Blockade of L-type calcium channel in myocardium and calcium-induced contractions of vascular smooth muscle by CPU 86017[1]

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ABSTRACT

AIM: To assess the blockade by CPU 86017 on the L-type calcium channels in the myocardium and on the Ca^{2+}-related contractions of vascular smooth muscle. **METHODS:** The whole-cell patch-clamp was applied to investigate the blocking effect of CPU 86017 on the L-type calcium current in isolated guinea pig myocytes and contractions by KCl or phenylephrine (Phe) of the isolated rat tail arteries were measured. **RESULTS:** Suppression of the L-type current of the isolated myocytes by CPU 86017 was moderate, in time- and concentration-dependent manner and with no influence on the activation and inactivation curves. The IC_{50} was 11.5 μmol/L. Suppressive effect of CPU 86017 on vaso-contractions induced by KCl 100 mmol/L, phenylephrine 1 μmol/L in KH solution (phase 1), Ca^{2+} free KH solution (phase 2) , and by addition of $CaCl_2$ into Ca^{2+}-free KH solution (phase 3) were observed. The IC_{50} to suppress vaso-contractions by calcium entry via the receptor operated channel (ROC) and voltage-dependent channel (VDC) was 0.324 μmol/L and 16.3 μmol/L, respectively. The relative potency of CPU 86017 to suppress vascular tone by Ca^{2+} entry through ROC and VDC is 1/187 of prazosin and 1/37 of verapamil, respectively. **CONCLUSION:** The blocking effects of CPU 86017 on the L-type calcium channel of myocardium and vessel are moderate and non-selective. CPU 86017 is approximately 50 times more potent in inhibiting ROC than VDC.

INTRODUCTION

CPU 86017 (*p*-chloro-benzyl-tetrahydroberberine), a derivative of berberine, blocked multiple ion channels evaluated by standard microelectrode electrophysiological study[1] and patch-clamp techniques. CPU 86017 is produced by attaching *p*-chlor-benzyl-side chain and hydrogenation on the moiety of berberine[2,3]. The bioavailability of berberine is very poor. So modification of moiety of berberine will be helpful to improve the solubility and bioavailability of berberine for oral medication. The potency of CPU 86017 was 7 times stronger than berberine to suppress the ouabain-induced arrhythmias in guinea pigs[4]. The inhibitory effects of CPU 86017 on phenylephrine induced contractions of rat anococcygeus had no significant difference compared with berberine[5]. The potency of CPU 86017 to suppress contractions of rat thoracic aortic ring induced by high K^+ in normal KH solution is as 3 times as ber-

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berine[6]. However the capability of CPU 86017 to suppress the contractions via the voltage dependent channel (VDC) is 10 time more potent than berberine[4]. It is implied that the L-type channels in myocardium was blocked by CPU 86017. The L-type Ca^{2+} channels in myocytes of guinea pigs are enhanced by chronic medication of L-thyroxin[7] and suppressed by propranolol[8]. CPU 86017 suppresses the electrical activity of pacing cells in the sino-atrial node[1] where the I_{Ca} is the main inward current to depolarize membrane and the developed force for atrial and papillary muscle contraction[5]. A reduction in heart rate and blood pressure after CPU 86017 intravenous treatment were observed[7,8].

The present study aimed to confirm the inhibitory effect of CPU 86017 on I_{Ca} current in isolated guinea-pig myocytes and to determine whether its inhibitory effects on Ca^{2+}-related contractions of the rat tail artery.

MATERIALS AND METHODS

Animals and chemical Guinea pigs 250-300 g, either sex, were provided by the Animal House of the Life Science Institute. SD rats, Grade II of either sex, 200±12 g, were supplied by the Experimental Animal Center of China Pharmaceutical University. The compound CPU 86017 was synthesized by the Center of New Drug Research of China Pharmaceutical University (Fig 1).

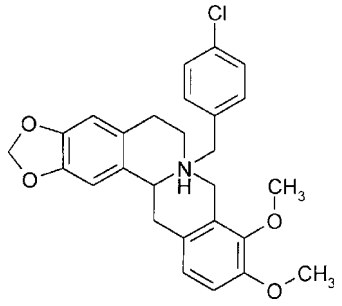

Fig 1. The chemical structure of CPU 86017.

Isolation of myocytes from guinea pig heart[7,8] In brief , the heart was taken quickly after a blow at the head, and mounted and infused at the Langendorff's apparatus at 37 ºC with O_2 gassed in series of solutions. The perfusion with the solution A was made at 6 mL/min for 5 min and followed by perfusion of an enzyme containing solution for 3 min to soften the heart. The ventricle was cut into small pieces at a size of 1 mm^3 at 37 ºC in the solution B for 3-5 min, then, the precipitate was moved into an enzyme containing solution B for 5 min. The supernatant was added with solution C 1.5 mL and striated myocytes with rode shape were selected for whole-cell patch-clamp to determine the I_{Ca} currents.

Reagents and solutions for patch clamp (mmol/L): 1) Solution A: NaCl 116, KCl 5.4, NaH_2PO_4 1.4, $MgSO_4$ 1.2, $NaHCO_3$ 15, glucose 15 , pH adjusted to 7.4 by NaOH. 2) Solution B: NaCl 116, KCl 5.4, NaH_2PO_4 1.4, $MgSO_4$ 1.2, $CaCl_2$ 0.15,$NaHCO_3$ 15, glucose 15, XIV type protease 0.1 g/L, BSA 1 g/L. 3) Solution C: NaCl 116, KCl 5.4, NaH_2PO_4 1.4, $MgSO_4$ 1.2, $CaCl_2$ 0.5, $NaHCO_3$ 15, glucose 15, XIV type protease 0.1 g/L, BSA 0.5 g/L. 4) The electrode solution: CsCl 140, $MgCl_2$ 2, $CaCl_2$, egtazic acid 11, Na-ATP 5, HEPES 10. 5) The cell bath solution: NaCl 116, KCl 5.4, NaH_2PO_4 1.4, $MgSO_4$ 1.2, $CaCl_2$ 0.5, $NaHCO_3$ 15, glucose, pH adjusted to 7.4 with NaOH.

Pronase E, HEPES, TEA-Cl, 4-AP, egtazic acid, Na-ATP, Tris, XIV protease, and BSA were products of Sigma. Tetrodotoxin (TTX) was from the Beijing Institution of Chemical Protection.

Whole-cell patch-clamp The patch clamp was conducted with the following instruments: the inverse microscopy (Nikon 810185, Japan), the microelectrode controller (Narishige PP-83, Japan), the liquid pressed processor (Narishige MD-320, Japan), the patch-clamp amplifier (EPC-7, List-Medical, Germany), the DMA sealing system (Labmaster Model TL-1, Axon Instruments, USA), computer (Casper, PC-386/IBM, USA) were used.

The CsCl electrode was applied to hold the myocyte at -40 mV and the stimulate potential was set from -40 mV up to +50 mV with an interval of 10 mV. Under this condition the inward I_{Na} and the T-type current were inactivated and the potassium current was suppressed by CsCl[11].

Contractions of rat tail artery The tail artery was carefully dissected and 3-mm length ring was mounted in 3 mL organ bath with a load of 0.6 g. The tail artery was treated with norepinephrine 1 μmol/L twice to stabilize the arterial contractile activity and washed and balanced for approximately 2 h. The contraction induced by phenylephrine 1 μmol/L or KCl 100 mmol/L in three phases: in the normal KH solution (phase 1) , the Ca^{2+} free KH solution (phase 2), and after addition of Ca^{2+} into the Ca^{2+} free medium (phase 3), respectively[12,13].

The IC_{50} of CPU 86017 on the contractions in the

three phases was determined separately in comparison with positive reference drug prazosin (Sigma) and verapamil (Lianyungang Pharmaceutical Factory) to show the potency to suppress intracellular Ca^{2+} release (phase 2), the Ca^{2+} channels of receptor operated channel (ROC) and voltage-dependent channel (VOC) (phase 3) and the mixed action (phase 1), functionally and respectively[12,13]. The constituents of KH solution and the Ca^{2+} free KH solution were as the previous description[12].

Statistic analysis The Student *t*-test was applied to test the difference between groups and the statistic significance was set at $P<0.05$ and $P<0.01$.

RESULTS

The L-type currents of isolated myocytes from guinea pigs An inward L-type Ca^{2+} current was recorded when the holding potential was set at the -45 mV to block the I_{Na} and 4-AP and CsCl were added to block the I_{to} and I_K, separately. An inward current which showed its maximal current at 0 mV and was suppressed completely by 4-min exposure to verapamil 1 μmol/L and recovered to a large extent after washing (data not shown). It was recognized and confirmed as the L-type Ca^{2+} current. The run-down phenomena of the L-type current was observed at maximum by 25 % in the first 20 min and the suppressive effect of CPU 86017 was observed during matched period and the run down was cleared (Fig 2).

CPU 86017 suppressed Ca^{2+} current in a time-dependent manner CPU 86017 10 μmol/L caused a reduction of the Ca^{2+} current at 6 min and further reduction at 12 min. The suppression was only partially recovered after washing (Fig 2A).

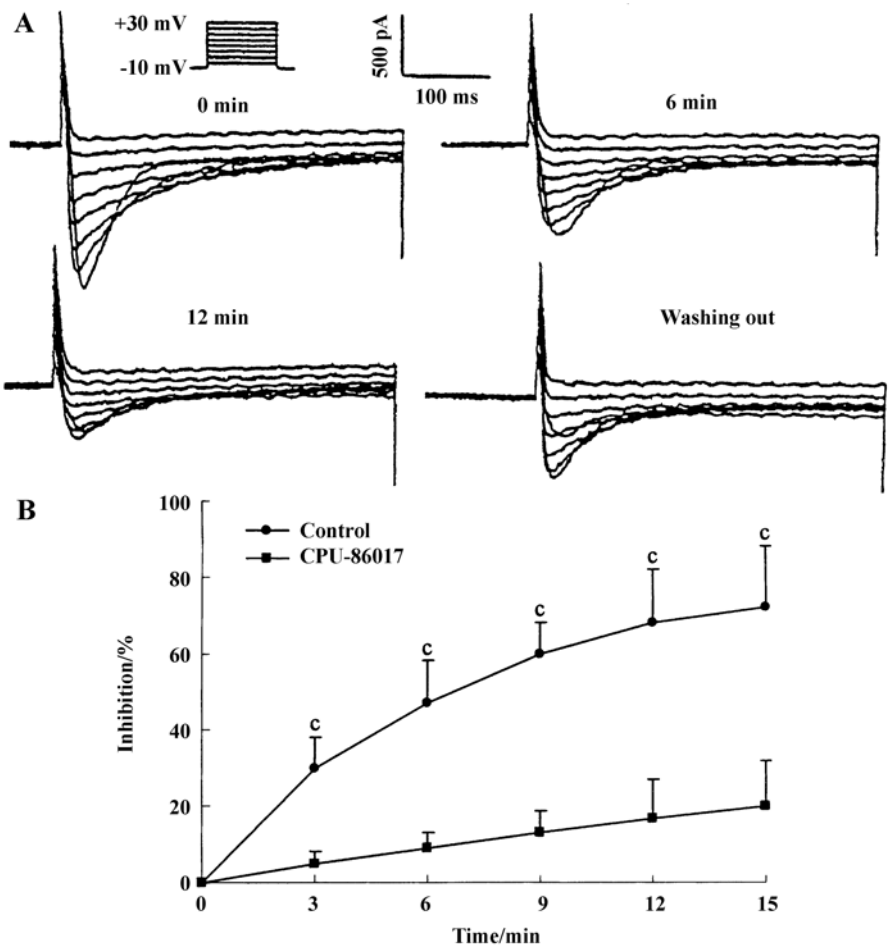

Fig 2. Effect of CPU 86017 10 μmol/L on L-type calcium current in ventricular myocytes of guinea pig. A) Calcium currents were elicited by the voltage protocol illustrated in the inset. Current traces are shown before (0 min) and after application of CPU 86017 at 6 min, 12 min and washing out. B) Time dependent effect of I_{Ca} blockade by CPU 86017. Percent blockade is determined and voltage step to 0 mV, according to the formula $100\times(1-I_a/I_b)$, where I_b and I_a are the current amplitude before and after application of CPU 86017, respectively. n=4-5 from 4 guinea pigs. $^cP<0.01$ *vs* control.

In another experiment after exposure to CPU 86017 suppression was initiated at 1-2 min , and the inhibition on the peak current at the 0 mV was enhanced along with time and reached the maximam at 15 min (Fig 2B) with half blocking time of (7.2±2.1) min.

CPU 86017 suppressed L-type Ca^{2+} current in a concentration-dependent manner CPU 86017 1, 3.3, 10, and 33 μmol/L suppressed L-type Ca^{2+} current by 20 %±11 %, 37 %±8 %, 66 %±6 %, and 79 %± 3 % at 15 min, respectively (Fig 3B). The IC_{50} value was 11.5 μmol/L.

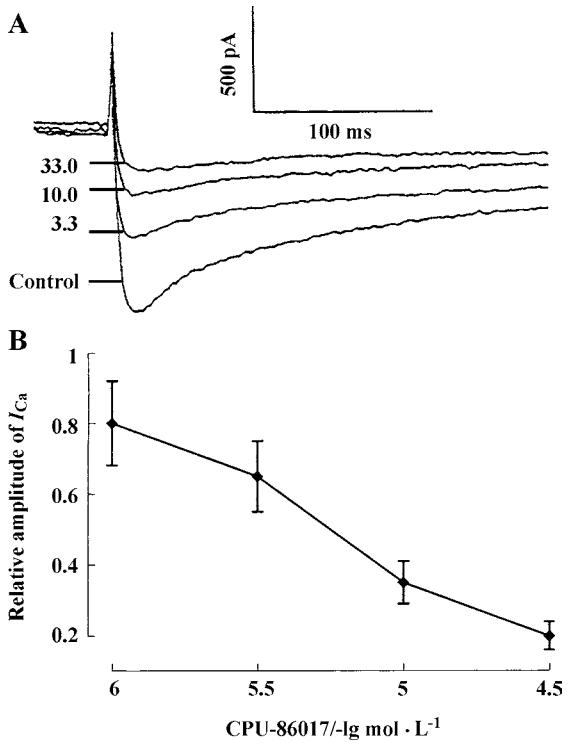

Fig 3. Effects of CPU 86017 3.3-33 μmol/L on L-type calcium currents. A) Ca^{2+} currents elicited by a test pulse to 0 mV from HP of -40 mV. B) CPU 86017 inhibited L-type current in a concentration-dependent manner. *n*=4~5 cells from 4 animals. Mean±SD. (subtraction of run down).

In voltage-current relationship the suppression by CPU 86017 reached the maximam at the 0 mV. CPU 86017 shifted the *I-V* curve upward but did not change the shape (Fig 4).

CPU 86017 had no effect on activation and inactivation curve of L-type calcium current Taking the maximal current as a unity the plot of I/I_{max} against potential yielded a stable activation curve which was fitted with the Boltzmann equation: $I/I_{max}=I/\{1+\exp[-(V-V_{1/2})/k]\}$, where V=the testing potentials, $V_{1/2}$ as the activation potential and k is the slope factor. $V_{1/2}$

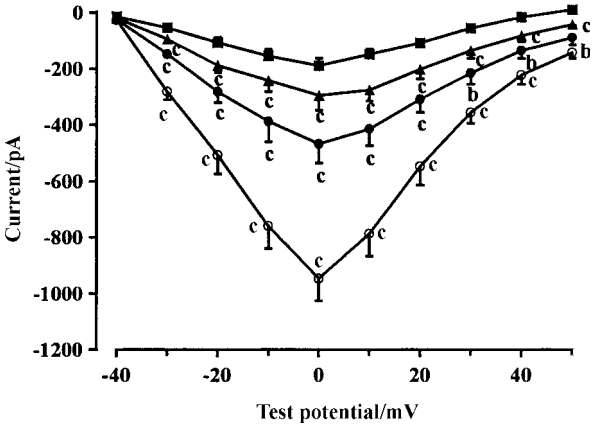

Fig 4. Effect of CPU 86017 on *I-V* relationship. I_{Ca} was evoked by the step pulses from 40 mV to +20 mV (HP of -40 mV). Control (○) and CPU 86017 3.3 (●), 10.0 (▲), 33.0 μmol/L (■). *n*=5-6 cells from 4 animals. $^bP<0.05$, $^cP<0.01$ *vs* control.

was (–22.7±4.3) mV and (–22.6±4.9) mV and the k value was 6.6±1.7, and 6.0±1.4 in control and CPU 86017 10 μmol/L group, respectively (n=6, P>0.05). The difference was not significant.

The decay of the L-type calcium current was to follow the bi-exponential equation and the time constant t_1 and t_2 of the inactivation of the maximal current at 0 mV were (88±24) ms and (18±6) ms in the control and (77±20) ms and (15±5) ms in CPU 86017 10 μmol/L group (n=8, P>0.05). CPU 86017 did not affect time constant of the inactivation (Fig 5).

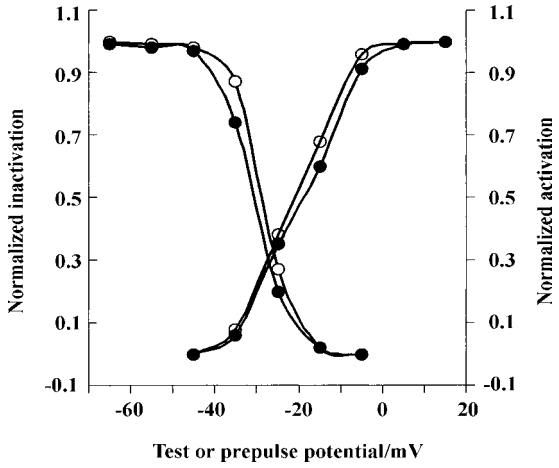

Fig 5. Effect of CPU 86017 10 μmol/L on steady state activation and inactivation curves of I_{Ca}. Control (○) and CPU 86017 3.3 (●).

CPU 86017 inhibited contractions of rat caudal artery CPU 86017 markedly inhibited vaso-

contractions of the caudal artery induced by phenylephrine 1 μmol/L (Fig 6, A-C) and KCl 100 mmol/L (Fig 6, D-F). The effects of CPU 86017 were stronger in phase 3 than that in phase 1 and 2 (Fig 6C and F, Tab 1).

CPU 86017 inhibited contractions induced by phenylephrine with IC_{50} of 0.465 μmol/L in phase 1, 0.459 μmol/L in phase 2, and 0.324 μmol/L in phase 3. The IC_{50} of prazosin in the three phases were 0.00246, 0.00302, and 0.00173 μmol/L, respectively. The potency of CPU 86017 was 1/187 of prazosin in terms of inhibiting intracellular Ca^{2+}-induced contractions (phase 3).

CPU 86017 inhibited contractions induced by KCl 100 mmol/L with IC_{50} value of 50.2, 28.1, and 16.3 μmol/L in phase 1, 2, and 3, respectively. The IC_{50} of verapamil was 1.16, 0.60, and 0.44 μmol/L in phase 1, 2, and 3, respectively. The relative potency of CPU 86017 was 1/37 of verapamil.

The vasorelaxative effects of CPU 86017 on Ca^{2+} induced contractions via the α-receptor-operated Ca^{2+} channels was 50 times potent than that via voltage dependent calcium channels (Fig 6).

DISCUSSION

CPU 86017 suppressed the L-type current in isolated myocytes from guinea pigs in time- and concentration-dependent manner and did not affect the activation and inactivation curve of the current. The potency of CPU 86017 is the strongest among berberine derivatives (Tab 1). The inhibitory effects of CPU 86017 (IC_{50} 13.7 μmol/L) on L-type calcium current determined by patch-clamp are coincided with that of pacing calcium current in the sinoatrial node measured by standard microelectrode[1], and more potent than its negative inotropic effect on atrium (IC_{50} 36 μmol/L)[5]. In contrast, the effect of berberine on myocardial L-type calcium current is controversial. Some observed a suppressive effect[2,3,14] and a change in inactivation curve but not activation curve[14]. Others reported an enhancement of the inward L-type Ca^{2+} current in myocardium by berberine[15,16] or no effect at all[17] (Tab 1) .

The vascular contractions caused by high K^+ are attributed to the consequence of depolarization of the membrane and can be separated into three phases[12]: 1) Phase 1 resulted from a mixed blockade on the phase

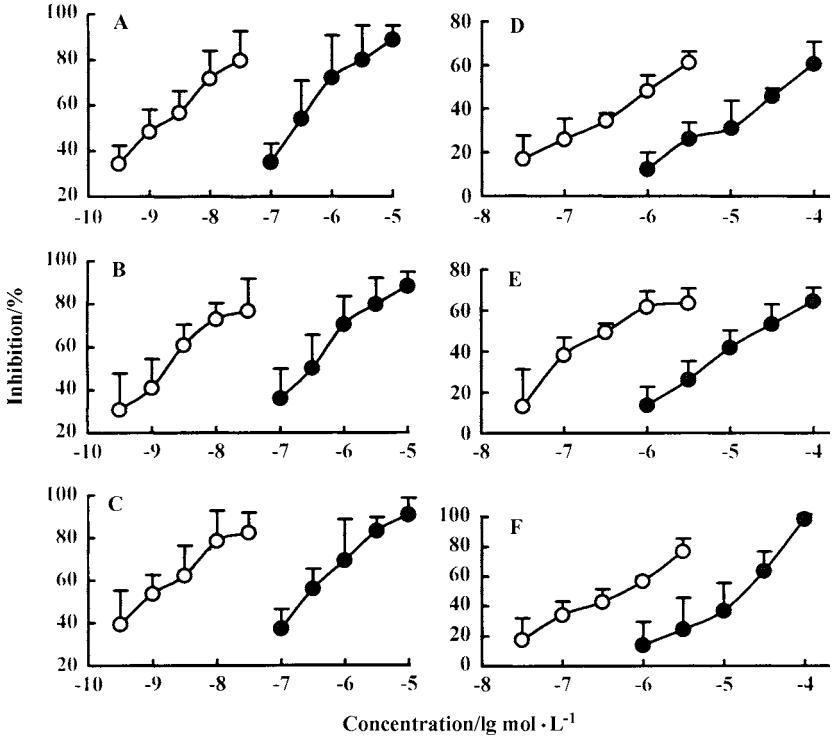

Fig 6. Inhibitory effects of CPU 86017 (●) on vaso-contraction of rat tail artery. Phenylephrine 1 μmol/L in normal KH solution (A), Ca^{2+} free KH solution (B), and after addition of Ca^{2+} 5 mmol/L into Ca^{2+} free medium (C). KCl 100 mmol/L in normal KH solution (D), Ca^{2+} free KH solution (E), and after addition of Ca^{2+} 5 mmol/L into Ca^{2+} free medium (F). Control (○).

Tab 1. Comparison of IC_{50} (μmol/L) of berberine and derivatives in suppression of the L-type calcium channels in myocardium.

Compounds	I_{Ca}	APD	Inotropic	Reference
CPU 86017	Suppress IC_{50} 11.5	Biphasic		Dai (2003)
CPU 86017	Suppressive/biphasic			Dai (1997)[2]
CPU 86017			Negative IC_{50} 36	Dai (1991)[5]
CPU 86035	Suppress IC_{50} 75			Li (2002)[26]
Berberine		Prolonged		Li (2001)[27]
Berberine	Suppressive, IC_{50} 30, (approximately)		Xu (1997)[14]	
Berberine	No effect;	Prolonged		Sanchez- (1996)[17]
Berberine	Enhance;	Prolonged		Wang (1997)[15]
Berberine	Enhance (single channel)			Zhou (1995)[16]
Berberine		Prolonged		Hua (1994)[28]
Berberine		Prolonged	Positive	Lau (2001)[3]
Berberine			Positive	Shaffer (1985)[2]
Berberine			Low concentration:positive High concentration: negative	Chang (1952)[2]
Berberine			iv infusion positive	Maroko (1983)[2]
8-Oxoberberine			Positive	Chi (1997)[30]

2 and 3. 2) Phase 2. The released calcium provokes an amplified Ca^{2-}-induced Ca^{2+} release (CICR) to implement the vasocontraction in Ca^{2+} free medium and possibly including more intracellular mechanisms[18]. 3) Phase 3, which is developed by voltage-gated calcium channels. Phase 3 is better than phase 1 to examine Ca^{2+} channel blocking effect of a novel compound.

The α-blocking effect of berberine on aorta induced by phenylephrine was the same as that for mesenteric artery[13,18]. The potency of CPU 86017 to suppress the contraction of anococcygeus muscle by phenylephrine[5] is almost as same as two other derivatives of berberine to suppress aortic contractions[19]. The potency of CPU 86017 on the caudal artery is also very closed to that on the mesenteric artery by berberine[18]. So it can be concluded that potency of α-blocking activity of CPU 86017 was not changed. The basic structure for the α-blocking activity is rigidity and possibly related to the skeleton of berberine (Tab 2).

The blocking effect by CPU 86017 in phase 3 is significant, however, it is much weaker compared with its α blocking activity. The fact that iv injection of CPU 86017 decreased blood pressure was mainly attributed to its α-blocking effect. This is the limitation of CPU 86017 in intravenous uses. On the other hand Ca^{2+} antagonism of CPU 86017 on the vascular contractions is enhanced significantly against berberine which exerts very weak effects to block calcium induced vascular contraction[20]. This is in agreement with the suppressive effect of CPU 86017 on L-type calcium current in myocardium. The chemical structure of protoberberines contributing to blockade of the calcium channels is flexible, not related to the skeleton of berberine but is sensitive to both the hydrogenation and a change in side chain. In general, the derivatives of berberine show more efficacy in blocking calcium channels of myocytes and vascular smooth muscles.

The effect of berberine to prolong APD is more prominent[2] *vs* CPU 86017. There are at least two determinants to control APD: $APD \propto I_{Ca}/I_K$ (I_{Kr} I_{KS}). Usually protoberberines prolong APD by suppression of the I_K current[2]. Berberine prolonged APD in concentration-dependent manner and an over-prolongation of APD is developed at high concentrations but it is not desired. CPU 86017 prolonged APD at low concentrations but shortened APD at higher concentration. The bi-phasic phenomena is stemmed from the two properties, reducing I_K at low concentration and blocking I_{Ca} blocking at higher concentration. The bi-phasic (or limited prolongation) pattern is an important characteristics of the novel, complex class III antiarrhythmic agents, such as Azimilide[21] and dronedarone (SR 33589)[22]. Complex class III agents do not induce *Torsards de pointes* (*Tdp*) very often, in contrast, the

Tab 2. Comparison of inhibitory effects of berberine and derivatives on vascular contractions by phenylephrine and high concentration of KCl.

Compounds	Artery (Rats)	α-Blockade (IC_{50} μmol/L)	Calcium antagonist (IC_{50} μmol/L)	References
CPU 86017	Caudal	0.324	16.3	Dai (2003)
CPU 86017	Aortic,		29.5	Dai (2000)[20]
CPU 86017	Aortic,		42.2	Dai (2000)[20]
	Aortic, diseased by *L*-thyroxin		34.3	Dai (1998)[12]
CPU 86017	Aortic	1.58		Dai (1999)[13]
CPU 86017	Aortic	2.14 (diseased by L-thyroxin)		Dai (1999)[13]
CPU 86017	Anococcygeus	pA_2 6.78		Dai (1991)[5]
Berberine	Anococcygeus	pD'_2 5.2		Dai (1991)[5]
Berberine	Mesenteric	1.48		Ko (2000)[18]
Berberine	Aortic		300 (approx)	Dai (2000)[20]
Berberine	Aortic	Potent	weak	Bova (1992)[31]
Berberine	Mesenteric	Potent	no effect	Chiou (1991)[32]
(+/-)-Govadine	Aortic	pA_2 6.57 (α1-blocker)		Ko (1996)[19]
(-/-)-THB	Aortic	pA_2 6.74 (α1-blocker)		Ko (1996)[19]
THB	Aortic		100 μmol/L (47 %)	Yang (1993)[33]

Govadine: derivative of tetrahydroberberine; THB: tetrahydroberberine.

pure class III agents, like dofetilide[23] and ibulilide[24], induce *Tdp* in clinic and this limits its uses in treating ventricular arrhythmias in diseased hearts. The *Tdp* which is identical to early after depolarization will induce ventricular fibrillation. At this point CPU 86017 is superior than berberine for preventing the occurence of *Tdp*.

Berberine possessed a positive inotropism which might be useful to treat heart failure[25]. For a long time positive inotropism has been adopted to combat congestive heart failure but this concept has been changed in recent years after application of ACEI and new generation of β-blockers which possess dominant negative inotropism[25]. The negative inotropism of CPU 86017 will be useful in treating congestive heart failure and pulmonary hypertension.

There are two new major merits of CPU 86017: 1) blocking the L-type channels in myocardium moderately and 2) suppressing vascular smooth muscle contraction mediated by calcium. Together with an improvement in the solubility and bioavailability, CPU 86017 will be a potential agent in new drug development.

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