British Journal of Pharmacology (2003) 138, 453–460

www.nature.com/bjp

Contribution of Na^+-Ca^{2+} exchanger to pinacidil-induced relaxation in the rat mesenteric artery

[1]Suk Ying Tsang, [1]Xiaoqiang Yao, [1]Chi Ming Wong, [1]Chak Leung Au, [2]Zhen Yu Chen & *,[1]Yu Huang

[1]Department of Physiology, Chinese University of Hong Kong, Hong Kong, China and [2]Department of Biochemistry, Chinese University of Hong Kong, Hong Kong, China

1 Pinacidil relaxes blood vessels through opening the K_{ATP} channels with a resultant membrane hyperpolarization and inhibition of Ca^{2+} influx. The aim of this study was to examine the mechanisms thereby pinacidil induces K^+ channel-independent relaxation in isolated endothelium-denuded rat mesenteric artery.

2 Pinacidil-induced relaxation was inhibited by glibenclamide (1–10 μM) in phenylephrine-preconstricted rings, but was unaffected by glibenclamide after inhibition of K^+ channels and VGCCs. Pinacidil-induced K^+ channel-independent relaxation remained unchanged after treatment with cyclopiazonic acid (10 μM), thapsigargin (1 μM), ouabain (100 μM), propranolol (10 μM), Rp-cAMPS triethylamine (30 μM), L-NNA (100 μM), or ODQ (10 μM).

3 Pinacidil induced more relaxant effect in the presence of nifedipine than in the presence of 60 mM K^+ plus nifedipine. Pretreatment with Na^+-Ca^{2+} exchanger inhibitors, nickel (30–300 μM) or benzamil (20 μM) attenuated pinacidil-induced relaxation in normal or in nifedipine-containing solution. Pinacidil (1 μM) produced less relaxant effect with decreasing extracellular Na^+ concentration. Na^+-free condition abolished the inhibitory effect of benzamil. Both nickel and benzamil inhibited pinacidil-induced relaxation in the presence of glibenclamide (10 μM). Nickel (300 μM) did not affect the relaxant response to sodium nitroprusside.

4 Pinacidil relaxed the rings preconstricted by active phorbol and U46619 with similar potency.

5 The present results indicate that stimulation of the forward mode Na^+-Ca^{2+} exchange pathway is in part responsible for pinacidil-induced K^+ channel-independent vasorelaxation. Pinacidil also induces K^+ channel-dependent but VGCCs-independent relaxation. The PKC-mediated cellular pathway may be a target site for pinacidil only in higher concentrations.

British Journal of Pharmacology (2003) **138,** 453–460. doi:10.1038/sj.bjp.0705062

Keywords: Pinacidil; Na^+-Ca^{2+} exchanger; relaxation; smooth muscle; artery; rat

Abbreviations: CPA, cyclopiazonic acid; IP_3, inositol 1,4,5-trisphosphate; K_{ATP} channel, ATP-sensitive K^+ channel; L-NNA, N^G-nitro-L-arginine; ODQ, 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one; PDA, phorbol 12,13-diacetate; PKC, protein kinase C; Tg, thapsigargin; U46619, 9,11- dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$; VGCC, voltage-gated Ca^{2+} channel

Introduction

Pinacidil, an antihypertensive drug, lowers blood pressure *via* direct vasodilation (Ahnfelt-Ronne, 1988; Friedel & Brogden, 1990; Quast, 1992). Many *in vitro* studies have demonstrated that direct activation of the K^+ channel is the primary mechanism for pinacidil-induced vasodilation. Pinacidil was more effective in inhibiting the contraction induced by noradrenaline than by elevated extracellular K^+ (Videbaek *et al*., 1988). Pinacidil stimulated an increase in outward K^+ current and hyperpolarized the cell membrane, which were sensitive to glibenclamide (Itoh *et al*., 1992); and pinacidil-induced vasorelaxation was also inhibited by glibenclamide and other K_{ATP} channel blockers (Standen *et al*., 1989). Pinacidil inhibited noradrenaline-induced inositol 1,4,5- trisphosphate (IP_3) production and Ca^{2+} release resulting from membrane hyperpolarization in rabbit mesenteric arteries and this effect was antagonized by glibenclamide (Itoh *et al*., 1992). On the other hand, large-conductance Ca^{2+}-activated K^+ channels were activated by pinacidil at 100 μM in smooth muscle cells of the rat cerebral arteries (Stockbridge *et al*., 1991). The glibenclamide-insensitive voltage-dependent K^+ current activated by pinacidil (1–20 μM) was sensitive to iberiotoxin in human coronary vascular smooth muscle cells (Bychkov *et al*., 1997).

The K^+ channel-independent vascular effects were also reported for pinacidil when its concentration used was higher than 1 μM. For example, pinacidil-induced K^+ channel-independent relaxant effect may be due to a stimulatory effect on plasmalemmal Ca^{2+} extrusion mechanism (Meisheri *et al*., 1991). The IC_{50} of K^+ channel-independent relaxation was $\sim$59 μM for the porcine coronary artery (Gollasch *et al*., 1995) and $\sim$50 μM for the human internal mammary artery (Gojkovic Bukarica *et al*., 1997).

Even though dissociation of K^+ channel opening and vasorelaxation by the K^+ channel openers exists in several

*Author for correspondence at: Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, Hong Kong, China; E-mail: yu-huang@cuhk.edu.hk

kinds of arteries from different species, including human resistance arteries (Quast, 1993), no specific mechanism has been provided to explain the K^+ channel-independent component of the pinacidil-induced relaxation. The possible mechanisms may include stimulation of Na^+-K^+ pump or the forward mode of Na^+-Ca^{2+} exchanger, promotion of intracellular Ca^{2+} uptake into the endoplasmic reticulum, direct inhibition of plasmalemmal Ca^{2+} channels, activation of cyclic nucleotide-dependent signalling pathway or protein kinase C-mediated contraction. The present study therefore attempts to investigate some cellular mechanisms that may underlie a K^+ channel-independent vasorelaxant response to pinacidil with various pharmacological interventions in the isolated endothelium-denuded rat mesenteric arteries.

Methods

Vessel preparation and mounting

This study was approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong. Male Sprague–Dawley rats (supplied by Animal Services Center, the Chinese University of Hong Kong) weighing 250–300 g were killed by cervical dislocation and bled. The main branch of the superior mesenteric artery was dissected out and cut into four 3-mm-wide ring segments following removal of the surrounding connective tissues. The endothelial layer was mechanically removed by gently rubbing the luminal surface of the artery back and forth several times with plastic tubing. Each ring was mounted between two stainless wire hooks in a 10-ml organ bath filled with Krebs solution. The upper wire was connected to a force-displacement transducer (Grass Instruments, U.S.A.) and the lower one fixed at the bottom of the organ bath. Krebs solution had the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, KH_2PO_4 1.2, and *D*-glucose 11. The bath solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 37°C to give rise to a relatively constant pH of 7.2–7.4. All rings were placed under an optimal resting tension of 5 mN, which had been determined by length-tension relationship experiments. The rings were allowed to equilibrate for 90 min during which time the bath solution was replaced every 20 min with pre-warmed and oxygenated Krebs solution. Baseline tone was readjusted when necessary. Functional removal of endothelium was verified by lack of a relaxant response to 0.3 μM acetylcholine at the beginning of each experiment. Each experiment was performed on rings prepared from different rats.

Force measurement

Thirty minutes after being set up in the organ baths, the rings were first contracted with 5 μM phenylephrine to test their contractile responses, and subsequently challenged by 0.3 μM acetylcholine to confirm denuation of the endothelium. They were then washed several times in Krebs solution to restore vessel tension to baseline level. In the first set of experiments, each ring was contracted submaximally with a constrictor (10 nM U46619, 5 μM phenylephrine, or 60 mM K^+). Once a plateau contraction was obtained, a cumulative concentration-response curve to pinacidil was constructed. In the second set of experiments, the ring was contracted with 60 mM K^+ (to annul the effect of K^+ channel activation) and subsequently relaxed by 1 μM nifedipine (to block the effect of Ca^{2+} channel activation). The ring was then contracted with U46619, or phenylephrine in the case of using glibenclamide. Once a plateau contraction was obtained, pinacidil was added to the bathing solution cumulatively. Individual inhibitor was applied 30 min prior to addition of the constrictor. These inhibitors included glibenclamide, cyclopiazonic acid (CPA), thapsigargin (Tg), ouabain, propranolol, Rp-cAMPS triethylamine, N^G-nitro-L-arginine (L-NNA), 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one (ODQ), nickel chloride, and benzamil. The high-K^+ solution was prepared by replacing Na^+ with an equimolar concentration of K^+ to maintain constant ion strength in the bath solution. In the third group of experiments, pinacidil was added cumulatively in the U46619-preconstricted rings in the absence and presence of 1 μM nifedipine, and the effects of nickel or benzamil were examined. The effect of nickel was also tested on the relaxation induced by sodium nitroprusside. In the final set of experiments, effect of pinacidil (1 μM) was investigated in a Na^+-free Krebs solution in the absence and presence of benzamil (20 μM). When Na^+-free solution was used in the experiments it contained the following compositions (mM): 119 N-methyl-D-glucamine chloride, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 choline bicarbonate, 1.2 KH_2PO_4, and 11 *D*-glucose. Since agonist-evoked tension could not be maintained in Na^+-free solution or in the presence of 10 μM glibenclamide, the effect of pinacidil at a single concentration was tested on the U46619-induced contraction in Na^+-free solution or phenylephrine (5 μM)-induced contraction in the presence of glibenclamide. The initial tone in different experiments was similar in magnitude by adjusting the constrictor concentration. All experiments were performed on endothelium-denuded rings.

Drugs

Phenylephrine hydrochloride, acetylcholine hydrochloride, 9, 11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619), pinacidil, nifedipine, glibenclamide, cyclopiazonic acid (CPA), thapsigargin, ouabain, propranolol, Rp-cAMPS triethylamine, N^G-nitro-L-arginine (L-NNA), phorbol 12,13-diacetate (PDA), sodium nitroprusside (Sigma, St. Louis, MO, U.S.A.), 1H- [1,2,4]oxadizolo[4,3-a]quinoxalin-1-one (ODQ) (Tocris Cookson Ltd., U.K.), nickel chloride (Merck, Germany), and benzamil chloride (RBI, U.S.A.). U46619, pinacidil, nifedipine, glibenclamide, CPA, thapsigargin, ouabain, propranolol, ODQ, PDA were dissolved in dimethyl sulphoxide (DMSO) and others in double distilled water. DMSO at 0.2% (v v^{-1}) did not affect the U46619- or high K^+-induced vessel tone.

Data analysis

Data are mean ± s.e.mean of *n* vessel rings prepared from separate rats. Four rings prepared from the same artery were studied in parallel, a concentration-response curve was established in each ring. The relaxant effect of pinacidil was expressed as the percentage reduction of the agonist-induced initial tone in each ring. Cumulative concentration–relaxation relationship was analysed by non-linear regression curve fitting using GraphPad software (version 3.0). pD_2 is the negative

logarithm of drug concentration that produced a half-maximum relaxation and E_{max} is the maxmum relaxation. Student's t two-tailed test or analysis of variance followed by Newman-Keuls test was used to compare pD_2 values. P values less than 0.05 were considered as statistically significant.

Results

Pinacidil-induced relaxation

Figure 1a shows that in phenylephrine-preconstricted rings, pinacidil induced concentration-dependent relaxant responses with a pD_2 of 6.42 ± 0.04 ($n=6$). Pretreatment with glibenclamide attenuated pinacidil-induced relaxation (pD_2: 5.00 ± 0.03, 4.82 ± 0.04, and 4.75 ± 0.04 in 1, 3 and 10 μM glibenclamide, respectively, $n=5-6$, $P<0.05$ as compared with the control) without affecting the maximum relaxation (Figure 1a). In U46619 (10 nmol/l)-preconstricted rings, pinacidil produced relaxations with a pD_2 of 6.73 ± 0.05 ($n=6$); but it induced significantly less inhibitory effect on the contraction induced by 60 mM K^+ (pD_2: 4.79 ± 0.07, $n=6$, $P<0.05$ as compared with that obtained with U46619 or phenylephrine, Figure 1b), indicating both K_{ATP} channel-dependent and -independent components of pinacidil-induced relaxation.

Pinacidil-induced relaxation in the presence of high K^+ and nifedipine

Pinacidil-induced K^+ channel-dependent vasorelaxation is likely mediated through inhibition of voltage-gated Ca^{2+} channels (VGCCs) in vascular smooth muscle. In order to minimize the influence of both K_{ATP} channels and VGCCs, the rings were first contracted with 60 mM K^+ and subsequently relaxed completely by 1 μM nifedipine. Under this condition, pinacidil still reduced phenylephrine-induced tone with the maximum response achieved. The relaxation was unaffected by 10 μM glibenclamide (pD_2: 4.87 ± 0.04 in control, and 4.85 ± 0.02 for glibenclamide, $n=5-6$, $P>0.05$, Figure 2a,b). Figure 2b shows the pD_2 values for pinacidil-induced relaxation under various conditions. Pinacidil-induced relaxation was the same in rings contracted by phenylephrine and by high K^+ in the presence of 10 μM glibenclamide or by U46619 following inhibition of K^+ channels and VGCCs ($n=5-6$, Figure 2b).

Figure 2c shows the concentration-dependent effects of pinacidil in U46619-contracted rings under three conditions. The presence of 1 μM nifedipine (inhibition of VGCCs) caused a significant rightward shift of the pinacidil concentration-response curve (pD_2: 5.85 ± 0.06, $n=5$). Following inhibition of K^+ channels and VGCCs, pinacidil-induced relaxation was further inhibited (pD_2: 4.69 ± 0.13, $n=6$, Figure 2c,d). This value was similar to that obtained in glibenclamide-treated high K^+-contracted rings (Figure 2b). These data clearly indicate that the present protocol could effectively eliminate the involvement of the K_{ATP} channels and VGCCs, thus enabling assessment of the K_{ATP} channel-independent vascular effect of pinacidil.

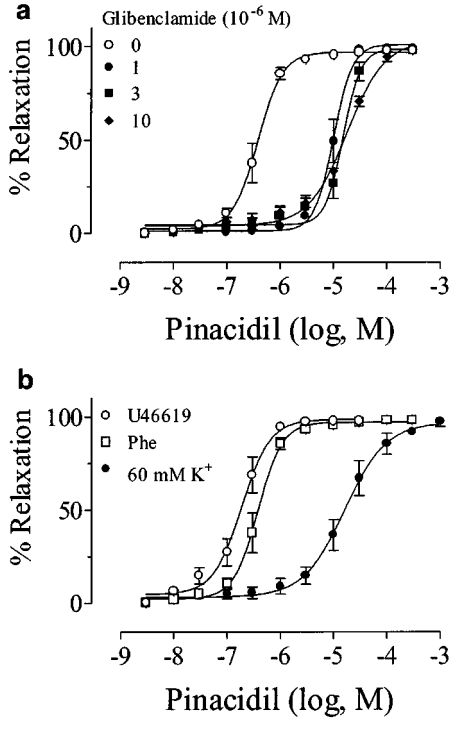

Figure 1 (a) Effect of glibenclamide (1–10 μM) on pinacidil-induced relaxation in endothelium-denuded rings preconstricted by phenylephrine. (b) The relaxant effect of pinacidil in rings preconstricted by 10 nM U46619, 5 μM phenylephrine, and 60 mM extracellular K^+. Data are mean of s.e.mean of 5–6 experiments.

Effects of cyclopiazonic acid, thapsigargin and ouabain

Following inhibition of K^+ channels and VGCCs, the pinacidil-induced vasorelaxant response was not modified by pretreatment with the endoplasmic reticulum Ca^{2+}-ATPase inhibitors, CPA (10 μM) and thapsigargin (Tg, 1 μM), or by the Na^+-K^+-ATPase inhibitor, ouabain (100 μM). The pD_2 values were 4.69 ± 0.13 in control, 4.71 ± 0.11 in CPA, 4.84 ± 0.23 in Tg, and 4.64 ± 0.23 in ouabain, respectively ($n=5-6$, $P>0.05$ as compared with the control, data not shown). None of these inhibitors influenced baseline tone.

Effects of propranolol, Rp-8-cAMPs, and nitric oxide inhibitors

Treatment with propranolol (10 μmol l^{-1}) did not alter pinacidil-induced relaxation following inhibition of K^+ channel and VGCCs (pD_2: 4.69 ± 0.13 in control, 4.36 ± 0.13 in propranolol, $n=5-6$, $P>0.05$). L-NNA (100 μM), ODQ (10 μM) or Rp-cAMPS (3 μM) had no effect on the relaxant response to pinacidil (pD_2: 4.69 ± 0.13 in control, 4.34 ± 0.27 in L-NNA, 4.44 ± 0.17 in ODQ, and 4.46 ± 0.19 in Rp-cAMPS, $n=5-6$, $P>0.05$ as compared with the control, data not shown). None of these agents influenced baseline tone.

Effects of Na^+-Ca^{2+} exchanger inhibitors

Pretreatment with Ni^{2+} (30 μM), a putative Na^+-Ca^{2+} exchanger inhibitor significantly inhibited the pinacidil-induced relaxation following inhibition of K^+ channels and VGCCs. Figure 3a shows that presence of Ni^{2+} caused rightward shift of the concentration-relaxation curve for

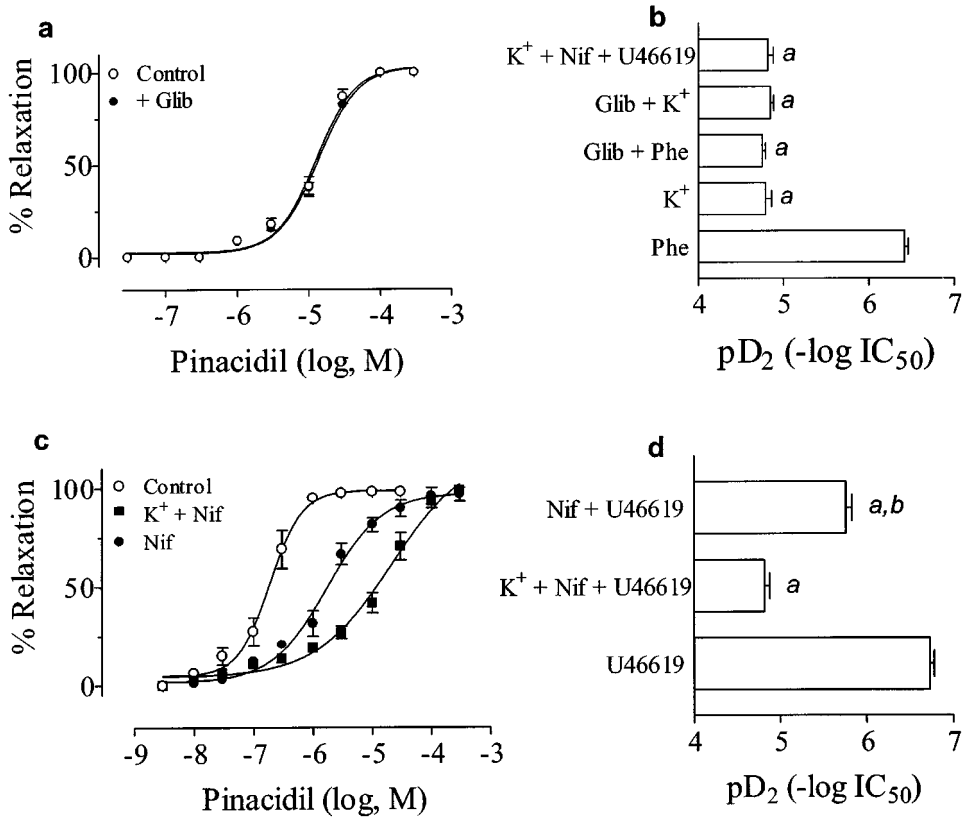

Figure 2 (a) The relaxant effect of pinacidil in phenylephrine-preconstricted rings using a protocol for inhibition of K^+ channels and VGCCs in the absence and presence of 10 μM glibenclamide. (b) The pD_2 values for pinacidil-induced relaxation under various conditions. (c) Pinacidil-induced relaxation of U46619-contracted rings in control, in the presence of 1 μM nifedipine and in the presence of 60 mM K^+ plus 1 μM nifedipine. (d) pD_2 values for pinacidil-induced relaxation in data presented in (c). Data are mean$\pm$s.e.mean of six experiments. A significant difference ($P<0.05$) is indicated by *a* between control and treatment groups, and *b* between Nif+U46619 and K^++Nif+U46619 groups (one-way ANOVA).

pinacidil (pD_2: 4.69$\pm$0.13 in control; 4.09$\pm$0.10 in nickel, $n=6$, $P<0.05$). In order to exclude the possibility that the effect of Ni^{2+} may be associated with membrane depolarization in a high K^+-containing solution, it was worthwhile testing whether Ni^{2+} should have a similar effect in a normal Krebs solution in the presence or absence of 1 μM nifedipine. In the presence of nifedipine, pinacidil caused relaxations with a pD_2 of 5.85$\pm$0.06 ($n=6$) in U46619-contracted rings. Pretreatment with Ni^{2+} significantly attenuated the relaxant response to pinacidil (pD_2: 5.33$\pm$0.05 and 4.89$\pm$0.14 in 30 and 300 μM Ni^{2+}, respectively, $n=5$, $P<0.05$ as compared with the control, Figure 3b). Besides, benzamil, another inhibitor of the Na^+-Ca^{2+} exchanger also inhibited pinacidil-induced relaxation in the presence of 1 μM nifedipine (pD_2: 4.88$\pm$0.08, $n=5$, $P<0.05$, Figure 3c). Traces in Figure 4 show the inhibitory effect of 300 μM Ni^{2+} and 20 μM benzamil on the relaxant responses to pinacidil in normal Krebs solution without nifedipine. Treatment with Ni^{2+} and benzamil attenuated pinacidil-induced relaxation (pD_2: 6.73$\pm$0.05 in control; 6.23$\pm$0.05 in 30 μM Ni^{2+}; 5.61$\pm$0.12 in 300 μM Ni^{2+}, $n=5-6$, Figure 4d; and 5.59$\pm$0.13 in 20 μM benzamil, $n=5-6$, Figure 4e; $P<0.05$ as compared with the control). In addition, Ni^{2+} at 300 μM and benzamil at 20 μM also inhibited pinacidil (20 μM)-induced relaxation in the presence of 10 μM glibenclamide by 45% and 20%, respectively, in the phenylephrine-contracted rings ($n=6$, $P<0.05$, Figure 5).

If pinacidil-induced K_{ATP} channel-independent relaxation is partially mediated through stimulation of Na^+-Ca^{2+} exchanger as suggested by these data, removal of extracellular Na^+ should be expected to exert a similar effect to the Na^+-Ca^{2+} exchanger inhibitors. Indeed, traces in Figure 6a–c show that the pinacidil (1 μM)-induced relaxation was inhibited in 25 mM Na^{2+}-containing solution and further reduced in Na^+-free Krebs solution. In a Na^+-free solution, benzamil at 20 μM failed to inhibit the pinacidil-induced relaxation (Figure 6d). The percentage relaxation induced by 1 μM pinacidil in U46619-contracted rings under different conditions is summarized in Figure 6e, $n=5-6$).

Effect of nickel on nitroprusside-mediated relaxation

Pretreatment with Ni^{2+} (300 μM) did not affect the nitroprusside-induced relaxation (pD_2: 9.02$\pm$0.32 in control and 8.68$\pm$0.12 in Ni^{2+}, $n=5$, $P>0.05$) (data not shown).

Effects of protein kinase C-mediated contraction

Phorbol 12,13-diacetate (PDA, 1 μM) induced steady contraction following inhibition of K^+ channels and VGCCs. Pinacidil produced concentration-dependent relaxations with

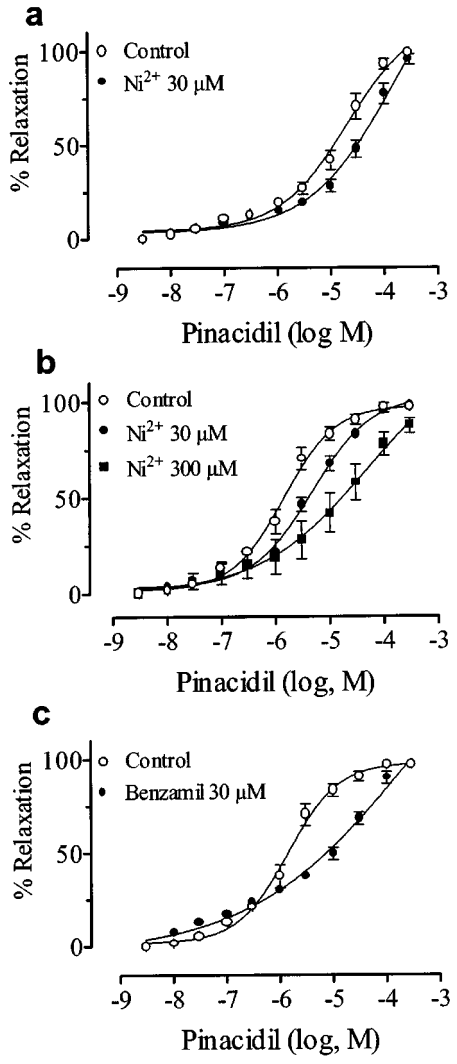

Figure 3 The inhibitory effect of Ni^{2+} (30 μM) on pinacidil-induced relaxation following inhibition of K^+ channels and VGCCs (a). The inhibitory effect of (b) Ni^{2+} (30–300 μM) or (c) Benzamil (20 μM) on pinacidil-induced relaxation in normal Krebs solution containing 1 μM nifedipine. Data are mean ± s.e.mean of 5–6 experiments.

a pD_2 of 4.82 ± 0.06 ($n = 6$). This value was not different from that obtained in U46619-contracted rings (pD_2: 4.69 ± 0.13, $n = 6$, $P > 0.05$).

Discussion

The present results show both K^+ channel-dependent and -independent relaxant responses to pinacidil in isolated rat mesenteric artery rings. We have provided novel evidence suggesting a role of the forward mode Na^+-Ca^{2+} exchange pathway in the K^+ channel-independent relaxation to pinacidil within the therapeutic doses in endothelium-denuded rings.

Pinacidil relaxed the artery rings preconstricted by U46619, phenylephrine, elevated extracellular K^+ and active phorbol ester with decreasing potency while the maximum response remained unaltered. There is ~86 fold (IC_{50} values) reduction for the pinacidil effect in high K^+- contracted rings as compared with that in U46619-contracted rings. Glibenclamide at 10 μM maximally attenuated pinacidil-induced relaxation. The IC_{50} value for the relaxant effect of pinacidil in the presence of glibenclamide, a potent blocker of vascular K_{ATP} channels (Standen *et al*., 1989; Nelson *et al*., 1990) is the same as that in the presence of 60 mM K^+ (16.2 μM versus 17.6 μM). Glibenclamide failed to influence the relaxant effect of pinacidil in high K^+-contracted rings. These results suggest that both experimental conditions could eliminate the K^+ channel-dependent component of pinacidil-induced relaxation, thus validating our protocol used for examining a K^+ channel-independent effect.

Sarcolemmal Na^+-Ca^{2+} exchange plays a significant role in regulation of $[Ca^{2+}]_i$ in smooth muscle cells and thus vessel tone (Motley *et al*., 1993). The activity of the Na^+-Ca^{2+} exchanger is coupled to $[Na^+]_i$, which is primarily regulated by the membrane permeability to Na^+ ions and the Na^+-K^+-ATPase activity. Decreased permeability to Na^+ and/or increased activity of Na^+-K^+ pump results in a reduction in $[Na^+]_i$, which then stimulates the forward mode of the Na^+-Ca^{2+} exchanger. A defect in the Na^+-Ca^{2+} exchange translocation pathway may contribute to altered $[Ca^{2+}]_i$ in the renal arterioles in salt-sensitive hypertension (Nelson *et al*., 1999; Bell *et al*., 2000).

Treatment with Ni^{2+}, a putative Na^+-Ca^{2+} exchanger inhibitor significantly inhibited pinacidil-induced relaxation following inhibition of K^+ channels and VGCCs. This finding points to the possibility that a K^+ channel-independent relaxation induced by pinacidil may be partly mediated through stimulation of Ca^{2+} efflux via the Na^+-Ca^{2+} exchanger. Although Ni^{2+} could inhibit the VGCCs in some vascular smooth muscle cells (Petkov *et al*., 2001), the attenuated relaxation should not be due to the VGCCs blocking effect of Ni^{2+} in the presence of nifedipine, a selective blocker of L-type of VGCCs. Instead, inhibition of the VGCCs, if any, would enhance pinacidil-induced relaxation. In order to investigate whether the effect of Ni^{2+} is related to the membrane depolarization in the high K^+-containing solution, similar experiments were conducted in normal K^+-containing solution in the presence or absence of 1 μmol l^{-1} nifedipine. Ni^{2+} (30–300 μM) again significantly inhibited the pinacidil-induced relaxation. Besides, both benzamil and Ni^{2+} also attenuated pinacidil-induced relaxation in the presence of glibenclamide at 10 μM, a concentration that maximally inhibited the K_{ATP} channel-dependent effect of pinacidil (see Figure 1a). The effect of Ni^{2+} is unlikely non-specific. This is supported by the following three additional pieces of evidence. Firstly, Ni^{2+} (300 μM) did not modify the cyclic GMP-mediated relaxant response to nitroprusside in the same preparations. Secondly, benzamil, another inhibitor of Na^+-Ca^{2+} exchanger (Schweda *et al*., 2001) also inhibited pinacidil-induced relaxation in normal Krebs solution with or without nifedipine. Lastly, the pinacidil-induced relaxation was markedly impaired in Na^+-free solution, a condition that abolishes the influence of Na^+-Ca^{2+} exchanger in vessel tone.

Lack of effect of ouabain, a Na^+-K^+-ATPase inhibitor indicates that stimulation of the Na^+-K^+-pump activity is unlikely to be involved in the K^+ channel-independent relaxant effect of pinacidil. It is conceivable that the Na^+-K^+ pump activity may be low at resting or contracted states since ouabain did not increase the baseline tone nor

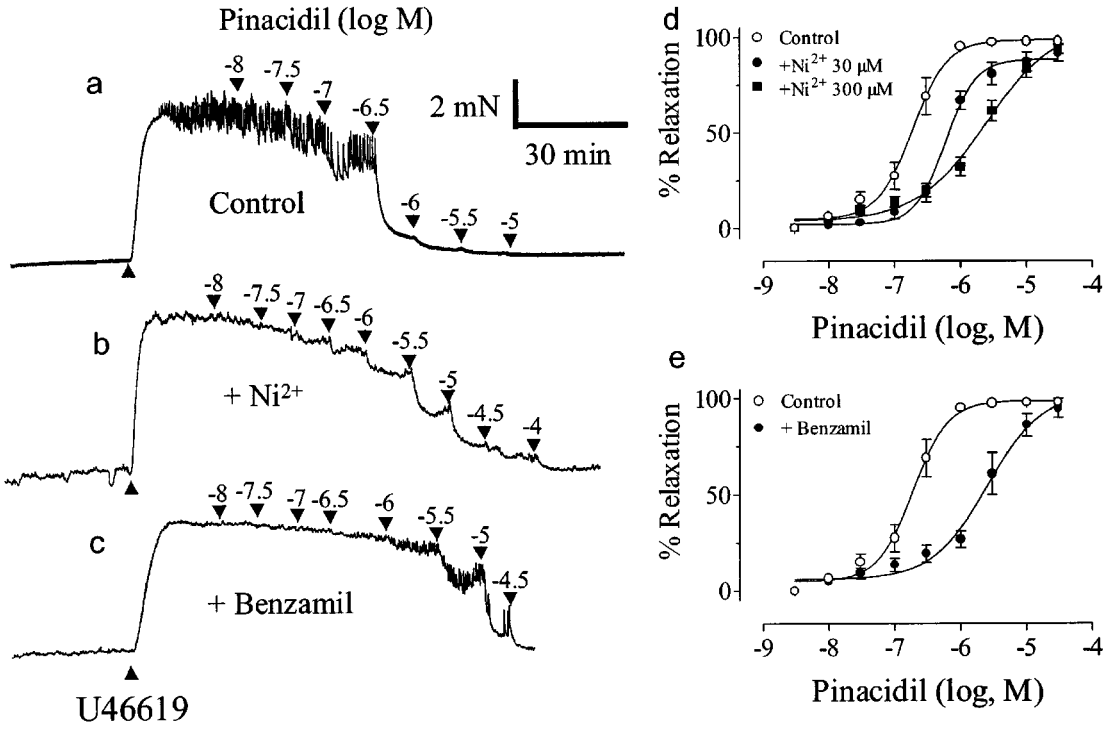

Figure 4 Traces showing pinacidil-induced relaxations of U46619-constricted rings in control (a) and in the presence of 300 μM Ni^{2+} (b) or 20 μM benzamil (c) in normal Krebs solution. Concentration-response curves for pinacidil-induced relaxation in the presence of (d) Ni^{2+} (30–300 μM) or (e) benzamil (20 μM). Data are mean±s.e.mean of six experiments.

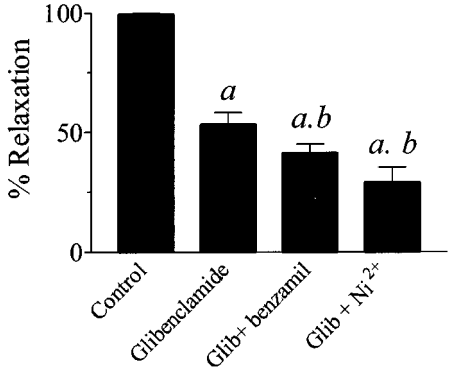

Figure 5 The inhibitory effect of 300 μM Ni^{2+} and 20 μM benzamil on pinacidil (20 μM)-induced relaxation in the presence of 10 μM glibenclamide in phenylephrine-constricted rings. A significant difference ($P<0.05$) is indicated by *a* between control and treatment groups and *b* between glibenclamide and other treatment groups (One-way ANOVA). Data are mean±s.e.mean of six experiments.

augmented the U46619-induced contraction as would be expected if this pump plays a significant role in maintaining the cell membrane potential.

The primary mechanism whereby the K^+ channel openers relax smooth muscle cells is the activation of K^+ channel and subsequent membrane hyperpolarization that in turns inhibits the VGCCs. Pinacidil displayed a reduced potency by ~10 fold in relaxing U46619-contracted rings in 60 mM K^+-plus nifedipine-containing solution (blocked K^+ conductance and VGCCs) than in nifedipine-containing normal K^+ solution (intact K^+ conductance). It appears that other hyperpolarization-dependent intracellular mechanisms are also operative in addition to inhibition of Ca^{2+} influx. These may include several reported hyperpolarization-related effects of pinacidil in smooth muscle cells. Pinacidil inhibited noradrenaline-induced Ca^{2+} release from internal stores through an inhibition of IP_3 formation resulting from its hyperpolarization action in rabbit mesenteric artery (Itoh *et al.*, 1992). Pinacidil inhibited the ryanodine-sensitive oscillatory outward K^+ current induced by Ca^{2+} released from an intracellular store and glibenclamide prevented the action of pinacidil (Xiong *et al.*, 1991). This indicates the presence of an additional site to K^+ channels for the vasodilator actions of pinacidil at which glibenclamide can act as an antagonist. It was suggested that pinacidil may decrease Ca^{2+} sensitivity of the contractile proteins (Meisheri *et al.*, 1991), but another research group had argued against this possibility (Anabuki *et al.*, 1990).

Pinacidil potentiated the β_1-adrenoceptor-mediated coronary vasodilation *in vivo* (Katsuda *et al.*, 1996). Propranolol, a non-selective β-adrenoceptor antagonist was reported to produce an antagonistic effect on pinacidil-induced coronary relaxation in the presence of glibenclamide (Kalsner, 1994). It is possible that pinacidil may stimulate vascular β-adrenoceptors or potentiate β-adrenoceptor-mediated activation of vascular K^+ channels (Randall & McCulloch, 1995; Huang & Kwok, 1997). However, our study clearly ruled out this likelihood since propranolol did not affect pinacidil-induced relaxation. Propranolol was indeed reported to inhibit both inward rectifier and K_{ATP} currents in isolated neonatal rat cardiac myocytes (Xie *et al.*, 1998).

The role of cyclic AMP seems unlikely because treatment with Rp-cAMPS, a potent membrane-permeable inhibitor of cyclic AMP-dependent protein kinase did not alter the

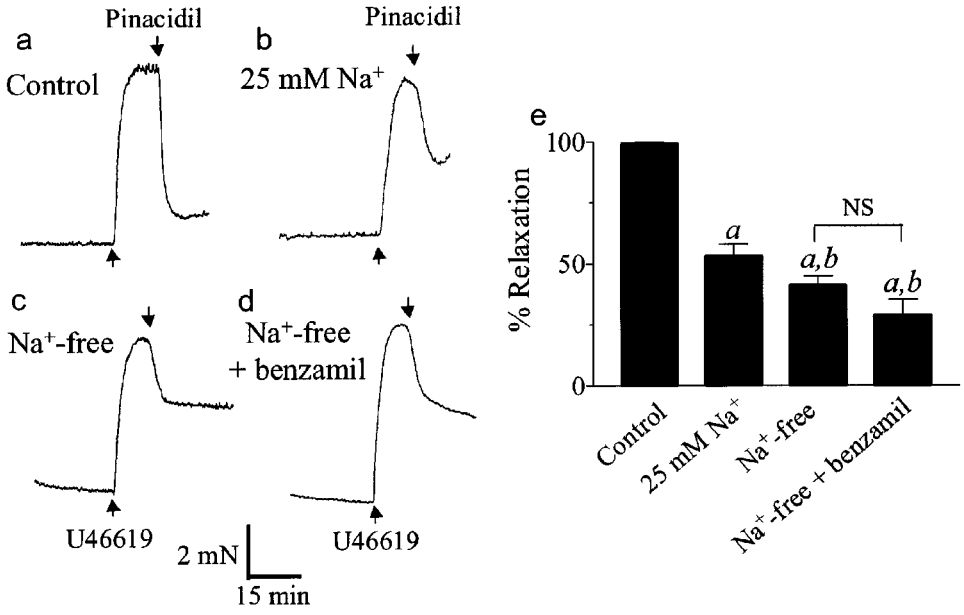

Figure 6 Traces showing pinacidil (1 μM)-induced relaxation in U46619-constricted rings in the control (a), in the presence of 25 mM extracellular Na^+ (b), in Na^+-free solution (c), and in Na^+-free solution containing 20 μM benzamil (d). The pinacidil-induced relaxation under various conditions (e, $n=5-6$). A significant difference ($P<0.05$) is indicated by *a* between control and treatment groups, and *b* between 25 mM Na^+ and other treatment groups (One-way ANOVA). Data are mean$\pm$s.e.mean of 5–6 experiments.

relaxant response to pinacidil. Neither L-NNA nor ODQ (a selective guanylate cyclase inhibitor) reduced the relaxant effect of pinacidil, thus discounting the involvement of nitric oxide or cyclic GMP.

CPA and thapsigargin, the endoplasmic reticulum Ca^{2+}-ATPase inhibitors (which could activate the store-operated Ca^{2+} entry) did not affect pinacidil-induced relaxation. These results indicate that pinacidil neither stimulated Ca^{2+} uptake into the endoplasmic reticulum nor inhibited the store-operated Ca^{2+} entry as other possible K^+ channel-independent mechanisms. Although pinacidil was described to inhibit intracellular Ca^{2+} mobilization, this action was thought to be indirect, resulting from a decrease in IP_3 production and was hyperpolarization-dependent and glibenclamide-sensitive (Itoh *et al*., 1992; Yanagisawa *et al*., 1993). Pinacidil relaxed the rings preconstricted by U46619 and PDA to the same extent following inhibition of K^+ channels and VGCCs, suggesting that PKC-mediated VGCCs-independent intracellular cascade may also be a target for the action of pinacidil. However, this occurs only if the concentration of pinacidil exceeds 1 μM, normally beyond the suggested therapeutic concentration range (80–300 $\mu g\,l^{-1}$, equivalent to 0.3–1.3 μM) (McBurney *et al*., 1987).

Taken together, this study provides some new findings on the cellular mechanisms underlying the vasorelaxant response to pinacidil. Apart from opening of the vascular K^+ channels, stimulation of the Na^+-Ca^{2+} exchange as a novel mechanism whereby pinacidil relaxes the rat mesenteric artery at concentrations (0.1–1 μM) that fall into its therapeutic dose range in human plasma (McBurney *et al*., 1987; Goldberg *et al*., 1989). This effect is independent of K^+ channels, L-type VGCCs, or the cell membrane potential. However, it is yet to be determined whether the Na^+-Ca^{2+} exchange should also play a role in the vasodilator responses to other structurally related K^+ channel openers within their therapeutic doses.

This study was supported by CUHK Mainline Research Grant (44M4032). SY Tsang and CM Wong are the recipients of CUHK PhD Studentship.

References

AHNFELT-RONNE, I. (1988). Pinacidil: History, basic pharmacology, and therapeutic implications. *J. Cardiovasc. Pharmacol.,* **12** (Suppl. 2), S1–S4.

ANABUKI, J., HORI, M., OZAKI, H., KATO, I. & KARAKI, H. (1990). Mechanisms of pinacidil-induced vasodilatation. *Eur. J. Pharmacol.,* **190,** 373–379.

BELL, P.D., MASHBURN, N. & UNLAP, M.T. (2000). Renal sodium/calcium exchange; a vasodilator that is defective in salt-sensitive hypertension. *Acta Physiol. Scand.,* **168,** 209–214.

BYCHKOV, R., GOLLASCH, M., RIED, C., LUFT, F.C. & HALLER, H. (1997). Effects of pinacidil on K^+ channels in human coronary artery vascular smooth muscle cells. *Am. J. Physiol.,* **273,** C161–171.

FRIEDEL, H.A. & BROGDEN, R.N. (1990). Pinacidil. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in the treatment of hypertension. *Drugs* **39,** 929–967.

GOJKOVIC BUKARICA, L., KAZIC, T., SAJIC, Z., DJUKANOVIC, B., PANIC, G., PERIC, M. & BOJIC, M. (1997). The effects of levcromakalim and pinacidil on the human internal mammary artery. *Fundam. Clin. Pharmacol.,* **11,** 550 – 560.

GOLDBERG, M.R., ROCKHOLD, F.W., THOMPSON, W.L. & DESANTE, K.A. (1989). Clinical pharmacokinetics of pinacidil, a potassium channel opener, in hypertension. *J. Clin. Pharmacol.,* **29,** 33 – 40.

GOLLASCH, M., BYCHKOV, R., RIED, C., BEHRENDT, F., SCHOLZE, S., LUFT, F.C. & HALLER, H. (1995). Pinacidil relaxes porcine and human coronary arteries by activating ATP-dependent potassium channels in smooth muscle cells. *J. Pharmacol. Exp. Ther.,* **275,** 681 – 692.

HUANG, Y. & KWOK, K.H. (1997). Effects of putative K^+ channel blockers on β-adrenoceptor-mediated vasorelaxation of rat mesenteric artery. *J. Cardiovasc. Pharmacol.,* **29,** 515 – 519.

ITOH, T., SEKI, N., SUZUKI, S., ITO, S., KAJIKURI, J. & KURIYAMA, H. (1992). Membrane hyperpolarization inhibits agonist-induced synthesis of inositol 1,4,5-trisphosphate in rabbit mesenteric artery. *J. Physiol.,* **451,** 307 – 328.

KALSNER, S. (1994). Propranolol antagonizes coronary artery relaxation by a potassium channel opener. *Life. Sci.,* **55,** 1109 – 1121.

KATSUDA, Y., EGASHIRA, K., UENO, H., ARAI, Y., AKATSUKA, Y., KUGA, T., SHIMOKAWA, H. & TAKESHITA, A. (1996). ATP-sensitive K^+ channel opener pinacidil augments β_1-adrenoceptor-induced coronary vasodilation in dogs. *Am. J. Physiol. Heart Circ. Physiol.,* **39,** H2210 – H2215.

MCBURNEY, A., FARROW, P.R. & WARD, J.W. (1987). Effects of formulation on the pharmacokinetics of orally administered pinacidil in humans. *J. Pharm. Sci.,* **76,** 940 – 941.

MEISHERI, K.D., SWIRTZ, M.A., PUROHIT, S.S., CIPKUS-DUBRAY, L.A., KHAN, S.A. & OLEYNEK, J.J. (1991). Characterization of K^+ channel-dependent as well as -independent components of pinacidil-induced vasodilation. *J. Pharmacol. Exp. Ther.,* **256,** 492 – 499.

MOTLEY, E.D., PAUL, R.J. & MATLIB, M.A. (1993). Role of Na^+-Ca^{2+} exchange in the regulation of vascular smooth muscle tension. *Am. J. Physiol. Heart Circ. Physiol.,* **264,** H1028 – H1040.

NELSON, L.D., UNLAP, M.T., LEWIS, J.L. & BELL, P.D. (1999). Renal arteriolar Na^+/Ca^{2+} exchange in salt-sensitive hypertension. *Am. J. Physiol. Renal Physiol.,* **45,** F567 – F573.

NELSON, M.T., HUANG, Y., BRAYDEN, J.E., HESCHELER, J. & STANDEN, N.B. (1990). Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. *Nature,* **344,** 770 – 773.

PETKOV, G.V., FUSI, F., SAPONARA, S., GAGOV, H.S., SGARAGLI, G.P. & BOEV, K.K. (2001). Characterization of voltage-gated calcium currents in freshly isolated smooth muscle cells from rat tail main artery. *Acta Physiol. Scand.,* **173,** 257 – 265.

QUAST, U. (1992). Potassium channel openers: pharmacological and clinical aspects. *Fund. Clin. Pharmacol.,* **6,** 279 – 293.

QUAST, U. (1993). Do the K^+ channel openers relax smooth muscle by opening K^+ channels? *TiPS,* **14,** 332 – 337.

RANDALL, M.D. & MCCULLOCH, A.I. (1995). The involvement of ATP-sensitive potassium channels in β-adrenoceptor-mediated vasorelaxation in the rat isolated mesenteric arterial bed. *Br. J. Pharmacol.,* **115,** 607 – 612.

SCHWEDA, F., KRAMER, B.K. & KURTZ, A. (2001). Differential roles of the sodium-calcium exchanger in renin secretion and renal vascular resistance. *Pflügers Arch.,* **442,** 693 – 699.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science,* **245,** 177 – 180.

STOCKBRIDGE, N., ZHANG, H. & WEIR, B. (1991). Effects of K^+ channel agonists cromakalim and pinacidil on rat basilar artery smooth muscle cells are mediated by Ca^{++}-activated K^+ channels. *Biochem. Biophys. Res. Commun.,* **181,** 172 – 178.

VIDEBAEK, L.M., AALKJAER, C. & MULVANY, M.J. (1988). Pinacidil opens K^+-selective channels causing hyperpolarization and relaxation of noradrenaline contractions in rat mesenteric resistance vessels. *Br. J. Pharmacol.,* **95,** 103 – 108.

XIE, L.H., TAKANO, M. & NOMA, A. (1998). The inhibitory effect of propranolol on ATP-sensitive potassium channels in neonatal rat heart. *Br. J. Pharmacol.,* **123,** 599 – 604.

XIONG, Z.L., KAJIOKA, S., SAKAI, T., KITAMURA, K, & KURIYAMA, H. (1991). Pinacidil inhibits the ryanodine-sensitive outward current and glibenclamide antagonizes its action in cells from the rabbit portal vein. *Br. J. Pharmacol.,* **102,** 788 – 790.

YANAGISAWA, T., YAMAGISHI, T. & OKADA, Y. (1993). Hyperpolarization induced by K^+ channel openers inhibits Ca^{2+} influx and Ca^{2+} release in coronary artery. *Cardiovasc. Drugs. Ther.,* **7** (Suppl 3), 565 – 574.

(Received June 12, 2002
Revised October 21, 2002
Accepted October 28, 2002)