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• BASIC RESEARCH •

# Effects of palmatine on potassium and calcium currents in isolated rat hepatocytes

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## Abstract

**AIM:** To study the effects of palmatine, a known inhibitor on delayed rectifier potassium current and L-type calcium current ($I_{Ca,L}$) in guinea pig ventricular myocytes, on the potassium and calcium currents in isolated rat hepatocytes.

**METHODS:** Tight-seal whole-cell patch-clamp techniques were performed to investigate the effects of palmatine on the delayed outward potassium currents ($I_K$), inward rectifier potassium current ($I_{K1}$) and $Ca^{2+}$ release-activated $Ca^{2+}$ current ($I_{CRAC}$) in enzymatically isolated rat hepatocytes.

**RESULTS:** Palmatine 0.3-100 μM reduced $I_K$ in a concentration-dependent manner with $EC_{50}$ of 41.62±10.11 μM and $n_H$, 0.48±0.07 ($n$=8). The effect of the drug was poorly reversible after washout. When the bath solution was changed to tetraethylammonium (TEA) 8 mM, $I_K$ was inhibited. Palmatine 10 μM and 100 μM shifted the I-V curves of $I_K$ downward, and the block of $I_K$ was voltage-independent. Palmatine 0.3-100 μM also inhibited $I_{CRAC}$ in a concentration-dependent manner. The fitting parameters were as follows: $EC_{50}$=51.19±15.18 μM, and $n_H$=0.46±0.07 ($n$=8). The peak value of $I_{CRAC}$ in the I-V relationship was decreased by palmatine 10 μM and 100 μM. But the reverse potential of $I_{CRAC}$ occurred at Voltage=0 mV in all cells. Palmatine 0.3-100 μM failed to have any significant effect on either inward or outward components of $I_{K1}$ at any membrane potential examined.

**CONCLUSION:** The inhibitory effects on $I_K$ and $I_{CRAC}$ could be one of the mechanisms that palmatine exerts protective effect on hepatocytes.

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## INTRODUCTION

There are many natural drugs for liver diseases currently used in popular medicine. For example, quaternary protoberberine alkaloids from *Flissitigma* and *Goniothalamus* have been used in popular medicine for hepatomegaly and hepatosplenomegaly[1,2]. The uses of alkaloids from *Berberis aristata* for liver injury induced by chemical carcinogenesis and alkaloids from *Enantica* for disorders of bilirubin have also been reported[3,4].

Palmatine, the protoberberine class of isoquinoline alkaloids, has been found in plants of various families, and mainly presents in the rhizomes of *Fibrarurea Tinctoria Lour*. These medicinal plants have been used as folk medicine in treatment of jaundice, dysentery, hypertension, inflammation and liver-related diseases[5,6]. The previous studies have shown that palmatine could block the delayed rectifier potassium current and had inhibition effect on L-type calcium current ($I_{Ca,L}$) in guinea pig ventricular myocytes[7-11]. Pauli *et al* reported a protoberberine alkaloids mixture from *Enantia chlorantha*, called Hepasor, containing palmatine, columbamine and jatrorrhizine prevented liver from chemically induced traumatization and also promoted the healing process in the hepatic injury models selected. Hepasor improved the blood flow and mitotic activity in thioacetamide-traumatized rat livers[12]. However, the hepatoprotective mechanism of palmatine still remains unknown. And there are no data available to the relationship between ion currents in hepatocytes and the hepatoprotective effect of palmatine.

In the present study, we investigated the effects of palmatine on whole-cell currents recorded from isolated rat hepatocytes to explore its mechanisms against liver injury. We tried to develop not only an effective hepatoprotective agent but also a promising leading compound against liver injury while maintaining a low side-effect profile.

## MATERIALS AND METHODS

### *Cell preparation*

The rat hepatocytes were enzymatically isolated from Sprague Dawley (SD) rats of either sex (150 to 200 g) by slightly modified procedures described previously[13-18]. Briefly, adult animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) in strict accordance to the guidelines established by the Institutional Animal Care and Use Committee, which follow all applicable state and federal laws. The portal vein and the inferior vena cava were cannulated. The liver was initially perfused at a flow rate of 25 $mL \cdot min^{-1}$ with a constant-flow system with modified oxygenated $Ca^{2+}$, $Mg^{2+}$-free Hanks' solution containing (in mM): NaCl 137, KCl 5.4, $NaH_2PO_4$ 0.5, $Na_2HPO_4$ 0.58, $NaHCO_3$ 4.16 and Glucose 5.5 (pH 7.3) for several minutes, followed by perfusion with a $Ca^{2+}$, $Mg^{2+}$-free Hanks' solution containing collagenase (0.3 $g \cdot L^{-1}$; type I) for 10 min. The solutions were gassed with 100 % $O_2$ and warmed to 37 ℃. After these perfusions, the liver was excised and then minced in $Ca^{2+}$, $Mg^{2+}$-free Hanks' solution at 0 ℃. The cells were filtered through a 200 μm nylon mesh, and washed three times by centrifugation at 50 g for 2 min. The cell pellets were resuspended in Kraft-bruhe (KB) solution containing (in mM): L-glutamic acid 70, KCl 130, taurine 15, $KH_2PO_4$ 10, $MgCl_2$ 0.5, Glucose 11, 4-(2-hydroxyethyl)-1-piperazine-*N*'-2-ethanesulfonic acid (HEPES) 10 and ethylene glycol-bis (β-aminoethyl ether)-*N*,

*N*, *N'*, *N'*-tetraacetic acid (EGTA) 0.5 (pH 7.4) that yielded approximately 85 % to 95 % viable hepatocytes. A small aliquot of the medium containing single cell was transferred into a 1 mL chamber mounted on the stage of an inverted microscope ($XD\text{-}101_{2B}$, Nanjing, China). The spherical, smooth cells were used for the whole-cell voltage-clamp studies. All experiments were performed at room temperature (20 to 22 ℃).

### *Voltage-clamp recording*

A programmable vertical puller (pp-83, Narishige, Japan) was used to pull the electrodes. The resistance of the capillary glass electrodes (GG-17, Nanjing, China) used was 2 to 4 MΩ when filled with internal solution. A patch-clamp amplifier (PC-II, Wuhan, China) was used to record whole-cell currents with four-pole Bessel filter set at 1 kHz, digitized at 5 kHz. The protocols for voltage clamp and data analysis were established with routines using software (pClamp 6.0, Wuhan, China) and data were stored on computer for subsequent analysis. Drug actions were measured only after steady-state-conditions were reached, which were judged by the amplitudes and time courses of currents remaining constant with further perfusion of drug.

### *Drugs and solutions*

Palmatine hydrochloride was obtained from Zhonglian Pharmaceutical Company of China as base powders, dissolved in distilled water and made a stock solution at 0.1 M. Palmatine was added to bath solution for extracellular application. All drugs were from Sigma Chemical Co unless otherwise indicated.

With studies of $I_K$, the bath solution was a modified Tyrode' s solution contained (in mM): NaCl 144, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.53, $Na_2HPO_4$ 0.33, HEPES 5 and Glucose 5.5 (pH 7.3). The patch pipette solution contained (in mM): KCl 130, $K_2ATP$ 5.0, creatine phosphate 5.0 and HEPES 5.0 (pH 7.4).

For experiments on $I_{K1}$, both the bath solution and the pipette solution contained (in mM): KCl 7, $MgCl_2$ 2, EGTA 1, K-glutamate 130 and HEPES 10 (pH 7.4).

For $I_{CRAC}$ recording, the bath solution was (in mM): NaCl 140, KCl 2.8, $CaCl_2$ 10, $MgCl_2$ 0.5, Glucose 11 and HEPES 10 (pH 7.4). The pipette solution used (in mM): K-glutamate 145, NaCl 8, $MgCl_2$ 1, MgATP 0.5, EGTA 10 and HEPES 10 (pH 7.2).

### *Statistics*

All values are expressed as mean ±S.E.M and error bars were plotted as S.E.M. Student' s *t* test was used to evaluate the statistical significance of differences between means. A value of $P<0.05$ was considered to be statistically significant. Concentration-response curves were fitted by the Hill equation: Inhibition of current (%)= $100/[1+(EC_{50}/C)^{nH}]$

Where $EC_{50}$ is the concentration of palmatine for half-maximum block, C is the concentration of palmatine, and $n_H$, the Hill coefficient.

## RESULTS

### *Effects of palmatine on $I_K$*

$I_K$ was evoked in isolated rat hepatocytes by depolarizing pulse to +140 mV for 900 ms from a holding potential of -50 mV. The current at the end point of the test pulse was measured as the amplitude of $I_K$[19].

To better the concentration of palmatine necessary for half-maximal effect, six concentrations (0.3-100 μM) were studied. The percentage block of $I_K$ was defined as $(I_{Control}-I_{palmatine})/I_{Control}$ and plotted as a function of logarithm [palmatine] in Figure 1B. At +140 mV, palmatine exerted a concentration-dependent inhibition of the current, which was poorly reversible after washout (Table 1). The data points are fitted according to the Hill equation with an $EC_{50}$ for palmatine on $I_K$ is 41.62±10.11 μM and $n_H$, 0.48±0.07 ($n$=8). When the bath solution was changed to tetraethylammonium (TEA) 8 mM, $I_K$ was inhibited.

Figure 1C shows the effects of palmatine 10 μM and 100 μM on the steady-state I-V relationship for $I_K$ generated by applying 12 depolarizing pulses from +30 mV to +140 mV for 900 ms with a 10 mV increment from a holding potential of -50 mV. In the presence of palmatine 100 μM, the amplitude of $I_K$ was significantly reduced from +70 mV through +140 mV ($n$=8, $P<0.05$ or $P<0.01$ *vs* control). The currents were inhibited in a voltage-independent manner at the potentials tested. For example, at +100 mV, $I_K$ was reduced by palmatine 100 μM from 1 319.52±192.60 to 575.37±133.29 pA (56.40 % reduction), and at +140 mV, current was reduced from 1 861.42±215.76 to 879.37±172.30 pA (52.76 % reduction). The relative reductions of $I_K$ were illustrated in Figure 1D. Currents after addition of 10 μM and 100 μM palmatine were normalized to currents under control conditions at a given voltage, and it can clearly be seen that the currents were inhibited to the same degree at all potentials tested.

**Table 1** Effects of palmatine on $I_K$ at a test potential of +140 mV and $I_{CRAC}$ at -100 mV

| Concentration (μM) | Inhibition of $I_K$ (%) | Inhibition of $I_{CRAC}$ (%) |
|---|---|---|
| 0.3 | 3.36±1.96 | 3.81±0.40 |
| 1 | 11.04±3.03 | 10.19±2.03 |
| 3 | 23.97±4.62 | 21.42±3.22 |
| 10 | 36.75±5.12 | 37.39±3.95 |
| 30 | 49.58±7.98 | 47.50±4.38 |
| 100 | 55.40±8.97 | 52.10±5.98 |

### *Effects of palmatine on $I_{K1}$*

Hyperpolarizing and depolarizing potentials over a range from -200 mV to +175 mV were applied from a holding level of 0 mV[20]. The absolute value at the end of test pulse was measured as the amplitude of $I_{K1}$. Palmatine 0.3-100 μM failed to have any significant effect on either inward or outward components of $I_{K1}$ at any membrane potential examined.

### *Effects of palmatine on $I_{CRAC}$*

When the holding potential was 0 mV, and the cells were depolarized to -100 mV for 200 ms at a frequency of 0.2 Hz, the $I_{CRAC}$ was evoked[21]. As shown in Figure 2, $I_{CRAC}$ also was blocked by palmatine in a concentration-dependent fashion, and the current was less sensitive to palmatine than $I_K$ with an $EC_{50}$ of 51.19±15.18 μM and $n_H$=0.46±0.07 ($n$=8). Table 1 also showed the effects of palmatine on $I_{CRAC}$ at a test potential of -100 mV. Figure 2C showed the effects of palmatine on the steady-stated I-V relationships generated by applying a series depolarizing pulses from a holding potential of 0 mV to different membrane potentials (-100 mV to +80 mV) with a 20 mV increment. The peak value of $I_{CRAC}$ in the I-V relationship was decreased by palmatine 10 μM and 100 μM ($n$=8, $P<0.05$ or $P<0.01$ *vs* control). But the reverse potential of $I_{CRAC}$ occurred at voltage=0 mV in all cells.

## DISCUSSION

In this study we have, for the first time, characterized the effects of palmatine on the hepatocyte $I_K$, $I_{K1}$ and $I_{CRAC}$ by patch-clamp techniques and demonstrated that palmatine effectively inhibited $I_K$ and $I_{CRAC}$ in isolated rat hepatocytes.

Membrane potential is important in regulating metabolic processes in the liver, including gluconeogenesis, amino acid transport, and the rate of uptake of bile salts[22,23]. Changes in

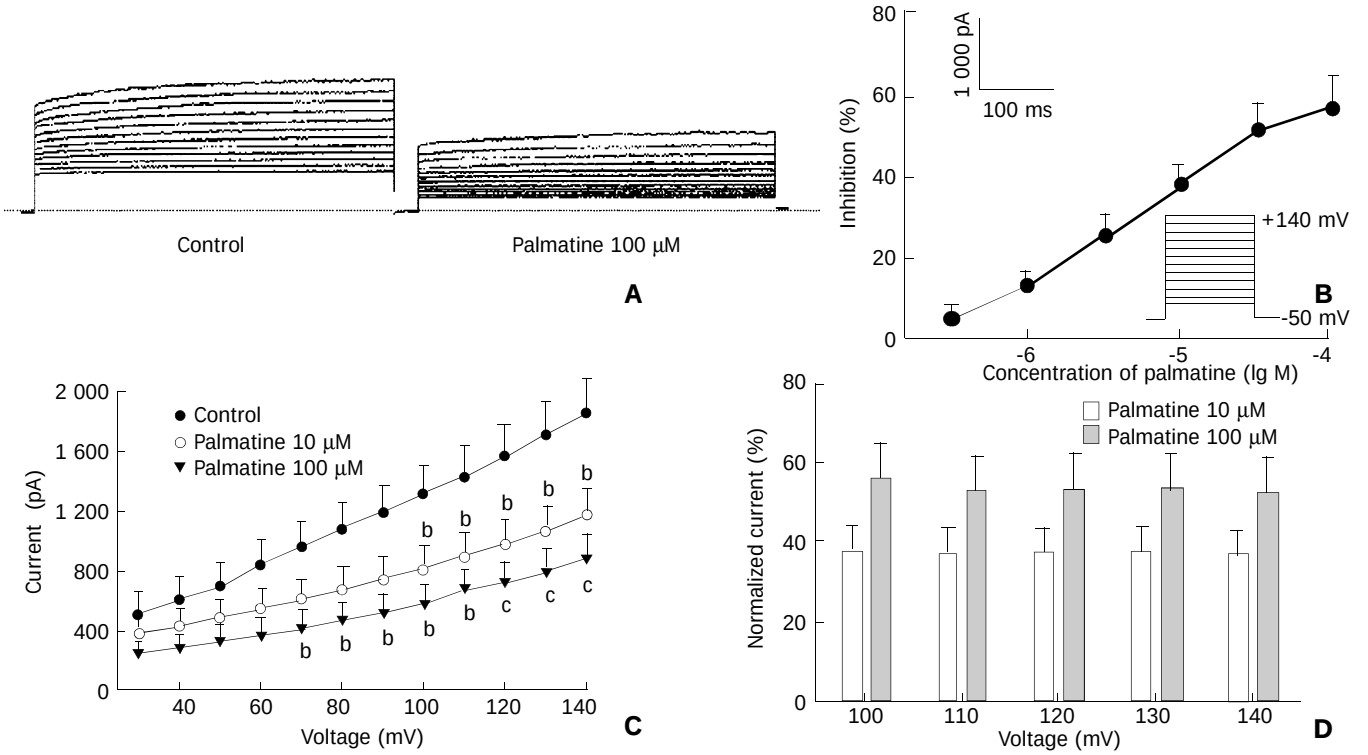


**Figure 1** Effects of palmatine on $I_K$. (A) Family of $I_K$ recorded with changes in the absent or present of palmatine 100 μM. Dotted line indicates zero current level. (B) Dose-response curve for the effects of palmatine on $I_K$. The data are mean values from $n$=8 cells. (C) I-V relationship of $I_K$ under control (●) and palmatine 10 μM (○), 100 μM (●). The voltage steps used to elicit $I_K$ are shown in the inset of panel (B). $^bP$<0.05, $^cP$<0.01 *vs* control ($n$=8). (D) Dependence of palmatine effects on test potential. The values for the mean percentage reductions in $I_K$ induced by palmatine 10 μM (□) and 100 μM (■) are plotted against the corresponding test potential. No significant voltage-dependence was observed for the blocks induced by palmatine.

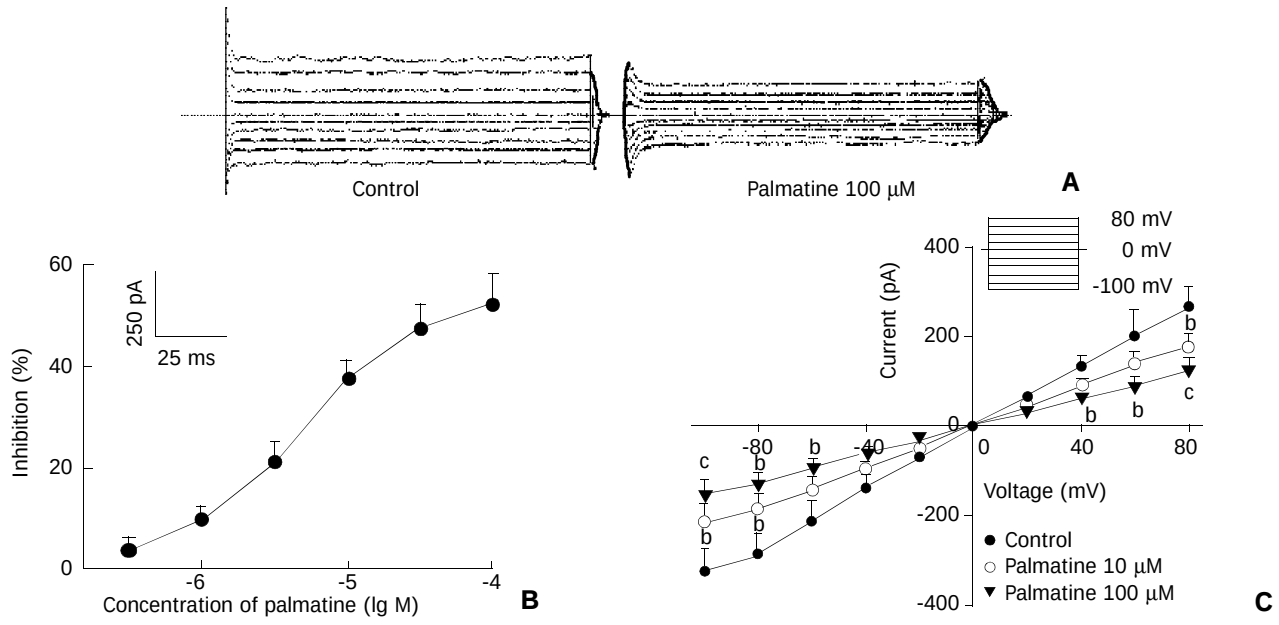


**Figure 2** Effects of palmatine on $I_{CRAC}$. (A) Family of $I_{CRAC}$ recorded with changes in the absent or present of palmatine 100 μM. Dotted line indicates zero current level. (B) Dose-response curve for effects of palmatine on $I_{CRAC}$. The data are mean values from $n$=8 cells. (C) I-V relationship of $I_{CRAC}$ under control (●) and palmatine 10 μM (○), 100 μM (●). The voltage steps used to elicit $I_K$ are shown in the inset of panel (B). $^bP$<0.05, $^cP$<0.01 *vs* control ($n$=8).

$K^+$ permeability can affect the transmembrane potential. Transcellular bile acid transport is integrated in the regulation of intracellular pH, $K^+$ homeostasis and membrane potential. Hepatocellular $K^+$-depletion can result in inhibition of bile acid secretion despite rising intracellular concentration[24-26].

During ischemia and hypoxia, hepatocellular volume and $K^+$ conductance are increased. It was reported that the extracellular $K^+$ increase would result in hyperpolarization and hyperexcitabillity of cells. This would lead to cell death[27-29]. Nietsch *et al* demonstrated membrane potential change by modulation of $K^+$ channel activity might be involved in the mechanism of apoptosis in human hepatoma cells[30,31]. The inhibition of $K^+$ channels could delay hepatocyte apoptosis and death.

Calcium has been demonstrated to play an important role in hepatocyte damage. Elevation of intracellular $Ca^{2+}$

concentration was associated with the development of cell damage and apoptosis[32-35].

Recent developments suggest that an early disturbance in hepatocellular $Ca^{2+}$ homeostasis might be involved in the hepatocellular damage induced by $CCl_4$[36-38].

Hepatocytes as the nonexcitable cells are short of the voltage-dependent $Ca^{2+}$ channels but possess plasma membrane $Ca^{2+}$ channels that have a high selectivity for $Ca^{2+}$, and are activated by a decrease in the concentration of $Ca^{2+}$ in intracellular stores, which named $I_{CRAC}$[39, 40]. The gating of $I_{CRAC}$ is independent of membrane voltage, there is, nevertheless, a strong dependence of $Ca^{2+}$ influx on the driving force exerted by the membrane potential, ie, the influx rate increases with hyperpolarization and decreases with depolarization, which is different from cardiac myocytes that $Ca^{2+}$ influx increases with depolarization and decreases with hyperpolarization[41].

Palmatine inhibits $I_{CRAC}$ with $EC_{50}$ of 51.19 μM, which is higher than the $EC_{50}$ of $I_{Ca,L}$ in cardiac myocytes[42]. The differential drug sensitivity of the two currents also provides further support for the idea that $I_{CRAC}$ is different from voltage-gated $Ca^{2+}$ channel.

In conclusion, palmatine blocks $K^+$ channel and decreases the extracellular $K^+$ to regulate the metabolic processes in the liver. Palmatine also inhibits $I_{CRAC}$ effectively and protects hepatocytes from calcium overload via the inhibition of $I_{CRAC}$. The inhibitory effects on $I_K$ and $I_{CRAC}$ may partly contribute to the hepatoprotective action of palmatine.

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