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Y-26763 protects the working rat myocardium from ischemia/reperfusion injury through opening of K_{ATP} channels

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Abstract

This investigation was undertaken to determine the possible protection against ischemia afforded by Y-26763, [(−)-(3*S*,4*R*)-4-(*N*-acetyl-*N*-hydroxyamino)-6-cyano-3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-3-ol], which has K^+ channel-opening properties, in isolated rat hearts under working conditions. This preparation was subjected to 28 min of global ischemia followed by 30 min of reperfusion. Drugs were injected into the aortic cannula prior to ischemia. Compared to control, Y-26763 (1 μM) resulted in a significant recovery of post-ischemic cardiac functions, significant reduction of cellular enzyme loss, and preserved significantly the stocks of cellular high-energy phosphates and the myocyte ultrastructure. These effects of Y-26763 were completely prevented by glibenclamide (10 μM), a specific K^+ channel blocker of K_{ATP} channels. In non-ischemic conditions, Y-26763 significantly increased coronary flow without affecting cardiac output and heart rate. The data were analyzed statistically by analysis of variance. The results clearly demonstrate that Y-26763 protects the myocardium from ischemic injury by opening K_{ATP} channels.

Keywords: Y-26763; K^+ channel opener; Glibenclamide; Cardioprotection; K^+ channel, ATP-sensitive

1. Introduction

ATP-sensitive K^+ channels (K_{ATP}) exist in cardiac muscles (Noma, 1983), pancreatic beta cells (Ashcroft et al., 1985), blood vessels (Standen et al., 1989), skeletal muscle cells (Spruce et al., 1985) and other types of smooth muscles (Escande et al., 1989). The discovery of K_{ATP} channels in the cardiac myocyte membrane (Noma, 1983) and their gating by intracellular ATP levels provide a basis for a better understanding of the pharmacology of certain classes of drugs. Protection by openers of these channels against ischemia-reperfusion injury has been demonstrated previously in several experimental models (Ohta et al., 1991; Grover et al., 1990).

A mechanism initially proposed to explain this finding was the dilatation of the coronary artery; however, currently direct myocyte protection is favored. Thus, K_{ATP} openers are expected to be potentially useful for treating certain clinical conditions, like angina pectoris, in addition to hypertension, intermittent claudication, cerebral ischemia, etc. (Quast and Cook, 1989).

This study was performed to examine the effects of Y-26763, [(−)-(3*S*,4*R*)-4-(*N*-acetyl-*N*-hydroxyamino)-6-cyano-3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-3-ol], a K_{ATP} channel opener, on isolated rat hearts. This compound is the active metabolite of Y-27152 (a benzopyran derivative) and has a prolonged antihypertensive action with a reduced incidence of tachycardia (Nakajima, 1991; Nakajima et al., 1992). The goal of this study was to evaluate the possible ability of Y-26763 to protect the rat working heart under in vitro conditions from ischemia-reperfusion injury.

Blockade of K_{ATP} channels in pancreatic beta cells promotes the secretion of insulin (Cook, 1988). This appears to be the mechanism of the hypoglycemic effects of glibenclamide, which is used to treat patients suffering from diabetes mellitus. Glibenclamide as well as other sulphonylureas have also an affinity for K_{ATP} channels in cardiac myocytes (Fosset et al., 1988) and have been already reported to inhibit the cardioprotective (Grover et al., 1989) and vasorelaxing activity of K_{ATP} channel openers (Cavero et al., 1989). For this reason, we used glibenclamide as a potential antagonist of Y-26763. These two compounds were evaluated by measuring mechanical function, intracellular enzyme loss, metabolic state and mor-

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phologic change produced by a prolonged ischemia and reperfusion period.

2. Materials and methods

2.1. Drugs and reagents

Y-26763 was synthesized in the research laboratories of Yoshitomi Pharmaceutical Industries (Fukuoka, Japan). Glibenclamide was obtained from Sigma (St. Louis, MO, USA). These compounds were separately dissolved in dimethyl sulfoxide and then diluted with 0.9% saline. Pentobarbital sodium was obtained from Abbott Laboratories (North Chicago, IL, USA). All other chemical reagents and materials were purchased from local commercial sources.

2.2. Experimental animals

A total of 86 male Wistar rats (body weight 300–420 g) were used. They were bred and supplied by Seiwa Experimental Animals (Fukuoka, Japan). The animals were kept for at least 6 weeks in our air-conditioned animal quarters. They were allowed free access to food and water until the day before they were used for the study.

2.3. Isolated heart preparations

After general anesthesia with sodium pentobarbital (30 mg/kg i.p.), heparin sodium (200 IU) was injected into the femoral vein for clot prevention. A minute later, the chest was opened in the midline and the heart was rapidly excised and immersed into an ice-cold Krebs-Henseleit bicarbonate buffer (KHB) solution (pH 7.4). The heart was then quickly cannulated with the help of two stainless steel cannulae, of which one was inserted into the aortic root and the other into the left atrium. An elastic chamber was placed above the heart to trap air bubbles before they entered the coronary system. A membrane filter made of cellulose nitrate (Advantec, Tokyo, Japan), of 5 μm pore and 47 mm diameter, was inserted into the flow circuit to prevent the entry of any microparticle into the perfusion fluid. The KHB solution was bubbled with 95% O_2 and 5% CO_2 for at least 15 min before being perfused into the isolated heart. The whole flow circuit was covered by a water-jacketed chamber to maintain a constant temperature of 37°C.

2.4. Study of Y-26763 and glibenclamide in ischemic condition

In the first protocol of this study, the isolated hearts were divided into four groups: (1) vehicle ($n = 7$), (2) Y-26763 ($n = 7$), (3) Y-26763 + glibenclamide ($n = 6$) and (4) glibenclamide ($n = 5$). The preparations were converted into a working mode by perfusing them for 10 min via the left atrium at a filling pressure of 18 cm H_2O. The left ventricle contracted against a pressure load set at 80 cm H_2O to generate aortic flow into the aortic cannula. Coronary flow (effluent exiting from the coronary sinus and pulmonary artery) was collected from an outlet at the bottom of the heart chamber. Cardiac output, the sum of the aortic and coronary flow, was measured with a calibrated cylinder. Heart rate was measured with a tachometer (NEC San-ei, Tokyo, Japan) triggered by aortic pressure pulses. Mean aortic pressure was obtained with an appropriate amplifier (NEC San-ei, Tokyo, Japan). Preischemic values were recorded after the hearts were apparently steady for at least 10 min. Global ischemia (28 min) was produced by simultaneously closing the aortic and left atrial cannulae. This was followed by 30 min of reperfusion during which the previously mentioned parameters were measured every 10 min. A needle, connected to a peristaltic pump (Perista Mini-pump, Atto, Tokyo, Japan), was inserted into the root of the aortic cannula to infuse Y-26763 (1 μM total concentration in 1 ml solution) over 1 min prior to ischemia. Similarly, in a separate group of preparations, glibenclamide (10 μM in 1 ml solution) was infused alone just before the infusion of Y-26763.

2.5. Measurement of enzyme loss

Creatine phosphokinase and lactate dehydrogenase were measured in coronary effluent collected in ice-chilled plastic vials before ischemia and every 10 min during the reperfusion period. These enzymes were measured immediately at the end of the experimental procedure by using an automatic analyzer (model 7250 Hitachi, Tokyo, Japan).

2.6. Electron microscopy

The preparations were divided into four groups: (1) pre-ischemia, (2) post-ischemia, (3) reperfusion and (4) reperfusion + Y-26763, each consisting of 3 hearts. Tissue samples from the left ventricle were divided into small pieces, and fixed firstly in 2% glutaraldehyde, then in 2% osmium tetraoxide and finally, after dehydration, were embedded in epoxy resin. After a polymerization procedure, semi-thin sections (1 μm thick) were cut with an ultramicrotome (ULTRACUT N, Tokyo, Japan), stained with methylene blue and observed under a low-power microscope. Subsequently, ultra-thin sections (70-nm thick) were cut with the same microtome, double-stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope operated at 75 kV (Hitachi H-600SS, Tokyo, Japan).

2.7. Measurement of high-energy phosphates (ATP, ADP and AMP)

The preparations were divided into five groups: (1) normoxia control ($n = 8$), (2) ischemia-Y ($n = 9$), (3) ischemia + Y-26763 ($n = 6$), (4) reperfusion-Y ($n = 6$) and (5) reperfusion + Y-26763 ($n = 6$). Each preparation was quickly frozen with Wollenberger tongs pre-cooled in liq-

uid nitrogen at the end of each observation period, freeze-dried for at least 16 h and stored at −80°C until use. Freeze-dried tissue of known weight was homogenized in 6% $HClO_3$ (2 ml for 50 mg dry weight). The homogenate was centrifuged at $12\,000 \times g$ for 10 min, and the supernatant was neutralized with NaOH (1N) and further centrifuged at $37\,000 \times g$ for 15 min. The supernatant was pipetted into Eppendorf tubes, chilled on dry-ice and stored at −30°C until high-performance liquid chromatography analysis. ATP, ADP and AMP were separated by high-performance liquid chromatography and determined by means of an ultraviolet detector as previously described (Sellevold et al., 1986). The energy charge potential (ECP) was calculated with the following equation:

$$ECP = (ATP + 0.5 \times ADP)/(ATP + ADP + AMP).$$

2.8. Study of Y-26763 in non-ischemic hearts

In this protocol, the effects of Y-26763 were studied under non-ischemic (normoxic) conditions. The hearts ($n = 7$) were freshly perfused with KHB solution for 10 min, and then with KHB containing Y-26763 (1 μM) for 15 min. Hemodynamic parameters were measured before and 3, 5, 10, 15 min and at wash-out after starting perfusion of Y-26763.

2.9. Data analysis

The results are expressed as means ± S.E.M. and were statistically analyzed using one-way (Tukey) and two-way (Dunnett) analyses of variance and *t*-test (unpaired). Differences in P values of < 0.05 were considered statistically significant.

3. Results

3.1. Studies on hemodynamic parameters under normoxic conditions: effects of 60 min of perfusion

Coronary and aortic flows, and heart rates remained virtually constant in the rat working heart during a 60-min period of continuous perfusion with oxygenated KHB solution. Indeed, at the end of the equilibration period, coronary and aortic flows and heart rates for 7 heart preparations were 21 ± 0.36 ml/min, 56 ± 0.59 ml/min and 299 ± 8 beat/min, respectively. These values at most were within ±10% of the baseline measurement at the end of the 60-min perfusion.

3.2. Studies on hemodynamic parameters during ischemia / reperfusion: effects of Y-26763, glibenclamide alone, glibenclamide followed by Y-26763 and their vehicle (control)

In control hearts, treated with 1 ml (over 1 min) of the vehicle (0.1% aqueous dimethyl sulfoxide) at the beginning of the 28-min period of ischemia, coronary flow recovered by approximately 30% of the pre-ischemia value 10 min after the start of reperfusion and no further change occurred during the subsequent 20 min. By contrast, aortic flow was strongly depressed (70%) 10 min after the start of reflow and 20 min later it was still 60% below the pre-ischemic value (Fig. 1).

Glibenclamide (10 μM) alone did not modify the poor recuperation of aortic flow measured in control preparations during reperfusion. Moreover, it depressed coronary flow significantly more than that of vehicle treatment (control) and failed to reduce the release of intracellular enzymes measured in the control group (Fig. 2).

Y-26763 (1 μM) enhanced the recovery of coronary and aortic flows as compared to that of control preparations. Indeed, 10 min after the start of reperfusion, coro-

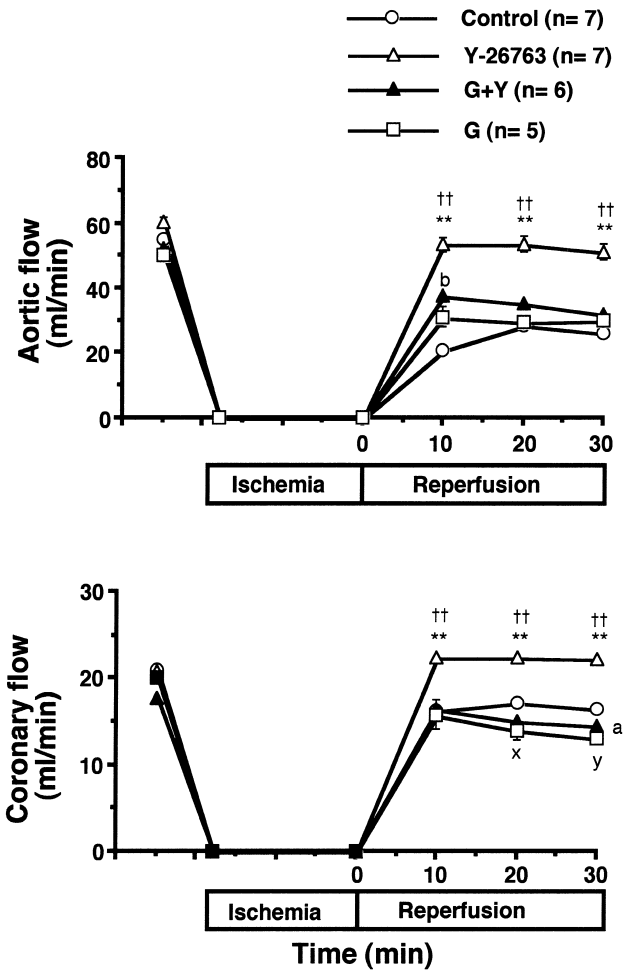

Fig. 1. Effects of Y-26763, glibenclamide, glibenclamide followed by Y-26763 on hemodynamic parameters. These parameters were determined in distinct groups of rat working hearts perfused with Y-26763, glibenclamide followed by Y-26763, glibenclamide alone and their vehicle for 1 min before starting a 28-min period of global ischemia followed by a 30-min period of reperfusion. Each point represents the mean ± S.E.M. G+Y, glibenclamide with Y-26763; G, glibenclamide alone. Significant differences: ** $P < 0.01$ (control vs. Y-26763). ^{a} $P < 0.05$; ^{b} $P < 0.01$ (control vs. G+Y), †† $P < 0.01$ (Y-26763 vs. G+Y), ^{x} $P < 0.05$; ^{y} $P < 0.01$ (control vs. G).

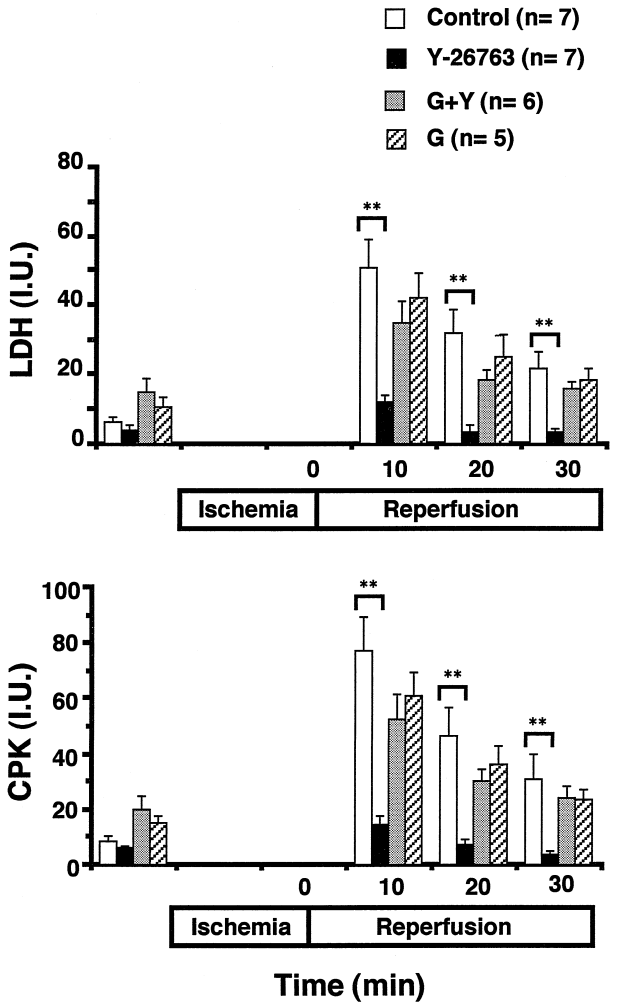

Fig. 2. Effects of Y-26763, glibenclamide, glibenclamide followed by Y-26763 on biochemical parameters. These parameters were determined in distinct groups of rat working hearts perfused with Y-26763, glibenclamide followed by Y-26763, glibenclamide alone and their vehicle for 1 min before starting a 28-min period of global ischemia followed by a 30-min period of reperfusion. Each point represents the mean ± S.E.M. G + Y, glibenclamide with Y-26763; G, glibenclamide alone. Significant differences: ** $P < 0.01$ (control vs. Y-26763).

nary flow was even 10% higher than pre-ischemic values and remained virtually so for the subsequent 20 min. Aortic flow was only 11% below the baseline pre-ischemic value after 10 min of reperfusion, whereas, in control preparations, it was strongly depressed. This recovery persisted until the end of the 30-min period of reperfusion (Fig. 1).

In preparations pre-treated with glibenclamide, the beneficial effects of Y-26763 were no longer seen. At the end of the 30-min reperfusion period, the aortic and coronary flows of preparations receiving glibenclamide + Y-26763 were 39 and 21%, respectively, lower than pre-ischemia levels, whereas in hearts pre-treated with Y-26763 alone, coronary flow was even higher than the baseline pre-ischemic value, and aortic flow was only marginally (14%) below the pre-ischemia values (Fig. 1). As shown in Fig. 2, pre-treatment with glibenclamide resulted in an increased leakage of lactate dehydrogenase and creatine phosphokinase into the coronary effluent during reperfusion, their net values being similar to those measured in the control group, suggesting that the protective effects of Y-26763 were completely antagonized by glibenclamide.

3.3. Studies on cell energy metabolism

The content of ATP in the tissue sampled from normally perfused hearts was approximately 4- and 30-fold higher than that of ADP and AMP (Fig. 3; panels A–C). This resulted in a calculated electric charge potential close to 1 (Fig. 3; panel D). At the end of 28 min of ischemia, the ATP content fell to a very low value (from 29.7 ± 1.4 to 2.5 ± 0.2 μmol/g dry tissue, $n = 9$) whereas the ADP content decreased very slightly and AMP increased by approximately 15-fold. After 30 min of reperfusion, there was no ATP recovery, a slight elevation in ADP and a return of AMP values closed to pre-ischemic levels.

In hearts treated with Y-26763, the values of ATP, ADP and AMP were similar to those measured in control prepa-

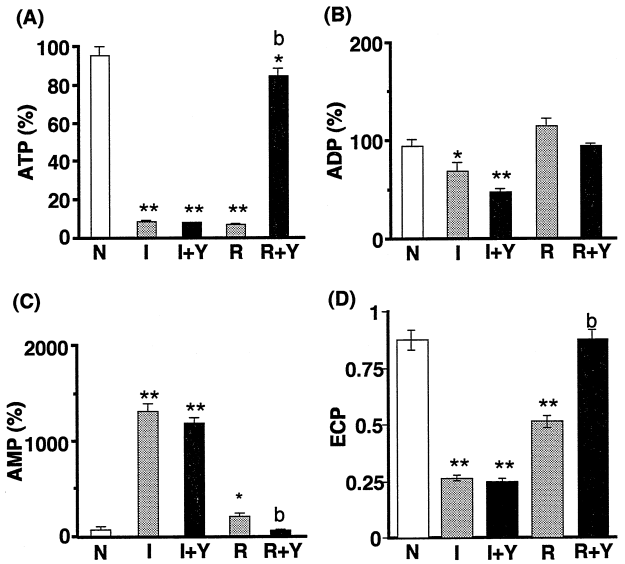

Fig. 3. Protective effect of Y-26763 on the loss of ATP after ischemia. Panels A to C: Levels of ATP, ADP and AMP, respectively, in heart preparations treated with Y-26763 and its vehicle ($n = 6–9$/group) at the end of a 28-min period of ischemia and after a 30-min period of reperfusion. The levels were reported as % change from values measured in a separate group studied under normoxic conditions. The values of ATP, ADP, and AMP during normoxic conditions were 29.72 ± 1.40, 7.63 ± 0.49 and 0.9 ± 0.27 μmol/g dry weight, respectively ($n = 8$). Panel D: ECP in hearts treated with Y-26763 or its vehicle at the end of 28 min of ischemia and at the end of 30 min of reperfusion. ECP is reported as % change for the values calculated for the normoxia group. The value for the normoxia group is 0.88 ± 0.01 ($n = 8$). Each point represents the mean ± S.E.M. N, normoxia; I, ischemia; I + Y, ischemia with Y-26763; R, reperfusion; R + Y, reperfusion with Y-26763; ECP, energy charge potential. Significant differences from the respective preischemic value: * $P < 0.05$; ** $P < 0.01$ vs. normoxia, ^{b} $P < 0.01$ vs. reperfusion.

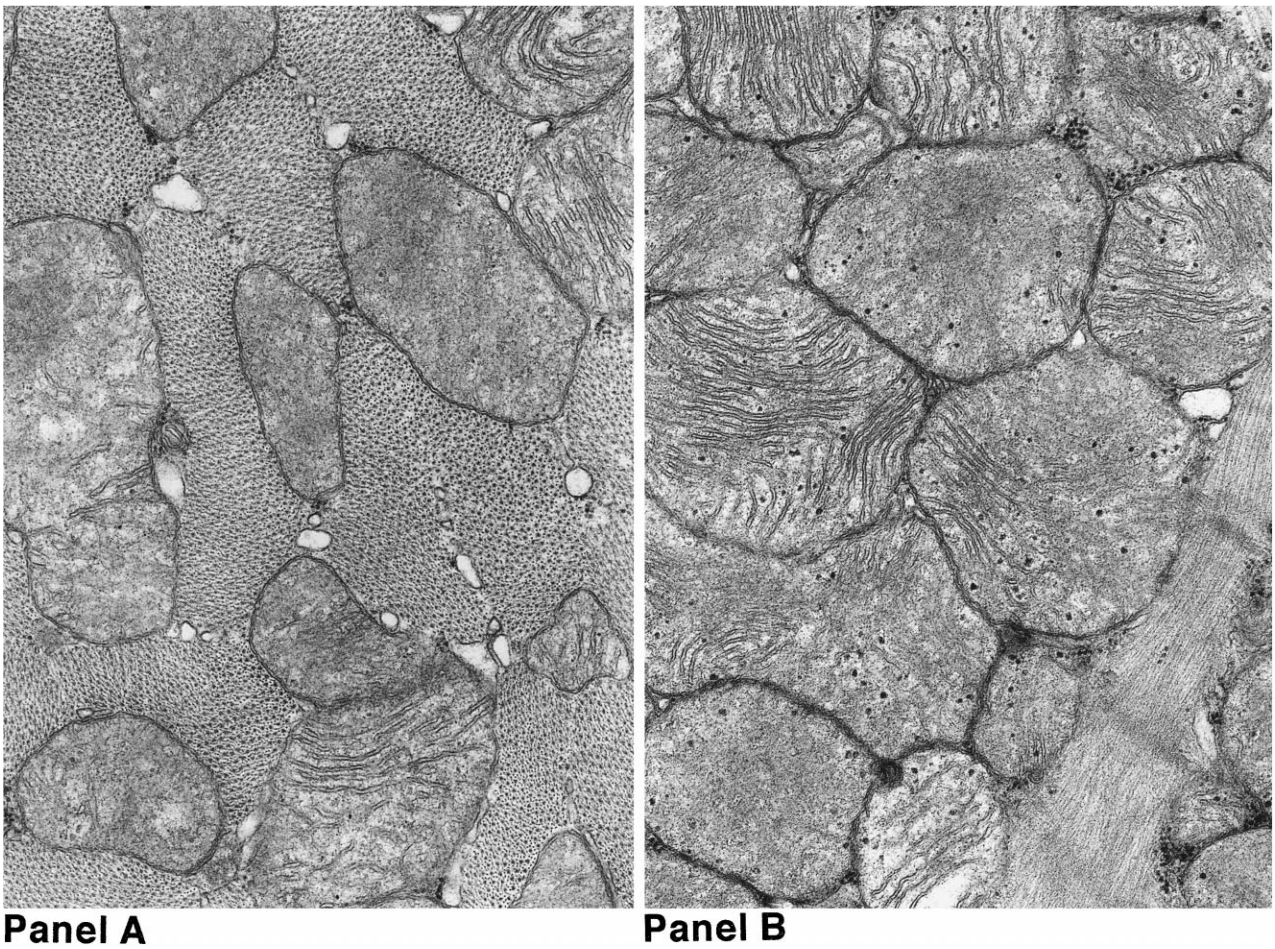

Fig. 4. Electron photomicrograph from left ventricle myocytes after 28-min of ischemia followed by a 30-min period of reperfusion in the vehicle- (A) and Y-26763-treated groups (B). Note the mitochondrial cristae are damaged, mitochondrial granules are absent and air vacuoles are collected adjacent to mitochondria in A. Note the preservation of mitochondrial cristae and granules and the presence of myofibril with A and I bands (×24 000) in situ.

rations at the end of the ischemic period (Fig. 3; panels A–C). However, after 30 min of reperfusion, the concentration of nucleotides as well as the electric charge potential recovered to the values measured under normoxic conditions.

3.4. Studies on cardiac myocyte ultrastructure

The ultrastructure of myocytes sampled from hearts perfused only with a normoxic medium exhibited a normal organization: well-demarcated mitochondria and cristae, and the intervening myofibrils and numerous mitochondrial granules were distributed throughout the cytosolic space. However, after a 28-min period of ischemia, only a few mitochondrial granules were present inside the cell and mitochondria appeared to be slightly damaged. Following 30 min of reperfusion (Fig. 4; panel A), mitochondrial cristae and granules were no longer present in the cell and mitochondria were filled with an amorphous matrix, as observed recently by other investigators (Monticello et al.,

Table 1
Effects of Y-26763 (1 μM) on cardiac hemodynamics under non-ischemic conditions

Parameters	Time after perfusion with Y-26763 (min)					
	Before	3	5	10	15	Wash-out
Coronary flow (ml/min)	21 ± 0.46	24 ± 0.80 [b]	23 ± 0.74 [b]	23 ± 0.80 [b]	24 ± 1.0 [b]	24 ± 0.97 [b]
Aortic flow (ml/min)	57 ± 1.7	56 ± 1.4	55 ± 1.3	55 ± 1.6	55 ± 1.5	55 ± 1.3
Heart rate (beats/min)	327 ± 40	349 ± 40	350 ± 38	331 ± 38	301 ± 23	301 ± 23
MAP (mm Hg)	111 ± 0.60	111 ± 0.70	111 ± 0.60	110 ± 0.60	110 ± 0.50	111 ± 0.50

Data showing the results of Y-26763 under non-ischemic conditions at different time intervals ($n = 7$). Y-26763 significantly increased coronary flow from 3 min onwards. Aortic flow, heart rate and mean aortic pressure (MAP) were similar to pre-drug values. [b] $P < 0.01$ (vs. pre-drug value).

1996). Furthermore, adjacent to swollen mitochondria there were air vacuoles and the myofibril distribution was abnormal. This serious structural ischemia/reperfusion damage was undetectable in the hearts pre-treated with Y-26763 before initiating the ischemia/reperfusion procedure (Fig. 4; panel B). Indeed, the ultrastructure of myocytes from these hearts revealed the presence of numerous mitochondrial granules scattered within the cellular space. Myofibrils with distinct A and I bands and Z lines could also be clearly distinguished within these cells.

3.5. Effects of Y-26763 on non-ischemic myocardium

In the preparations perfused with normoxic KHB solution containing Y-26763 (1 μM), there was a significant increase in coronary flow 3 min after infusion until the end of the experimental procedure (Table 1). Within this period, aortic flow, heart rate and mean aortic pressure remained close to pre-drug infusion levels.

4. Discussion

The results of this study provide clear evidence that Y-26763 can protect the isolated rat myocardium from ischemia/reperfusion damage. Indeed, hearts exposed to Y-26763 at the beginning of prolonged ischemic stress recovered fully their function and ATP content by the end of the reperfusion period. In addition, they lost only a minor amount of intracellularly located enzymes and the ultrastructural organization of their myocytes was preserved. By contrast, matched control hearts exhibited serious ischemic lesions. Y-26763, while affording such a remarkable protection against ischemic injury, did not modify cardiac output but increased coronary flow when administered to hearts perfused with normoxic medium for 15 min. Thus, the beneficial effects of Y-26763 against ischemia can be observed with a concentration of this compound which does not negatively affect the normal pumping function of the heart. Moreover, Y-26763 can enhance coronary flow. The latter property is characteristic of this class of pharmacological agent which, like Y-26763, open K_{ATP} channels. In relation to this study, protection of the myocardium by K_{ATP} without affecting cardiac work was observed previously in another study (Grover et al., 1991).

Y-26763 is the active metabolite and is formed by the liver cytochrome *P*-450 system from an inactive form, Y-27152. Y-27152 lowers blood pressure in hypertensive animals, but this effect is not accompanied by a substantial increase in heart rate (Nakajima et al., 1992). There is no major difference between Y-26763 and other potassium channel openers in vitro tests, but the prolonged duration of action and gradual onset of action of Y-27152 in vivo are desirable properties for a potential drug to treat cardiovascular disease.

The protection afforded by Y-26763 against ischemia/reperfusion injury in our isolated working heart preparation was antagonized by glibenclamide, a blocker of K_{ATP} channels. From these results, Y-26763 is hypothesized to work by activating K_{ATP} channels. These findings confirm several previous reports (Auchampach et al., 1991; Ohta et al., 1991; Sargent et al., 1991) on the ability of glibenclamide to negate the salutary effects of various K_{ATP} channel openers in in vitro and in vivo models of ischemia/reperfusion damage.

Although the present study was carried out under in vitro conditions, the functional, biochemical and ultrastructural ischemia/reperfusion damage resembles that seen clinically following PTCA (percutaneous transluminal coronary angioplasty), aortocoronary bypass graft, organ transplantation or reperfusion injury after cardiopulmonary arrest. Therefore, the effects of Y-26763 were investigated with the help of a working heart model in this study.

Y-26763 increases the outward conductance for K^+ and thereby accelerates the efflux of K^+ out of the cell. The resulting hyperpolarization could accelerate cardiac arrest during ischemia and thus saves ATP for delaying the appearance of injury and for accelerating recovery of cardiac function at reperfusion. We measured ATP before and just after ischemia and then at the end of reperfusion, as other investigators (Auchampach et al., 1991; Grover et al., 1991; Sargent et al., 1991; Mcpherson et al., 1993; Ohta et al., 1993) did before us. It has been reported that continued contractile activity and termination of oxidative metabolism during global ischemia (Hearse et al., 1977), impairment of mitochondrial ATP synthesis (Opie, 1989) and sustained myofibrillar contraction due to Ca^{2+} overload at reperfusion, are responsible for ATP depletion. We observed a sharp reduction in the total myocardial ATP content at the end of ischemia and reperfusion in control preparations. This effect was significantly less in preparations pre-treated with Y-26763 in which the recovery of ATP ($84 \pm 4\%$) was very high and that of ADP and AMP was low.

The mechanism of ATP sparing by Y-26763 and other K^+ channel openers is not yet clearly understood. An accelerated down-regulation of contractile function at the onset of ischemia by K^+ channel openers has been hypothesized to lead to energy sparing and thus cardioprotection (Escande and Cavero, 1992). However, recently Cavero et al. (1996) have provided direct experimental evidence against this mechanism, since in the isolated right ventricle wall of guinea-pigs the cardioprotective effects of the K^+ channel opener aprikalim and ischemic pre-conditioning occurred independently of an accelerated loss of myocardial contractility during the ischemic episode. Indirect evidence favoring this conclusion was also given by Yao and Gross (1994), who reported that K^+ channel openers can produce cardioprotection without enhancing the shortening of the cardiac action potential associated with ischemia.

A significant leakage (vehicle group) of creatine phosphokinase and lactate dehydrogenase occurred during

reperfusion in control preparations. This was probably due to an increased membrane permeability during the reperfusion period. Hearse (1977) reported that this effect can result from the Ca^{2+} overload-induced damage of the sarcolemma and membranes of other organelles during reperfusion. In the present study, electron microscopy analysis demonstrated Y-26763 to prevent the ischemia-induced alterations of the basic cellular ultrastructure of the myocyte and, in particular, to preserve the cell membrane integrity, and thus attenuated enzyme leakage into the extracellular fluid. Very recently, BMS-180448 was reported to ameliorate morphological evidence of ischemia/reperfusion myocardial damage in the isolated rat heart model (Monticello et al., 1996).

In conclusion, this study demonstrates that Y-26763 protects myocytes against ischemia-induced functional, biochemical and morphological lesions. The mechanism of this salutary effect could be attributed to K_{ATP} channel activation, since glibenclamide completely prevented the cardioprotective activity of Y-26763 against ischemia. These results further support the idea that Y-26763 is a promising specific K_{ATP} channel opener for the treatment of ischemic heart disease.

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