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ATP-sensitive Potassium-channel-opening Activity of CL-065, a 3-[(Substituted-carbonyl)amino]-2*H*-1-benzopyran, in the Rat

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Abstract

The pharmacological activity of CL-065 (*trans*-3-acetamido-2,2-dimethyl-4-hydroxy-3,4-dihydro-2*H*-1-benzopyran-6-carbonitrite) was investigated in anaesthetized spontaneously hypertensive rats (SHR) and isolated thoracic aorta of Sprague-Dawley rats.

The intravenous administration of CL-065 (0·1–2·0 mg kg^{-1}) to anaesthetized SHR induced a dose-dependent reduction of mean arterial pressure (MAP) with maximum effect approximately 5 min after injection and which persisted for over 3 h. CL-065 also induced a reflex tachycardia which seemed to parallel the time course of the hypotensive effect. The hypotensive effect of CL-065 was blocked by pretreatment with glibenclamide (5 mg kg^{-1}, i.v.), a specific ATP-sensitive potassium (K_{ATP}) channel blocker. Moreover, CL-065 (0·01–10 μM) resulted in dose-dependent vasodilatory effects on phenylephrine (0·3 μM)-induced vasoconstriction in isolated thoracic aorta. The vasorelaxation elicited by CL-065 was antagonized competitively by pretreatment with glibenclamide (0·1–1·0 μM; $pA_2 = 6{\cdot}90 \pm 0{\cdot}09$; slope $= 1{\cdot}03 \pm 0{\cdot}18$). Similarly, the other two K_{ATP}-channel openers cromakalim (1·0 nM–1·0 μM) and nicorandil (0·1–30 μM) also induced vasorelaxation in thoracic aorta. The EC50 of cromakalim, CL-065 and nicorandil (i.e. the doses having half the maximum effect) were approximately 0·083, 0·17, and 4·5 μM, respectively, for phenylephrine (0·3 μM)-induced vasoconstriction in isolated thoracic aorta. Moreover, increased extracellular potassium levels (20–60 mM) resulted in concentration-dependent attenuation of the vasodilator effect of CL-065.

In conclusion, CL-065 induces a depressor effect via activation of K_{ATP} channels.

ATP-sensitive potassium (K_{ATP}) channels, which are blocked by intracellular ATP and activated when intracellular ATP levels decrease, are found in a wide variety of tissues including heart and skeletal muscle, the brain and the pancreas (Ashcroft 1988). It has been suggested that K_{ATP} openers might be of potentially great value as therapeutically useful agents (Quast & Cook 1989; Atwal 1994). They are principally characterized by their ability to relax muscle tissues via the opening of K^+ channels leading to outward flow of K^+ ions and to membrane hyperpolarization, which results in a reduction of intracellular calcium by blocking both of voltage-regulated calcium channels and intracellular calcium release (Weston & Edwards 1992). Their vasodilating effects are thought to be their main clinical utility. However, in clinical and preclinical studies the use of K_{ATP} openers for the treatment of hypertension has been generally demonstrated not to be more advantageous than that of the widely used anti-hypertensive drugs because the potent vasodilation of K_{ATP} openers caused side-effects such as reflex tachycardia, oedema, headache and flushing (Atwal 1994; Colatsky & Hamilton 1996). Therefore, additional work must be performed to find new drugs of this class which have greater clinical potential for the treatment of various diseases.

Since the discovery of cromakalim (Hamilton & Weston 1989) one kind of K_{ATP}-channel opener containing a benzopyran ring system, a series of novel compounds based on the structure of cromakalim, has been shown to relax blood vessels and reduce blood pressure (Ashwood et al 1986, 1991; Evans & Stemp 1996) and pinacidil and nicorandil are further structural analogues of cromakalim now used in clinical treatment (Atwal 1994; Haeusler & Lues 1994). Recently, when screening a series of synthesized benzopyran deri-

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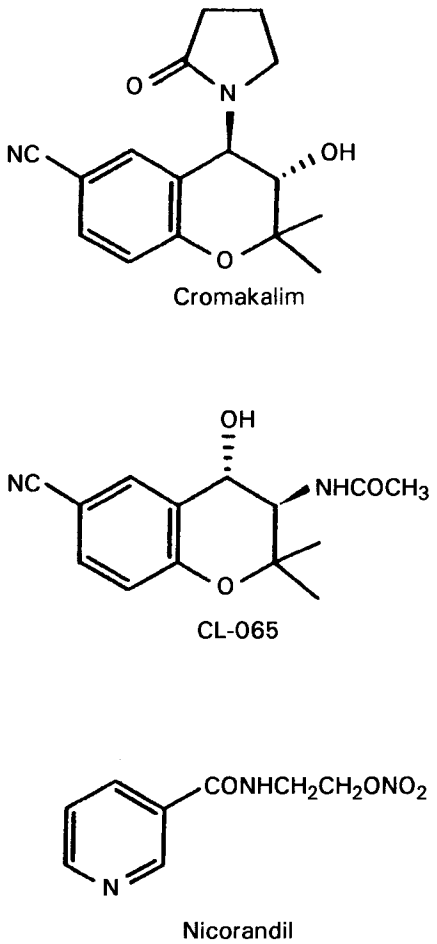

Figure 1. The chemical structures of cromakalim, CL-065 and nicorandil.

vatives for anti-hypertensive effects, we found that the derivative *trans*-3-acetamido-2,2-dimethyl-4-hydroxy-3,4-dihydro-2*H*-1-benzopyran-6-carbonitrite (CL-065) (Figure 1), which is a mixture of enantiomers, has K_{ATP}-channel-opening activity. In previous studies, this chemical has been synthesized and reported to have anti-hypertensive activity in conscious spontaneously hypertensive rats (SHR; Cassidy et al (1992)) which could be attenuated by pretreatment with glibenclamide, a well-known specific K_{ATP}-channel blocker (Ashcroft 1988). Therefore, the purpose of this study was to evaluate more extensively the mechanism of the hypotensive and vasorelaxant activity of CL-065, and to compare its potency with that of the other K_{ATP}-channel openers cromakalim and nicorandil.

Materials and Methods

Drugs

CL-065 was synthesized by Dr C. W. Chen, Department of Pharmacy, College of Medicine, National Taiwan University, and dissolved in 0·1% dimethylsulphoxide (DMSO). Glucose, magnesium chloride and phenylephrine were obtained from Sigma (St Louis, MO) and sodium chloride, potassium chloride, sodium hydrogen carbonate and monobasic potassium phosphate from Wako (Japan). Cromakalim was from Biomol and prepared in DMSO. Nicorandil was from Chugai and dissolved in distilled water. Glibenclamide was purchased from RBI and dissolved in DMSO. Atropine sulphate salt and propranolol hydrochloride were purchased from Sigma and dissolved in distilled water.

Measurement of blood pressure in spontaneously hypertensive rats

The study was performed on the Wistar strain of SHR developed through selective breeding techniques by Okamoto & Aoki (1963). The close resemblance of haemodynamics in essential hypertension in man and in SHR justifies the widespread investigations on this experimental model (Udenfriend 1972; Page 1974). Male SHR, 250–350 g, 12–16 weeks, (mean arterial pressure 155 ± 12 mmHg; heart rate 360 ± 15 beats min^{-1}), were used in this study. The rats, whose stock originated from the Charles River Breeding Laboratories in Japan, were purchased from the Department of Laboratory Animal Science of the National Defense Medical Center, were caged individually in clear plastic cages kept in an environmentally controlled room maintained at 23°C, relative humidity 55%, and a 12-h light–dark cycle. Rats were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) and the left femoral artery and vein were cannulated with polyethylene-50 tubing to monitor blood pressure and for drug administration (CL-065, nicorandil and cromakalim). Blood pressure was measured with a pressure transducer (Statham P23D; Gould, CA) via a polyethylene-50 cannula placed in the right femoral artery. The heart rate was measured through a tachography pre-amplifier (Grass Model 7P4; Grass Instruments, MA) triggered with pulses of arterial blood pressure. All data were recorded on a polygraph (Grass Model 7D). The body temperature was maintained at $37 \pm 0{\cdot}5$°C with a heating pad and monitored with a rectal thermometer.

Preparation of rat isolated aortae

Experiments were performed on normotensive Sprague-Dawley rats, 270–350 g, of both sexes. The animals were anaesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.), the thoracic aortae were isolated, and excess fat and connective tissue were removed. Vessels were cut into rings

approximately 3–4 mm in length and mounted in organ baths containing Krebs solution of composition (mM): NaCl, 118; KCl, 4·7; $NaHCO_3$, 25; KH_2PO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 2·5 and glucose, 11. The tissue bath solution was maintained at 37°C and oxygenated with a mixture of 95% O_2 and 5% CO_2. Two stainless steel hooks were inserted into the aortic lumen in tissue organ baths containing 20 mL Krebs solution; one was fixed and the other was connected to a transducer (Grass FT03). Aortae were equilibrated in the medium for 90 min with three changes of Krebs solution. Each ring was progressively stretched to the optimum point on its length–tension curve as determined by the active tension developed to phenylephrine (1 μM) and maintained under an optimum resting tension (2 g) before specific experimental processes were initiated. Vasocontractions were recorded isometrically via a force-displacement transducer connected to a Grass Model 7D polygraph. Aorta was pre-contracted with phenylephrine (0·3 μM) or high levels of potassium ion (20–60 mM). When this contraction reached a steady state different concentrations of cromakalim (1·0 nM–1·0 μM), CL-065 (0·01–10 μM) or nicorandil (0·1–30 μM) were added cumulatively to the tissue bath to produce a concentration–response curve. In other experiments, concentration–response curves of cromakalim, CL-065 and nicorandil were studied in aortae pretreated with different concentrations of glibenclamide (0·1, 0·3 and 1·0 μM) before addition of phenylephrine (0·3 μM) to trigger vasoconstriction.

Haemodynamic analysis

Haemodynamic analysis was performed with six male SHR, 14–16 weeks, as previously described (Hu et al 1994). In brief, animals were anaesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}) and a tracheotomy was performed to provide artificial ventilation with a tidal volume of 3–5 mL and respiratory rate of 50–70 breaths min^{-1}. The left femoral artery was cannulated for the recording of femoral arterial pressure and the left femoral vein for the administration of supplemental anaesthetics and drugs. The chest was opened through the left third intercostal space. An electromagnetic flow probe (Carolina Medical Electronics, Model 100 series, internal circumference 9 mm) was placed around the ascending aorta for measurement of the aortic blood flow. A Millar catheter with one high-fidelity pressure sensor (Millar Instruments, Model SPR-407, Size 2F) was used to measure aortic pressure. To minimize baseline drift the catheter was soaked in saline at room temperature for at least 1 h before insertion. The Millar catheter was inserted via the isolated right carotid artery into the ascending aorta until the catheter tip reached a position just distal to the flow-probe.

The aortic pressure and flow waves were continuously monitored with a polygraph recorder (Gould, model 2800S) and also recorded on a tape recorder (TEAC, model MR-30) at a recording speed of 4·8 cm s^{-1} for off-line analysis. All data were recorded after the pressure and flow signals had been stable for 3–5 min. The pressure and flow signals were digitized at 1-ms intervals using a 12-bit analogue-to-digital converter (Microstar Laboratories, model DAP 1200/4) interfaced to a personal computer. All haemodynamic parameters were calculated beat by beat. The average value from four beats was used as an individual data point. Heart rate, stroke volume, mean aortic pressure and systemic vascular resistance were also determined for each beat. Cardiac output was the product of stroke volume and heart rate. All the data and derived haemodynamic parameters were analysed by computer programs developed in our laboratory. The data were expressed as means ± s.e.m. Haemodynamic parameters of SHR were compared by use of the paired *t*-test.

Procedures for measurement of aortic pressure and flow in rats were essentially similar to those described previously (Zuckerman & Yin 1989; Hu et al 1994). Because the Millar catheter (size 2F) used in small animals is only equipped with a pressure sensor for monitoring the aortic pressure, the measurement of aortic flow requires open-chest surgery to place an electromagnetic flow probe around the aorta. The procedures caused a fall in arterial pressure, as reported in other studies (Zuckerman & Yin 1989; Hu et al 1994). The extent to which the surgical procedures and blood-pressure reduction affect haemodynamic data is not known for certain. We thus discarded data for which the fall in arterial pressure was > 20 mmHg after thoracotomy and flow-probe placement in the SHR. Although the selection could minimize the effects of haemodynamic perturbation, the results only pertained to measurement under open-chest conditions in SHR.

Data analysis

In all experiments the vasorelaxant effects of different concentrations of cromakalim, CL-065 and nicorandil were expressed as percentages of the maximum response. The slopes of the Schild plots were used to evaluate the competitive antagonism and pA_2 values were calculated for each concentration of cromakalim, CL-065 and nicorandil according to $pA_2 = -\log([\text{antagonist}][\text{dose ratio}]^{-1} - 1)$ (Mackay

1978). The experimental results are expressed as means ± s.e.m. The significance of in-vivo and in-vitro data was assessed by analysis of variance, then by a Dunnett's test for significant differences. P values < 0.05 were considered as indicative of significance.

Results

Relationships between the doses of K_{ATP} openers and MAP or heart-rate responses in anaesthetized SHR are depicted in Figures 2 and 3. Administration of CL-065 (0·1, 0·5, 1·0 and 2·0 mg kg^{-1}, i.v. bolus) induced dose-related reduction of MAP, which reached a maximum and steady state at 5 min. A significant reduction in blood pressure persisted for over 3 h (Figure 2A). Meanwhile, all doses of CL-065 induced reflex tachycardia in SHR which gradually returned to baseline values 2 h after injection. Intravenous administration of nicorandil (0·25, 0·5, 1·0 and 2·0 mg kg^{-1}) caused a similar dose-related reduction of MAP (Figure 2B). Peak responses were observed within 5 min of nicorandil injection. At the highest dose of nicorandil (2·0 mg kg^{-1}), the hypotensive effect persisted for approximately 1 h after injection and then returned to baseline. The effect of nicorandil on HR was not significant after injection of the lowest dose (0·25 mg kg^{-1}) but intermediate doses (0·5 and 1·0 mg kg^{-1}) also induced reflex tachycardia (Figure 2B). At the highest dose (2·0 mg kg^{-1}) there was an obvious decrease in HR ($P < 0.05$) which paralleled the drug's hypotensive effect. All heart rate changes elicited by nicorandil returned to normal about 1 h after injection. Moreover, intravenous administration of cromakalim (0·025, 0·05 and 0·1 mg kg^{-1}) also induced a dose-related reduction of MAP (Figure 2C); peak responses were obtained within 5 min. The depressor response of 0·1 mg kg^{-1} cromakalim could persist for over 3 h after injection. Cromakalim 0·025 mg kg^{-1} did not change HR significantly. In contrast, the intermediate dose (0·05 mg kg^{-1}) increased HR initially, but it then decreased progressively over a period of at least 2 h ($P < 0.05$) whereas the highest dose (0·1 mg kg^{-1}) of cromakalim induced a significant tachycardia which persisted for 3 h. On the other hand, pretreatment with glibenclamide (5 mg kg^{-1}), a selective K_{ATP}-channel blocker (Cavero et al 1989; Clapham et al 1991), completely blocked the hypotensive effects of CL-065 (Figure 3) and cromakalim, but only partially inhibited the hypotensive effect of nicorandil; these results were not influenced by pretreatment with atropine, propranolol or antihistamines (data not shown).

CL-065 (0·1 mg kg^{-1}, i.v.) gradually caused haemodynamic changes which reached a steady state 5 min after injection. Haemodynamic data were collected 10 min after administration of the drug. CL-065 induced a significant ($P < 0.01$) reduction in mean aortic pressure and an increase in stroke volume ($P < 0.05$). Heart rate was not altered statistically and so cardiac output increased by approximately 17% ($P < 0.05$). The peripheral resistance (Rp) of vessels was significantly ($P < 0.05$) reduced by approximately 51% in rat thoracic aorta (Table 1).

In rat thoracic aorta, phenylephrine (0·3 μM) caused a phasic contraction and then a tonic contraction maintained for at least 30 min. Treatment with CL-065 (0·01–10 μM), nicorandil (0·1–30 μM) or cromakalim (0·1 nM–1 μM) caused concentration-dependent vasorelaxation in the phenylephrine-induced vasoconstriction (Figure 4). At 10 μM, CL-065 almost abolished the vasoconstriction induced by phenylephrine. The EC50 of CL-065, cromakalim and nicorandil against phenylephrine (0·3 μM)-induced vasoconstriction were approximately 0·17, 0·083 and 4·5 μM, respectively.

Concentration–response curves were plotted for the effects of CL-065, nicorandil and cromakalim on phenylephrine (0·3 μM)-induced vasoconstriction of thoracic aorta after pre-incubation with different concentrations of glibenclamide (0·1–1 μM). Pretreatment with glibenclamide produced parallel rightward shifts of the relaxation curves (Figure 5). The pA_2 values of glibenclamide against CL-065, cromakalim and nicorandil were approximately 6.90 ± 0.09 (slope 1.03 ± 0.18), 7.55 ± 0.09 (slope 0.97 ± 0.08) and 6.800 ± 0.004 (slope 0.87 ± 0.01), respectively. There were no significant differences between the slopes, which were not significantly different from 1·0. These results indicated that CL-065 antagonized the effect of glibenclamide competitively and was more potent than nicorandil.

The vasorelaxant effects of CL-065 (1 μM), cromakalim (0·1 μM) and nicorandil (10 μM) against high potassium ion (K^+; 20–60 mM)-induced vasoconstriction in thoracic aorta are shown in Table 2. Tension of aorta was developed after the addition of 20–60 mM K^+ solution. This condition of vasoconstriction was associated with membrane depolarization induced by high extracellular K^+ concentrations leading to calcium influx via voltage-dependent calcium channels. Treatment with CL-065 (1 μM) dramatically attenuated the vasoconstriction induced by 20 mM K^+ solution. Progressively increasing the concentration of K^+ (30 and 40 mM) resulted in significant concentration-dependent reduction of the vasorelaxation induced

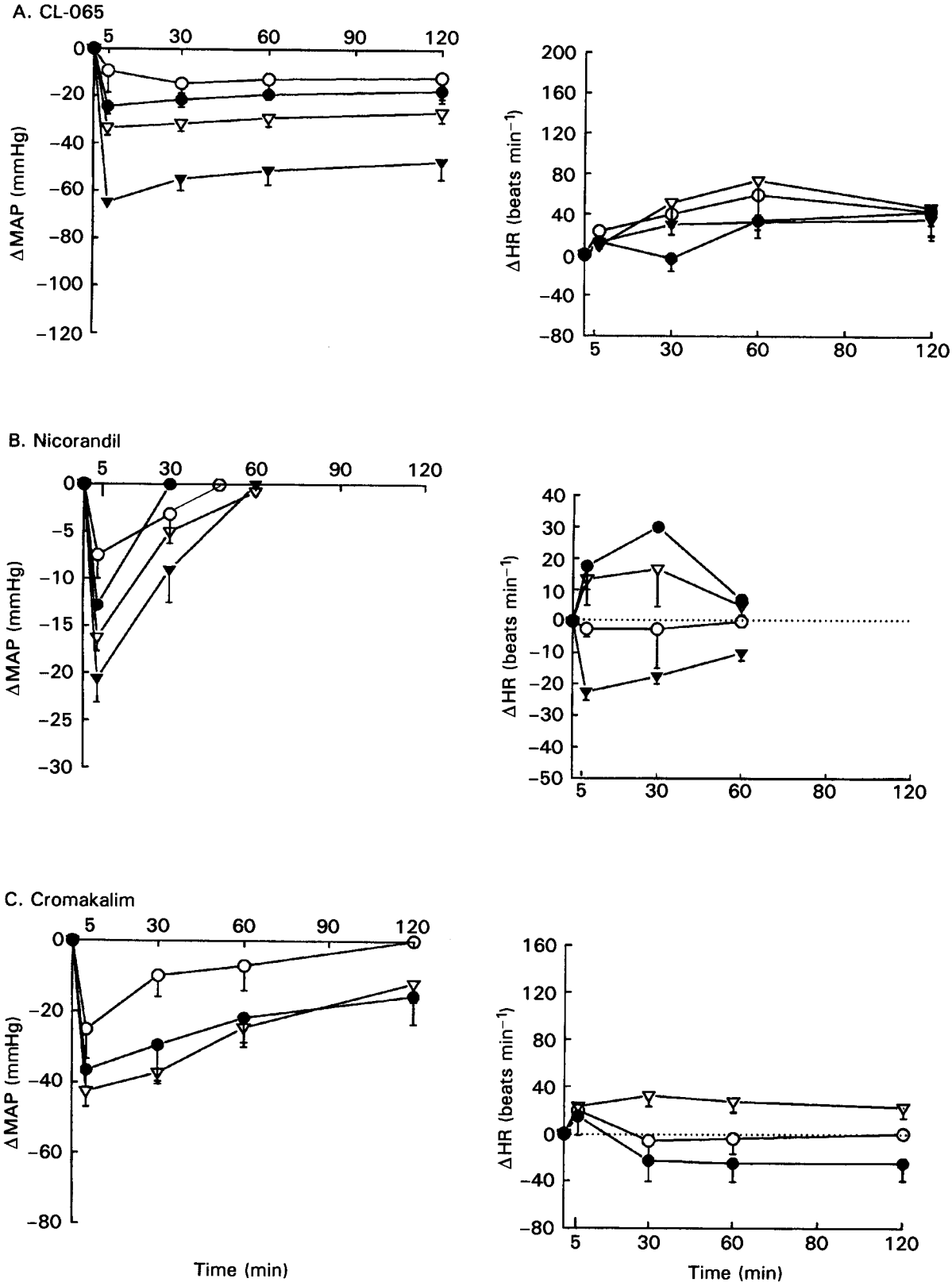

Figure 2. The effects of CL-065, nicorandil and cromakalim (i.v. bolus) on mean blood pressure and heart rate in SHR. Changes in mean arterial pressure (MAP) and heart rate (HR) caused by intravenous injection of different doses of (A) CL-065 (○, 0·1 mg kg^{-1}; ●, 0·5 mg kg^{-1}; ▽, 1·0 mg kg^{-1}; ▼, 2·0 mg kg^{-1}), (B) nicorandil (○, 0·25 mg kg^{-1}; ●, 0·5 mg kg^{-1}; ▽, 1·0 mg kg^{-1}; ▼, 2·0 mg kg^{-1}) and (C) cromakalim (○, 0·025 mg kg^{-1}; ●, 0·05 mg kg^{-1}; ▽, 0·1 mg kg^{-1}). Data are presented as means ± s.e.m. (n = 8). The baseline values of MAP and HR were 152 ± 12 mmHg and 360 ± 15 beats min^{-1}, respectively.

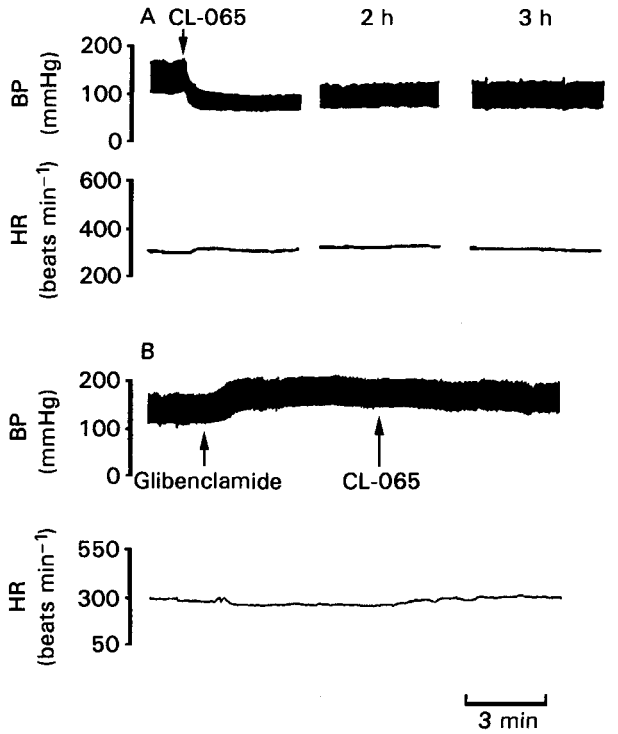

Figure 3. The effects of (A) CL-065 (0·5 mg kg^{-1}; i.v. bolus injection) and (B) glibenclamide (5 mg kg^{-1}) on blood pressure (BP) and heart rate (HR) in anaesthetized SHR.

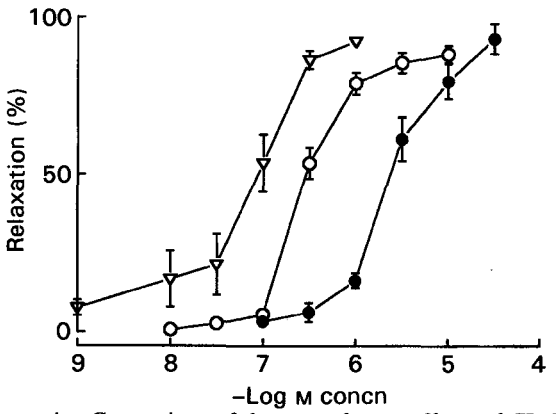

Figure 4. Comparison of the vasorelaxant effects of CL-065 (○; 0·01–10 μM), nicorandil (●; 0·1–30 μM) and cromakalim (▽; 1·0 nM–1·0 μM) on thoracic aorta pre-contracted with phenylephrine (0·3 μM). Values are presented as means ± s.e.m. (n = 8).

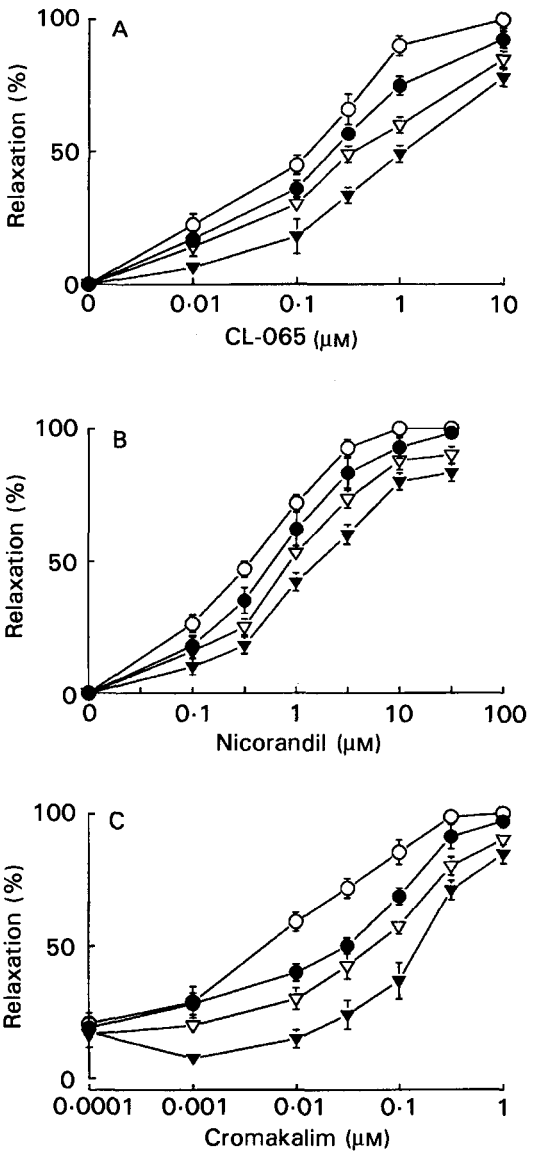

Figure 5. Effects of pretreatment with different concentrations of glibenclamide on antagonism of the vasorelaxation of (A) CL-065, (B) nicorandil and (C) cromakalim in phenylephrine (0·3 μM)-triggered aortic vasoconstriction: ○, control; ●, 0·1 μM glibenclamide; ▽, 0·3 μM glibenclamide; ▼, 1 μM glibenclamide. Data are presented as means ± s.e.m. (n = 12).

Table 1. Effect of administration of CL-065 on haemodynamics in spontaneously hypertensive rats.

	Mean aortic pressure (mmHg)	Heart rate (beats min^{-1})	Stroke volume (mL)	Cardiac output (mL min^{-1})	Peripheral resistance (dyne s cm^{-5})
Control	155 ± 10	370 ± 14	0·201 ± 0·014	75·0 ± 3·5	174333 ± 12334
CL-065	86 ± 10	378 ± 16	0·235 ± 0·016	87·4 ± 3·5	85034 ± 9021
Change (%)	45 ± 7	2 ± 2	17 ± 6	17 ± 6	−51 ± 4
P value	0·01	–	< 0·05	< 0·05	< 0·001

Values are means ± s.e.m.

Table 2. Comparison of the vasorelaxant effects of CL-065 (1 μM), nicorandil (10 μM) and cromakalim (0·1 μM) on isolated thoracic aorta pre-contracted with different concentrations of KCl (20–60 mM).

	Concentration of K^+ (mM)			
Drug	20	30	40	60
CL-065	−95·0 ± 2·4	−72·5 ± 4·4	−28·3 ± 4·8*	8·0 ± 2·0
Nicorandil	−41·0 ± 15·2	−23·0 ± 9·9	−13·8 ± 7·0*	9·0 ± 5·0
Cromakalim	−54·0 ± 11·9	−36·2 ± 5·7	−17·3 ± 5·7*	13·0 ± 2·0

*$P < 0·05$, significantly different from the vasorelaxant effect induced by these three K^+ channel openers on 20 mM KCl-induced contraction. Data are presented as means ± s.e.m. (n = 14).

by CL-065. However, in the presence of 60 mM K^+, CL-065 caused slight vasoconstriction ($P > 0·05$). On the other hand, the vasoreactivity to cromakalim (0·1 μM) and nicorandil (10 μM) were influenced by high K^+ (Table 2) similarly to CL-065.

Discussion

Experimental results have demonstrated that CL-065, a benzopyran derivative, resulted in dose-dependent inhibition of the contractile responses of rat thoracic aorta to phenylephrine. Pretreatment with the K_{ATP}-channel inhibitor glibenclamide almost completely blocked the hypotension and vasorelaxation of CL-065, indicating that CL-065 is a K_{ATP}-channel opener. On the inhibition of vasoconstriction elicited by phenylephrine the potency of CL-065 was greater than that of nicorandil but less than that of cromakalim.

CL-065, in common with the other K_{ATP}-channel openers cromakalim and nicorandil, induced a significant dose-dependent reduction of blood pressure in anaesthetized SHR. Similar results have been reported in conscious SHR (Cassidy et al 1992). After intravenous administration of these drugs, marked reflex tachycardia was observed; this might be triggered by the fall in blood pressure and mediated through the baroreceptor reflex system. In addition, analysis of the haemodynamic and vasodilator effects of CL-065 revealed that the peripheral resistance was significantly reduced. These results suggest that CL-065 exerts the anti-hypertensive effect by acting directly on the peripheral blood vessels. Furthermore, CL-065 did not affect the pressor response to angiotensin II and phenylephrine and the depressor response to isoproterenol and acetylcholine in rats (data not shown). On the basis of these findings, we suggest that the anti-hypertensive effect of CL-065 results neither from antagonizing adrenoceptors or blocking the renin–angiotensin pathway nor by activation of muscarinic receptors.

The vasodilator responses of CL-065 were significantly attenuated by high concentrations of K^+ implying that the CL-065-induced hyperpolarization was counteracted by increasing extracellular potassium concentration. This further supports the hypothesis that the vasodilator effect of CL-065 was mediated by opening of the K^+ channels.

In conclusion, CL-065, by activating K_{ATP} channels, induces a depressor effect in-vivo and is a potent vasorelaxant in-vitro, its potency is less than that of cromakalim but greater than that of nicorandil.

Acknowledgements

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