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# Mechanisms responsible for the in vitro relaxation of ligustrazine on porcine left anterior descending coronary artery

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**Abstract**

In this study, we have evaluated the underlying mechanisms responsible for the relaxation response of ligustrazine (2,3,5,6-tetra-methyl-pyrazine; 2,3,5,6-MP) and its structural analogues (2-methyl-pyrazine (2-MP); ethyl-pyrazine (EP); 2,3-di-methyl-pyrazine (2,3-MP); 2,5-di-methyl-pyrazine (2,5-MP); 2,6-di-methyl-pyrazine (2,6-MP) and 2,3,5-tri-methyl-pyrazine (2,3,5-MP)) in porcine left anterior descending coronary artery (tertiary branch, O.D. $\leq$ 1 mm). In 5-hydroxytryptamine (3 μM) precontracted preparations, cumulative administration (0.1–300 μM) of all pyrazine analogues caused an endothelium-independent, concentration-dependent relaxation. The relative inhibitory potency, as compared at concentration with which 50% relaxation occurred, was 2,3,5,6-MP>2,3,5-MP>EP>2,5-MP $\geq$ 2,6-MP $\geq$ 2,3-MP>2-MP. Besides, salbutamol and forskolin caused an endothelium-independent relaxation. The relaxation response of ligustrazine, salbutamol and forskolin was blunted in the presence of *cis*-*N*-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine (MDL 12330A) (10 μM, an adenylate cyclase inhibitor) and *N*-[2-((bromocinnamyl)amino)ethyl]-5-isoquinoline-sulphonamide (H-89, a protein kinase A inhibitor, 3 μM). Patch-clamp, whole-cell electrophysiological studies using single smooth muscle cells of the left anterior descending coronary artery revealed that ligustrazine (300 μM), salbutamol (30 μM) and forskolin (1 μM) inhibited the nifedipine-sensitive L-type $Ca^{2+}$ channels, and the inhibitory effect was eradicated by MDL 12330A (10 μM) and H-89 (1 μM). However, neither the $Ca^{2+}$-dependent $K^+$ channel nor the ATP-dependent $K^+$ channel was modified by ligustrazine (300 μM). In conclusion, our results indicate that ligustrazine-mediated left anterior descending coronary artery relaxation is due to the activation of adenylate cyclase/protein kinase A cascade and the subsequent inhibition of nifedipine-sensitive, voltage-dependent L-type $Ca^{2+}$ channels. However, opening of $K^+$ channels seems to play no role in mediating the relaxation effect of ligustrazine.


*Keywords:* Ligustrazine; Coronary artery, porcine; Adenylate cyclase; $Ca^{2+}$ channel, L-type

## 1. Introduction

Ligustrazine (2,3,5,6-tetra-methyl-pyrazine, 2,3,5,6-MP) is one of the active principles found in the extract of the traditional Chinese medicinal herb Chuanxiong (*Ligusticum wallichii*), *Jatropha podagrica* and in cultures of *Bacillus subtilis* (Sutter and Wang, 1993). In China, ligustrazine has been used to treat a variety of cardiovascular disorders, e.g., myocardial and cerebral infarction (Mao, 1989; Zhang et al., 1994; Lin et al., 1997). It has been reported that ligustrazine causes an increase in coronary blood flow and reduce myocardial ischaemia in animal study (Dai and Bache, 1985). In addition, the relaxation effect of ligustrazine is mediated through the elevation of cAMP level (but not cGMP), due to the inhibition of cAMP-phosphodiesterase activity, in dog and human isolated coronary artery preparation (Lin et al., 1993). However, in rat isolated aorta, soluble guanylate cyclase activation seems to play a role in ligustrazine-mediated relaxation (Tsai et al., 2002).

Similar to most compounds that are isolated from herbs, the reported activities of ligustrazine (e.g. the vascular relaxation effect and the inhibition of cAMP-phosphodiesterase cascade) are less potent than other synthetic compound, e.g., theophylline (Lin et al., 1993). In hopes of searching for a structural analogue that may be more potent than ligustrazine itself, the first aim of this study was to perform a structure–activity relationship on the vascular relaxation of a range of commercially available ligustrazine

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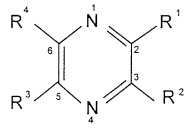
analogues: 2-methyl-pyrazine (2-MP), 2,3-di-methyl-pyrazine (2,3-MP), 2,5-di-methyl-pyrazine (2,5-MP), 2,6-di-methyl-pyrazine (2,6-MP), 2,3,5-tri-methyl-pyrazine (2,3,5-MP) and ethyl-pyrazine (EP) (Fig. 1).

Depending on the species and tissues employed, it has been shown that ligustrazine has multiple actions (Kwan et al., 1991), and modified the activities of different ion channels. For instance, the vasorelaxing effect of ligustrazine on rat isolated aorta involved the opening of small conductance $Ca^{2+}$-activated $K^+$ channel and ATP-dependent $K^+$ channels (Tsai et al., 2002). In rat isolated pulmonary artery and mesenteric artery, ligustrazine-induced relaxation involved the endothelium-dependent nitric oxide generation (Peng et al., 1996). Ligustrazine also possesses calcium antagonist-like action in vascular tissue, smooth muscle cells (Wu et al., 1989; Kwan, 1994; Pang et al., 1996) and in rat ventricular myocytes (Zou et al., 2001). So far, most studies were performed in rodents that may not truly represent men. The second aim of this study was to examine the vascular effect of ligustrazine using the coronary artery of pig's heart, an animal model that its anatomy and physiology is comparable with humans (Riquet et al., 2000; Allan et al., 2001).

Despite a wide spectrum of activities (related to modulation of different ion channels) of ligustrazine that has been reported, most previous studies were performed on multicellular preparations (Wu et al., 1989; Kwan et al., 1990; Tsai et al., 2002). It has made the interpretation of results sometimes difficult, compared to experiments using single cells (Zou et al., 2001) with which a particular ion channel type can be "isolated" for a detailed examination. Hence, the third aim of this study was to employ the whole-cell patch-clamp technique, using enzymatic dissociated single smooth muscle cells, to characterize pharmacologically which membrane ion channels as well as the intracellular signalling pathways are involved in ligustrazine-mediated vascular relaxation.

A



| Compound | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| pyrazine | H | H | H | H |
| 2-methylpyrazine | $CH_3$ | H | H | H |
| ethylpyrazine | $CH_3CH_2$ | H | H | H |
| 2,3-dimethylpyrazine | $CH_3$ | $CH_3$ | H | H |
| 2,5-dimethylpyrazine | $CH_3$ | H | $CH_3$ | H |
| 2,6-dimethylpyrazine | $CH_3$ | H | H | $CH_3$ |
| 2,3,5-trimethylpyrazine | $CH_3$ | $CH_3$ | $CH_3$ | H |
| 2,3,5,6-tetramethylpyrazine | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ |

B

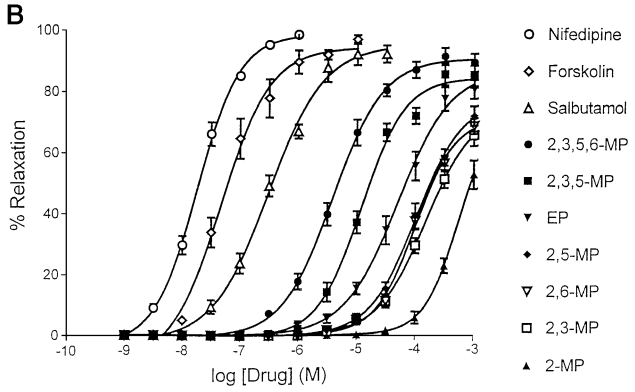



Fig. 1. (A) Chemical structures of pyrazine analogues. (B) Summary of the relaxation effect of nifedipine (○), forskolin (◇), salbutamol (△), 2,3,5,6-tetra-methyl-pyrazine (2,3,5,6-MP, ●), 2,3,5-tri-methyl-pyrazine (2,3,5-MP, ■), ethyl-pyrazine (EP, ▼), 2,5-di-methyl-pyrazine (2,5-MP, ♦), 2,6-di-methyl-pyrazine (2,6-MP, ▽), 2,3-di-methyl-pyrazine (2,3-MP, □) and 2-methyl-pyrazine (2-MP, ▲) on 5-hydroxytryptamine (3 μM) precontracted left anterior descending coronary artery (endothelium intact). Results are expressed as mean ± S.E.M., $n = 5–7$.

## 2. Materials and methods

### 2.1. Preparation of porcine coronary artery

Fresh heart was obtained from pig (either sex, weight ~ 25 kg) in the morning of the experiment day at a local slaughterhouse. The heart was immediately immersed in an ice-cold physiological salt solution before transported to the laboratory. Segment of the left anterior descending coronary artery (tertiary branch, O.D. ≤ 1 mm) was isolated/dissected within an hour after the animal was slaughtered. Fat and connective tissue were carefully removed under the dissecting microscope. Care was taken not to touch the lumen of the coronary artery during dissection. Three to four arterial rings (each ~ 1 mm in length) were obtained from each heart and only one ring was used for each drug treatment.

### 2.2. Isometric tension measurement

The arterial ring was mounted in a 5-ml vertical organ-bath containing Krebs' solution (gassed with 95% $O_2$–5% $CO_2$; pH 7.4, 37 ± 1 °C) of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 2.5. In some preparations, endothelium was carefully removed by gently rubbing the intima of the blood vessel with a wire, and confirmed by the failure of acetylcholine (10 μM)-induced relaxation. Isometric tension change was measured using the MacLab Chart v 3.6 program (AD Instruments, Australia). The preparations were equilibrated under the resting tension of 10 ± 1 mN in the bath solution for 90 min. Resting tension was readjusted, if necessary, before commencing the experiments. Relaxation in response to ligustrazine and its analogues was expressed as percentage of 5-hydroxytryptamine (3 μM)-induced tone and 100% relaxation was considered when the active tone returned to baseline level. In the preliminary study ($n = 5$), 3 μM 5-hydroxytryptamine yielded a sustained contraction which is ~ 80% of 5-

hydroxytryptamine-induced maximum contraction of the left anterior descending coronary artery. Manoeuvres such as administration of 4-aminopyridine, barium chloride, $N^G$-nitro-L-arginine methylester (L-NAME), iberiotoxin, 6-anilino-5,8-quinolinedione (LY 83583) and endothelium denudation resulted in an enhanced 5-hydroxytryptamine (3 μM)-induced tone ( ~ 34% increase), concentration of 5-hydroxytrptamine was therefore adjusted accordingly (0.6–1 μM) in order to have a similar magnitude of action tone (normalized to 80 mM $[K^+]_o$-induced maximum contraction).

Due to the lipophilicity of ligustrazine and its structural analogues (dissolved in/diluted with dimethyl sulphoxide), only one concentration–response curve of individual agent was constructed in each preparation. A 30-min incubation time was allowed in experiments with specific inhibitor, which was left in contact with the preparations before the precontraction of the left anterior descending coronary artery with 5-hydroxytryptamine and further during the construction of the concentration–response curves of ligustrazine or its analogue.

### 2.3. Enzymatic dissociation of the coronary artery smooth muscle cells

Stripe of the left anterior descending coronary artery (1 × 1 mm) was placed in an Eppendorff tube containing a low $Ca^{2+}$ dissociation medium of the following composition (mM): NaCl 110, KCl 5, $NaH_2PO_4$ 0.5, $KH_2PO_4$ 0.5, $NaHCO_3$ 10, HEPES 10, Phenol Red 0.015, taurine 10, creatine 5, Na pyruvate 5, glucose 11, $CaCl_2$ 0.16, $MgCl_2$ 2 and ethylenediaminetetraacetic acid (EDTA) 0.5 (pH 7.0 with NaOH). The arterial stripe was incubated for 45–60 min at 37 °C of the above solution (volume: 1 ml) with the supplement of 6.4 mg dithiothreitol (Sigma-Aldrich, USA), 2.0 mg papain (Fluka, Switzerland), 7 mg collagenase (Type 2, Worthington Biochemicals, USA) and 2 mg bovine serum albumin (Sigma-Aldrich). After incubation, the digested tissue was transferred to enzyme-free dissociation medium, and gently triturated with a fire-polished pasture pipette to dislodge smooth muscle cells. Exclusive use of freshly dissociated cells avoided the real-time confirmation of smooth muscle identity using techniques that required cell fixation. Electrophysiological measurements were carried out only on cell that exhibited morphological features of vascular smooth muscle cells (an elongated, spindle shape, ~ 100 μm in length) when observed under the microscope. Moreover, parallel observations were made using aliquots of cells (relaxed) and demonstrating a reversible contractile response to high $[K^+]_o$ (80 mM).

### 2.4. Electrophysiology experiments

A drop of cell suspension was placed in a glass-bottom recording chamber (volume: 0.5 ml), mounted on the stage of an inverted microscope (Nikon Diaphot 200, Japan) equipped with a Hoffman Modulation Contrast condenser, containing a physiological salt solution of the following composition (mM): NaCl 132, KCl 4.8, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) 10, $MgCl_2$ 2, glucose 5 and $CaCl_2$ 1 (pH 7.4 with NaOH). Cells that were relaxed and have a cleared edge observed under the microscope were used in this study. Voltage-clamp and voltage pulse generation were controlled with an Axopatch 200 A patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), and data were acquired and analysed with pCLAMP softwares (Axon Instruments). To facilitate the measurement of a particular type of ion channel activity, different voltage protocols with appropriate external bathing and internal pipette solutions were employed, as previously reported for a particular type of ion channel of the vascular smooth muscle cells (Brzezinska et al., 2000; Gerzanich et al., 2001; Zhao et al., 2001). To investigate the effect of drugs on the steady-state inactivation of $I_{CaL}$, the double-pulse protocol was used (Kwan and Kass, 1993). A test pulse ($P_1$) to + 10 mV (duration 100 ms) was preceded by 5-s conditioning prepulses ($P_2$) to various potentials (from − 60 to + 50 mV). A gap of 5 ms was present between $P_2$ and $P_1$. The relative amplitude of $I_{CaL}$ (normalized by taking value at − 60 mV as unity) was plotted against conditioning potentials, and the data were fitted using the Boltzmann equation: $I/I_{max} = 1/\{1 + \exp[(V_m - V_{1/2})/K]\}$ where $I/I_{max}$ is the relative amplitude of $I_{CaL}$, $V_m$ is the conditioning potential ($P_2$), $V_{1/2}$ is the voltage of half-inactivation and $K$ is a slope factor.

In all experiments, the current amplitude was recorded at room temperature ( ~ 22 °C) using the single micropipette "gigaseal" patch-clamp technique in the whole-cell configuration (Hamill et al., 1981). Series resistance was compensated to provide the fastest decay of the capacitative current with no sign of ringing. Pipettes were fabricated from haematocrit glass capillaries (Accu-Fill 90 Micropet, Clay Adams, Parsippany, NJ, USA) and pulled on a two-stage vertical pipette puller (PP-83; Narishige Scientific Instruments, Japan). The resistance of the micropipettes (Clay Adams) was 1–3 MΩ when filled with a particular type of internal pipette solution. The pipette tip was positioned near the nuclear region of the coronary smooth muscle cell (dimension ~ 10 μm) using an oil-based hydraulic micromanipulator (Narishige Scientific Instruments). Currents were filtered at 5 kHz and sampled at 3–10 kHz. Cell membrane capacitance was estimated, as previously described (Matsuda et al., 1990), and it was 19.2 ± 2.3 pF ($n$ = 33). Cells with visible change in leakage currents during the course of study were discarded and excluded from analysis. External solution was delivered, through gravity, and controlled by solenoid valves coupled to a four-channel valve driver (General Valve, USA). Solution change ( ~ 5 ml, which is 10 times the volume of the recording chamber) could be completed in 15–20 s. Current amplitude was recorded before, during and after the administration of a particular drug. Only one concentration of drug was tested in each cell.

Where stated, concentration of inhibitor employed in this study was the reported effective concentration of individual agent based on our previous studies (Kwan et al., 1999; Choy et al., 2002) and other groups (Ramillard and Leblanc, 1996, Yamamura et al., 1999, Zhao et al., 2001) on vascular smooth muscle.

### 2.5. Chemicals

All chemicals for preparing the physiological salt solution, dissociation medium, different external bathing solutions and internal pipette solutions were purchased from Sigma-Aldrich. Ethyl-pyrazine, 2-methyl-pyrazine, 2,3-di-methyl-pyrazine, 2,5-di-methyl-pyrazine, 2,6-di-methyl-pyrazine and 2,3,5-trimethyl-pyrazine were purchased from Acros Organics (Belgium). 2,3,5,6-Tetra-methyl-pyrazine, 5-hydroxytryptamine hydrochloride, 4-aminopyridine, glibenclamide, barium chloride, $N^G$-nitro-L-arginine methylester hydrochloride (L-NAME), sodium nitroprusside, nifedipine, salbutamol and forskolin were obtained from Sigma-Aldrich. Iberiotoxin and apamin were purchased from Alomone Labs (Israel). Pinacidil was purchased from Research Biochemicals (USA). 6-Anilino-5,8-quinolinedione (LY 83583), dibutyryl cyclic AMP sodium, *cis*-*N*-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine hydrochloride (MDL 12330A) and *N*-[2-((bromocinnamyl)amino)ethyl]-5-isoquinoline-sulphonamide dihydrochloride (H-89) were obtained from Calbiochem-Novabiochem. (USA).

### 2.6. Statistical analysis

All data were expressed as mean ± S.E.M. Statistical significance was determined by Student's *t*-test and one-way analysis of variance, where appropriate. In organ-bath experiment, $n$ = no. of heart, whereas in patch-clamp electrophysiology study, $n$ = no. of single cells used. $P < 0.05$ was considered significantly different from controls.

## 3. Results

### 3.1. Comparison of the relaxation effect of ligustrazine and the structural analogues

Chemical structure of ligustrazine and its structural analogues are illustrated in Fig. 1. Cumulative administration of ligustrazine (2,3,5,6-tetra-methyl-pyrazine; 2,3,5,6-MP) (0.1–300 μM) yielded a concentration-dependent relaxation of 5-hydroxytryptamine (3 μM) precontracted left anterior descending coronary artery (endothelium intact). Maximum relaxation that occurred at 300 μM of ligustrazine was 93.3 ± 4.7% (Fig. 1). Endothelium denudation did not alter the relaxation response of ligustrazine (maximum relaxation that occurred at 300 μM of ligustrazine was 90.1 ± 6.2%, $P$>0.05, $n$ = 5). Ligustrazine analogues (2-methyl-pyrazine (2-MP), 2,3-di-methyl-pyrazine (2,3-MP), 2,5-di-methyl-pyrazine (2,5-MP), 2,6-di-methyl-pyrazine (2,6-MP), 2,3,5-tri-methyl-pyrazine (2,3,5-MP) and ethyl-pyrazine (EP)) also elicited a concentration-dependent relaxation (Fig. 1). Similar to ligustrazine, endothelium denudation was without effect on all pyrazine analogues-elicited relaxation ($n$ = 5–6 for each analogue) (data not shown). There was, however, no maximum relaxation observed at the highest concentration tested (1 mM) with 2,5-MP, 2,6-MP, 2,3-MP and 2-MP. Hence, the relative inhibitory potency was compared at concentration with which 50% relaxation observed: 2,3,5,6-MP>2,3,5-MP>EP>2,5-MP ≥ 2,6-MP ≥ 2,3-MP>2-MP (Fig. 1). Similar to ligustrazine, administration of salbutamol (0.1–30 μM, a $\beta_2$-adrenoceptor agonist) ($n$ = 6), forskolin (0.03–10 μM, an adenylate cyclase activator) ($n$ = 5) and nifedipine (0.01–1 μM, a 1,4-dihydropyridine L-type $Ca^{2+}$ channel blocker) ($n$ = 6) resulted in a concentration-dependent relaxation (Fig. 1) of the precontracted left anterior descending coronary artery.

### 3.2. Mechanism responsible for the relaxation effect of ligustrazine

As ligustrazine was found to be the most potent pyrazine analogue, it was decided to examine this agent in details. In addition to 5-hydroxytryptamine (3 μM), high $[K^+]_o$ was also used as the contractile agent for comparison. In the preliminary study, 55 mM $[K^+]_o$ caused a similar magnitude of contraction ($n$ = 5) as of 3 μM 5-hydroxytryptamine. In high $[K^+]_o$ precontracted preparations, ligustrazine caused a similar degree of relaxation as that observed in preparations precontracted with 5-hydroxytryptamine (300 μM ligustrazine, endothelium intact: 92.7 ± 5.5%; endothelium denuded: 90.3 ± 6.1%, $P$>0.05, $n$ = 5).

The presence of *cis*-*N*-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine (MDL 12330A) (10 μM, an adenylate cyclase inhibitor) markedly attenuated the magnitude of relaxation induced by ligustrazine (300 μM, relaxation: 7.1 ± 2.2%, $P < 0.001$ compared to control, $n$ = 6), salbutamol (30 μM, relaxation: 6.3 ± 3.4%, $P < 0.001$ compared to control, $n$ = 6) and forskolin (10 μM, relaxation: 8.3 ± 3.6%, $P < 0.001$ compared to control, $n$ = 5). In addition, a similar degree of inhibition, as that caused by MDL 12330A, was observed with *N*-[2-((bromocinnamyl)amino)ethyl]-5-isoquinoline-sulphonamide (H-89, a protein kinase A inhibitor, 3 μM) ($n$ = 5–6) (data not shown). MDL 12330A and H-89, on its own, did not alter the resting tension and the raised tone of the coronary artery (data not shown).

Neither glibenclamide (3 μM, $n$ = 6), iberiotoxin (300 nM, $n$ = 4), apamin (500 nM, $n$ = 4), $[Ba^{2+}]_o$ (10 μM, $n$ = 6), 4-aminopyridine (3 mM, $n$ = 6), indomethacin (1 μM, $n$ = 6), 6-anilino-5,8-quinolinedione (LY 83583) (3 μM, $n$ = 5) nor $N^G$-nitro-L-arginine methylester (L-NAME) (50 μM, $n$ = 6) altered the relaxation response of ligustrazine observed in both endothelium-intact and endothelium-denuded preparations (data not shown).

### 3.3. Modulation by ligustrazine of ion channel activities

In experiments that were designed to study the nifedipine-sensitive L-type $Ca^{2+}$ channel ($I_{CaL}$), 10 mM $[Ba^{2+}]_o$ instead of $[Ca^{2+}]_o$ was used as the charge carrier in order to enhance the signal-to-noise ratio. Results from the current–voltage relationship (holding potential = − 40 mV, from − 50 to 60 mV with a 10-mV increment, pulse duration = 100 ms, stimulated at 0.2 Hz) indicated that the peak amplitude of the recorded basal $I_{CaL}$ occurred at 10 mV ($n = 5$). Administration of ligustrazine markedly inhibited the peak amplitude of the $I_{CaL}$ (control, 39 ± 5 pA; 300 μM ligustrazine, 6 ± 4 pA, $P < 0.001$ compared to control, $n = 5$) (Fig. 2), with no apparent change in the reversal potential (58.3 ± 4.2 vs. 61.6 ± 3.8 mV, $P > 0.05$, $n = 5$) and peak potential (10 mV) ($n = 5$). Effect of ligustrazine (3 μM) on the steady-state inactivation of $I_{CaL}$ was examined using the double-pulse protocol. Ligustrazine (3 μM) failed to alter the steady-state inactivation kinetics of $I_{CaL}$ (half-inactivation potential ($V_{1/2}$): 25.3 ± 1.3 vs. 23.4 ± 1.8 mV, $P > 0.05$, $n = 6$; slope factor $K$: 6.7 ± 0.6 vs. 6.1 ± 0.5 mV, $P > 0.05$, $n = 6$) (Fig. 2).

Using a train protocol designed to measure the rate of onset of and the recovery from block of $I_{CaL}$, an immediate recovery from block by nifedipine (1 μM) was observed when holding potential was switched to − 80 mV (nifedipine was still present in the solution) instead of − 40 mV ($n = 4$) (Fig. 3). In contrast, switching to a hyperpolarized holding potential (− 80 mV) did not alleviate the block of $I_{CaL}$ by ligustrazine (7 ± 3 pA, $n = 5$) (Fig. 3), salbutamol (11 ± 4 pA, $n = 6$) and forskolin (8 ± 3 pA, $n = 5$), and no apparent recovery from block was observed after washout (Fig. 4).

Similar to ligustrazine, forskolin (10 μM; 43 ± 3 vs. 6 ± 3 pA, $P < 0.001$, $n = 6$), salbutamol (30 μM, 44 ± 4 vs. 5 ± 2 pA, $P < 0.001$, $n = 5$), nifedipine (1 μM, 39 ± 3 vs. 3 ± 2 pA, $P < 0.001$, $n = 4$) and dibutyryl cAMP (100 μM, 41 ± 6 vs. 7 ± 3 pA, $P < 0.001$, $n = 6$) inhibited the basal $I_{CaL}$ peak amplitude (Fig. 4) with no obvious change in the reversal potential (control, 57 ± 4 mV, $n = 6$; salbutamol, 58 ± 4 mV, $P > 0.05$ compared to control, $n = 5$; nifedipine, 60 ± 3 mV, $P > 0.05$ compared to control, $n = 4$; dibutyryl cAMP, 62 ± 4 mV, $P > 0.05$ compared to control, $n = 5$).

The inhibition caused by ligustrazine, forskolin and salbutamol (but not dibutyryl cAMP) of the basal $I_{CaL}$ amplitude was abolished by MDL 12330A (10 μM) (ligustrazine: 43 ± 4 vs. 38 ± 5 pA, $P > 0.05$, $n = 5$; forskolin: 40 ± 2 vs. 37 ± 4 pA, $P > 0.05$, $n = 6$; salbutamol: 43 ± 5 vs. 37 ± 7 pA, $P > 0.05$, $n = 5$; dibutyryl cAMP: 44 ± 5 vs. 6 ± 3 pA, $P < 0.001$, $n = 5$) (Fig. 4). The presence of protein kinase A inhibitor H-89 (1 μM) eradicated ligustrazine-, forskolin-, salbutamol- ($n = 5–6$) (data not shown) and dibutyryl cAMP-mediated inhibition of $I_{CaL}$ (42 ± 4 vs. 39 ± 6 pA, $P > 0.05$, $n = 5$). On its own, MDL 12330A (10 μM) (Fig. 4) and H-89 (1 μM) ($n = 6$) did not significantly alter the magnitude of $I_{CaL}$.

In addition to $I_{CaL}$, effect of ligustrazine on $K^+$ channels was evaluated. At a holding potential of − 40 mV, depolarisation pulses (− 60 mV to 100 mV with a 20 mV increment, duration of 600 ms, stimulated at 0.1 Hz) elicited a sustained outward current that was sensitive to 100 nM iberiotoxin (magnitude of current measured at 595 ms, 2.47 ± 0.19 vs. 0.37 ± 0.11 nA, $P < 0.001$, $n = 4$), suggesting that the recorded current is the large-conductance $Ca^{2+}$-dependent $K^+$ channels ($BK_{Ca}$). However, ligustrazine (300

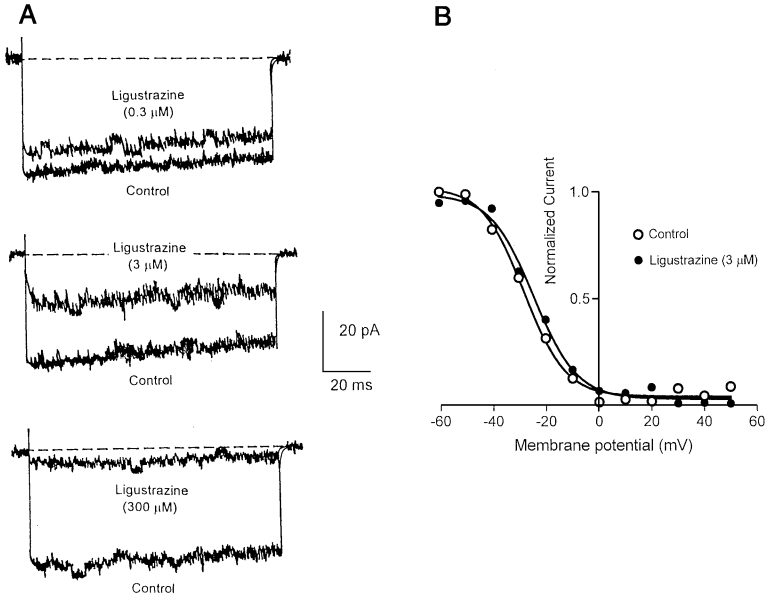



Fig. 2. (A) Effect of ligustrazine on $I_{CaL}$ peak amplitude. Inset shows representative current traces of $I_{CaL}$ recorded in the presence of 0.3, 3 and 300 μM ligustrazine. $I_{CaL}$ was elicited with a test potential of + 10 mV for 100 ms from a holding potential of − 40 mV at 0.2 Hz. (B) Effect of ligustrazine (●, 3 μM) on the steady-state inactivation of $I_{CaL}$, using the double-pulse protocol, of a representative smooth muscle cell.

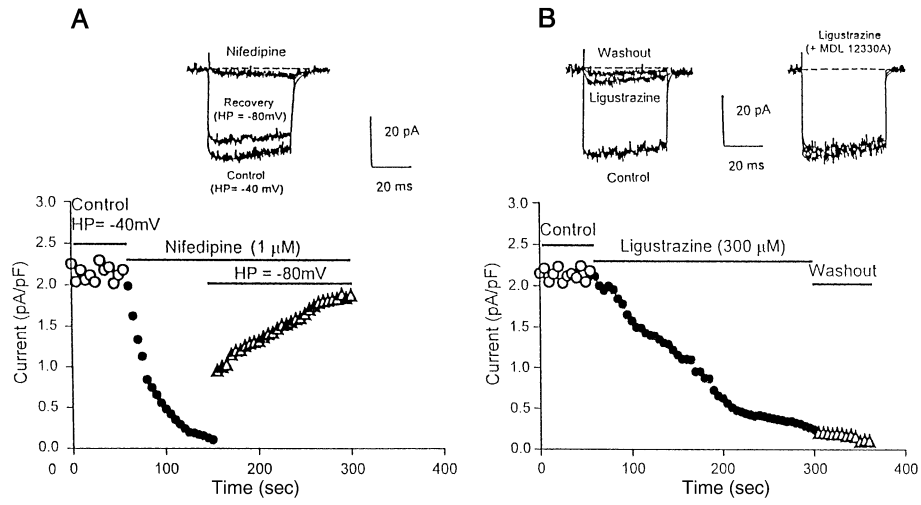



Fig. 3. (A) Time course of the inhibitory effect of nifedipine (1 μM) on $I_{CaL}$ amplitude. Insets are the representative traces of $I_{CaL}$ recorded in control (○, holding potential = − 40 mV), nifedipine (●, 1 μM) and recovery (△, in the continuous presence of nifedipine, holding potential = − 80 mV). $I_{CaL}$ was elicited with a test potential of + 10 mV for 40 ms from a holding potential of − 40 mV at 0.2 Hz. (B) Time course of the inhibitory effect of ligustrazine (300 μM) on $I_{CaL}$ amplitude. Insets are the representative traces of $I_{CaL}$ recorded in control (○), ligustrazine (●, 300 μM) (with and without MDL 12330A (10 μM)) and washout (△). $I_{CaL}$ was elicited with a test potential of + 10 mV for 40 ms from a holding potential of − 40 mV at 0.2 Hz.

μM) did not significantly modify the $BK_{Ca}$ channels (2.13 ± 0.07 vs. 1.87 ± 0.18 nA, $P>0.05$, $n=6$). Application of sodium nitroprusside (300 nM, a nitric oxide donor) markedly enhanced the magnitude (2.46 ± 0.35 vs. 3.33 ± 0.48 nA, $P<0.01$, $n=5$) of the recorded $BK_{Ca}$ channels.

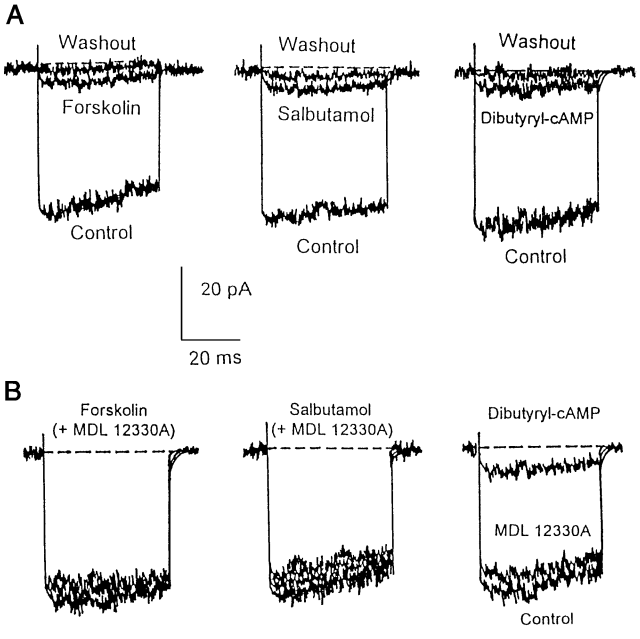



Fig. 4. (A) Effect of forskolin (10 μM), salbutamol (30 μM) and dibutyryl-cAMP (100 μM) on $I_{CaL}$ peak amplitude. Insets are the representative traces of $I_{CaL}$ recorded in control, the maximum effect of individual agent and washout. $I_{CaL}$ was elicited using a test potential of + 10 mV for 40 ms from a holding potential of − 40 mV at 1 Hz. (B) Effect of MDL 12330A (10 μM) on forskolin (10 μM)-, salbutamol (30 μM)- and dibutyryl-cAMP (100 μM)-mediated inhibition of $I_{CaL}$ peak amplitude. Insets are the representative traces of $I_{CaL}$ recorded in control, the maximum effect of individual agent and washout. $I_{CaL}$ was elicited with a test potential of + 10 mV for 40 ms from a holding potential of − 40 mV.

In another set of experiment, the modulatory effect of ligustrazine (300 μM) on glibenclamide-sensitive, ATP-dependent $K^+$ channel ($IK_{ATP}$) was also evaluated. The cell was held at a holding potential of − 60 mV, and stimulated at 0.1 Hz ( − 60 mV to 80 mV with a 20-mV increment, pulse duration of 500 ms). The recorded current was enhanced by pinacidil (10 μM, an $IK_{ATP}$ opener) (magnitude of current measured at 495 ms, 213.62 ± 22.77 vs. 322.82 ± 31.04 pA, $P<0.01$, $n=6$), and the effect was eradicated by 3 μM glibenclamide (244.38 ± 19.91 pA, $P>0.05$ compared to control, $n=5$). However, application of ligustrazine (300 μM) failed to alter $IK_{ATP}$ (232.24 ± 18.61 vs. 241.02 ± 11.13 pA, $P>0.05$, $n=6$) and pinacidil (10 μM)-enhanced $IK_{ATP}$ ($n=6$) (data not shown).

## 4. Discussion

The root of *L. wallichii* (Chuanxiong) is used in traditional Chinese medicine as a cardiac stimulant (Chen et al., 1987) and hypotensive agent (Mashour et al., 1998). It has been demonstrated that ligustrazine (2,3,5,6-tetra-methylpyrazine, 2,3,5,6-MP), the active alkaloid extracted from *L. wallichii* (Cao et al., 1983), inhibits platelet aggregation in vitro (Zhou et al., 1985) and lowers blood pressure by vasodilation in dogs (Dai and Bache, 1985) and in conscious rat (Tsai et al., 2001).

In this study, the in vitro coronary vascular effect of ligustrazine and some of its structural analogues in pig's heart (an organ which is anatomically and physiologically similar to humans) (Riquet et al., 2000; Allan et al., 2001)

was evaluated. Ligustrazine elicited a concentration-dependent relaxation of precontracted (by 5-hydroxytryptamine) left anterior descending coronary artery. Compared to its structural analogues tested, ligustrazine was found to be the most potent in eliciting coronary artery relaxation (Fig. 1). In dog isolated saphenous vein preparation (Wang et al., 1991), it has been reported that the presence of four "bulky" hydrophobic ethyl groups attached to the pyrazine moiety has made the compound (i.e. tetra-ethyl-pyrazine) more potent than tetra-methyl-pyrazine (ligustrazine) in causing vascular relaxation. In our study, the magnitude of relaxation of pyrazine analogues seems to correlate fairly well with the number of methyl group present on the basic pyrazine structure. Our results demonstrate that ligustrazine (it contains four methyl groups attached symmetrically to the planar pyrazine structure) (Fig. 1) possesses the greatest ability in relaxing the left anterior descending coronary artery (ligustrazine is more hydrophobic than 2-methylpyrazine) (Yamagami et al., 1991; Vauthey et al., 2000). In addition to the number and type of substituents, steric hindrance between substitute groups (e.g. 2,3-disubstituted derivative has a greater steric hindrance than the 2,5-disubstituted derivative) (Yamagami et al., 1991) can alter the biological activity by lowering the partition coefficient (log *P*, an estimation of lipophilicity) of individual agent. It is interesting to note that the relative vascular relaxation potency of all pyrazine analogues (2,3,5,6-MP was the most potent whereas 2-MP was the least one) agrees fairly well with the estimated log *P* value reported (2,3,5,6-MP, 1.28; 2,3,5-MP, 0.95; EP, 0.69; 2,5-MP, 0.63; 2,6-MP, 0.54; 2,3-MP, 0.54; 2-MP, 0.21) (Yamagami et al., 1990; Yamagami et al., 1991). Among all agents tested, nifedipine (log *P* of 2.5) (van der Lee et al., 2000) was found to be the most potent ( ~ 300 times more potent than ligustrazine).

The reported underlying mechanisms responsible for the vascular relaxation effects of ligustrazine are very complicated, as it has multiple actions (Kwan et al., 1991). The obligatory role of nitric oxide/endothelium in mediating ligustrazine vascular responses is confusing (Oddoy et al., 1991; Lin et al., 1993; Cao et al., 1994; Peng et al., 1996). However, all pyrazine analogues examined in our study elicited an endothelium-independent relaxation. In contrast to rat pulmonary artery (Peng et al., 1996), pretreatment with $N^G$-nitro-L-arginine methylester (L-NAME, a common nitric oxide synthase inhibitor) did not alter the relaxation response, supporting our conclusion that the nitric oxide/endothelium cascade plays no role in mediating the coronary artery relaxation of all pyrazine analogues. In addition, both cyclo-oxygenase and guanylate cyclase activation were not involved as indomethacin (a cyclo-oxygenase inhibitor) and 6-anilino-5,8-quinolinedione (LY 83583, a guanylate cyclase inhibitor) pretreatment failed to alter ligustrazine-mediated relaxation.

It has been reported that inhibition of vasoconstriction of canine vascular smooth muscle (multicellular preparation) by ligustrazine was partly due to an inhibition of influx of extracellular $Ca^{2+}$ through L-type $Ca^{2+}$ channel ($I_{CaL}$), and ligustrazine behaves as the classical organic $Ca^{2+}$ channel blocker (Kwan et al., 1990; Wang et al., 1991; Pang et al., 1996). Using single smooth muscle cells, we have evaluated the modulatory effect of ligustrazine on nifedipine-sensitive $I_{CaL}$. Ligustrazine caused an inhibition of the basal $I_{CaL}$ with no apparent recovery after washout. In rat ventricular myocyte (Zou et al., 2001), ligustrazine inhibited $I_{CaL}$ in a manner (binds preferentially to the inactivated $I_{CaL}$) similar to other neutral 1,4-dihydropyridine derivatives, e.g., nisoldipine and nifedipine. In contrast, switching to a hyperpolarized holding potential ( − 80 mV) could not alleviate the inhibitory effect of ligustrazine on $I_{CaL}$ of the coronary artery smooth muscle cells. These results strongly suggest that the inhibitory effect of ligustrazine of $I_{CaL}$ is distinct from that produced by nifedipine. It is interesting to note that in rat ventricular myocyte (Zou et al., 2001), ligustrazine at 250 μM and 1 mM caused ~ 60% and ~ 68% inhibition of $I_{CaL}$, respectively. In our study, ligustrazine (300 μM) caused >90% inhibition of $I_{CaL}$. In contrast to rat ventricular myocyte (Zou et al., 2001), ligustrazine was without effect on the steady-state inactivation of $I_{CaL}$ of the coronary smooth muscle cells. Taken together, our results demonstrate that ligustrazine acts differently from the dihydropyridine derivative in inhibiting $I_{CaL}$.

Activation of adenylate cyclase and the subsequent increase in intracellular cAMP level have been shown to activate protein kinase A in pig coronary arteries (Jiang et al., 1992). In organ-bath experiments, our results indicate that the relaxation effect of ligustrazine is *cis*-*N*-(2-phenylcyclopentyl) azacyclotridec-1-en-2-amine (MDL 12330A, an adenylate cyclase inhibitor)- and *N*-[2-((bromocinnamyl)amino)ethyl]-5-isoquinoline-sulphonamide (H-89, a protein kinase A inhibitor)-sensitive. To strengthen our conclusion on the involvement of adenylate cyclase/protein kinase A cascade, salbutamol (a $\beta_2$-adrenoceptor agonist acting through the adenylate cyclase pathway) and forskolin (an adenylate cyclase/protein kinase A activator) were also examined. Both salbutamol and forskolin mimicked ligustrazine, and caused MDL 12330A- and H-89-sensitive relaxation. In smooth muscle cell, ligustrazine-, salbutamol- and forskolin-mediated inhibition of $I_{CaL}$ was markedly attenuated by MDL 12330A and H-89. In addition, only H-89, but not MDL 12330A, abolished the inhibition of $I_{CaL}$ by dibutyryl cAMP. Taken together, our results indicate that ligustrazine-mediated coronary artery relaxation involved the activation of adenylate cyclase/cAMP/protein kinase A cascade, and the subsequent inhibition of $I_{CaL}$.

Although ligustrazine, salbutamol and forskolin caused porcine coronary artery relaxation via the same intracellular pathway, ligustrazine is the least potent one. It has been reported that cAMP/adenylate cyclase-mediated arterial relaxation involved multiple cellular mechanisms. In smooth muscle cells of guinea pig basilar artery (Tewari

and Simard, 1994), rat mesenteric artery (Taguchi et al., 1997), rat aortic myocytes (Neveu et al., 1994), rat portal vein (Viard et al., 2001) and porcine epicardial right coronary artery (diameter: 1.5–2.5 mm) (Fukumitsu et al., 1990), activation of β-adrenoceptor/adenylate cyclase cascade resulted in an enhancement of $I_{CaL}$. However, our study and in rat tail artery smooth muscle cells (Wang et al., 2000) clearly indicated that elevation of cAMP consistently lead to an inhibition of $I_{CaL}$. The underlying reasons responsible for the discrepancy between our observations and results reported by Fukumitsu et al. (1990) are not known. It was, however, admitted by Fukumitsu et al. (1990) that the physiological significance of an increase in inward current ($I_{CaL}$) by β-adrenoceptor activation was difficult to assess and does not seem to occur physiologically. Perhaps, it may be related to the anatomical location of coronary artery used (we used tertiary branch of the left anterior descending coronary artery (O.D. ~ 1 mm) which is deeply embedded in the heart muscle), and the different recording conditions (e.g. concentration of charge carrier) of the $I_{CaL}$. Nonetheless, it is important to point out that in the same vascular muscle (rabbit ear artery), potentiation (Benham and Tsien, 1988) and inhibition (Droogmans et al., 1987) by catecholamines of inward current ($I_{CaL}$) have been reported.

It has been reported that ligustrazine-mediated vascular relaxation involved both $Ca^{2+}$-dependent and ATP-dependent $K^+$ channels (Tsai et al., 2002). However, in our organ-bath experiments, $[Ba^{2+}]_o$ (a nonselective $K^+$ channel blocker), 4-aminopyridine (4-AP) (3 mM, a voltage-dependent $K^+$ channel blocker) (in vascular tissue, $IC_{50}$: 1.37 mM) (Ramillard and Leblanc, 1996), glibenclamide (an ATP-dependent $K^+$ channel blocker), iberiotoxin (a $BK_{Ca}$ blocker) and apamin (a small-conductance $Ca^{2+}$-activated $K^+$ channel inhibitor) all failed to modify ligustrazine-elicited relaxation. Besides, the magnitude of ligustrazine relaxation observed in high $[K^+]_o$ precontracted preparation was indistinguishable from that observed in preparation precontracted with 5-hydroxytryptamine. In single cell experiments, ligustrazine (300 μM) failed to modify glibenclamide-sensitive, ATP-dependent $K^+$ channel and $BK_{Ca}$ channel. Taken together, our results strongly suggest that $K^+$ channel openings are probably not responsible for ligustrazine-induced coronary artery relaxation.

In conclusion, our study demonstrates that ligustrazine (2,3,5,6-tetramethylpyrazine) and its structural analogues elicited porcine coronary artery relaxation. The magnitude of relaxation correlates fairly well with the reported lipophilicity (log *P*) of individual pyrazine compound, as well as the number of methyl group attached to the pyrazine moiety. Ligustrazine is the most potent, whereas 2-methylpyrazine is the least effective agent. In addition, the relaxation effect of ligustrazine is solely due to the inhibition of nifedipine-sensitive $I_{CaL}$ through the activation of adenylate cyclase/cAMP/protein kinase A cascade inside the vascular smooth muscle cells.

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