British Journal of Pharmacology (2004) 141, 360–366
www.nature.com/bjp

Minoxidil opens mitochondrial K_{ATP} channels and confers cardioprotection

*,[1]Toshiaki Sato, [1,2]Yulong Li, [1]Tomoaki Saito & [1]Haruaki Nakaya

[1]Department of Pharmacology, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

1 ATP-sensitive potassium channel in the mitochondrial inner membrane (mitoK_{ATP} channel) rather than in the sarcolemma (sarcK_{ATP} channel) appears to play an important role in cardioprotection. We examined the effect of minoxidil, a potent antihypertensive agent and hair growth stimulator, on sarcK_{ATP} and mitoK_{ATP} channels in guinea-pig ventricular myocytes.

2 Minoxidil activated a glybenclamide-sensitive sarcK_{ATP} channel current in the whole-cell recording mode with an EC_{50} of 182.6 μM. Minoxidil reversibly increased the flavoprotein oxidation, an index of mitoK_{ATP} channel activity, in a concentration-dependent manner. The EC_{50} for mitoK_{ATP} channel activation was estimated to be 7.3 μM; this value was notably $\approx$25-fold lower than that for sarcK_{ATP} channel activation.

3 Minoxidil (10 μM) significantly attenuated the ouabain-induced increase of mitochondrial Ca^{2+} concentration, which was measured by loading cells with rhod-2 fluorescence. Furthermore, pretreatment with minoxidil (10 μM) before 20-min no-flow ischaemia significantly improved the recovery of developed tension measured after 60 min of reperfusion in coronary perfused guinea-pig ventricular muscles. These cardioprotective effects of minoxidil were completely abolished by the mitoK_{ATP} channel blocker 5-hydroxydecanoate (500 μM).

4 Our results indicate that minoxidil exerts a direct cardioprotective effect on heart muscle cells, an effect mediated by the selective activation of mitoK_{ATP} channels.
British Journal of Pharmacology (2004) **141,** 360–366. doi:10.1038/sj.bjp.0705613

Keywords: K_{ATP} channel; minoxidil; mitochondria; cardioprotection

Abbreviations: $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; DNP, 2,4-dinitrophenol; 5-HD, 5-hydroxydecanoate; K_{ATP}, ATP-sensitive potassium; mitoK_{ATP}, mitochondrial K_{ATP}; sarcK_{ATP}, sarcolemmal K_{ATP}

Introduction

Cardiac myocytes contain ATP-sensitive potassium (K_{ATP}) channels in both sarcolemmal plasma membrane (sarcK_{ATP} channels) and in mitochondrial inner membrane (mitoK_{ATP} channels) (Noma, 1983; Garlid *et al*., 1996; Liu *et al*., 1998). SarcK_{ATP} channels have been molecularly defined as an octameric complex of four pore-forming Kir6.2 and four SUR2A sulphonylurea receptors (Inagaki *et al*., 1996; Clement *et al*., 1997). On the other hand, the molecular cloning of mitoK_{ATP} channel has not yet been achieved, although recent studies using Kir6.1- and Kir6.2-deficient mice suggest that neither of these subunits is an essential component of the cardiac mitoK_{ATP} channel in mice (Miki *et al*., 2002; Suzuki *et al*., 2002).

MitoK_{ATP} channels possess a distinct pharmacological profile, while sharing some pharmacological properties with sarcK_{ATP} channels. Notably, diazoxide opens mitoK_{ATP} channels $\approx$2000-fold more potently than sarcK_{ATP} channels in cardiac myocytes (Garlid *et al*., 1996). Consistent with this, Liu *et al*. (1998) have demonstrated that diazoxide oxidizes the mitochondrial matrix redox potential *via* opening of mitoK_{ATP} channels in rabbit hearts, whereas sarcK_{ATP} channels are resistant to diazoxide. Zang *et al*. (2001) have also demonstrated that diazoxide increases the open probability of reconstituted myocardial mitoK_{ATP} channels in lipid bilayers. Using diazoxide as a pharmacological tool, recent studies have suggested that mitoK_{ATP} channels rather than sarcK_{ATP} channels are involved in cardioprotection (Garlid *et al*., 1997; Liu *et al*., 1998; Sato *et al*., 2000b). However, diazoxide has been reported to inhibit succinate dehydrogenase (Schäfer *et al*., 1969; Hanley *et al*., 2002), suggesting that the interpretation of the effect of diazoxide may not be straightforward. Accordingly, to further elucidate the functional role of mitoK_{ATP} channel, it is desirable to look at another mitoK_{ATP} channel-specific agent.

Minoxidil (chemical structure shown in Figure 1) is a potent K_{ATP} channel opener, and has been shown to act as a vasodilating agent (Campese, 1981; Leblanc *et al*., 1989), and the drug is used externally for treatment of androgenetic alopecia at present (DeVillez, 1990). Hayashi *et al*. (1993) reported that a relatively high concentration of minoxidil opened the sarcK_{ATP} channel in guinea-pig ventricular myocytes. Contrarily, the effect of minoxidil on cardiac mitoK_{ATP} channel remains unclear. Although minoxidil has been shown to improve the contractile function after ischaemia–reperfusion in dog hearts (Yamamoto *et al*., 2002), cardioprotective action of minoxidil is not well understood. In the present study, we therefore examined the effects of minoxidil on mitoK_{ATP} channels by measuring flavoprotein

*Author for correspondence; E-mail: tsato@faculty.chiba-u.jp
[2]Current address: Department of Physiology and Biophysics, University of Nebraska Medical Center, Omaha, NE 68198-4575, U.S.A.
Advance online publication: 22 December 2003

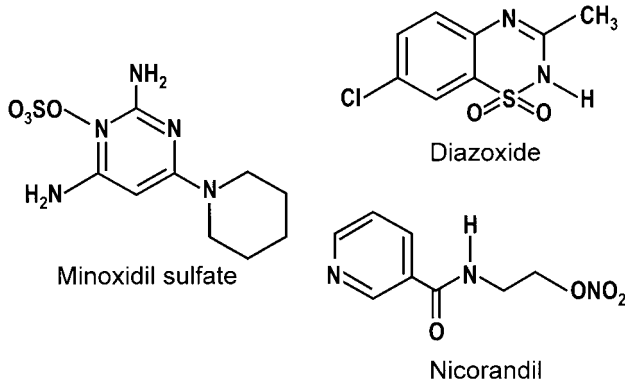

Figure 1 Chemical structures of minoxidil sulphate, diazoxide, and nicorandil.

fluorescence in guinea-pig ventricular myocytes. The results show that minoxidil confers cardioprotection *via* preferential activation of mitoK_{ATP} channels.

Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Cell preparation

Adult guinea-pig ventricular myocytes were isolated by collagenase digestion, as previously described (Tohse *et al*., 1992). Once isolated, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum at room temperature until use. The cells used in the present experiments had a regular shape with clear cross-striation.

Membrane current measurement

The patch-clamp technique was used in whole-cell patch or nystatin-perforated patch configuration, as previously described (Sakamoto *et al*., 1998). Single ventricular cells were superfused with HEPES-buffered Tyrode's solution containing (in mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, NaH_2PO_4 0.33, $MgCl_2$ 0.5, glucose 5.5, and HEPES 5 (pH 7.4) at 37°C. For whole-cell patch recording, the internal pipette solution (solution A) contained (in mM) K-aspartate 110, KCl 20, $CaCl_2$ 1.4, $MgCl_2$ 1, EGTA 10, HEPES 5, phosphocreatinine 1, and, unless otherwise noted, K_2-ATP 1 (pH 7.4). In a separate series of whole-cell clamp experiments, 100 μM ADP was added to the solution A. For nystatin-perforated patch recording, the pipette solution (solution B) contained (in mM) K-aspartate 110, KCl 20, $CaCl_2$ 1, $MgCl_2$ 1, EGTA 0.1, HEPES 5 (pH 7.4), and nystatin. A stock solution of nystatin was added to the pipette solution to a final concentration of 300 $\mu g\,ml^{-1}$ just before the experiments. In both whole-cell and nystatin-perforated patch recordings, the membrane potential was held at -40 mV and depolarized first to $+50$ mV and then hyperpolarized to -100 mV with a slope of $-60\,mV\,s^{-1}$. This ramp-pulse protocol was repeated every 5 s. The quasi-steady state membrane current was plotted against the membrane potential during hyperpolarizing voltage ramps. The current signals were filtered at 3 kHz with a digital Gaussian filter and digitized at 2 kHz for data analysis with pClamp software (Axon Instruments, Foster City, CA, U.S.A.). These experiments were performed at 36°C.

Flavoprotein fluorescence measurement

To index the mitoK_{ATP} channel activity, flavoprotein fluorescence was measured by a modification of method described by Sato *et al*. (1998). Briefly, the cells were superfused with a bath solution containing (mM): NaCl 143, KCl 5.4, $CaCl_2$ 1.8, NaH_2PO_4 0.33, $MgCl_2$ 0.5, and HEPES 5 (pH 7.4) at room temperature ($\approx$22°C). Flavoprotein fluorescence was excited at 480 nm (for 200 ms) and emitted at 520 nm. At the end of each experiment, cells were exposed to the mitochondrial uncoupler 2,4-dinitrophenol (DNP, 100 μM) to obtain maximal flavoprotein oxidation. The emitted fluorescence was monitored with a cooled charge-coupled device (CCD) digital camera (Hamamatsu Photonics, Hamamatsu, Japan). The imaging of flavoprotein was analysed for average pixel intensities of regions of interest drawn to include the whole cell, and expressed as a percentage of the DNP-induced maximal oxidation, using an Aquacosmos image-processing system (Hamamatsu Photonics).

$[Ca^{2+}]_m$ measurement

The Ca^{2+} fluorophore rhod-2 was used to measure the changes of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$). For rhod-2 loading, cells were plated on uncoated 35 mm Falcon culture dishes with a medium based on a 1 : 1 mixture of DMEM and HEPES-buffered Tyrode's solution, supplemented with 10% foetal calf serum. Then, cells were loaded with 10 μM rhod-2 acetoxymethyl ester for 120 min at 4°C. After cold loading, cells were incubated for 30 min at 37°C. This two-step cold loading/warm incubation protocol achieves exclusive loading of rhod-2 into the mitochondria (Trollinger *et al*., 2000). Cells loaded with rhod-2 were perfused with a HEPES-buffered Tyrode's solution containing 2.7 mM $CaCl_2$ at 37°C. Rhod-2 fluorescence was excited at 540 nm (for 100 ms), with emission monitored through a 605-nm (55-nm bandpass) barrier filter. The imaging of rhod-2 was analysed for the average pixel intensities of regions of interest drawn to include the whole cell, following correction for background, using an Aquacosmos image-processing system (Hamamatsu Photonics).

Coronary-perfused right ventricular myocardium

The isolated coronary-perfused guinea-pig right ventricular free wall was prepared as described previously (Shigematsu *et al*., 1995). In brief, the preparation was mounted in the recording chamber and pinned to the floor of the chamber. The coronary artery was perfused with oxygenated Tyrode's solution containing (in mM) NaCl 136.7, $NaHCO_3$ 11.9, KCl 5.4, NaH_2PO_4 0.42, $MgCl_2$ 0.5, $CaCl_2$ 1.8, and glucose 11 (pH 7.35–7.40 when gassed with 97% O_2 and 3% CO_2). The flow rate was maintained at $1.0\pm0.2\,ml\,min^{-1}\,g^{-1}$ wet weight using a roller pump (MP-3; Tokyo Rikakikai, Tokyo, Japan). The surface of the preparation was superfused with glucose-free hypoxic Tyrode's solution ($10\,ml\,min^{-1}$) to minimize direct O_2

diffusion from the surface of the preparations into the muscles. The composition of the hypoxic Tyrode's solution was the same as above, except that it contained no glucose and was gassed with 97% N_2 and 3% CO_2. The temperatures of these solutions were maintained at $37\pm0.5^{\circ}C$. The basal portion of the preparation was stimulated at 3 Hz throughout the experiment and contractile tension was recorded using a force transducer (TB-612T; Nihon Kohden, Tokyo, Japan) connected to the apical end of the preparation. Resting tension was adjusted to obtain the optimal developed tension. The contractile tension was monitored on a multibeam oscilloscope (VC-9A; Nihon Kohden) and recorded on a multichannel thermal array corder (WT-645G; Nihon Kohden).

After equilibration for 90 min, the preparations were assigned to the study groups. Control ($n=5$): the preparations were subjected to 20 min of no-flow ischaemia followed by 60 min of reperfusion. Minoxidil ($n=4$): the preparations were treated for 5 min with minoxidil (10 μM) followed by a 10-min washout before no-flow ischaemia. Minoxidil + 5-HD ($n=4$): pretreatment with the mitoK_{ATP} channel blocker 5-hydroxydecanoate (5-HD, 500 μM) (Sato *et al*., 1998) starting 5 min prior to and continued during minoxidil treatment. Minoxidil + HMR ($n=3$): pretreatment with the sarcK_{ATP} channel blocker HMR 1098 (HMR, 30 μM) (Sato *et al*., 2000b) starting 5 min prior to and continued during minoxidil treatment.

Chemicals

Minoxidil sulphate was a kind gift from Taisho Pharmaceutical (Omiya, Japan). HMR 1098 (HMR) was a kind gift from Aventis Pharma (Tokyo, Japan). Pinacidil, sodium 5-hydroxydecanoic acid (5-HD), glybenclamide, and ouabain were purchased from Sigma (St Louis, MO, U.S.A.). Rhod-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, U.S.A.). Nystatin and 2,4-dinitrophenol (DNP) were purchased from Wako Pure Chemical (Osaka, Japan). Minoxidil sulphate and glybenclamide were dissolved as a 100 mM stock solution in dimethyl sulphoxide, and the final concentration of solvent was $\leqslant 0.1\%$. Pinacidil was dissolved as a 50 mM stock solution in 0.1 N HCl + saline. A stock solution of nystatin dissolved in methanol at a concentration of 10 $mg^{-1}\,ml^{-1}$ was prepared fresh before each experiment. Ouabain, 5-HD, HMR, and DNP were dissolved in the perfusate.

Data analysis

Data are presented as mean $\pm$ s.e.m., and the number of cells or experiments is shown as n. Concentration–response data were fit with a four-parameter logistic equation:

$$Y = A1 + (A2 - A1)/(1 + 10(\hat{\log}EC_{50} - [\text{minoxidil}])^{n})$$

where Y is the current (Figure 2b) or the flavoprotein oxidation (Figure 5), A1 is the minimum current (Figure 2b) or the minimum flavoprotein oxidation (Figure 5), A2 is the maximum current (Figure 2b) or the maximum flavoprotein oxidation (Figure 5), [minoxidil] is the concentration of minoxidil, and n is the Hill coefficient. Curve fits were performed with Origin 7J software (OriginLab, Northampton, MA, U.S.A.). Intergroup comparisons are made by Student's t-test for two groups and by ANOVA followed by Fisher's *post hoc* test for multiple groups. A value of $P<0.05$ was regarded as significant.

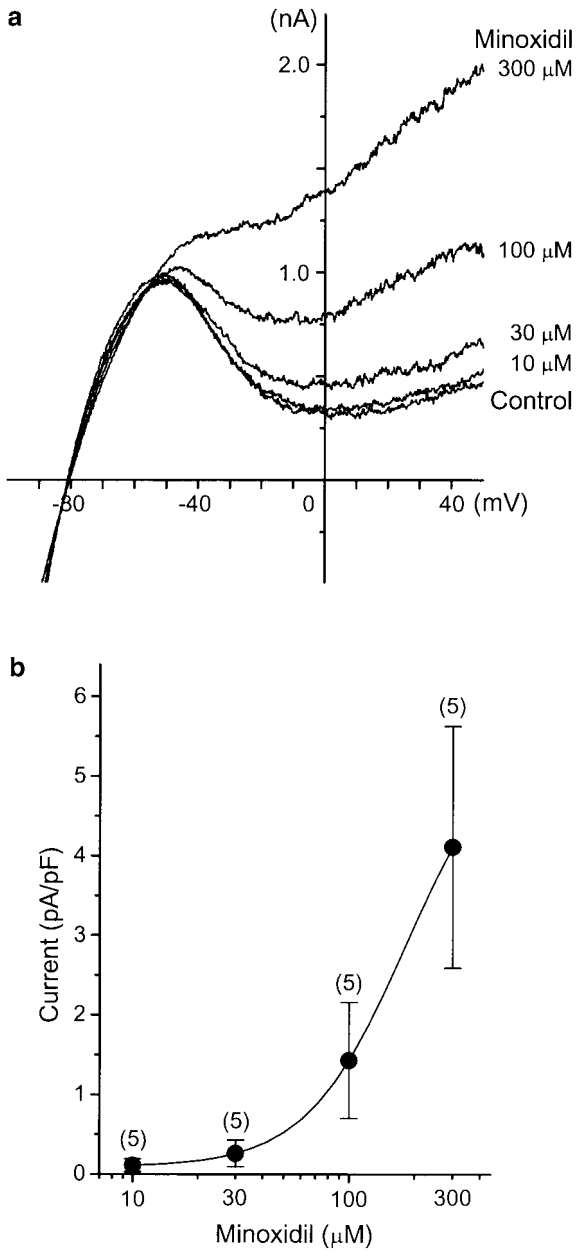

Figure 2 Effects of minoxidil on the sarcK_{ATP} channels. (a) Representative current–voltage relationships recorded under whole-cell patch clamp. Each concentration of minoxidil was applied for 5 min. (b) Dose–response curve for minoxidil-induced sarcK_{ATP} channel currents. The current traces obtained in each drug concentration were subtracted from the control current tracing. The amplitude of minoxidil-sensitive current at 0 mV was normalized to the cell capacitance of each cell. All data resented as mean $\pm$ s.e.m., with numbers of cells given in parentheses.

Results

Effect of minoxidil on membrane currents

To test the effect of minoxidil on sarcK_{ATP} channel, membrane current was recorded with patch-clamp techniques. Figure 2a

shows the representative current traces recorded in the whole-cell configuration. When 1 mM ATP was included in the pipette solution, cumulative application of minoxidil (10–300 μM) resulted in a concentration-dependent increase in the quasi-steady-state outward current. The minoxidil-induced outward current was completely inhibited by subsequent application of 10 μM glybenclamide (data not shown), indicating that minoxidil is an activator of sarcK_{ATP} channel. Figure 2b illustrates the dose–response curve for minoxidil-induced currents measured at the membrane potential of 0 mV. The estimated EC_{50} value for minoxidil in activating glybenclamide-sensitive outward current was 182.6 μM.

To determine if intracellular ADP modulates the effect of minoxidil, the quasi-steady-state membrane current was recorded by adding ADP to the internal pipette solution. ADP (100 μM) *per se* did not affect the outward current and the current amplitude measured at 0 mV was 2.7 ± 1.3 ($n = 11$) and 2.9 ± 1.2 pA pF^{-1} ($n = 5$) in the absence and presence, respectively, of ADP in the pipette. As shown in Figure 3a, there was no significant change in the amplitude of minoxidil-induced outward current, when the patch pipette contained ADP. In a nystatin-perforated patch configuration, as shown in Figure 3b, significant current activation could not be detected even at a high concentration of minoxidil (300 μM), whereas pinacidil at a concentration of 100 μM produced a robust increase in outward current.

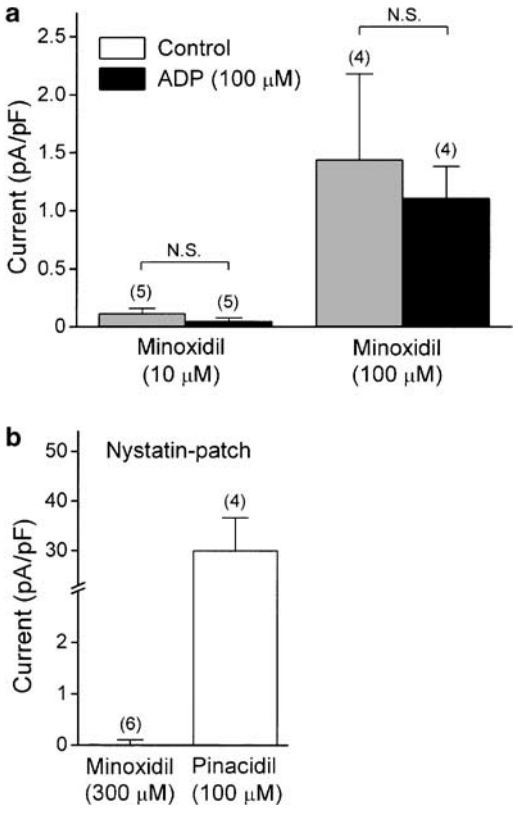

Figure 3 (a) Summarized effect of intracellular ADP on minoxidil-induced sarcK_{ATP} channel current. ADP (100 μM) was added to the pipette in the whole-cell recording mode. (b) Comparative effect of minoxidil and pinacidil on sarcK_{ATP} channel current recorded in the nystatin-perforated patch configuration. In each panel, the amplitude of sarcK_{ATP} channel at 0 mV was normalized to the cell capacitance of each cell. Each bar represents the mean $\pm$ s.e.m., with numbers of cells given in parentheses.

Effect of minoxidil on flavoprotein oxidation

Figure 4 shows the representative images of flavoprotein fluorescence in a cell exposed to minoxidil. Flavoprotein fluorescence was low under control condition (Figure 4b) in agreement with earlier reports (Liu *et al*., 1998; Romashko *et al*., 1998). Exposure to minoxidil (10 μM) oxidized flavoprotein and increased the fluorescence (Figure 4c), which was reversible on washout (Figure 4d). Subsequent exposure to DNP (100 μM) led to increase in flavoprotein fluorescence (Figure 4e). As summarized in Figure 5, minoxidil increased flavoprotein fluorescence in a concentration-dependent manner. The estimated EC_{50} value for minoxidil to induce flavoprotein oxidation was 7.3 μM. Coadministration of 5-HD (500 μM), a selective mitoK_{ATP} channel blocker (Sato *et al*., 1998), virtually abolished the minoxidil-induced flavoprotein oxidation. These results indicate that minoxidil is an opener of mitoK_{ATP} channels.

Effect of minoxidil on mitochondrial Ca^{2+} overload

A previous study demonstrated that the opening of mitoK_{ATP} channels by diazoxide attenuated the mitochondrial Ca^{2+} overload (Ishida *et al*., 2001). We therefore examined the effect of minoxidil on mitochondrial Ca^{2+} overload. As summarized in Figure 6, treatment of myocytes with ouabain (1 mM) evoked mitochondrial Ca^{2+} overload and the intensity of rhod-2 fluorescence significantly increased from 27.6 ± 1.3 to 75.7 ± 8.5 a.u. after 30-min exposure to ouabain ($P < 0.001$).

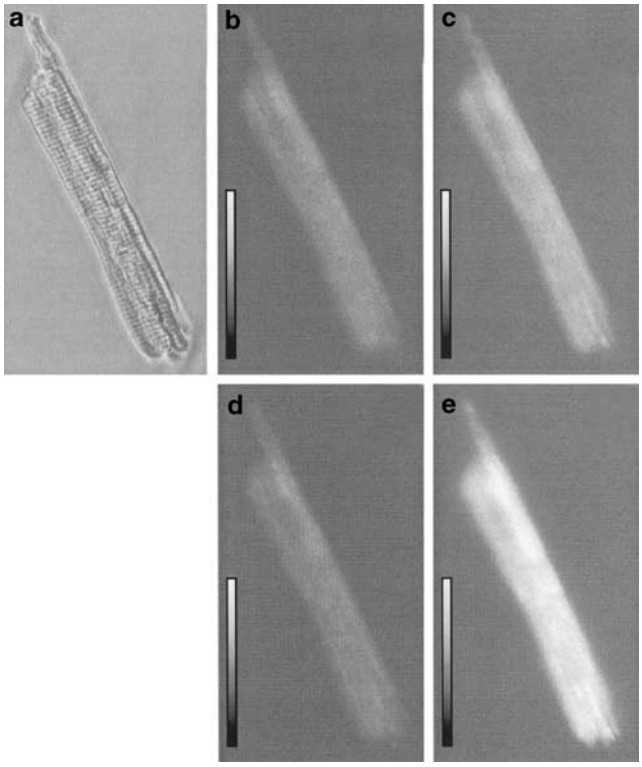

Figure 4 Imaging of flavoprotein fluorescence in guinea-pig ventricular myocyte. (a) Transmitted image. (b–e) A pseudocolour palette was applied to visualize the relative increase in flavoprotein oxidation, to yield images of cell at control (b), after 7-min exposure to 10 μM minoxidil (c), washing out of minoxidil (d), and 2 min after exposure to 100 μM DNP (e).

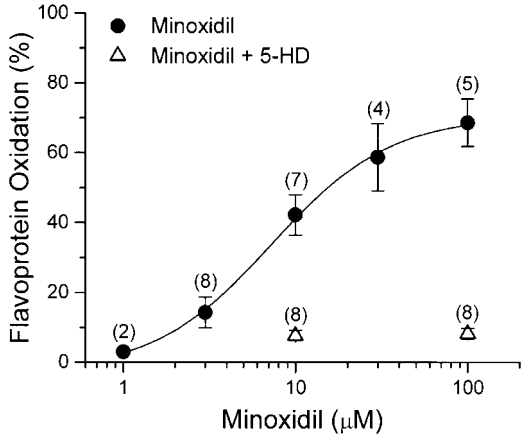

Figure 5 Summarized dose–response data for minoxidil-induced flavoprotein oxidation. Values are expressed as percents relative to those obtained with DNP. All data are presented as mean ± s.e.m., with numbers of cells given in parentheses.

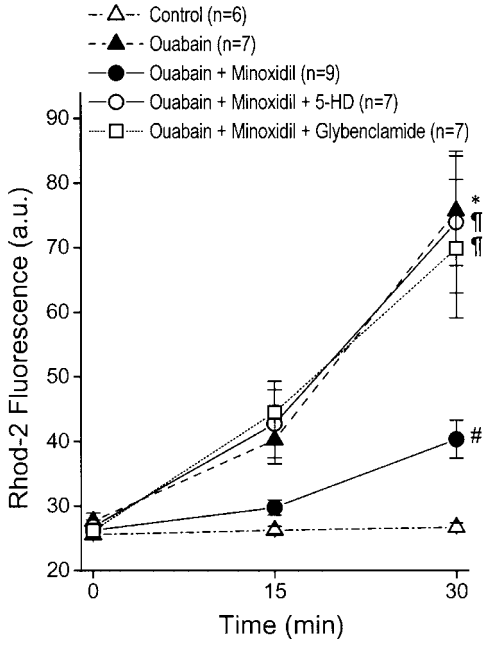

Figure 6 Summarized effect of minoxidil on ouabain-induced mitochondrial Ca^{2+} overload. In each group, drugs (minoxidil, 10 μM; 5-HD, 500 μM; glybenclamide, 10 μM) were applied together with ouabain (1 mM), and the resultant fluorescence was collected at 15 and 30 min after exposure to ouabain. Each point indicates the mean ± s.e.m. $^{*}P<0.001$ vs baseline; $^{\#}P<0.001$ vs ouabain; $^{\P}P<0.001$ vs ouabain + minoxidil.

Coadministration of minoxidil (10 μM) significantly prevented the ouabain-induced increase in rhod-2 fluorescence to 40.3 ± 3.0 a.u. ($P<0.001$ vs ouabain alone). The effect of minoxidil was antagonized by both 5-HD (500 μM) and glybenclamide (10 μM). These results indicate that opening of mitoK_{ATP} channels by minoxidil attenuates the ouabain-induced Ca^{2+} overload in mitochondria.

Effect of minoxidil on contractile function during ischaemia/reperfusion

To test whether minoxidil confers cardioprotection in guinea-pig hearts, coronary perfused right ventricular preparations were subjected to 20-min no-flow ischaemia, followed by 60-min reperfusion. Table 1 summarizes the changes in developed tension before ischaemia. Neither 5-HD (500 μM) nor HMR (30 μM) alone had any significant effect on developed tension. Although not statistically significant, the developed tension was slightly depressed by minoxidil (10 μM). Figure 7 shows the time courses of developed tension during ischaemia/reperfusion. Pretreatment with 10 μM minoxidil prior to ischaemia significantly improved the recovery of contractility after 60 min of reperfusion, compared with controls (64.0 ± 1.8 vs 34.8 ± 4.1%, $P<0.01$). This cardioprotective effect of minoxidil was blocked by 5-HD (39.7 ± 0.5%, $P<0.01$ vs minoxidil alone), but not by HMR (61.3 ± 3.1%, P = NS vs minoxidil alone), suggesting that the cardioprotective effect of minoxidil results from mitoK_{ATP} channel activation.

Discussion

Minoxidil is a potent K_{ATP} channel opener and has diverse actions ranging from vasodilation to promotion of hair growth (Campese, 1981; Leblanc *et al*., 1989; DeVillez, 1990). Although significant side effects, such as ventricular arrhythmia and severe hypotension, may limit the clinical utility of the K_{ATP} channel openers, these compounds are effective in protecting cells from ischaemic injury, and thus merit further investigation. The major finding of the present study is that minoxidil could open mitoK_{ATP} channels ≈25-fold more potently than sarcK_{ATP} channels in guinea-pig ventricular myocytes, and thereby attenuated mitochondrial Ca^{2+} overload and improved contractile recovery after ischaemia/reperfusion.

In agreement with the previous report (Hayashi *et al*., 1993), minoxidil activated the sarcK_{ATP} channels in a concentration-dependent fashion, with an EC_{50} value of 182.6 μM when assessed by whole-cell recording in the presence of 1 mM ATP. We further found that, in contrast to pinacidil, minoxidil

Table 1 Changes of developed tension before ischemia

		Drug		
Group	*Stabilization*	*5-HD or HMR*	*Minoxidil*	*Preischemia*
Control ($n=5$)	11.6 ± 0.8 mN	—	—	11.3 ± 0.9 mN
Minoxidil ($n=4$)	11.8 ± 0.8 mN	—	11.0 ± 0.9 mN	11.8 ± 0.8 mN
Minoxidil + 5-HD ($n=4$)	12.0 ± 0.8 mN	12.9 ± 1.0 mN	12.0 ± 0.8 mN	12.0 ± 0.8 mN
Minoxidil + HMR ($n=4$)	11.6 ± 1.0 mN	11.8 ± 1.0 mN	11.1 ± 0.9 mN	11.4 ± 1.0 mN

Values are mean ± s.e.m. Stabilization: at the end of 90 min of stabilization; 5-HD or HMR: 5 min after treatment with 5-hydroxydecanoate or HMR 1098; minoxidil; 5 min after treatment with minoxidil in the absence and presence of 5-hydroxydecanoate or HMR 1098; preischemia: immediately before the onset of ischaemia.

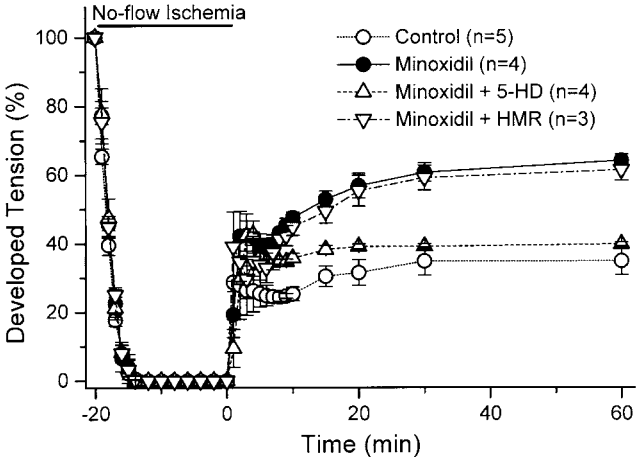

Figure 7 Time courses of changes in developed tension during 20-min no-flow ischaemia and 60-min reperfusion. Each point indicates the mean ± s.e.m. for 3–5 preparations, and is expressed as a percentage of the preischaemic value.

evoked only a small outward current even at high concentration (Figure 3b), when $sarcK_{ATP}$ current recordings were performed with nystatin in the pipette solution (nystatin-perforated patch). Therefore, minoxidil is less potent than pinacidil in activating $sarcK_{ATP}$ channel current. The perforated-patch technique allows the exchange of monovalent cations and anions, whereas it maintains intracellular metabolites intact (Horn & Marty, 1988). In this respect, the activity of minoxidil to open $sarcK_{ATP}$ channels is dependent on the intracellular ATP concentrations, and the drug can be expected to have no effect on the $sarcK_{ATP}$ channel under normal condition. Recently, diazoxide and nicorandil, a putative $mitoK_{ATP}$ channel opener (Liu *et al*., 1998; Sato *et al*., 2000a), have been shown to activate $sarcK_{ATP}$ channels during simulated ischaemia or when intracellular ADP is raised (D'hahan *et al*., 1999; Matsuoka *et al*., 2000). Such an ADP-dependent activation of $sarcK_{ATP}$ channel has been proposed to underlie the cardioprotective effect against ischaemia-induced contractile dysfunction of mouse heart (Suzuki *et al*., 2003). In the present study, we found that minoxidil did not enhance the $sarcK_{ATP}$ channel activity even when the patch pipette contained 100 μM ADP (Figure 3a). These results indicate that minoxidil may not open $sarcK_{ATP}$ channels even when intracellular ADP was considerably increased, a condition to be encountered, for example, during ischaemia.

$MitoK_{ATP}$ channel activity was indexed by measuring flavoprotein fluorescence (Liu *et al*., 1998). It is so far the only method that can be used to assess $mitoK_{ATP}$ channel activity in intact cells. However, two previous studies (Lawrence *et al*., 2001; Hanley *et al*., 2002) have failed to demonstrate the oxidation of flavoprotein by diazoxide. These discrepancies are likely to reflect the different experimental conditions. They used freshly isolated myocytes and measured flavoprotein fluorescence in the presence of glucose. In our experiments, the cells were kept in a culture medium until use to stabilize the mitochondrial redox state. Moreover, since $mitoK_{ATP}$ channel-induced flavoprotein oxidation is detectable only if uncompensated by increased production of electron donor such as NADH (Chance *et al*., 1972), we used the glucose-free Tyrode's solution for measurement of flavoprotein fluorescence. Under our experimental conditions, the $mitoK_{ATP}$ channel opener diazoxide oxidized flavoprotein in guinea-pig ventricular myocytes (Sato *et al*., 2003). The present study demonstrated that minoxidil reversibly oxidized the flavoprotein in a concentration-dependent manner (Figures 4, 5). Moreover, the $mitoK_{ATP}$ channel blocker 5-HD completely abolished the minoxidil-induced flavoprotein oxidation. The estimated EC_{50} value for minoxidil-induced flavoprotein oxidation was 7.3 μM. This value was notably $\approx$25-fold lower than that for $sarcK_{ATP}$ channel activation assessed by whole-cell recording (182.6 μM), suggesting that minoxidil primarily activates $mitoK_{ATP}$ channels in cardiac cell. Thus, a pharmacological profile of minoxidil is qualitatively similar to that of diazoxide and nicorandil.

Ca^{2+} uptake into mitochondria is driven primarily by the large negative electrical potential of the matrix (Gunter & Pfeiffer, 1990). Therefore, depolarization of the mitochondrial membrane *via* opening of $mitoK_{ATP}$ channels reduces the driving force for Ca^{2+} influx and, hence, results in the prevention of mitochondrial Ca^{2+} overload. In agreement with this hypothesis, we have reported that, in rat cardiomyocytes, opening of $mitoK_{ATP}$ channels by diazoxide attenuates the ouabain-induced mitochondrial Ca^{2+} overload, and such an effect is associated with the depolarization of the mitochondrial membrane (Ishida *et al*., 2001). In the present study, using the same experimental design, we found that minoxidil could prevent the ouabain-induced Ca^{2+} overload in mitochondria (Figure 6). The concentration of minoxidil used in these experiments (10 μM) was close to the EC_{50} for flavoprotein oxidation. The $mitoK_{ATP}$ channel blocker 5-HD completely abolished the effects of minoxidil. Moreover, the degree of protection conferred by minoxidil was comparable to that seen with diazoxide (data not shown). Here, we used diazoxide and 5-HD as the $mitoK_{ATP}$ channel-selective agents. However, it has also been claimed that diazoxide inhibits succinate dehydrogenase and 5-HD is converted to 5-HD-CoA (Schäfer *et al*., 1969; Hanley *et al*., 2002; Lim *et al*., 2002). Thus, the interpretation of our results may not be straightforward. So far, there is no report suggesting that minoxidil, like diazoxide, inhibits succinate dehydrogenase. The specific succinate dehydrogenase inhibitor malonate could not prevent the ouabain-induced mitochondrial Ca^{2+} overload (our unpublished data). Furthermore, the nonselective K_{ATP} channel blocker glybenclamide abolished the effect of minoxidil, in a manner similar to 5-HD (Figure 6). We therefore propose that cardioprotective effects of minoxidil are mediated by the opening of $mitoK_{ATP}$ channels, although $mitoK_{ATP}$ channel-independent action cannot be completely excluded.

We found that brief exposure to minoxidil prior to ischaemia improved the recovery of developed tension after reperfusion. In the present study, the coronary flow rate was maintained at $1.0 \pm 0.2\,ml\,min^{-1}\,g^{-1}$ wet weight using a roller pump. It is therefore reasonable to assume that, under our experimental condition, cardioprotective effects of minoxidil are not due to vasodilation resulting from vascular $sarcK_{ATP}$ channel activation. As described above, minoxidil can hardly open $sarcK_{ATP}$ channels at the concentration used. In addition, the putative $mitoK_{ATP}$ channel blocker 5-HD but not the putative $sarcK_{ATP}$ channel blocker HMR completely abolished the effect of minoxidil (Figure 7). These results further support the notion that minoxidil confers cardioprotection *via* preferential activation of $mitoK_{ATP}$ channels.

This study was supported in part by Grants-in-Aid for Scientific Research, the Mitsui Life Social Welfare Foundation, and the Mochida Memorial Foundation for Medical and Pharmaceutical Research. We thank M. Tamagawa, Y. Reien, and I. Sakashita for excellent technical and secretarial assistance.

References

CAMPESE, V.M. (1981). Minoxidil: a review of its pharmacological properties and therapeutic use. *Drugs,* **22,** 257–278.

CHANCE, B., SALKOVITZ, I.A. & KOVACH, A.G. (1972). Kinetics of mitochondrial flavoprotein and pyridine nucleotide in perfused heart. *Am. J. Physiol.,* **223,** 207–218.

CLEMENT JR, J.P., KUNJILWAR, K., GONZALEZ, G., SCHWANSTECHER, M., PANTEN, U., AGUILAR-BRRYAN, L. & BRYAN, J. (1997). Association and stoichiometry of K_{ATP} channel subunits. *Neuron,* **18,** 827–838.

DEVILLEZ, R.L. (1990). The therapeutic use of topical minoxidil. *Dermatol. Clin.,* **8,** 367–375.

D'HAHAN, N., MOREAU, C., PROST, A.L., JACQUET, H., ALEKSEEV, A.E., TERZIC, A. & VIVAUDOU, M. (1999). Pharmacological plasticity of cardiac ATP-sensitive potassium channels toward diazoxide revealed by ADP. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 12162–12167.

GARLID, K.D., PAUCEK, P., YAROV-YAROVOY, V., MURRAY, H.N., DARBENZIO, R.B., D'ALONZO, A.J., SMITH, M.A. & GROVER, G.J. (1997). Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels: possible mechanism of cardioprotection. *Circ. Res.,* **81,** 1072–1082.

GARLID, K.D., PAUCEK, P., YAROV-YAROVOY, V., SUN, X. & SCHINDLER, P.A. (1996). The mitochondrial K_{ATP} channel as a receptor for potassium channel openers. *J. Biol. Chem.,* **271,** 8796–8799.

GUNTER, T.E. & PFEIFFER, D.R. (1990). Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.,* **258,** C755–C786.

HANLEY, P.J., MICKEL, M., LÖFFLER, M., BRANDT, U. & DAUT, J. (2002). K_{ATP} channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart. *J. Physiol.,* **542,** 735–741.

HAYASHI, S., HORIE, M. & OKADA, Y. (1993). Ionic mechanism of minoxidil-induced shortening of action potential durations in guinea pig ventricular myocytes. *J. Pharmacol. Exp. Ther.,* **265,** 1527–1533.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.,* **94,** 145–159.

INAGAKI, N., GONOI, T., CLEMEMT Jr, J.P., WANG, C.Z., AGUILAR-BRYAN, L., BRYAN, J. & SEINO, S. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron,* **16,** 1011–1017.

ISHIDA, H., HIROTA, Y., GENKA, C., NAKAZAWA, H., NAKAYA, H. & SATO, T. (2001). Opening of mitochondrial K_{ATP} channels attenuates the ouabain-induced calcium overload in mitochondria. *Circ. Res.,* **89,** 856–858.

LAWRENCE, C.L., BILLUPS, B., RODRIGO, G.C. & STANDEN, N.B. (2001). The K_{ATP} channel opener diazoxide protects cardiac myocytes during metabolic inhibition without causing mitochondrial depolarization or flavoprotein oxidation. *Br. J. Pharmacol.,* **134,** 535–542.

LEBLANC, N., WILDE, D.W., KEEF, K.D. & HUME, J.R. (1989). Electrophysiological mechanisms of minoxidil-induced vasodilation of rabbit portal vein. *Circ. Res.,* **65,** 1102–1111.

LIM, K.H., JAVADOV, S.A., DAS, M., CLARKE, S.J., SULEIMAN, M.S & HALESTRAP, A.P. (2002). The effect of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat heart mitochondrial volume and respiration. *J. Physiol.,* **545,** 961–974.

LIU, Y., SATO, T., O'ROURKE, B. & MARBAN, E. (1998). Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation,* **97,** 2463–2469.

MATSUOKA, T., MATSUSHITA, K., KATAYAMA, Y., FUJITA, A., INAGEDA, K., TANEMOTO, M., INANOBE, A., YAMASHITA, S., MATSUZAWA, Y. & KURACHI, Y. (2000). C-terminal tails of sulfonylurea receptors control ADP-induced activation and diazoxide modulation of ATP-sensitive K^+ channels. *Circ. Res.,* **87,** 873–880.

MIKI, T., SUZUKI, M., SHIBASAKI, T., UEMURA, H., SATO, T., YAMAGUCHI, K., KOSEKI, H., IWANAGA, T., NAKAYA, H. & SEINO, S. (2002). Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. *Nat. Med.,* **8,** 466–472.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature,* **305,** 147–148.

ROMASHKO, D., MARBAN, E. & O'ROURKE, B. (1998). Subcellular metabolic transients and mitochondrial redox waves in heart cells. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 1618–1623.

SAKAMOTO, N., UEMURA, H., HARA, Y., SAITO, T., MASUDA, Y. & NAKAYA, H. (1998). Bradykinin B_2-receptor-mediated modulation of membrane currents in guinea-pig cardiomyocytes. *Br. J. Pharmacol.,* **125,** 283–292.

SATO, T., O'ROURKE, B. & MARBÁN, E. (1998). Modulation of mitochondrial ATP-dependent K^+ channels by protein kinase C. *Circ. Res.,* **83,** 110–114.

SATO, T., SASAKI, N., O'ROURKE, B. & MARBÁN, E. (2000a). Nicorandil, a potent cardioprotective agent, acts by opening mitochondrial ATP-dependent potassium channels. *J. Am. Coll. Cardiol.,* **35,** 514–518.

SATO, T., SASAKI, N., SEHARASEYON, J., O'ROURKE, B. & MARBÁN, E. (2000b). Selective pharmacological agents implicate mitochondrial but not sarcolemmal K_{ATP} channels in ischemic cardioprotection. *Circulation,* **83,** 110–114.

SATO, T., TAKIZAWA, T., SAITO, T., KOBAYASHI, S., HARA, Y. & NAKAYA, H. (2003). Amiodarone inhibits sarcolemmal but not mitochondrial K_{ATP} channels in guinea-pig ventricular cells. *J. Pharmacol. Exp. Ther.*, October 8, 2003; DOI:10.1124/jpet.103.055863.

SCHÄFER, G., WEGENER, C., PORTENHAUSER, R. & BOJANOVSKI, D. (1969). Diazoxide, an inhibitor of succinate oxidation. *Biochem. Pharmacol.,* **18,** 2678–2681.

SHIGEMATSU, S., SATO, T., ABE, T., SAIKAWA, T., SAKATA, T. & ARITA, M. (1995). Pharmacological evidence for the persistent activation of ATP-sensitive K^+ channels in early phase of reperfusion and its protective role against myocardial stunning. *Circulation,* **92,** 2266–2275.

SUZUKI, M., SAITO, T., SATO, T., TAMAGAWA, M., MIKI, T., SEINO, S & NAKAYA, H. (2003). Cardioprotective effect of diazoxide is mediated by activation of sarcolemmal but not mitochondrial ATP-sensitive potassium channels in mice. *Circulation,* **107,** 682–685.

SUZUKI, M., SASAKI, N., MIKI, T., SAKAMOTO, N., OHMOTO-SEKINE, Y., TAMAGAWA, M., SEINO, S., MARBÁN, E. & NAKAYA, H. (2002). Role of sarcolemmal K_{ATP} channels in cardioprotection against ischemia/reperfusion injury in mice. *J. Clin. Invest.,* **109,** 509–516.

TOHSE, N., NAKAYA, H. & KANNO, M. (1992). α_1 Adrenoceptor stimulation enhances the delayed rectifier K^+ current of guinea pig ventricular cells through the activation of protein kinase C. *Circ. Res.,* **71,** 1441–1446.

TROLLINGER, D.R., CASCIO, W.E. & LEMASTERS, J.J. (2000). Mitochondrial calcium transients in adult rabbit cardiac myocytes: inhibition by ruthenium red and artifacts caused by lysosomal loading of Ca^{2+}-indicating fluorophores. *Biophys. J.,* **79,** 39–50.

YAMAMOTO, A., SATOH, K., ICHINOSAWA, K., KANETA, S., KANO, S. & ICHIHARA, K. (2002). Effects of minoxidil on ischemia-induced mechanical and metabolic dysfunction in dog myocardium. *Jpn. J. Pharmacol.,* **90,** 173–180.

ZANG, D.X., CHEN, Y-F., CAMPBELL, W.B., ZOU, A-P., GROSS, G.J. & LI, P.-L. (2001). Characteristics and superoxide-induced activation of reconstituted myocardial mitochondrial ATP-sensitive potassium channels. *Circ. Res.,* **89,** 1177–1183.

(Received June 23, 2003
Revised October 9, 2003
Accepted November 7, 2003)