

## Cardioprotective Drugs Decrease the $\text{Na}^+$ Background Current

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Cardiac dysfunctions such as myocardial functional failure and ventricular arrhythmia have been largely attributed to intracellular  $\text{Ca}^{2+}$  overload. One of the mechanisms of intracellular  $\text{Ca}^{2+}$  overload involves a rapid influx of  $\text{Ca}^{2+}$  via  $\text{Na}^+-\text{Ca}^{2+}$  exchange during the reperfusion which utilizes the accumulation of  $\text{Na}^+$  in myocytes during ischemic cardiac arrest. Possible sources of the intracellular  $\text{Na}^+$  accumulation include  $\text{Na}^+$  channel,  $\text{Na}^+-\text{H}^+$  exchange,  $\text{Na}^+-\text{Ca}^{2+}$  exchange, and  $\text{Na}^+$  background current. In this study, we studied the role of the  $\text{Na}^+$  background current in intracellular  $\text{Na}^+$  accumulation during the cardiac arrest by measuring the  $\text{Na}^+$  background current in guinea pig ventricular myocytes with whole cell clamp method and evaluating the effects of cardioprotective drugs on the  $\text{Na}^+$  background current. The results were as follows: ① The  $\text{Na}^+$  background inward current at  $-40\text{ mV}$  membrane potential was larger at  $\text{Ca}^{2+}$  free solution than  $1.8\text{ mM}$   $\text{Ca}^{2+}$  solution. ② The  $\text{Na}^+$  background current was not affected by verapamil. ③  $2\text{ }\mu\text{M}$   $\text{O}(\text{N}, \text{N-hexamethylene})\text{-amiloride}$  (HMA) decreased the  $\text{Na}^+$  background current at negative membrane potential. ④ The new cardioprotective drug, R 56865, decreased the  $\text{Na}^+$  background current. These results suggest that the  $\text{Na}^+$  background current plays a role in increasing the intracellular  $\text{Na}^+$  activity during high  $\text{K}^+$  cardioplegia and the blocking effect of myoprotective drugs, such as R 56865, on the  $\text{Na}^+$  background current may contribute to myocardial protection after cardioplegia.

**Key Words:**  $\text{Na}^+$  background current, myocardial protection, amiloride, R 56865

Myocardial functional failure and ventricular arrhythmias can occur after high  $\text{K}^+$  cardioplegia and ischemia. These kinds of cardiac dysfunction have been called reperfusion injury. One of the hypotheses explaining the cause of reperfusion injury is the calcium paradox (Singal *et al.* 1986; Chapman and Tuns-

tall, 1987; Makino *et al.* 1988). The importance of the role of  $\text{Ca}^{2+}$  in the cardiac pathophysiology has been recognized and the effects of various cardioprotective drugs such as  $\text{Ca}^{2+}$  antagonists have been investigated (Farber, 1982; Baldermann *et al.* 1984; Hearse *et al.* 1984). But the series of cellular events leading to  $\text{Ca}^{2+}$  overload has yet to be elucidated.

Recently, a mechanism of the calcium paradox appears to involve a rapid influx of  $\text{Ca}^{2+}$  by  $\text{Na}^+-\text{Ca}^{2+}$  exchange during the reperfusion (Opie and DPhill, 1989; Rodrigo and Chapman, 1991; Jennings and Yellon, 1992). By this reason, the accumulation of  $\text{Na}^+$  in myocytes during ischemic cardiac arrest is postulated to be one of the main causes of the calcium paradox (Rodrigo and Chapman, 1991; Jennings and Yellon, 1992). The factors increasing the

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intracellular Na<sup>+</sup> activity include Na<sup>+</sup> current, Na<sup>+</sup>-H<sup>+</sup> exchange current, Na<sup>+</sup>-Ca<sup>2+</sup> exchange, and the Na<sup>+</sup> leak (Mullins, 1981; Gadsby *et al.* 1985; Kalia and Vaughan-Jones, 1987).

There are many possible procedures to lessen reperfusion injury. Since the intracellular Na<sup>+</sup> activity may be critically involved in the calcium paradox on reperfusion, preventions of the intracellular Na<sup>+</sup> accumulation could have cardioprotective effects. The reduction of the extracellular Na<sup>+</sup> concentration in cardioplegic solution can prevent early development of cellular edema and rapid influx of Ca<sup>2+</sup> during the reperfusion (Stinner *et al.* 1989; Kim *et al.* 1993). Amiloride is known to have a protective effect on ischemia-reperfusion injury by blocking Na<sup>+</sup>-H<sup>+</sup> exchange (Dennis *et al.* 1990; Meng and Pierce, 1991).

The new cardioprotective drug, R 56865 (N-[1-[4-(4-fluorophenoxy)butyl]-4-piperidiny]-N-methyl-2-benzothiazolamine), antagonizes the damaging effects of ischemia-reperfusion injury, including arrhythmia and mechanical deterioration. But until now, the exact site of the action of R 56865 has remained obscure. R 56865 has been known to inhibit the influx of Na<sup>+</sup> and prevent the Ca<sup>2+</sup> overload resulting from Na<sup>+</sup> overload (Luk and Carmeliet, 1990; Leyssens and Carmeliet, 1991; Verdonck *et al.* 1991; Borgers *et al.* 1992).

The Na<sup>+</sup> leak during cardioplegia or ischemia has been considered as one of the factors of intracellular Na<sup>+</sup> accumulation (Stinner *et al.* 1989; Rodrigo and Chapman, 1991). But the electrophysiological verification of Na<sup>+</sup> leak had been difficult until the group of Hagiwara measured the Na<sup>+</sup> background current in sinoatrial node cells in a rabbit heart (Hagiwara *et al.* 1992). In the present study, we examined the Na<sup>+</sup> leak by the measurement of Na<sup>+</sup> background current and evaluated the role of the Na<sup>+</sup> background current in intracellular Na<sup>+</sup> accumulation during K<sup>+</sup> cardioplegia.

The purpose of the present study was to examine ① the contribution of the Na<sup>+</sup> background current to the intracellular Na<sup>+</sup> accumulation during cardioplegia by measuring the Na<sup>+</sup> background current by whole cell clamp method and ② the effects of cardioprotective drugs, known to prevent the increase of intra-

cellular Na<sup>+</sup> activity on the Na<sup>+</sup> background current.

## METHODS

### Cell isolation

Guinea pigs were killed by intraperitoneal injection of sodium pentobarbital (50 mg/Kg) and heparin (2,000 IU/Kg). The heart was rapidly excised and placed in oxygenated (100% O<sub>2</sub>) Tyrode solution. Guinea pig hearts were perfused according to the Langendorff perfusion method with a normal Tyrode solution, Ca<sup>2+</sup> free solution for 5 min, and a solution containing 0.04% collagenase (Type I, Worthington) and 0.04% hyaluronidase (Sigma) for 20 min successively. After washout of the enzyme solution with a high K<sup>+</sup> and low Cl<sup>-</sup> solution (KB solution: taurine, 10; oxalic acid, 10; glutamic acid, 70; KCl, 25; KH<sub>2</sub>PO<sub>4</sub>, 10; glucose, 11; EGTA, 0.5; HEPES, 10 mM, pH 7.3~7.4), the ventricle was cut into small pieces, and the cells were released into the medium by gentle mechanical agitation. Myocytes were stored in KB solution at room temperature before use.

### Electrical recording

The whole cell membrane currents of guinea pig ventricular myocytes were recorded by a patch clamp method which was similar to that described by Hamill *et al.* (1981). Glass electrodes with a resistance of 2~3 MΩ were used. Current-voltage (I-V) curves were constructed from voltage clamp tracings by slowly ramping the voltage from +40 to -100 mV with dV/dt of 1V/s using a holding potential of -40 mV (Hagiwara *et al.* 1992). Axopatch 1C amplifier (Axon Ins.) was employed to record both the membrane current and potential. Voltage clamp and data analysis were performed using pCLAMP software (Axon Ins.). The membrane capacitance was determined from the whole cell capacitance compensation.

The voltage ramping was applied with and without 140 mM NaCl in the bath solution (see Table 1) and the Na<sup>+</sup> background current was defined as the amount of the membrane

current with 140 mM NaCl minus the one with 140 mM NMG Na<sup>+</sup> free solution.

### Solutions

The composition of the external solutions was as follows. The normal Tyrode solution contained (mM) NaCl, 140; KCl, 4.0; CaCl<sub>2</sub>, 1.8; MgCl<sub>2</sub>, 1.0; NaH<sub>2</sub>PO<sub>4</sub>, 1.0; Glucose, 5.5; *N*-2-Hydroxy-ethylpiperazine-*N'*-ethanesulphonic acid (HEPES) 5 (pH = 7.4 with NaOH). The 140 mM NaCl and 140 mM NMG (N-methyl-D-glucamine) Na<sup>+</sup> free solutions had the composition listed in Table 1. BaCl<sub>2</sub> 1 mM was added to block the delayed rectifier K<sup>+</sup> channel ( $I_K$ ); NiCl<sub>2</sub> 4 mM to block the Na<sup>+</sup>-Ca<sup>2+</sup> exchange current as well as the Ca<sup>2+</sup> current ( $I_{Ca}$ ); and 5  $\mu$ M strophanthidin (Sigma) to block the Na<sup>+</sup>-K<sup>+</sup> ATPase activity. The composition of the internal pipette solution is listed in Table 1. In several experiments, 2  $\mu$ M verapamil (Sigma) was used to block Ca<sup>2+</sup> channels. To block Na<sup>+</sup> channel and Cl<sup>-</sup> channel separately, 10  $\mu$ M tetrodotoxin (TTX) and 4,4'-dinitrostilbene-2,2'-disulphonic acid disodium salt (DNDS; Tokyo Kasei Kogyo) were used respectively. To study the effect of cardioprotective drugs on the Na<sup>+</sup> background current, 10  $\mu$ M 0-(*N,N*-hexamethylene)-amiloride (HMA, Sigma) and R 56865 (donated by Janssen Research Foundation, Belgium) were used.

## RESULTS

### Measurement of the Na<sup>+</sup> background current

The Na<sup>+</sup>-dependent background current was measured as previously described by Hagiwara *et al.* (1992). Fig. 1 shows the Na<sup>+</sup> background currents recorded with 140 mM Na<sup>+</sup> and 140 mM NMG Na<sup>+</sup> free external solutions. It was remaining membrane current after all the known time-dependent currents were blocked. To test whether or not the measured Na<sup>+</sup> background current contains the Na<sup>+</sup> influx through the fast Na<sup>+</sup> channel, we added 10  $\mu$ M TTX to the 140 mM Na<sup>+</sup> solution and Na<sup>+</sup> free solution. There were no significant effects on the I-V relation of the current (Fig. 2).

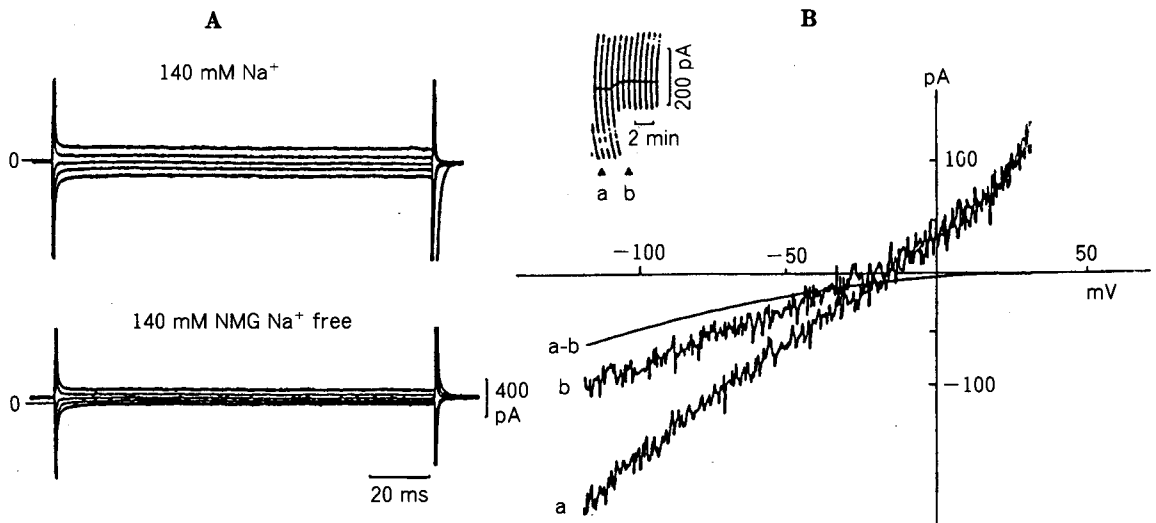
The addition of a Cl<sup>-</sup> channel blocker (DNDS) failed to affect the I-V relation of the Na<sup>+</sup> background current (Fig. 3). This result indicates that the Cl<sup>-</sup> current does not contribute to the Na<sup>+</sup> background current.

### Contribution of the Na<sup>+</sup> background current during high K<sup>+</sup> cardioplegia

In previous studies, we measured the membrane potential during perfusion with 25 mM K<sup>+</sup> cardioplegic solution in papillary muscle of guinea pig (Kim *et al.* 1993). The averaged depolarized membrane potential was about -40

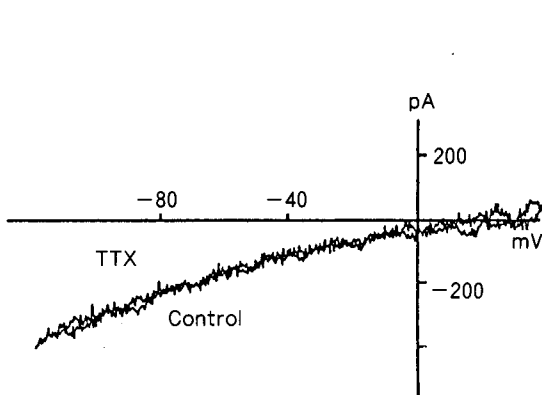
Table 1. Composition of experimental solutions (in mM)

External solutions								
	NaCl	NMGCl	MgCl <sub>2</sub>	HEPES	NiCl <sub>2</sub>	BaCl <sub>2</sub>	Strophanthidin	
140 mM NaCl	140	—	1	5	4	1	0.005	
140 mM NMG	—	140	1	5	4	1	0.005	
pH 7.4 with NaOH or NMGCl								
Internal solution								
CsOH	CsCl	TEA-Cl	EGTA	HEPES	MgCl <sub>2</sub>	Na <sub>2</sub> -creatinine phosphate	TrisATP	Aspartate
120	20	20	10	5	2	5	5	80
pH 7.4 with CsOH								



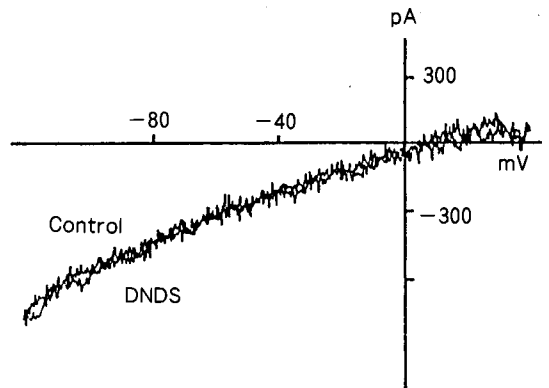
**Fig. 1.** Measurement of the  $\text{Na}^+$  background current.

A. Traces of current elicited by changing clamp pulses from a holding potential of  $-40$  mV to  $10$ ,  $-20$ ,  $-50$ ,  $-80$  and  $-110$  mV, respectively, in  $140$  mM  $\text{Na}^+$  solution and  $140$  mM NMG  $\text{Na}^+$  free solution. The bath solutions contained  $\text{Ni}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cs}^+$  and strophanthidin. Note that the inward current increased in response to  $140$  mM  $\text{Na}^+$  solution and the current showed no time dependence as reported by Hagiwara *et al.* (1992). B. I-V relations recorded by the ramp clamp method. Trace a: I-V curve obtained in  $140$  mM  $\text{Na}^+$  solution, trace b: I-V curve in  $140$  mM NMG  $\text{Na}^+$  free solution, a-b: difference curve of the two I-V curves.



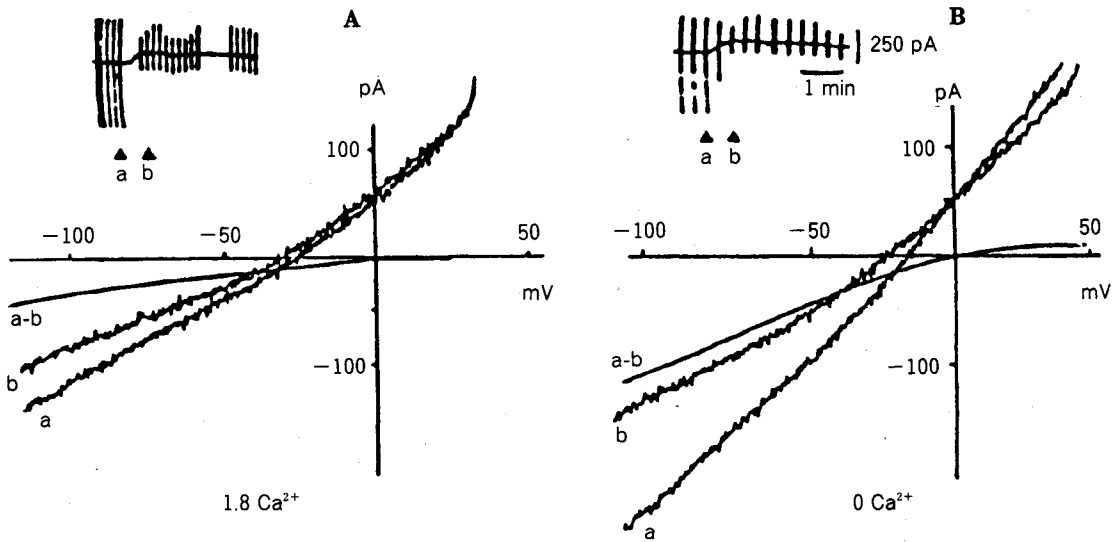
**Fig 2.** Effect of tetrodotoxin on the  $\text{Na}^+$  background current.

The  $\text{Na}^+$  background current was  $-0.84$  pA/pF at  $-40$  mV. In the presence of  $10$   $\mu\text{M}$  tetrodotoxin (TTX), there was no significant change in the I-V relation. The possibility of the contribution of fast  $\text{Na}^+$  channels to this background current could be excluded.



**Fig 3.** Effect of  $\text{Cl}^-$  channel blocker on the  $\text{Na}^+$  background current.

The addition of  $1$  mM DNDS (4,4-dinitro-2,2-disulphonic acid disodium salt) did not affect the  $\text{Na}^+$  background current.



**Fig 4.** Effects of external  $\text{Ca}^{2+}$  concentration on the  $\text{Na}^{+}$  background current

A. illustrates I-V relations in the 140 mM  $\text{Na}^{+}$  solution (a) and 140 mM NMG  $\text{Na}^{+}$  free solution (b) at 1.8 mM  $\text{Ca}^{2+}$  external solution. The difference curve a-b gives the  $\text{Na}^{+}$  dependent background current. B. illustrates I-V relations in the 140 mM  $\text{Na}^{+}$  solution (a) and 140 mM NMG  $\text{Na}^{+}$  free solution (b) at  $\text{Ca}^{2+}$  free external solution. The difference curve a-b gives the  $\text{Na}^{+}$  background current. In  $\text{Ca}^{2+}$  free solution, the  $\text{Na}^{+}$  background current at  $-40$  mV increased from  $-0.43$  pA/pF to  $-0.71$  pA/pF.

mV. The current density of the  $\text{Na}^{+}$ -dependent inward current obtained by subtracting the current in NMG  $\text{Na}^{+}$  free solution from that in 140 mM  $\text{Na}^{+}$  solution was  $0.31 \pm 0.08$  pA/pF ( $n=8$ ) at  $-40$  mV.

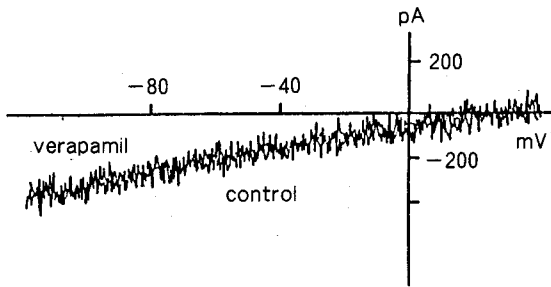
To study the effect of external  $\text{Ca}^{2+}$  concentration on the  $\text{Na}^{+}$  background current, the  $\text{Ca}^{2+}$  concentration of external perfusion solution was changed (Fig. 4). The  $\text{Na}^{+}$  background currents at  $-40$  mV membrane potential were  $-0.43$  pA/pF in 1.8 mM  $\text{Ca}^{2+}$  solution and  $-0.71$  pA/pF in  $\text{Ca}^{2+}$  free solution. This result suggests that the role of  $\text{Na}^{+}$  background current in the intracellular  $\text{Na}^{+}$  accumulation is increased in the  $\text{Ca}^{2+}$  free cardioplegic solution.

#### Effects of cardioprotective drugs on the $\text{Na}^{+}$ background current

To investigate the effects of cardioprotective drugs on the  $\text{Na}^{+}$  background current, we added cardioprotective drugs to the 140 mM  $\text{Na}^{+}$  solution and  $\text{Na}^{+}$  free solution. Calcium

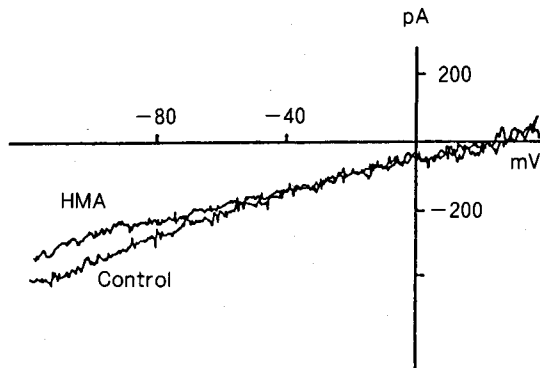
channel blockers have been used in cardioplegic solution to improve the recovery of function in myocardium (Baldermann *et al.* 1984; Tyres, 1988). However, it is unclear whether these beneficial effects reflected a direct protective action, or whether they were mediated by indirect action (Przyklenk and Kloner, 1988). To test whether the increased  $\text{Na}^{+}$  background current during  $\text{Ca}^{2+}$  free solution was due to the increased  $\text{Na}^{+}$  permeability through the  $\text{Ca}^{2+}$  channel,  $2 \mu\text{M}$  verapamil was added. There was no significant effect on the I-V relation. The possibility of the leak of  $\text{Na}^{+}$  through  $\text{Ca}^{2+}$  channel during perfusion with  $\text{Ca}^{2+}$  free solution could be excluded (Fig. 5).

We examined whether or not amiloride influence the  $\text{Na}^{+}$  background current (Fig. 6). The  $\text{Na}^{+}$  background current was  $-0.84$  pA/pF at  $-40$  mV.  $10 \mu\text{M}$  HMA depressed the  $\text{Na}^{+}$  background current at negative membrane potential, but there was no significant effect at  $-40$  mV.



**Fig. 5.** Effect of verapamil on the Na<sup>+</sup> background current.

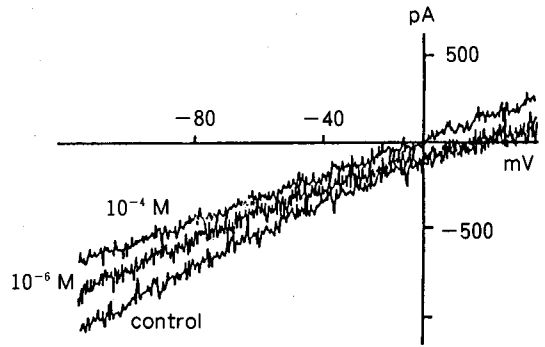
The Na<sup>+</sup> background current was  $-0.80$  pA/pF at  $-40$  mV. In the presence of  $2 \mu\text{M}$  verapamil, there was no significant effect on the I-V relation. The possibility of the leak of Na<sup>+</sup> through Ca<sup>2+</sup> channel during Ca<sup>2+</sup> free solution could be excluded.



**Fig. 6.** Effect of amiloride on the Na<sup>+</sup> background current.

The Na<sup>+</sup> background current was  $0.84$  pA/pF at  $-40$  mV. The  $10 \mu\text{M}$  HMA (O-(N,N-hexamethylene)-amiloride) depressed the Na<sup>+</sup> background current at negative membrane potential, but there was no significant effect at  $-40$  mV membrane potential.

R 56865 is a new cardioprotective drug but controversy exists on the role of intracellular Na<sup>+</sup> in protective effect. We investigated the



**Fig. 7.** Effect of R 56865 on the Na<sup>+</sup> background current.

The Na<sup>+</sup> background current at  $-40$  mV was  $385$  pA. R 56865 reduced the Na<sup>+</sup> background current at negative membrane potential. At  $-40$  mV,  $10^{-6}$  M and  $10^{-4}$  M R 56865 decreased Na<sup>+</sup> background currents respectively to  $288$  pA (75% control) and  $231$  pA (60% control).

effects of R 56865 on the Na<sup>+</sup> background current to elucidate its mechanism underlying the prevention of Na<sup>+</sup> loading (Fig. 7). The Na<sup>+</sup> background current at  $-40$  mV was  $385$  pA. R 56865 reduced the Na<sup>+</sup> background current at negative membrane potential. At  $-40$  mV,  $10^{-6}$  M R 56865 decreased the Na<sup>+</sup> background current to  $288$  pA (75% control), and  $10^{-4}$  M R 56865 decreased that current to  $231$  pA (60% of control).

## DISCUSSION

Intracellular Ca<sup>2+</sup> overload is considered to represent the final common pathway leading to the cell death under the pathological condition such as ischemia-reperfusion injury (Farber, 1982; Koch, 1990; Tani, 1990). It occurs frequently under a variety of pathological conditions such as cardioplegia-reperfusion injury, ischemia-reperfusion injury, and digitalis intoxication (Bourdillon and Poole-Wilson, 1982; Bigger, 1985). Therefore, examination of the cellular events leading to Ca<sup>2+</sup> overload and investigations of various cardioprotective drugs are

very important.

However, the exact route of  $\text{Ca}^{2+}$  entry and the site of action of cardioprotective drugs remain obscure.  $\text{Ca}^{2+}$  channel blockers have shown their efficiency in reperfusion injury but were found to be unable to antagonize intracellular  $\text{Ca}^{2+}$  overload directly in the reperfused ischemic myocardium (Bourdillon and Poole-Wilson, 1982; Poole-Wilson *et al.* 1984). The recent studies have shown that an intracellular  $\text{Na}^+$  accumulation during ischemia or cardioplegia triggers  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange leading to  $\text{Ca}^{2+}$  overload (Jennings and Yellon, 1992; Kim *et al.* 1993).

This hypothesis of  $\text{Na}^+$ -mediated  $\text{Ca}^{2+}$  overload is supported by recent data. An improved electrophysiological recovery was demonstrated in the reoxygenated hypoxic brain after the fast  $\text{Na}^+$  channels had been blocked by TTX (Prenen *et al.* 1988; Duff *et al.* 1989). Amiloride,  $\text{Na}^+$ - $\text{H}^+$  exchange inhibitor and/or  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange inhibitor, could effectively preserve the cardiac contractile function and normalize the intracellular  $\text{Na}^+$  activity during reperfusion even in the presence of ouabain (Dennis *et al.* 1990; Meng and Pierce, 1991). In our previous result, the low  $\text{Na}^+$  cardioplegic solution had the protective effect of myocardium during reperfusion (Kim *et al.* 1993).

In this study, we investigated the contribution of  $\text{Na}^+$  leak measured by the form of  $\text{Na}^+$  background current, among the several factors of increasing  $\text{Na}^+$  activity during cardioplegia. The role of the  $\text{Na}^+$  background current in the intracellular  $\text{Na}^+$  accumulation during cardioplegia or ischemia has not been proved. The current density of the  $\text{Na}^+$  background current in guinea pig ventricular myocyte was  $0.31 \pm 0.08$  pA/pF ( $n=8$ ) at  $-40$  mV ( $-40$  mV was the averaged membrane potential in 25 mM  $\text{K}^+$  cardioplegic solution). And that current was increased to 170% of control in  $\text{Ca}^{2+}$  free solution. In our previous results, the external  $\text{Ca}^{2+}$  concentration of cardioplegic solution was important to the recovery of myocardium after cardioplegia and the intracellular  $\text{Na}^+$  activity was higher in  $\text{Ca}^{2+}$  free solution than in the 1.8 mM  $\text{Ca}^{2+}$  solution (Kim *et al.* 1993). These results suggest that the  $\text{Na}^+$  background current can be one of the factors

that increase the intracellular  $\text{Na}^+$  activity during cardioplegia and plays a role in the calcium paradox.

The increased  $\text{Na}^+$  permeability through the  $\text{Ca}^{2+}$  channel in  $\text{Ca}^{2+}$  free solution may induce an increase of the  $\text{Na}^+$  background current. But as the  $\text{Na}^+$  background current was not influenced by verapamil, the possibility of the leakage of  $\text{Na}^+$  through  $\text{Ca}^{2+}$  channel in  $\text{Ca}^{2+}$  free solution could be excluded.

The  $\text{Na}^+$ - $\text{H}^+$  exchange inhibitor, O-(N,N-hexamethylene)-amiloride (HMA) could effectively protect the myocardium against ischemia-reperfusion injury by the prevention of intracellular  $\text{Na}^+$  increase (Dennis *et al.* 1990; Meng and Pierce, 1991). Our result suggests that amiloride may block the  $\text{Na}^+$  background current in a voltage-dependent manner, but the effect on the  $\text{Na}^+$  background current may be minimal during the high  $\text{K}^+$  cardioplegia.

R 56865 has recently been shown to have cardioprotective properties in various experimental models (Farber, 1982; Koch *et al.* 1990; Tani, 1990; Borgers *et al.* 1992). But until now, the exact cellular site of action of R 56865 has remained to be clarified. R 56865 prevents cell death by inhibition of the non-inactivating  $\text{Na}^+$  current in depolarized cells (Verdonck *et al.* 1991) and has antiarrhythmic effect by suppression of oscillatory  $\text{Ca}^{2+}$  release and action potential shortening (Luk and Cameleit, 1990). By its unique combination of effects, R 56865 is considered as a new cytoprotective principle which inhibits  $\text{Na}^+$  and  $\text{Ca}^{2+}$  overload (Ver Donck and Borger, 1991; Ver Donck *et al.* 1992). In our results,  $10^{-6}$  M and  $10^{-7}$  M R 56865 reduced the  $\text{Na}^+$  background current at  $-40$  mV. But in  $10^{-7}$  M and  $10^{-8}$  M R 56865, there was no significant changes in the  $\text{Na}^+$  background current (not shown). The new cardioprotective drug R 56865 decreased the  $\text{Na}^+$  background current at negative membrane potential. This blocking effect of R 56865 may contribute to cardioprotection during cardioplegia by decreasing intracellular  $\text{Na}^+$  loading.

In conclusion, the  $\text{Na}^+$  background current might be one of the factors of increasing intracellular  $\text{Na}^+$  activity during high  $\text{K}^+$  cardioplegia and the calcium paradox. The method or drugs which can decrease the  $\text{Na}^+$  back-

ground current might enhance myocardial protection.

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