken together, Na^+ channel blockade of calcium antagonists during ischemia appears to be related to cardioprotection in rat hearts. This hypothesis is consistent with the claim of other investigators that Ca^{2+} channel blockade does not result in cardioprotection, rather blockade of Na^+ channel may play an important role in cardioprotection (Ver Donck et al., 1993; Silverman and Stern, 1994).

Several agents that can exert negative inotropic effects before or during ischemia such as β-adrenoceptor and Ca^{2+} channel antagonists are capable of restoring contractile function during reperfusion. This is considered to be due to reduction of energy expenditure or energy sparing effects during ischemia. Apparently, our findings of the improvement of post-ischemic function of diltiazem- and nicardipine-treated hearts cannot rule out this possibility.

In conclusion, our results suggest that K^+ channel blockade contributes to the protection against ischemia and reperfusion-induced contractile dysfunction to a minimum degree, and rather attenuation of sodium overload during ischemia by ion channel blockers plays a critical role in the prevention of ischemia and reperfusion-induced contractile dysfunction.

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