Role of an ATP-Sensitive Potassium Channel Opener, YM934, in Mitochondrial Energy Production in Ischemic/Reperfused Heart

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ABSTRACT

We examined a possible mechanism of action of an ATP-sensitive potassium (KATP) channel opener, YM934, for the improvement of energy metabolism in hearts subjected to 35-min ischemia and 60-min reperfusion. The treatment with 30 nM YM934 for the final 15 min of preischemia enhanced postischemic recovery of left ventricular developed pressure, attenuated the postischemic rise in left ventricular end-diastolic pressure, and suppressed the release of creatine kinase and ATP metabolites during reperfusion. The treatment also restored myocardial ATP and creatine phosphate contents and attenuated the decrease in mitochondrial oxygen consumption rate during reperfusion. The higher mitochondrial function was also seen in YM934-treated hearts at the end of ischemia. In another set of experiments, myocardial skinned bundles were incubated for 30 min under hypoxic conditions in the presence and absence of YM934, and then mitochondrial oxygen consumption rate was determined. Hypoxia decreased the mitochondrial oxygen consumption rate of skinned bundles to approximately 40% of the prehypoxic value. In contrast, the treatment of skinned bundles with 30 nM YM934 preserved the mitochondrial oxygen consumption rate during hypoxia. The effect of YM934 on the hypoxic skinned bundles was abolished by combined treatment with either the KATP channel blocker glyburide or the mitochondrial KATP channel blocker 5-hydroxydecanoate in a concentration-dependent manner. The results suggest that YM934 is capable of attenuating ischemia/reperfusion injury of isolated perfused hearts due to preservation of mitochondrial function during ischemia, probably through opening of mitochondrial KATP channels.

YM934, a benzopyran derivative, was developed as an ATP-sensitive potassium (KATP) channel opener. Earlier, Uchida et al. (1994) showed that YM934 induced a stronger relaxation of contracted rabbit aorta pretreated with potassium chloride, norepinephrine, or prostaglandin F2α compared with lemakalim and that treatment of anesthetized dogs with YM934 induced an increase in coronary blood flow, a reduction in total peripheral resistance, and a decrease in mean arterial blood pressure. These effects of YM934 were abolished by combined treatment with the KATP channel blocker glyburide, suggesting that YM934 is a potent KATP channel opener. Opening of KATP channels by this agent in guinea pig cardiomyocytes has also been observed with the patch-clamp technique (Yamada et al., 1993).

Several reports have shown that KATP channel openers protected the myocardium from ischemia/reperfusion injury (Grover et al., 1989; Challinor-Rogers and McPherson, 1994). As for YM934, Taguchi et al. (1999) showed that treatment with this agent attenuated myocardial stunning of anesthetized dogs. Because these effects were abolished by treatment with glyburide, YM934 is considered to exert cardioprotective effects via KATP channel opening. Recently, several investigators proposed that the cardioprotective effect is attributed to opening of mitochondrial KATP channels rather than sarcolemmal KATP channels (Garlid et al., 1997; Jovanovic et al., 1998; Liu et al., 1998). The effects of KATP channel opener on the mitochondria might indirectly link to improvement of energy production of reperfused hearts. However, it is unclear whether YM934 affects mitochondrial KATP channels in the myocardium.

Materials and Methods

Animals. Male Wistar rats, weighing 220 to 260 g, were used in the present study. The animals were conditioned at 23 ± 1°C with a constant humidity of 55 ± 5% and a 12-h light/dark cycle and were

ABBREVIATIONS: KATP, ATP-sensitive potassium; CK, creatine kinase; CP, creatine phosphate; 5-HD, 5-hydroxydecanoate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure.
The supernatant fluid was neutralized with 2.5 M K$_2$CO$_3$ and centrifuged. The ventricle was pulverized by a mortar-driven homogenizer with mainly inosine and hypoxanthine. The release of ATP metabolites was determined by the HPLC method described previously (Takeo et al., 1996). Myocardial CP was converted to ATP according to the enzymatic method of Lowry and Passonneau (1972) and then determined by the same HPLC method as used for ATP as described above.

**Mitochondrial Oxygen Consumption Rate.** The mitochondrial oxygen consumption rate was determined according to the method of Sanbe et al. (1993), which is a modification of the method of Saks et al. (1989). This method has been extensively characterized by the latter group (Saks et al., 1989) and defined as a measure of the maximal mitochondrial oxygen consumption capacity in cardiac tissue. After perfusion, the hearts were quickly removed from the perfusion apparatus. Myocardial bundles, 0.3 to 0.4 mm in diameter and 3 to 4 mm in length, were prepared from the left ventricular free wall by use of a McIlwain Tissue Chopper (Mickle Lab. Engineering Co., Westbury, NY) and transferred into relaxing solution A composed of 10 mM EGTA, 3 mM MgSO$_4$, 20 mM taurine, 0.5 mM dithiothreitol, 20 mM imidazole, 160 mM potassium 2-(N-morpholino)-ethanesulfonate, 5 mM ATP, and 15 mM CP, pH 7.0. Eight to 10 bundles were incubated for 20 min in 1 ml of solution A containing 75 µg/ml saponin. After incubation, the bundles (skinned bundles) were washed for 10 min in fresh solution A to remove the saponin. All procedures were carried out at 4°C. The oxygen consumption rate of the skinned bundles was determined by means of a Clark-type electrode connected to an Oxygraph (Central Kagaku, Tokyo, Japan) containing 7 to 10 skinned bundles in 1.0 ml of solution B (a solution A without ATP and CP but supplemented with 0.5% BSA) at 30°C with continuous and gentle stirring. The basal oxygen consumption rate was measured by the addition of 5 mM glutamate, 3 mM malate, and 2 mM KH$_2$PO$_4$. Total oxygen consumption rate was measured after the further addition of 1 mM ADP and 7.5 mM creatine. The maximal velocity of oxygen consumption rate ($V_{oxygen}$) of the skinned bundles was taken as the difference between total and basal oxygen consumption rates. After determination of oxygen consumption rate, the skinned bundles were transferred to a test tube and washed with saline to remove the BSA and dithiothreitol. The skinned bundles were solubilized with 0.5 ml of 2 N NaOH for 30 min at 60°C, and then the protein concentration was determined according to the method of Lowry et al. (1951). The mitochondrial oxygen consumption rate was expressed as nano-atoms of oxygen consumed/min/mg protein.

**Hypoxic Incubation of Skinned Bundles.** In another set of experiments, skinned bundles were prepared from the left ventricular free wall of normal rats to determine whether YM934 directly affect mitochondria. Hypoxia was induced by incubating the skinned bundles in the solution B for 30 min in an atmosphere of 100% nitrogen gas in a tightly sealed chamber at 30°C. The skinned bundles were exposed to the hypoxic conditions as above in the absence and presence of various concentrations of YM934, glyburide, or 5-hydroxydecanoate (5-HD) alone or in combination. After a 30-min hypoxic or normoxic incubation, the skinned bundles were quickly transferred to the glass cell, and then their oxygen consumption rates were determined as described above.

In a preliminary study, we measured the oxygen content in the atmosphere and incubation medium in the present study. The oxygen content of the normoxic buffer was 7.51 to 7.53 mg/liter, when determined with the Oxylgraph (Oxylgraph 8; Central Kagaku, Tokyo, Japan) equipped with Clark-type oxygen electrode. The oxygen content of atmosphere in the chamber rapidly decreased to less than 0.1 mg/liter within 1 min. The oxygen content of the incubation buffer decreased to less than 0.1 mg/ml within 2.5 min after the onset of hypoxia.

**Agents.** YM934 was kindly provided by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Glyburide and 5-HD were purchased from Sigma Chemical Co. (St. Louis, MO).

**Statistical Analysis.** The results were expressed as the mean ± S.E. The statistical significance of differences in LVDP at the end of reperfusion between the hearts treated with YM934 and untreated.
hearts and that in the release of CK and ATP metabolites from the reperfused heart was evaluated with ANOVA, followed by Dunnett’s multiple comparison. Differences in LVDP recovery of the hearts at the end of reperfusion and release of CK and ATP metabolites during reperfusion among hearts treated with different agents were estimated by ANOVA, followed by Scheffé’s multiple comparison. Differences with probability of 5% or less were considered to be statistically significant (p < .05).

Results

Cardiac Function of Perfused Heart. Changes in LVDP and LVEDP of ischemic/reperfused hearts untreated or treated with 30 nM YM934 are shown in Fig. 1. Ischemia induced a rapid decline in LVDP. The LVDP dropped to zero within 2.5 min after the onset of ischemia; thereafter, it was not generated during ischemia. LVDP of the heart recovered to approximately 20% of the preischemic value by the end of the reperfusion period.

When the hearts were treated with 3 to 30 nM YM934 for the last 15 min of the preischemic period, their LVDPs at the onset of ischemia (Fig. 1, 0 min, top panel) were similar to the LVDP value of the untreated heart. In contrast, LVDPs at the end of reperfusion were significantly recovered to approximately 30 to 85% of the preischemic value. Treatment with 100 nM YM934 resulted in a recovery similar to that with 30 nM YM934, and therefore we used 30 nM YM934 in the subsequent experiments.

The LVEDP of the untreated heart began to rise at 10 min after the onset of ischemia and reached its peak level approximately 20 min of ischemia (Fig. 1, bottom). The LVEDP of the heart was further increased on reperfusion; the maximum level reached, approximately 80 mmHg, occurred at 5 min after the onset of reperfusion. Although the LVEDP was gradually declined during reperfusion, this high level of LVEDP was sustained throughout the reperfusion period. In contrast, treatment with various concentrations of YM934 attenuated the rise in LVEDP during reperfusion in a concentration-dependent manner but not during ischemia.

Release of CK and ATP Metabolites. To determine the release of CK from perfused hearts, we collected the perfusate from the hearts and measured the CK activity in the perfusate (Fig. 2, left). During the 15-min period of preischemic perfusion, CK activity in the perfusate was less than 1 nmol NADPH/min/g wet tissue regardless of the presence or absence of YM934 (n = 6 each). CK activity in the perfusate of the heart perfused for 95 min under normoxic conditions was less than 5 nmol NADPH/min/g wet tissue regardless of treatment with or without 30 nM YM934. CK activity in the perfusate from the untreated heart markedly increased during reperfusion. Treatment with YM934 attenuated the release of CK from the perfused heart.

The amount of ATP metabolites released during reperfusion was also determined (Fig. 2, right). The release of ATP metabolites was less than 0.1 μmol/g wet tissue during preischemia (n = 6). No appreciable release of ATP metabolites was observed from hearts perfused for 95 min under normoxic conditions (n = 6). The release of ATP metabolites was markedly increased during reperfusion. Treatment with YM934 partially suppressed the release of ATP metabolites during reperfusion (to approximately 60% of the value for the untreated heart, n = 6, p < .05).

Myocardial Energy Metabolites. Myocardial energy metabolites such as ATP and CP were determined in the heart untreated or treated with YM934 to examine the myocardial energy profile (Fig. 3). Myocardial ATP and CP contents at the end of the preischemia period were 26.75 ± 2.71 and 36.70 ± 1.80 μmol/g dry tissue, respectively (n = 6). There were no differences in the metabolite content at 95 min of normoxia compared with the preischemic value (at 0 min). Myocardial ATP and CP contents at the end of the ischemia
were approximately 3 and 15% of the preischemic values, respectively (Fig. 3). Reperfusion of the ischemic heart resulted in little restoration of myocardial ATP and CP contents (approximately 12 and 25% of the preischemic values, respectively). Treatment with 30 nM YM934 for the last 15 min of the preischemia period did not alter the preischemic value of these metabolites (data not shown). Treatment with YM934 did not prevent the decreases in myocardial ATP and CP during ischemia. During reperfusion, however, myocardial ATP and CP contents were restored to approximately 40 and 80% of the preischemic values, respectively, by treatment with YM934 (n = 6).

**Mitochondrial Oxygen Consumption Rate of Perfused Heart.** The mitochondrial oxygen consumption rate of the untreated left ventricular muscle and of that treated with YM934 were also determined (Fig. 4). The preischemia rate was 57.02 ± 1.27 nano-atoms O/min/mg protein (n = 6). There were no significant differences in the mitochondrial oxygen consumption rate of perfused hearts under normoxic conditions regardless of treatment without or with YM934. Mitochondrial oxygen consumption rate of the untreated heart under ischemic conditions was significantly lower than that of the normoxic heart (approximately 45% of the value for normoxic hearts, n = 6). A further decline in the mitochondrial oxygen consumption rate was observed on reperfusion (approximately 25% of the value for normoxic hearts, n = 6). In contrast, treatment with YM934 preserved the mitochondrial oxygen consumption rate at the end of both ischemia and reperfusion (approximately 90 and 75% of the value for normoxic hearts, respectively, n = 6 each).

**Mitochondrial Oxygen Consumption Rate under Hypoxic Conditions.** To determine whether YM934 may preserve the mitochondrial oxygen consumption capacity from hypoxic injury, we prepared skinned bundles from the left ventricular free wall of normal rats and incubated them under hypoxic conditions. At first, to determine the experimental conditions of hypoxic incubation of skinned bundles, we measured the mitochondrial oxygen consumption rate at 15 to 120 min of hypoxia. The relationship between time exposed to hypoxia and mitochondrial oxygen consumption rate is shown in Fig. 5. The rate was reduced in a time-dependent manner between 15 and 30 min of hypoxia. Thus, we used 30-min hypoxia in subsequent experiments. Under this condition, we measured mitochondrial oxygen consumption rate in the absence and presence of 3 to 100 nM YM934 (Fig. 6). After the 30-min hypoxic incubation, the mitochondrial oxygen consumption rate was decreased to approximately 40% of the value for the normoxic skinned bundles (n = 6). When the skinned bundles were incubated in the presence of 3, 10, and 30 nM YM934 under hypoxic condi-
tions, the hypoxia-induced decrease in mitochondrial oxygen consumption rate was attenuated in a concentration-dependent manner (n = 3, 4, and 6, respectively). The rate for skinned bundles treated with 100 nM YM934 was similar to that of those treated with 30 nM YM934. The preservation of mitochondrial oxygen consumption capacity by treatment with 30 nM YM934 was significantly abolished by combined treatment with either 1 to 10 μM glyburide (n = 3, 6, and 4; Fig. 7, left) or 10 and 30 μM 5-hydroxydecanoate (n = 3 and 5; Fig. 7, right).

**Discussion**

In the present study, we observed that treatment with YM934 during preischemia markedly enhanced the postischemic contractile recovery of ischemic/reperfused hearts. This improvement was associated with restoration of myocardial high-energy phosphates during reperfusion. Because it is well recognized that cardiac contraction basically requires myocardial high-energy phosphates (Katz, 1977), appreciable levels of high-energy phosphates in the YM934-treated, reperfused heart may be substantially beneficial for the recovery of myocardial contractility of the reperfused heart.

Generally, agents that have negative inotropic action, such as calcium antagonists and β blockers, are capable of enhancing the recovery of postischemic contractile function of perfused hearts, probably as a result of an energy-preserving effect or energy-sparing effect (Nayler et al., 1978, 1980). In the case of YM934, this agent did not have a negative inotropic action during the 15-min infusion in the preischemic period. Furthermore, there were no significant differences in high-energy phosphate levels between YM934-treated and untreated hearts at the end of the ischemia. These findings suggest that the energy-preserving effect during ischemia is unlikely to be the mechanism for cardioprotection against ischemia/reperfusion injury.

Treatment with YM934 suppressed the release of CK from the reperfused heart into the perfusate, whereas this treatment attenuated the release of ATP metabolites to a lesser degree. This implies that an ischemia-induced increase in membrane permeability of macromolecules such as CK protein across cell membranes and/or induction of cardiac cell necrosis in the reperfused heart was suppressed by treatment with YM934. The finding also suggests that relatively small restoration of myocardial ATP by treatment with the agent may be due to the loss of ATP metabolites during reperfusion, because ATP metabolites, such as adenosine and inosine, are substrates for salvage synthesis of ATP in hearts. In contrast, the myocardial CP content of the heart treated with YM934 was restored to 80% of the preischemic value during reperfusion. These results suggest that the ability to produce energy in mitochondria during reperfusion is retained by pretreatment with YM934.

Several researchers have proposed that cardiac K<sub>ATP</sub> channel in the sarcolemma may be involved in cardioprotection against ischemia/reperfusion-induced injury (McPherson et al., 1993; Grover, 1994; Gross, 1995; Liang, 1997). In particular, K<sub>ATP</sub> channel opening has been shown to reduce infarct size, to mimic ischemic preconditioning, and to enhance postischemic recovery of cardiac contractile force (McPherson et al., 1993; Hearse, 1995; Grover, 1997; Schwarz et al., 1997). Numerous studies have suggested that K<sub>ATP</sub> channel blockers such as glyburide abolish the cardioprotective effects of K<sub>ATP</sub> channel openers. However, the degree of action potential shortening was divorced from the extent of protection (Grover et al., 1995a,b). Despite no effects on action potential duration, a K<sub>ATP</sub> channel opener reduced the myocardial
Investigators have suggested that mitochondrial KATP channels may be involved in cardioprotection against ischemia/reperfusion injury (Inoue et al., 1991; Paucek et al., 1992). Several investigators have suggested that mitochondrial KATP channel blockers may be involved in cardioprotection against ischemia/reperfusion injury (Garlid et al., 1997; Joanovic et al., 1998; Liu et al., 1998). These findings suggest that the opening of sarcolemmal KATP channels appears not to be a major mechanism for cardioprotection against ischemia/reperfusion injury.

Recently, it has been shown that there is another isoform of KATP channel on the mitochondrial inner membrane, where the channel regulates mitochondrial volume and energetics (Inoue et al., 1991; Paucek et al., 1992; Garlid et al., 1996; Holmuhamedev et al., 1998). This channel, which differs from the sarcolemmal KATP channel (Yarov-Yarovoy et al., 1997; Lorenz et al., 1998), regulates electron transport in mitochondria and is blocked by the KATP channel blocker glyburide (Inoue et al., 1991; Paucek et al., 1992). Several investigators have suggested that mitochondrial KATP channel blockers may be involved in cardioprotection against ischemia/reperfusion injury (Garlid et al., 1997; Joanovic et al., 1998; Liu et al., 1998). Thus, we measured the oxygen consumption rate of saponin-skinned myocardial bundles to determine whether YM934 may protect the cardiac mitochondria against ischemia/reperfusion injury. At the end of the ischemia, the oxygen consumption rate of myocardial skinned bundle decreased, whereas this decrease was partially restored by reperfusion. YM934 preserved this mitochondrial function of the heart during ischemia as well as during reperfusion. The experimental conditions for determination of mitochondrial oxygen consumption rate were the same as those used to measure the ability for mitochondrial oxidative phosphorylation (Saks et al., 1989). Thus, the results suggest that YM934 is capable of preserving mitochondrial oxidative phosphorylation activity during ischemia.

Although we observed that treatment with YM934 attenuated the ischemia-induced decrease in mitochondrial activity, it remained to be elucidated whether YM934 may directly affect the mitochondria. Therefore, this possibility was addressed in another set of experiments. Skinned myocardial bundles were prepared from the left ventricular free wall and then exposed to 30-min hypoxia in the presence and absence of YM934. Incubation of the skinned bundles under hypoxic conditions resulted in a decrease in mitochondrial oxygen consumption rate. When skinned bundles were incubated under the hypoxic conditions in the presence of YM934, however, the hypoxia-induced decrease in the rate was attenuated in a concentration-dependent manner. This effect of YM934 on mitochondrial oxygen consumption rate was abolished by the combined treatment with KATP channel blocker glyburide. Because glyburide can block KATP channels on both cell membrane and mitochondrial inner membrane (Paucek et al., 1992; Szewczyk et al., 1995; Garlid et al., 1996), we incubated skinned bundles in the presence of combination of YM934 and 5-HD under hypoxic conditions. 5-HD is reported to block sarcolemmal KATP channels of guinea pigs (Notsu et al., 1992). In contrast, McCullough et al. (1991) have shown that 5-HD did not affect cromakalim-activated sarcolemmal KATP currents of rat cardiomyocytes. Furthermore, Liu et al. (1998) suggested that 5-HD was an effective blocker for mitochondrial KATP channels. Although the discrepant conclusion as above remains to be elucidated, this agent is at least effective in the blockade of mitochondrial KATP channels in rats. The treatment of the skinned bundle with 5-HD also abolished the effect of YM934 on the mitochondrial oxygen consumption rate of hypoxic skinned bundles in a concentration-dependent manner. In addition, the cell membrane of the bundles was partially permeated by pretreatment with saponin. Accordingly, the agents used in the skinned bundle experiment may act preferentially on mitochondrial KATP channels rather than on sarcolemmal KATP channels. Thus, sarcolemmal KATP channels of skinned bundles appear not to be functioning under the present experimental conditions. In a preliminary study, we observed that the mitochondrial oxygen consumption rate under normoxic conditions was not altered by the presence of either glyburide or 5-HD per se. We also observed that combined treatment with either YM934 and glyburide, or YM934 and 5-HD, did not alter the mitochondrial oxygen consumption rate under normoxic conditions. An electrophysiological study has shown that YM934 activated opening of KATP channels in isolated cardiac cells at concentrations similar to those used in the present study (T. Taguchi, unpublished observation). Thus, it is likely that the observed preservation of mitochondrial consumption capacity in the ischemic heart by treatment with YM934 was exerted via mitochondrial KATP channel opening.

In a preliminary study, when perfused hearts were treated with YM934 only during reperfusion, the postsischemic recovery of LVDP was not enhanced by this treatment (data not shown). The mitochondrial oxygen consumption rate of the untreated heart was also decreased at the end of ischemia. These findings indicate that mitochondrial oxygen consumption capacity of the heart has already decreased under ischemic conditions before reperfusion injury and suggests that the presence of YM934 during ischemia is necessary to elicit the improvement in the recovery of contractility of the reperfused heart. If so, ischemia/reperfusion-induced damage to cardiac function and metabolism, at least in part, might be initiated and/or promoted by impairment in mitochondrial function during ischemia but not during reperfusion. The results also suggest that the reduction in mitochondrial oxygen consumption capacity in the ischemic heart may be one of the causes rather than effects of ischemia/reperfusion injury.

Liu et al. (1998) showed that oxidation of endogenous flavoprotein fluorescence of cardiac cells, a marker of mitochondrial redox state, was increased by a mitochondrial KATP channel opener, diazoxide, and suggested that mitochondrial KATP channels may mediate the protection from KATP channel openers. If this is the case, KATP channel opener YM934 might exert cardioprotective effects in the ischemic/reperfused heart due to enhancement of the mitochondrial oxidation and reduction. However, this reaction can be achieved in the presence of oxygen. Thus, it is unclear whether the cardioprotective effects of YM934 are related to the alterations in the mitochondrial redox state.

In conclusion, the present study has shown that YM934 is capable of protecting the myocardium against ischemia/reperfusion injury and enhancing the recovery of postsischemic myocardial contractile function associated with restoration of myocardial high-energy phosphate content. The mechanism underlying cardioprotective effect of YM934 may be attributed to preservation of mitochondrial function dur-
ing ischemia, probably via activation of mitochondrial $K_{ATP}$ channel opening.

References


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