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Blocking effect of rhynchophylline on calcium channels in isolated rat ventricular myocytes[1]

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ABSTRACT The effect of rhynchophylline (Rhy) on *L*-type Ca^{2+} channel current was studied in rat ventricular myocytes using patch-clamp whole-cell recording technique. When the holding potential was −40 mV, cells were depolarized to 0 mV for 250 ms at frequency of 0.5 Hz, Rhy 1.0 and 5.0 μmol·L^{-1} reduced verapamil-sensitive Ca^{2+} inward current by 60 % and 80 %, respectively, without affecting the voltage-dependency of the maximal activation of Ca^{2+} current. This indicated that the effect of Rhy on the activation of Ca^{2+} channel was voltage-independent. The effect of Rhy on Ca^{2+} current reached the maximum at 10 min and was partially recovered by washout of Rhy. A pD_2 value of 5.91 and Hill coefficent of 1.1 were obtained from the concentration-response curve.

KEY WORDS *rhynchophylline; verapamil;* calcium channels; electrophysiology; myocardium; cultured cells

Rhynchophylline (Rhy) is a major effective ingredient extracted from *Uncaria rhynchophylla*, which has been used to treat hypertension, epilepsy, asthma, and some other diseases in China. Rhy showed hypotensive effect[1], bradycardic effects[2], and might reduce calcium inward current[3,4]. In this study the effect of Rhy on calcium channels in rat ventricular myocytes was investigated by using the patch-clamp whole-cell recording techniques.

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MATERIALS AND METHODS

Isolation of ventricular myocytes Ventricular myocytes were isolated from male adult rats (weighing 240±*s* 21 g) by enzymatic disaggregation[5,6]. Langendorff hearts were perfused with a calcium-free solution containing NaCl 116, KCl 5.4, NaH_2PO_4 1.1, $NaHCO_3$ 15, $MgSO_4$ 1, and glucose 15 mmol·L^{-1}, for 5 min, and then with the Ca-free solution containing collagenase type I (Sigma) 0.3 mg·ml^{-1} and type IA (Sigma) 0.15 mg·ml^{-1}, and $CaCl_2$ 150 μmol·L^{-1} for 15 min. The ventricles were cut into small chunks and incubated in the above enzyme solution for 10 min. Cells were harvested after filtration and washed once with the calcium-free solution containing albulmin 0.5 mg·ml^{-1}.

Voltage clamp experiments Transmembrane currents were recorded using the single electrode (whole-cell patch-clamp) technique[7]. The current was measured with a List L/M EPC-7 amplifier (List Medical, Germany). Computer program pCALMP 5.51 was used to produce voltage clamping signals. Acquisition and analysis of membrane currents were also done with the program. The electrode resistances ranged from 2—3 MΩ. They were filled with the solution containing KCl 140, $MgCl_2$ 0.5, egtazic acid (EGTA) 10, and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 10 mmol·L^{-1}. The pH was adjusted to 7.2 with KOH. All experiments were carried out at the room temperature (24—6 ℃). The chamber was perfused at a rate of 1.5 ml·min^{-1} with the modified Tyrode's solution containing NaCl 150, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 2, glucose 10, HEPES 5 mmol·L^{-1}. The pH of the solution was adjusted to 7.4 with NaOH.

Drugs Rhy was isolated in our Institute. The purity was 99.8 %. (−)-Verapamil was purchased from Sigma Co.

Analysis of data Data were presented as $\bar{x}\pm s$.

Paired or unpaired *t* tests were used.

RESULTS

Effect of Rhy on Ca^{2+} inward currents Calcium currents were determined by depolarization of individual ventricular cells from a holding potential of −40 mV to 0 mV. Under this condition the activity of Na^+ channels was completely inhibited[8], and a Ca^{2+} inward current can be measured. Verapamil decreased the amplitude of peak Ca^{2+} inward currents. When cells were exposed to verapamil 1 $\mu mol \cdot L^{-1}$ for 5 min, the Ca^{2+} inward current was completely abolished, indicating the characteristic of the *L*-type Ca^{2+} channel current. Under the same condition, Rhy 1.0 or 5.0 $\mu mol \cdot L^{-1}$ inhibited the amplitude of peak Ca^{2+} inward current by 60 % and 80 %, respectively (Fig 1). After washout of Rhy with Tyrode's solution, the peak inward currents partially increased again.

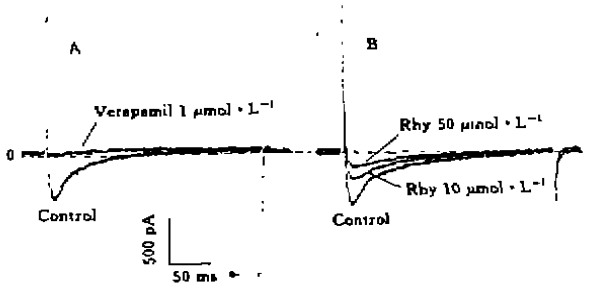

Fig 1. Inhibition of Ca^{2+} inward currents by verapamil (A) and rhynchophylline (B).

Effect of Rhy on the current-voltage relationship Cells were depolarized from a holding potential of −40 mV to +50 mV in steps of 10 mV, resulting in a progressive activation of Ca^{2+} current. The current-voltage relationships (*I-V* curve) of Ca^{2+} current in control conditions and in the presence of Rhy 50 $\mu mol \cdot L^{-1}$ are showed in Fig 2A. Each point represents average current amplitude from 4 cells.

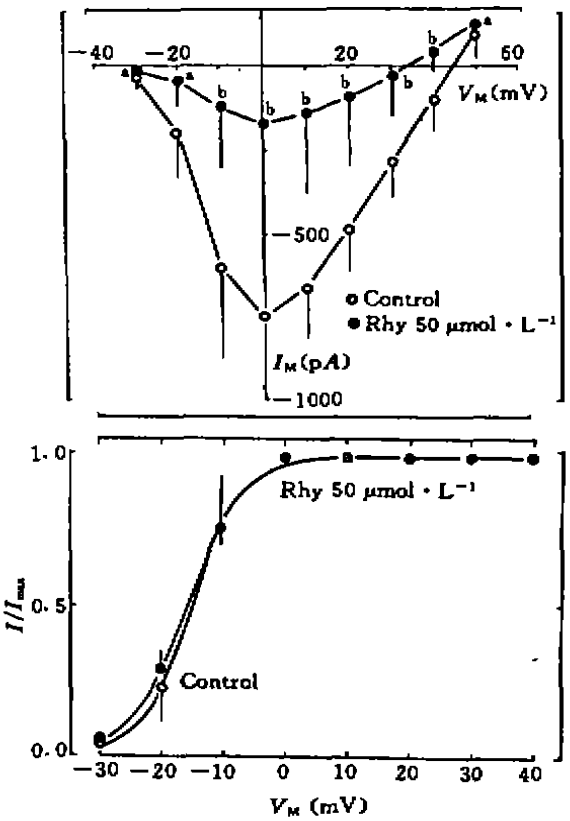

Fig 2. A) Relationship between Ca^{2+} inward current and membrane potential (*I-V* curve). B) Steady-state activation curve obtained from the *I-V* curve in A. $n=4$, $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

The maximal activation of Ca^{2+} current appeared at 0 mV in the presence of Rhy. This was not different from the control, despite that the levels of Ca^{2+} current were obviously decreased by Rhy. The steady-state activation of the *L*-type Ca^{2+} channels in the absence and presence of Rhy is showed in Fig 2B. Dividing the currents in Fig 2A in the range of −40 to 40 mV by the fully activated current, which was estimated by extrapolation of a linear regression through the currents between 0 and 40 mV, we obtained a steady-state activation curve fitted to Boltzmann func-

tion given in equation:

$I/I_{max} = 1/(1 + \exp(-(V - V_{1/2})/s))$

where V = test potential (mV), $V_{1/2}$ = potential of half-maximal activation (mV), and s = slope parameter (mV).

In control, the potential of half-maximal activation was -15.1 ± 0.2 mV, and the slope parameter was 4.2 ± 0.2 mV. In the presence of Rhy, $V_{1/2}$ and s were -15.9 ± 0.3 and 4.8 ± 0.2, respectively. This result demonstrates that Rhy dose not significantly affect the steady-state activation curve of $I_{Ca\cdot L}$.

Time course of Ca^{2+} channel blocking effect of Rhy Due to the "run down" of Ca^{2+} current during the measurement, the amplitude of Ca^{2+} inward current in the presence of Rhy was compared with that in control group not exposed to Rhy (Fig 3). The amplitude

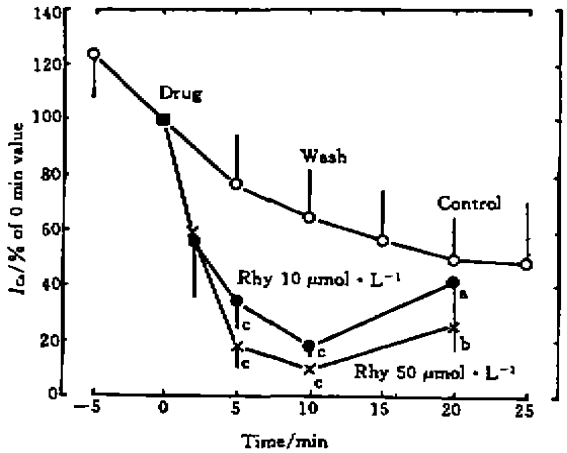

Fig 3. Ca^{2+} inward currents after Rhy was added at 0 time and washed out at 10 min. $n = 4 - 7$, $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. At 0 min, the calcium currents were 355 ± 104, 385 ± 164 and 391 ± 114 pA, in control, Rhy 10 μmol·L^{-1} and 50 μmol·L^{-1} treated cells, respectively.

of peak inward currents just before addition of Rhy was considered as 100 % activity of Ca^{2+} channel. It was found that the Ca^{2+} currents were decreased to 65 ± 17 % in 10 min in control cells. In the presence of Rhy 10 or 50 μmol·L^{-1} for the same period, the Ca^{2+} inward currents were reduced to 18.1 ± 3.4 % and 9.8 ± 2.3 %, respectively, which were lower than the time-matched controls. After 10-min washout of Rhy with perfusion solution, the peak Ca^{2+} inward currents partially recovered. This result demonstrated that the reduction of Ca^{2+} channel currents was mainly due to the effect of Rhy.

Potence of Rhy in blocking Ca^{2+} current To avoid the influence of "run down" on determination of Ca^{2+} current, the differences between Ca^{2+} current levels in the presence of Rhy and in the time-matched controls were used to represent the responses of cells to Rhy. The effect of Rhy on Ca^{2+} current was clearly concentration-dependent. Rhy 1, 10, and 50 μmol·L^{-1} reduced the Ca^{2+} currents to 62.2 ± 17.6 %, 21.0 ± 12.9 % and 15.2 ± 3.6 %, respectively. The concentration-response curve (not shown) was fitted by using the nonlinear regression technique to the data points with the computer program "GraphPAD" (iSi Software). A pD_2 value was calculated to be 5.91 ± 0.02. The Hill coefficient was 1.1.

DISCUSSION

Our study demonstrated that Rhy inhibited cardiac L-type Ca^{2+} channels obviously. Because, under the condition of the present study the L-type Ca^{2+} channle currents were activated, meanwhile, T-type Ca^{2+} channels and Na^+ channels were inactivated[8,9], the currents recorded could be completely inhibited by verapamil (1 μmol·L^{-1}) — a typical Ca^{2+} channel antagonist.

Current-voltage relationship (I-V curve) showed that the peak amplitude of Ca^{2+} inward currents were attenuated by Rhy at all depolarizing steps, but the steady-state activation curve was not changed by Rhy; the potential

of halp-maximal activation ($V_{1/2}$) and the time constant of activation (s) were not significant different from the control in the presence of Rhy, indicating that the blocking effect of Rhy on Ca^{2+} channels was voltage-independent.

In the present study it was found that the "run down" phenomenon of Ca^{2+} current was obviously. It has been reported very often by other laboratories[10-11], but the mechanisms are not very clear up to now. Therefore, a time- matched control is very important to evaluate the effect of drugs on Ca^{2+} current in whole-cell patch-clamp study.

Our results have shown that the effect of Rhy on Ca^{2+} current is dose-dependent. The Hill coefficient closed to 1 suggests a simple one-to-one binding of Rhy with its affecting sites. The pD_2 value of 5.91 indicating that the blocking effect of Rhy on Ca^{2+} channel currents is apparent, but the effect is lower than that of verapamil. We have also found that the effect of Rhy was relatively easy to reverse by washout of the drug. Therefore, one of the mechanisms of Rhy in treatment of hypertension and arrhythmia in animal experiments could be explained by Ca channel blocking effect.

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钩藤碱对大鼠心肌细胞钙离子通道的阻滞作用*

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A **摘要** 用膜片钳全细胞记录的方法研究了钩藤碱(Rhy)对大鼠心肌细胞钙离子通道的阻滞作用。Rhy明显抑制verapamil敏感的L型钙通道，减少钙离子内向电流，但不改变最大激活时的膜电位水平以及激活时间常数，提示Rhy非电压依赖地抑制钙通道，其最大反应出现在给药10分钟，为浓度依赖的作用。pD_2值为5.91，Hill系数1.1。

关键词 钩藤碱；维拉帕米；钙离子通道；电生理学；心肌；培养的细胞