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# Cardiovascular effects of CPU-23, a novel L-type calcium channel blocker with a unique molecular structure

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**1** The cardiovascular effects of CPU-23 (1-{1-[(6-methoxy)-naphth-2-yl]}-ethyl-2-(1-piperidinyl)-acetyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline), a cleavage product of tetrandrine, were investigated using the whole cell perforated patch-clamp technique, *in vitro* tension measurements and *in vivo* haemodynamic recordings.

**2** CPU-23 (1 and 10 $\mu$M) dose-dependently reduced concentration–response curves for KCl and phenylephrine (PE) in the rat tail artery; inhibition of KCl-induced contraction was much more potent than for PE. At the same concentrations, CPU-23 inhibited the inward $Ba^{2+}$ currents in single smooth muscle cells isolated from the rat tail artery, while CPU-23 (10 $\mu$M) produced 95% vasorelaxation of the rat middle cerebral artery preconstricted with BayK 8644.

**3** CPU-23 (10 and 30 $\mu$M) inhibited the noradrenaline-induced phasic contraction of the rat tail artery in the absence of extracellular $Ca^{2+}$ from 40% of control to 23% and 14%, respectively ($P<0.01$) and tonic contraction of the artery after addition of $Ca^{2+}$ (2 mM) from 100% of control to 83% and 75%, respectively ($P<0.01$). In the presence of extracellular $Ca^{2+}$ the PE-induced contraction was reduced by CPU-23 (30 and 100 $\mu$M) to 27% and 37%, respectively.

**4** The haemodynamic profile of CPU-23 in the rat was very similar to diltiazem. At 5 mg $kg^{-1}$ CPU-23 induced a rapid onset and long-lasting decrease in left ventricular systolic pressure (LVSP), maximal velocity of pressure increase ($dP/dt_{max}$), systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR). When haemodynamic actions of CPU-23, verapamil, diltiazem and nifedipine were compared at equidepressor doses, the order of potency for reducing LVSP and $dP/dt_{max}$ was verapamil > CPU-23 = diltiazem > nifedipine and the order of potency for decreasing HR was verapamil = CPU-23 = diltiazem > nifedipine.

**5** These data indicate that CPU-23 is a novel calcium channel blocker with unique molecular structure, which exerts antihypertensive and cardiac depressant effects due primarily to its action on L-type voltage-gated calcium channels.

**Keywords:** CPU-23; calcium channel blocker; calcium currents; vasorelaxation; haemodynamics; rat tail artery

## Introduction

The clinical use of calcium channel blockers has become increasingly popular for the treatment of various cardiovascular disorders such as hypertension, angina pectoris and supraventricular arrhythmias. Currently, three distinct classes of L-type calcium channel blockers have been accepted into clinical use, namely the dihydropyridines (e.g. nifedipine, nitrendipine and nimodipine), the phenylalkylamines (e.g. verapamil) and the benzothiazepines (e.g. diltiazem) (Fleckenstein, 1977; Conti *et al*., 1985). The prototypic dihydropyridine calcium channel blocker, nifedipine, can induce vasorelaxation and lower blood pressure by reducing peripheral vascular resistance; however, it can also produce a reflex tachycardia which somewhat limits its clinical use. While numerous new dihydropyridine analogues are currently being developed by different laboratories to provide better benefit-to-risk ratios than nifedipine (Freeman & Waters, 1987; Ohtsuka *et al*., 1989), we and others have been attempting to develop novel calcium channel blockers based on lead compounds isolated from Chinese medicinal herbs (Huang *et al*., 1990; Dong *et al*., 1992; 1993).

The alkaloid tetrandrine is isolated from the Chinese medicinal herb *Radix stephania tetrandrae* and has been used traditionally in China to treat angina and hypertension with good results (Gao *et al*., 1965). Recent pharmacological studies have demonstrated that tetrandrine blocks inward calcium currents through the L-type voltage-gated calcium channel (VGCC) (Liu *et al*., 1995). As it competitively inhibits [$^3$H]-diltiazem binding, partly inhibits [$^3$H]-verapamil binding and stimulates [$^3$H]-nitrendipine binding to cardiac sarcolemmal membranes in a manner very similar to diltiazem, it is likely that tetrandrine acts at the benzothiazepine site of the L-type calcium channel (King *et al*., 1988). Chemical synthesis of tetrandrine is difficult due to its complicated structure. Additionally, the pharmacological profile of tetrandrine indicates that in comparison to the dihydropyridine calcium channel blockers, tetrandrine has less potency and selectivity for blood vessels (Dong, 1994). Therefore, the clinical use of tetrandrine has been limited.

To address these problems, we have synthesized and screened, using [$^3$H]-nitrendipine binding and biological assays, a series of benzyl-tetrahydroisoquinolines (cleavage products of tetrandrine). CPU-23 (Figure 1), the most potent compound identified in this series, was found to inhibit KCl-induced contraction of rat aorta and displace [$^3$H]-nitrendipine binding in rat cerebral cortical membranes with a similar potency. CPU-23 also behaves as a simple competitive inhibitor at [$^3$H]-nitrendipine binding sites (Dong *et al*., 1992), inhibits KCl-induced $Ca^{2+}$ influx, alters the action potential characteristics of myocardial preparations *in vitro* and induces hypotension and bradycardia in rats *in vivo*. We suggested that CPU-23 may exert its cardiovascular effects by inhibiting calcium influx via specific calcium channels, probably L-type VGCC (Dong *et al*., 1992; 1993). Therefore, the aim of the present study was to test the hypothesis that CPU-23 inhibits L-type VGCC in isolated vascular smooth muscle cells. The cardiovascular effects of CPU-23 were also investigated using both *in vitro* isolated tissue studies of the rat tail artery and *in vivo* haemodynamic recordings in rats.

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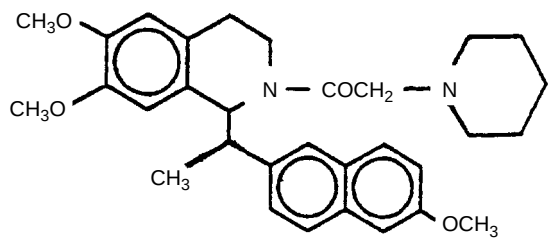


**Figure 1** Chemical structure of CPU-23 (1-{1-[(6-methoxy)-naphth-2-yl]}-ethyl-2-(1-piperidinyl)-acetyl-6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline).

## Methods

### *Tension measurements*

Sprague-Dawley rats (300–350 g) of either sex were killed according to a research protocol consistent with the standards of the Canadian Council on Animal Care and approved by the local Animal Care Committee of The University of Calgary. The tail artery and middle cerebral artery were rapidly removed and placed in cold physiological salt solution (PSS) of the following composition (in mM): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; $KH_2PO_4$ 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 12.5; dextrose 11.1. The pH of the solution after saturation with 95% $O_2$ + 5% $CO_2$ gas mixture was 7.4. In $Ca^{2+}$-free PSS, $CaCl_2$ was omitted from the PSS. Adherent connective tissue was removed and a 2 mm segment of either artery was then prepared for recording isometric force development in a wire myograph as previously described (Mulvany & Halpern, 1976). Briefly, two tungsten wires (40 $\mu$m diameter) were inserted through the lumen of the vessel and the tissue placed in a 10 ml myograph chamber containing gassed PSS. One wire was then attached to a force transducer and the other connected to a micrometer. Endothelial cells were removed from the vessels by repeatedly passing a stainless steel cannula through the vessel lumen. Cannulae with external diameters slightly larger than the lumenal diameter of the artery were used. Destruction of the endothelium was confirmed pharmacologically by loss of a relaxant response to acetylcholine (10 $\mu$M). After a 30 min equilibration period, arteries were stretched in a stepwise fashion to a resting tension of 10 mN (tail artery) or 2 mN (middle cerebral artery) as preliminary studies had demonstrated that these preloads were optimal for force development. Tissues were allowed to equilibrate for 1 h before the start of the experiments. Isometric tension was directly downloaded to a hard disc via an A/D converter (Axon Instruments, Foster City, U.S.A.) and analysed using commercial software (Axotape 2.0, Axon Instruments) with a 486 IBM clone computer.

The first series of experiments was performed to determine the effects of CPU-23 on KCl- or phenylephrine (PE)-induced tension in segments of rat tail arteries. After equilibration in PSS for 1 h, arterial rings were constricted by the stepwise addition of either KCl or PE to construct control concentration–response curves. The tissues were then treated with CPU-23 (1–10 $\mu$M) prior to a repeated exposure to KCl or PE. The concentration–response curves were always performed after 30 min of washing with PSS and CPU-23 was added to the tissues 40 min prior to the addition of the agonist. Three concentration–response curves were obtained for an individual tissue in these experiments. In preliminary experiments, control curves to KCl or PE were performed in the absence of antagonists to determine the effects of time and repeated exposure to KCl or PE on the magnitude of the vasoconstriction. No significant difference was identified during three sequential concentration–response curves. In a second series of experiments, noradrenaline (NA) was used to induce contraction in nominally $Ca^{2+}$-free PSS. This was followed by addition of $CaCl_2$ (2 mM). After control experiments, the tissues were preincubated with CPU-23 for 40 min before a repeated exposure to NA and $CaCl_2$.

### *Rat tail artery smooth muscle cell isolation*

Freshly dispersed vascular smooth muscle cells from the rat tail artery were isolated from male Sprague-Dawley rats weighing 350–450 g using a technique developed by Loutzenhiser *et al.* (unpublished results). Briefly, rats were anaesthetized using halothane and maintained with methoxyflurane applied via a nose cone. The lower abdomen was opened and the digestive system gently moved to one side. An i.v. injection of 0.1 ml heparin sodium was given into the femoral artery. The descending aorta was located above its bifurcation into the common iliac arteries and cleaned of connective tissue. A suture was placed around the aorta but not tied and the two femoral arteries were located and tied off. Three 10 ml syringes were prepared: two were filled with Dulbecco's Modified Eagle Medium (DMEM), the third was filled with agarose (SeaPrep ultralow gelling agarose, FMC Bioproducts, Rockland, Maine, U.S.A.) in Hank's Balanced Salt Solution (Gibco BRL, Life technologies, Grand Island, New York, U.S.A.). All solutions were maintained at 37°C. A modified 18 guage needle with a tapered end was connected to a three-way stopcock valve. The descending aorta was then clamped above and below the site of the suture thread and a small cut was made in the aorta. The cannula was inserted into the vessel, tied off with suture thread and the lower clamp removed. DMEM was infused into the descending aorta to remove blood from the tail artery followed by agarose gel (at 37°C) taking care not to apply excess pressure. The tail was placed on ice for 5 min to allow the agarose to solidify, the tail was removed and the animal was killed by laceration of the heart. The tail artery was placed into a nominally calcium-free dissection solution (SMDS, composition (mM): 120 NaCl, 25 $NaHCO_3$, 4.2 KCl, 0.6 $KH_2PO_4$, 1.2 $MgCl_2$, 11 glucose), cleaned of its connective tissue, cut into approximately 5 cm pieces and maintained at 37°C in SMDS. Enzyme containing DMEM was prepared with 2 mg $ml^{-1}$ collagenase type IV, 2 mg $ml^{-1}$ dispase II (neutral protease), 40 $\mu$g $ml^{-1}$ elastase III, 20 $\mu$g $ml^{-1}$ collagenic protease and 3.8 $\mu$g $ml^{-1}$ DNAse I. The tissue pieces were incubated at 37°C for 50 min then moved to a 2 mM EGTA solution for 5 min and stored on ice in DMEM until use. When required, single vascular smooth muscle cells were liberated from the tissue by gentle trituration and placed in a perfusion chamber where they adhered to a coverslip comprising the base of the chamber.

### *Electrophysiology*

Barium current recordings using the perforated patch clamp technique were obtained using a bath solution containing (mM): 130 NaCl, 5.4 KCl, 10 glucose, 10 $BaCl_2$, 0.1 EGTA, 10 HEPES pH 7.4 adjusted with NaOH and a pipette solution of composition (mM): 130 CsCl, 1 $MgCl_2$, 5 EGTA, 5 $Na_2ATP$, 5 HEPES, pH7.2 adjusted with CsOH. Under these conditions amphotericin was used to obtain electrical access to the cell without dialysis of the intracellular compartment. Twenty-five milligram pluronic F-68 was dissolved in 1 ml DMSO, sonicated and stored in the fridge for up to 1 week. Five milligram amphotericin was dissolved in 100 $\mu$l of this pluronic stock and sonicated; 20 $\mu$l of this solution was added to 4.98 ml of internal solution containing 5 mg BSA and sonicated to give a final concentration of amphotericin of 200 $\mu$g $ml^{-1}$. Electrodes were pulled with a Model P-87 Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, U.S.A.) from borosilicate glass and fire polished (Narishige Scientific Instrument Lab., Tokyo, Japan). The tip of the electrode (typically 3–6 MΩ) was briefly dipped into amphotericin-free solution prior to backfilling with amphotericin internal solution. After gigaseal formation, access resistance in the 6–10 MΩ range was typically obtained within 5–15 min. Series resistance was compensated 60% using an

Axopatch 200 A amplifier. Signals were filtered at 1 kHz, digitized using Axon Instruments DigiData 1200 interface, acquired and analysed using Axon Instruments pClamp 6.0. Whole cell barium currents were recorded at room temperature (21–23°C) and solutions were perfused through the bath using a Multi-Flo RVA-6 controller (Norscan Instruments Winnipeg). From a holding potential of −80 mV, current-voltage (I–V) relations were determined by stepping the voltage from −60 mV, in 10 mV steps for 200 ms, up to +70 mV, under control conditions and in the presence of the drug. CPU23 was dissolved in distilled water and added to the bath solution to give the final concentrations indicated. During drug application the cell was stepped from a holding potential of −80 mV to 0 mV for 200 ms every 10 s. Only cells of a spindle-shaped, optically refractive and relaxed nature were chosen for study.

### *Haemodynamic recordings*

Male Sprague-Dawley rats weighing 350–450 g were anaesthetized with pentobarbitone sodium (75 mg $kg^{-1}$, i.p.) and the trachea was cannulated to allow artificial ventilation. For the recording of the left ventricular systolic pressure (LVSP), a cardiac catheter (PE50) filled with heparin (145 IU $ml^{-1}$) was introduced via the right carotid artery. The catheter was connected to a pressure transducer (Gould P23 ID) and the maximal velocity of pressure increase ($\pm dP/dt_{max}$) was determined by an electronic differentiation. A catheter (PE50) connected to another pressure transducer was inserted into the left femoral artery to measure the systonic (SBP) and diastolic (DBP) blood pressure. Heart rate (HR) was recorded with a cardiac tachometer (Model 7P4) triggered by the arterial blood pressure pulse. All the cardiovascular parameters were recorded continuously with a polygraph data recording system (Grass, model 7D). A catheter was placed in the right femoral vein for administration of drugs and constant rectal temperature was maintained at about 37°C with a table lamp during the entire experiment. After surgery, rats were left for 30 min before beginning the experiment by bolus injection of drugs. Drug administration volume was less than 0.4 ml. All drugs were dissolved in saline except nifedipine, which was dissolved in ethanol and diluted in saline before use. In preliminary experiments, these haemodynamic parameters varied less than 5% from baseline values over a 30 min period after bolus injection of vehicles.

### *Chemicals*

L-phenylephrine, noradrenaline, nifedipine, collagenase type IV, elastase III, pluronic F-68 and amphotericin were purchased from Sigma Chemical Company (St. Louis, U.S.A.). Dispase II (Neutral protease), collagenic protease and DNAse I were purchased from Boehringer Mannheim (Quebec, Canada). SK&F96365 was purchased from Calbiochem-Novabiochem Corporation (California, U.S.A.). Verapamil, diltiazem, nimodipine and BayK 8644 were purchased from Research Biochemicals, Inc. (Natick, U.S.A.). CPU-23 was synthesized as previously described (Hunag *et al*., 1990) and

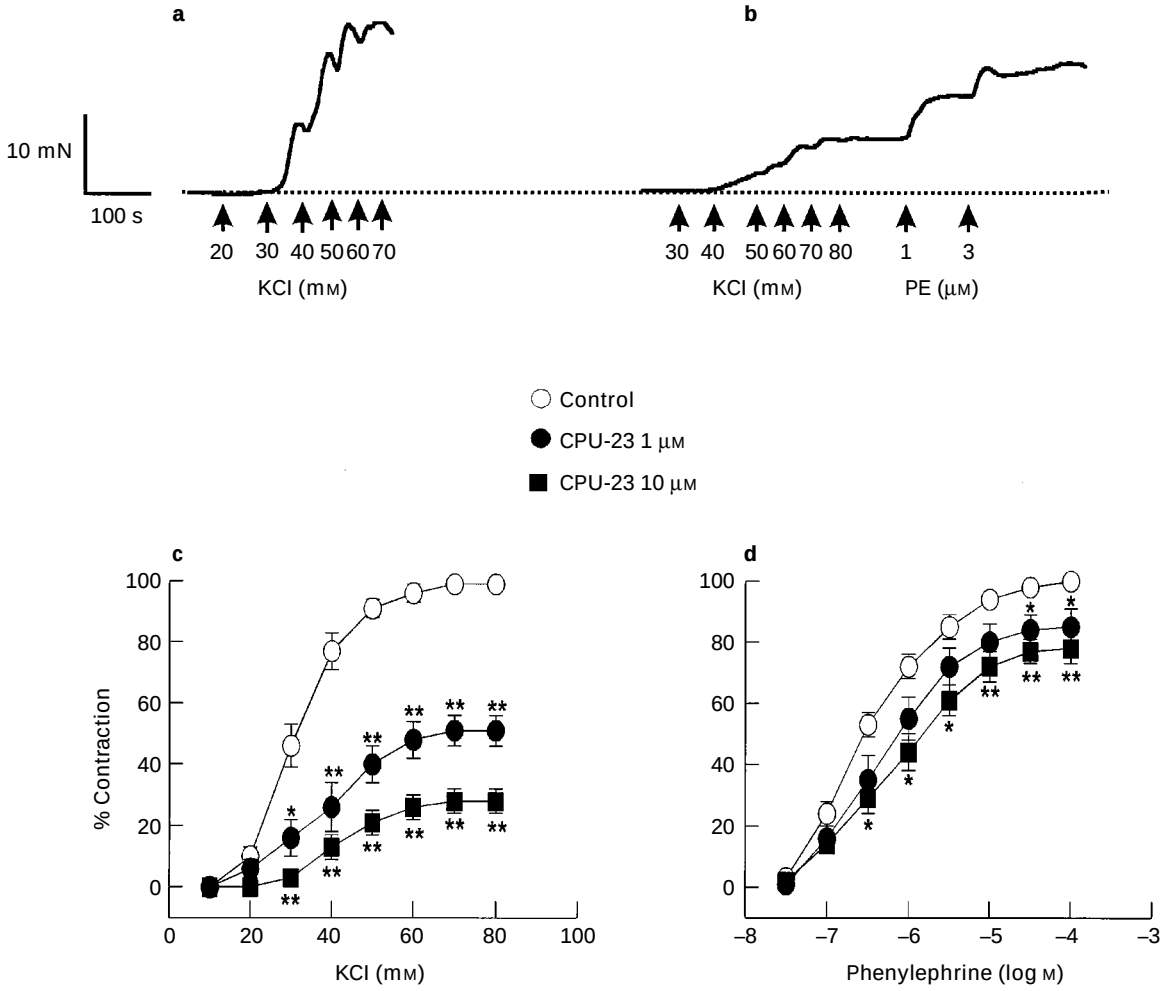


**Figure 2** Original tracings show the concentration–response curves of the rat tail artery for KCl and phenylephrine in the absence (a) and the presence of CPU-23 (10 $\mu$M, b). CPU-23 mediated inhibition of the concentration–response curves for KCl (c) and phenylephrine (d), plotted as mean±s.e.means from six to seven separate experiments. *$P<0.05$; **$P<0.01$ compared to control.

generously provided by Professor S.X. Peng of the China Pharmaceutical University.

Stock solutions of nifedipine, nimodipine and BayK 8644 were prepared in ethanol and diluted in PSS for *in vitro* or in saline for *in vivo* experiments before use. All other compounds dissolved freely in distilled water. At the final concentrations employed (<0.1%), none of the solvents used had any effect on the vessels during preliminary experiments.

### *Data analysis*

All statistical data are presented as means ± s.e.means and analysed using Student's *t* test or ANOVA where appropriate. Statistical significance was considered when $P<0.05$. Current traces were adjusted for junction potential. When necessary, $pD_2'$ value was determined by the method of Van Rossum (1963).

## Results

### *Effects of CPU-23 on KCl-induced contractions in the presence of extracellular $Ca^{2+}$*

Following equilibration in PSS for 1 h, addition of KCl induced a concentration-dependent contraction in segments of the rat tail artery in the presence of extracellular $Ca^{2+}$ (Figure 2a). Maximal contraction observed with KCl (80 mM) was 18.1 ± 1.1 mN ($n=7$). After washout and equilibration of tissues in PSS for 30 min, the addition of CPU-23 (10 $\mu$M) did not affect the resting tension of the rat tail arteries, but after preincubation with CPU-23 for 40 min, the concentration–response curves for KCl were markedly reduced: maximal contraction with KCl (80 mM) following preincubation with CPU-23 (1–10 $\mu$M) was reduced to 9.2 ± 0.7 mN and 5.0 ± 0.5 mN ($P<0.01$, $n=7$ for both), respectively. However, addition of PE still induced contraction of the rat tail artery (Figure 2b). Figure 2c shows CPU-23 (1–10 $\mu$M) noncompetitively inhibited the concentration–response curves for KCl in a concentration-dependent manner. The maximal response to KCl was reduced to 51 ± 5% and 28 ± 4% by CPU-23 at concentrations of 1 and 10 $\mu$M ($P<0.01$, $n=7$), providing a $pD_2'$ value of 5.70 ± 0.02 ($n=7$).

To address whether CPU-23 affects VGCCs, Bay K 8644 (1 $\mu$M), an L-type VGCC opener, was used to induce force development in the rat middle cerebral artery. At the plateau phase of BayK 8644-induced contraction, nimodipine (1 $\mu$M), a L-type VGCC blocker produced relaxation by 98 ± 5% of contraction ($n=6$). Similarly, CPU-23 (10 $\mu$M) produced relaxation by 95 ± 2% of BayK 8644-induced contraction in the rat middle cerebral artery ($n=6$).

### *Effects of CPU-23 on PE-induced contraction in the presence of extracellular $Ca^{2+}$*

PE induced a concentration-dependent vasoconstriction in rat tail artery rings in the presence of extracellular $Ca^{2+}$. After recovery of tissues in PSS for 30 min, pretreatment with CPU-23 (1–10 $\mu$M) for 40 min noncompetitively inhibited the concentration–response curves for PE (Figure 2d). The PE-induced maximal contraction was reduced to 85 ± 6% ($P<0.05$, $n=6$) by CPU-23 at the concentration of 1 $\mu$M and to 78 ± 5% ($P<0.01$, $n=6$) at the concentration of 10 $\mu$M. $pD_2'$ value for CPU-23 inhibition of PE-induced vasoconstriction could not be calculated as with a high concentration (10 $\mu$M) of CPU-23 the contraction to PE was greater than 50% of that in the absence of CPU-23: maximal contraction with PE (100 $\mu$M) was 21.3 ± 1.2 mN, reduced to 16.6 ± 0.8 mN by 10 $\mu$M CPU-23 ($n=6$).

In order to further investigate the cellular sites of action of CPU-23, the effects of CPU-23 on PE-induced contractions in the rat tail artery were compared to those evoked by verapamil, a L-type VGCC blocker, and SK&F96365, a putative inhibitor of receptor-operated calcium channels (Merritt *et al*., 1990; Moritoki *et al*., 1996). SK&F96365 (100 $\mu$M) completely relaxed the plateau phase of PE-induced contraction in the rat tail artery (Figure 3). The addtion of CPU-23 (1 $\mu$M) during the plateau phase of PE-induced contraction did not induce

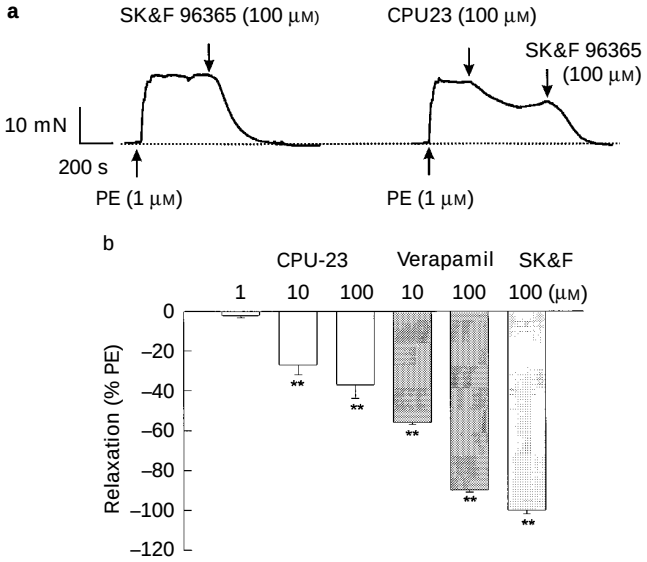


**Figure 3** Comparison of CPU-23, verapamil and SK&F 96365-mediated inhibition of phenylephrine (PE, 1 $\mu$M)-induced contraction in the rat tail artery. After the phenylephrine-induced contraction reached a steady state, various concentrations of CPU-23, verapamil or SK&F 96365 were added cumulatively. Data presented as the mean ± s.e.means from six to eight separate experiments. $^{**}P<0.01$ compared to control.

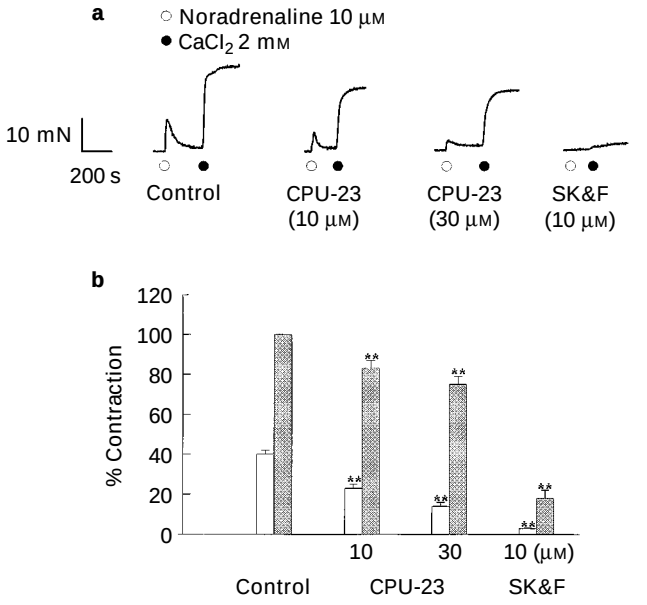


**Figure 4** Comparison of CPU-23 and SK&F 96365 on noradrenaline-induced contractions in the rat tail artery. (a) Original tracings: noradrenaline (10 $\mu$M) induced a phasic contraction in the absence of extracellular $Ca^{2+}$, which was followed by a tonic contraction after addition of extracellular $CaCl_2$ (2 mM). Preincubation with CPU-23 (10–30 $\mu$M) and SK&F 96365 (10 $\mu$M) resulted in a reduction in the contraction induced by noradrenaline and $CaCl_2$. (b) Comparison of CPU-23 and SK&F 96365 on noradrenaline-induced phasic contractions (□) in the absence of extracellular $Ca^{2+}$ and on tonic contractions (■) induced after addition of extracellular $CaCl_2$. Data represented as mean ± s.e.means from five to six separate experiments. $^{**}P<0.01$ compared to control.

significant relaxation of the tissue (2±1%, $P>0.05$, $n=7$); however, at concentrations of 10 and 100 μM, CPU-23 did induce relaxation by 27±5% and 37±7%, respectively ($P<0.01$, $n=7$). At high concentrations (100 μM), CPU-23 did not completely relax the plateau phase of PE-induced contraction in the rat tail artery; however, SK&F96365 (100 μM) completely relaxed the CPU-23 insensitive portion (Figure 3a). Verapamil and SK&F96365 (100 μM) produced 90±1% ($P<0.01$, $n=4$) and 100±2% ($P<0.01$, $n=8$) relaxation, respectively (Figure 3b).

### *Effects of CPU-23 on NA-induced contraction in the absence of extracellular $Ca^{2+}$*

In rat tail arterial ring preparations NA (10 μM) induced a phasic contraction in the absence of extracellular $Ca^{2+}$, followed by a tonic contraction after addition of $CaCl_2$ (2 mM) (Figure 4a). In nominally $Ca^{2+}$-free PSS this phasic contraction under control conditions was 40±2% of that induced by the addition of $CaCl_2$. After preincubation with CPU-23 (1 μM) for 40 min, both phasic and tonic contractions of the arterial rings were not significantly affected. However, after preincubation with higher concentrations of CPU-23 (10 and 30 μM), contraction was significantly reduced in $Ca^{2+}$-free PSS to 23±2% ($P<0.01$, $n=6$) and 14±2% ($P<0.01$, $n=5$) of control and in the presence of $Ca^{2+}$ to 83±4% and 75±4% ($P<0.05$), respectively. SK&F96365 (10 μM) significantly inhibited both the NA-induced contraction in $Ca^{2+}$-free PSS (3±1%, $P<0.01$, $n=6$) and the contraction induced by addition of $CaCl_2$ (18±4%, $P<0.01$, $n=6$, Figure 4b).

### *Effects of CPU-23 on the voltage-gated $Ca^{2+}$ channel*

To determine if the inhibitory effects of CPU-23 observed in this study were due to direct effects of the drug on VGCC, we undertook voltage clamp experiments using the perforated patch clamp technique. Utilizing freshly dispersed vascular smooth muscle cells from the rat tail artery, we recorded whole cell barium currents in the absence and presence of CPU-23 (1 and 10 μM). Cell capacitance was 52±10.8 pF ($n=9$), average peak inward current amplitude at +10 mV varied from −482.5 to −77.6 pA (mean±s.e.means was −215.6±117.9 pA, $n=9$) as is evident in Figure 4a vs b and c, giving values for pA/pF of −1.7 to −8.5 (mean±s.e.means was −4.3±2.4 pA/pF, $n=9$).

The inward currents displayed characteristics typical of the L-type VGCC; they were inhibited by the dihydropyridine calcium channel blocker, nifedipine (0.1 μM), and potentiated by the dihydropyridine calcium channel activator, BayK 8644 (1 μM) (Figure 5a), displaying a shift peak current. In all cases, we observed a dose-dependent decrease in the inward currents in the presence of CPU-23: Figure 5b and c show a representative recording and I–V curve for $n=6$–9 cells, obtained under control conditions and during exposure to 1 and 10 μM of the compound. Figure 5d describes the effects of CPU-23 on inward current measured at +10 mV, the potential at which peak current was observed: CPU-23, unlike the dihydropyridines, did not shift the voltage at which peak current was observed. CPU-23 (1 μM) inhibits peak current by 30.9±6% ($n=9$) whereas CPU-23 (10 μM) inhibits peak current by 80.4±14% ($n=6$), resulting in a significant inhibition of the $Ba^{2+}$ current at both concentrations compared to control

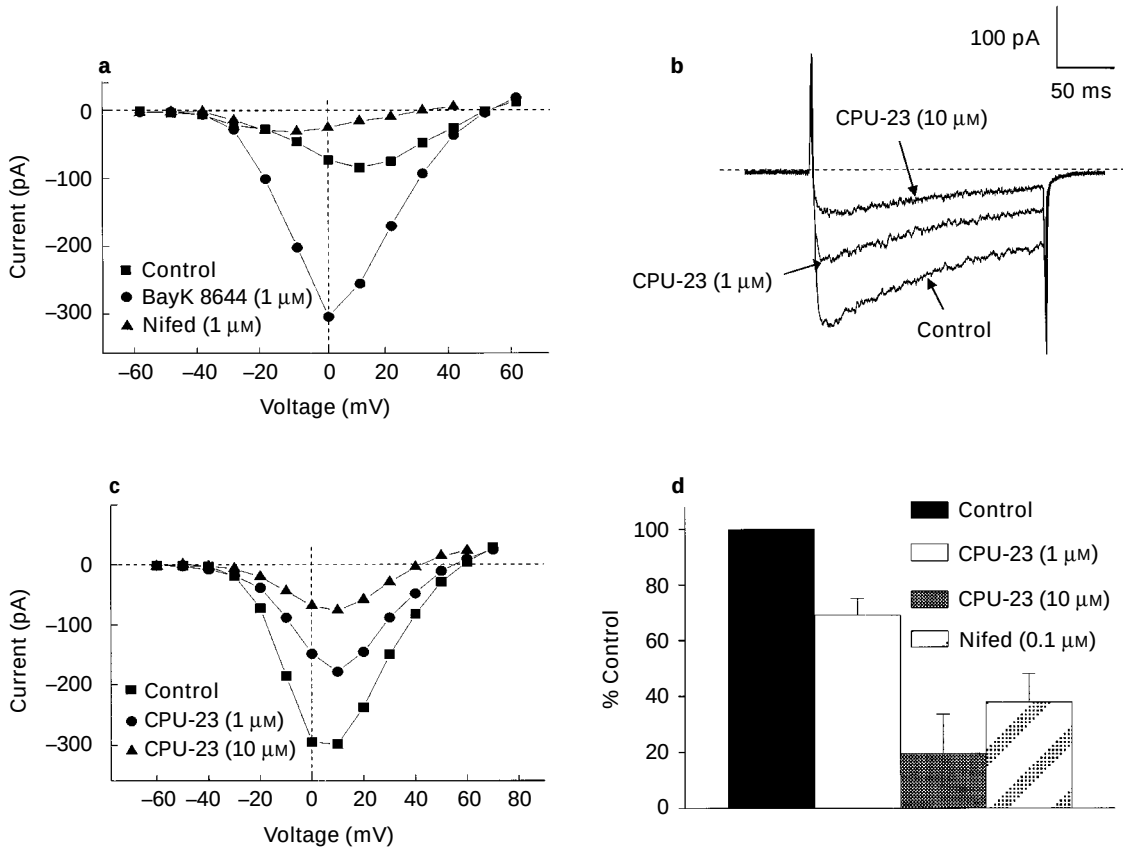


**Figure 5** Inward currents recorded using $Ba^{2+}$ (10 mM), in freshly dispersed vascular smooth muscle cells from the rat tail artery. (a) Effects of the dihydropyridines, nifedipine (0.1 μM) and BayK 8644 (1 μM) on the current-voltage relation. Similar effects were observed in $n=5$ cells. (b) Representative trace showing the effects of CPU-23 on the inward curent elicited by stepping to 0 mV from a holding potential of −80 mV. A dose-dependent inhibition of the current was observed. (c) Representative trace showing the inhibitory effects of CPU-23 (1 and 10 μM) on the I–V relation for this current. (d) Bar graph showing inhibition of the inward current with CPU-23 plotted as mean±s.e.means from six to nine cells at +10 mV, the potential at which peak current was observed in all cells. CPU-23 inhibits peak current by 30.9±6% (1 μM) and by 80.4±14% (10 μM). *$P<0.05$ compared to control.

**Table 1** Haemodynamic effects of CPU-23 (5 mg kg$^{-1}$, i.v.) in anaesthetized Sprague-Dawley rats

| | | *Time after administration* (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Control* | *1* | *3* | *5* | *10* | *15* | *20* | *25* | *30* |
| LVSP | 156±8 | 109±7** | 120±7** | 124±7** | 133±6** | 143±7** | 145±6* | 148±7** | 149±7 |
| $+dP/dt_{max}$ | 6060±387 | 3757±373** | 4242±405** | 4485±403** | 5151±412** | 5394±375** | 5576±362** | 5637±375** | 5697±375* |
| $-dP/dt_{max}$ | 5515±441 | 2970±275** | 3455±281** | 3697±260** | 4182±299** | 4667±311** | 4950±341* | 4950±341* | 5091±351* |
| SBP | 144±8 | 87±7** | 88±7** | 105±8** | 120±6** | 128±6** | 134±6* | 137±6 | 138±6 |
| DBP | 102±6 | 60±5** | 69±5** | 72±5** | 78±6** | 85±5** | 85±5** | 89±6** | 90±5** |
| HR | 374±17 | 300±15** | 287±19** | 287±19** | 305±17** | 321±17** | 336±17** | 342±17** | 345±17** |

Values are mean±s.e.mean, $n=11$, $^*P<0.05$, $^{**}P<0.01$ vs control. LVSP: left ventricular systolic pressure (mmHg); $\pm dP/dt_{max}$: maximal velocity of pressure increase (mmHg s$^{-1}$); SBP: systolic blood pressure (mmHg); DBP: diastolic blood pressure (mmHg); HR: heart rate (bpm).

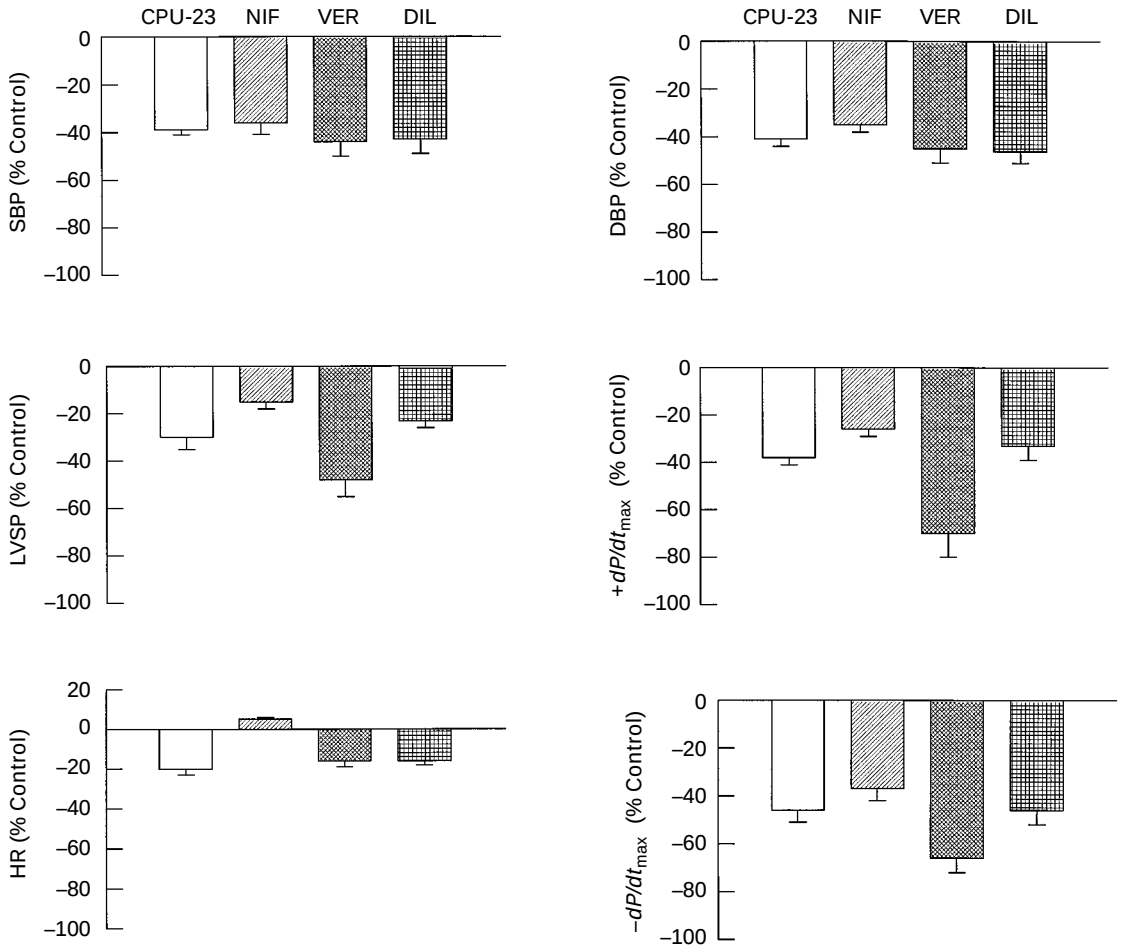


**Figure 6** Comparison of CPU-23, nifedipine (NIF), verapamil (VER) and diltiazem (DIL) on systolic blood pressure (SBP), diastolic blood pressure (DBP), left ventricular sytolic pressure (LVSP),maximal velocity of pressure increase ($\pm dP/dt_{max}$) and heart rate (HR) in rats. Columns are the mean±s.e.means from 7 to 11 animals.

($P<0.05$). Similar dose-dependent effects were also observed in isolated cells from the rabbit portal vein (data not shown).

### *Haemodynamic effects of CPU-23*

Prior to drug administration, haemodynamic parameters of anaesthetized rats were stable. Acute administration of CPU-23 (5 mg kg$^{-1}$, i.v.) induced a rapid onset decrease in haemodynamic parameters which were apparent 1 min after CPU-23 administration (Table 1). CPU-23 decreased LVSP, $+dP/dt_{max}$, $-dP/dt_{max}$, SBP, DBP and HR by 30±5%, 38±3%, 46±4%, 39±2%, 41±3% and 20±2%, respectively. The inhibitory effects of CPU-23 on these haemodynamic parameters were long lasting and all except LVSP and SBP remained significantly depressed 30 min after drug administration (Table 1). The effects of CPU-23 were compared with three other classes of calcium channel blockers in clinical use, the dihydropyridines, the phenylalkylamines and benzothiazepines. Equidepressor doses of these drugs were administered: CPU-23 (5 mg kg$^{-1}$), nifedipine (0.3 mg kg$^{-1}$), verapamil (1 mg kg$^{-1}$), diltiazem (2 mg kg$^{-1}$). At these equidepressor doses, which lowered SBP and DBP by approximately 40%, the order of potency for reducing LVSP and $dP/dt_{max}$ was verapamil > CPU-23 = diltiazem > nifedipine and the order of potency for reducing heart rate was verapamil = CPU-23 = diltiazem > nifedipine (Figure 6).

## Discussion

CPU-23 has been shown to reduce heart rate and blood pressure in normotensive WKY and spontaneously hyperten-

sive rats, to inhibit KCl-induced contraction of rat aorta and to displace [$^3$H]-nitrendipine binding in rat cerebral cortical membranes, all with similar potency (Dong *et al*., 1992). In addition, CPU-23 inhibited KCl-induced $Ca^{2+}$ influx and altered action potential characteristics of myocardial preparations. Consequently, we suggested that the cardiovascular effects of CPU-23 were due primarily to inhibition of calcium influx via specific calcium channels, probably L-type VGCC (Dong *et al*., 1993). In the present study we have explored, in rat tail arterial rings, the mechanisms whereby CPU-23 induces either inhibition of KCl- and PE-induced contraction or vasodilation of α-adrenoceptor agonist-induced tone. More specifically, we have determined the effects of this compound on dihydropyridine-sensitive calcium channels in isolated tail artery cells and provide direct evidence that CPU-23 inhibits current flow through the L-type VGCC.

It is generally assumed that $Ca^{2+}$ influx from the extracellular medium is mainly responsible for contraction of vascular smooth muscle; contraction is quickly reduced or abolished by removal of external calcium or administration of calcium channel blockers. Although calcium channel blockers predominantly suppress high $K^+$-induced contraction, several of them such as verapamil, have little effect on the contraction induced by agonists at cholinoceptors or adrenoceptors. Therefore, the main mechanisms of action of calcium channel blockers are explained by selective blockade of $Ca^{2+}$ influx through the L-type VGCC (Conti *et al*., 1985; Godfraind *et al*., 1986). Furthermore, some calcium channel blockers also have other mechanisms of action, such as interaction with α-adrenoceptors, inhibition of intracellular $Ca^{2+}$ release and/or influx of $Ca^{2+}$ through receptor-operated calcium channels (ROCC) (Bolton, 1979; Godfraind *et al*., 1986). The greater inhibition of CPU-23 on the contraction induced by KCl compared to that induced by PE and its vasodilation of Bay K8644-induced tone are similar to those of classical L-type calcium channel blockers, such as verapamil and nifedipine (Godfraind *et al*., 1986; Hagiwara *et al*., 1993). In both the absence and presence of extracellular $Ca^{2+}$, CPU-23 had a significant effect on the NA-induced contraction. These data suggest that this compound, in addition to inhibiting influx of $Ca^{2+}$ via the L-type calcium channels, also affects contractility via other, as yet undefined mechanisms. In the present study, to more directly test the effects of CPU-23 on L-type VGCCs, we utilized the perforated patch clamp technique to record inward currents in isolated cells from the rat tail artery and observed a dose-dependent decrease in the amplitude of the dihydropyridine-sensitive VGCC; CPU-23 (1 μM) reduced peak current by $30.9 \pm 6\%$ whereas at 10 μM, peak current was reduced by $80.4 \pm 14\%$. These data indicate that CPU-23 can inhibit the opening of L-type VGCC. To our knowledge this is the first report of a direct action of this compound on VGCC.

Like some classical L-type calcium channel blockers, CPU-23, at concentrations exceeding 10 μM, also inhibited contraction induced by α-adrenoceptor agonists, although with less potency than those of verapamil and SK&F96365, a putative blocker of ROCC (Merritt *et al*., 1990; Moritoki *et al*., 1996). There are several explanations for these actions of CPU-23. First, CPU-23 may interact directly with α-adrenoceptors. To address this possibility, we performed binding studies in membrane preparations isolated from rat brain. CPU-23 was found to displace [$^3$H]-prazosin binding from $\alpha_1$-adrenoceptors with an $IC_{50}$ value of 3.2 μM (H. Dong, unpublished results). This $IC_{50}$ value is seven times higher than that of CPU-23 displacement of [$^3$H]-nitrendipine binding to L-type calcium channels (Dong *et al*., 1992). These data may, in part, explain the inhibitory effects of CPU-23 on the contractile response to α-adrenoceptor agonists observed in the present study. These pharmacological properties of CPU-23 are consistent with those of verapamil and diltiazem (Godfraind *et al*., 1986). CPU-23 was not found to interact with $\alpha_2$-adrenoceptor binding sites. Second, CPU-23 may inhibit intracellular $Ca^{2+}$ release and/or influx of $Ca^{2+}$ via VGCC-independent pathways. To address these possibilities, functional tests were performed in rat tail arteries. The initial phasic component of the response to α-adrenoceptor stimulation is thought to be mediated via inositol trisphosphate-induced release of intracellular $Ca^{2+}$, whereas the more sustained, tonic component of the response is most likely the result of activation of a $Ca^{2+}$ influx pathway (Ruffolo *et al*., 1991), which may be VGCC or perhaps ROCC (Bolton, 1979; Bülbring & Tomita, 1987). In our previous studies, verapamil was found to inhibit both phasic and tonic components of the response to α-adrenoceptors and 5-HT receptor stimulation (Dong *et al*., 1990; 1991). In the present study, SK&F96365, a putative blocker of the ROCC, also inhibited α-adrenoceptor-induced phasic and tonic contraction. It has recently been reported that tetrandrine and closely related analogues can affect non-voltage operated $Ca^{2+}$ entry as well as intracellular release in HL 60 cells (Leung *et al*., 1996). In the present study we have also shown that although CPU-23 inhibits both the phasic and tonic components of the normotensive response elicited in the absence of extracellular $Ca^{2+}$ the inhibition of the phasic response is greater, which may reflect an intracellular action of CPU-23 on the release of intracellular $Ca^{2+}$. However, these actions of CPU-23 will require further study. Like tetrandrine, the effects of CPU-23, at the whole tissue and single cell level, were not easily reversible.

In anaesthetized rats, we demonstrate a rapid onset and long lasting decrease in LVSP, $\pm dP/dt_{max}$, SBP, DBP and HR with CPU-23 (5 mg $kg^{-1}$, i.v.). The haemodynamic profile of CPU-23 was similar to the more classical calcium channel blockers (Waite *et al*., 1990). As with previous studies in anaesthetized Sprague-Dawley (SD), spontaneously hypertensive (SHR) and normotensive (WKY) rats (Dong *et al*., 1992), CPU-23 lowered DBP and SBP; the decrease in SBP was short lived when compared to the decrease in DBP (Table 1). The haemodynamic profile of CPU-23 *in vivo* was consistent with our observations *in vitro* from cardiac tissue (Dong *et al*., 1993). Therefore we propose that the cardiodepressant effects of CPU-23 observed in the present study may be due in part to VGCC blockade. It is well established that the effects of the dihydropyridines such as nifedipine on cardiac muscle are not as pronounced as those on vascular tissue. Here we show that nifedipine was less potent than CPU-23, verapamil and diltiazem on cardiac contractility and caused a small increase in HR, consistent with reports by Waite *et al*. (1990). Our results suggest that the haemodynamic effects of CPU-23 resemble those of the benzothiazepines rather than the dihydropyridines, although clearly we cannot account for all of our data without considering cellular actions of CPU-23 on sites other than the L-type VGCC.

In summary, we have demonstrated that a cleavage product of tetrandrine, CPU-23, has both hypotensive and cardiodepressant actions, which are similar to diltiazem, but distinct from nifedipine. We show for the first time a direct action of this compound on extracellular $Ca^{2+}$ entry via the VGCC in vascular smooth muscle cells isolated from the rat tail artery. We propose that the antihypertensive and cardiac depressant effects of CPU-23 are due primarily to inhibition of L-type VGCC, although additional effects of CPU-23 at high concentration cannot be ruled out from the present study.

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