

©2003, Acta Pharmacologica Sinica
Chinese Pharmacological Society
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
<http://www.ChinaPhar.com>

Perforated patch recording of L-type calcium current with β -escin in guinea pig ventricular myocytes

FU Li-Ying¹, WANG Fang, Chen Xue-Song², ZHOU Hong-Yi, YAO Wei-Xing³, XIA Guo-Jin, JIANG Ming-Xing

Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China

KEY WORDS patch-clamp techniques; escin; myocardium; L-type calcium channels

ABSTRACT

AIM: To establish a perforated patch recording (PPR) mode with β -escin and compare L-type calcium current ($I_{Ca,L}$) recorded under PPR and normal whole-cell recording (WCR) condition in isolated guinea-pig ventricular myocytes. **METHODS:** Single myocytes were dissociated by enzymatic dissociation method. β -escin was added to the pipette solution to perforate the cell membrane and obtain PPR mode. $I_{Ca,L}$ was recorded using PPR and WCR techniques. **RESULTS:** β -Escin 20, 25, and 30 $\mu\text{mol/L}$ could permeabilize the cell membrane and obtain PPR mode. With β -escin 25 $\mu\text{mol/L}$, the success rate was highest (16/17, 94 %) and the time required for permeabilization was 2-15 (8 \pm 4) min. Run-down of $I_{Ca,L}$ was considerably slower in PPR than in WCR condition. The amplitude of $I_{Ca,L}$ was decreased by 36 % at 20 min after the formation of WCR, while it was slowly decreased by 8 % at 30 min after the formation of PPR. The current-voltage relation (I - V) curves, activation and inactivation curves of $I_{Ca,L}$ were not significantly different between WCR and PPR. The inactivation rate of $I_{Ca,L}$ was slower in PPR than in WCR, the faster inactivation time constant (τ_f) was longer in PPR than in WCR at membrane potentials of -20 mV - +10 mV ($n=6$, $P<0.05$), and the slower time constant (τ_s) was also longer in PPR than in WCR at membrane potentials of -10 mV - +10 mV ($n=6$, $P<0.05$). There was no significant difference between the activation rate in WCR and PPR. **CONCLUSION:** Using β -escin 25 $\mu\text{mol/L}$ can easily obtain stable PPR in isolated guinea-pig ventricular myocytes, and this method is useful in dealing with channels, which show run-down under normal WCR such as L-type Ca channel.

INTRODUCTION

Whole cell recording (WCR) is an important technique in electrophysiological experiments, especially in studies of isolated mammalian cells. Although the

method has several well-known advantages, one disadvantage is the run-down of ion currents, ie, ion currents decreased rapidly during the recording course because of the diffusional exchange between the cytoplasm and the contents of the pipette. The run-down of voltage-dependent calcium current (I_{Ca}) is particularly severe, which makes it difficult for long-term recording of I_{Ca} ^[1,2].

Perforated-patch recording (PPR) is an effective means to resolve this disadvantage of WCR. PPR is characterized by the formation of small pores in cell membrane, usually, these small pores are impermeable

Now in ¹Department of Pharmacology, ²Department of Internal Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA.

E-mail liyingfu215@yahoo.com.cn

³ Correspondence to Prof YAO Wei-Xing.

Phn 86-27-8369-2033. E-mail wxyao2001@yahoo.com.cn

Received 2003-02-25

Accepted 2003-03-31

to large molecules. PPR does not change the inner environment of the cell, so it permits long term maintenance of labile currents^[3].

Some antibiotics, such as nystatin and amphotericin B are used for PPR^[4,5]. But there are some inconvenient aspects in using them, such as they are insoluble in water, and may reduce the success rate for obtaining seals^[6]. β -escin is a major constituent of the saponins obtained from the seed of a chestnut tree. It was used initially to permeabilize smooth muscle^[7]. It can interact with cholesterol in the lipid bilayer and form pores, and was used to permeabilize the smooth muscle cells in recording Ca-dependent K channels^[8]. The extent of cell membrane permeability by β -escin is very concentration dependent, and it can form holes permeable to molecules of various sizes at different concentration^[9]. It was also reported to be used in PPR of calcium current in isolated rat ventricular myocytes and have some advantages over nystatin and amphotericin B^[10].

In our experiment, we established a perforated patch recording (PPR) mode with β -escin and compared L-type calcium current ($I_{Ca,L}$) recorded under PPR with that recorded under whole-cell recording (WCR) condition in isolated guinea-pig ventricular myocytes. We try to develop a useful method to deal with channels that show run-down under the normal WCR such as voltage-dependent Ca channel.

MATERIALS AND METHODS

Drugs and animals β -escin, collagenase I, protease E, bovine serum albumin (BSA), Na_2ATP , HEPES, CsCl, $CdCl_2$, and CsOH were products of Sigma Co. Egtazic acid was purchased from Fluka Biochemica. Tetrodotoxin (TTX) was purchased from Hebei Aquatic Product Research Institute. Other analytical reagents were products of Shanghai Chemical Reagent Plant. Male or female guinea pigs weighing (290±40) g were supplied by the Medical Experimental Animal Center of Tongji Medical College of Huazhong University of Science and Technology, Grade II, Certificate No 19-023.

Cell isolation Single ventricular cells were isolated from guinea pig hearts using a double-enzyme method similar to that previously described^[8].

$I_{Ca,L}$ recording technique $I_{Ca,L}$ was recorded using WCR and PPR technique with a patch-clamp amplifier (PC-II, Huazhong University of Science and Technology). Pclamp 6.0 software (P-6, Huazhong University of Science and Technology) was used to produce the signal, collect and process the data. The

resistances of the glass electrodes used were 2-5 M Ω when they were filled with the pipette solution and immersed in the extracellular solution.

The extracellular solution was normal Tyrode's solution, and contained (mmol/L): NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.33, HEPES 10, Glucose 10, and pH was adjusted to 7.4 with NaOH. In the extracellular solution we added 30 μ mol/L TTX to block I_{Na} . The pipette solution for $I_{Ca,L}$ measurements contained (mmol/L): CsCl 120, $CaCl_2$ 1, $MgCl_2$ 5, Na_2ATP 5, egtazic acid 11, HEPES 10, Glucose 11, and the pH was adjusted to 7.3 with CsOH. β -escin was prepared as a 50 mmol/L stock solution in water, and was added into the pipette solution to a final concentration when used. All pipette solutions were back filled into the pipette. The experiments were performed at room temperature (20-21 °C).

Data analysis The data were analyzed by using Sigmaplot (Jandel scientific) software, and were presented as mean \pm SD and compared with *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Selection of the concentrations of β -escin β -escin 20, 25, and 30 μ mol/L were tried to obtain PPR. During each PPR, several well-established steps were followed. First, a gigaohm seal (>10 M Ω) was formed, and the capacitive transient was compensated, then gradual perforation was observed as a progressive increase in the amplitude and speed of capacitance current transients (I_c) and a progressive decrease of series resistance (R_s) during voltage steps from the holding potential of -70 mV to -50 mV. We never observed abrupt changes indicative of breakthrough to WCR. At these concentrations, the success rate for obtaining seals was not reduced by β -escin. Changes in R_s were used to monitor the time course of perforation of the patches following seal formation. PPR was judged to have occurred when the I_c and R_s were stabilized, and in most cells R_s was stabilized in 20 M Ω -30 M Ω . This course needed about 15 min with β -escin of 25 and 30 μ mol/L, longer time was needed with β -escin of 20 μ mol/L (Fig 1). With β -escin 30 μ mol/L, the success rate at forming perforated patches was 4/8 (50 %), with β -escin 25 μ mol/L, the success rate was 16/17 (94 %), while with β -escin 20 μ mol/L, the success rate was 6/9 (67 %). So, we used β -escin 25 μ mol/L to obtain PPR in the following experiments.

Run-down of $I_{Ca,L}$ Under normal WCR condition,

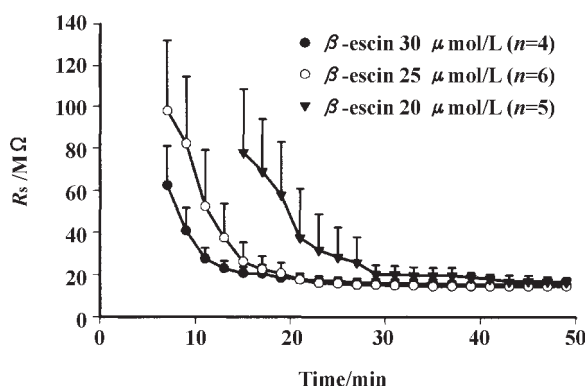


Fig 1. Series resistance (R_s) changes under perforated patch recording (PPR) condition with different concentrations of β -escin. R_s was determined by a 30 ms pulse to -50 mV from -70 mV. Time=0 represents time of gigaseal formation. Mean \pm SD.

when the holding potential was -40 mV, and the cells were depolarized to 0 mV for 150 ms at a frequency of 0.2 Hz, the inward $I_{Ca,L}$ was evoked. Under PPR condition, an inward current was evoked by the same depolarizing pulse, this current can be apparently enhanced by Bay K 8644 5 μ mol/L and inhibited by nifedipine (Nif) 20 μ mol/L, therefore, this current was $I_{Ca,L}$ (data not shown). Under WCR condition, $I_{Ca,L}$ of isolated guinea pig ventricular myocytes ran down quickly, 20 min after the formation of WCR, the amplitude of $I_{Ca,L}$ was decreased by 36 % (Fig 2). In contrast, run-down was considerably slower under PPR condition, $I_{Ca,L}$ was stable or slowly decreased during

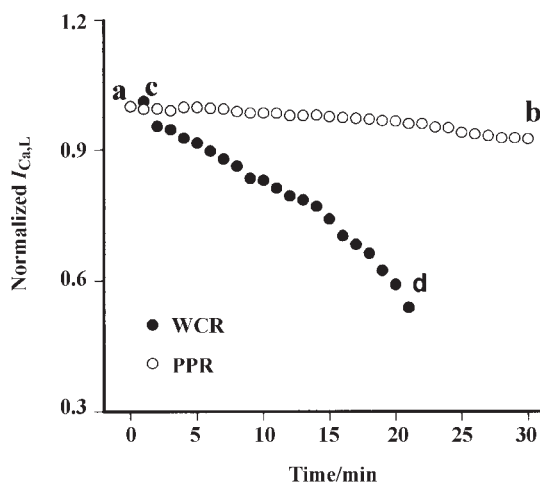


Fig 2. Comparison of run-down of $I_{Ca,L}$ in whole cell recording (WCR) and PPR with β -escin 25 μ mol/L. $I_{Ca,L}$ was evoked by a 150 ms depolarizing pulse to 0 mV from the holding potential of -40 mV. Time =0 in the PPR corresponds to 15 min after the formation of a gigaseal, by which time R_s had decreased to a minimum. a and b: $I_{Ca,L}$ recorded at 0 min and 30 min in PPR. c and d: $I_{Ca,L}$ recorded at 0 min and 20 min in WCR. $n=8$.

the course of recording, the amplitude of $I_{Ca,L}$ was decreased by 8 % at 30 min after the formation of PPR (Fig 2).

Current-voltage relationships of $I_{Ca,L}$ Current-voltage relationship ($I-V$) curves were generated by applying a series of depolarizing pulses from a holding potential of -40 mV to different membrane potentials (-40 mV to +50 mV) with a 10 mV increment. There was no significant difference between the peak $I_{Ca,L}$ under WCR and PPR in different membrane potentials. The average maximal amplitude of $I_{Ca,L}$ both occurred at $V_m=0$ mV under these two recording conditions (Fig 3).

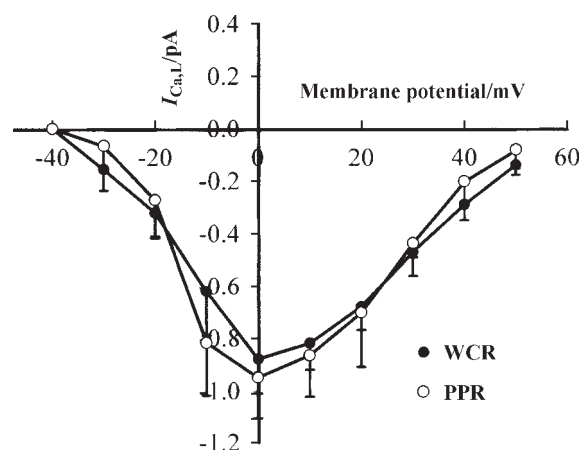
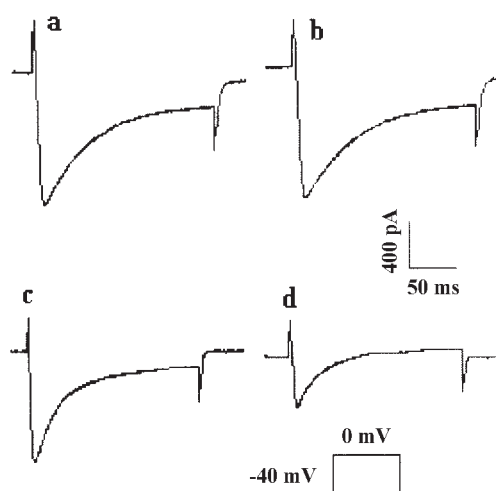


Fig 3. Current-voltage relationship ($I-V$) curves of $I_{Ca,L}$ in WCR and PPR with β -escin 25 μ mol/L. $I_{Ca,L}$ was evoked by a step pulses from -40 mV to +50 mV at the holding potential of -40 mV. $n=6$. Mean \pm SD.



Steady-state activation and inactivation of $I_{Ca,L}$

On the basis of data obtained from current-voltage relationship, activation curves were obtained (Fig 4). They were fitted by the Boltzmann function: $G/G_{max} = 1/[1+\exp(V-V_{1/2})/k]$, where G was membrane conductance transferred from membrane current, G_{max} was the maximal membrane conductance, $V_{1/2}$ was the membrane potential of half maximal activation and k was slope factor. $V_{1/2}$ of $I_{Ca,L}$ under WCR and PPR was (-10 ± 3) mV and (-12 ± 4) mV, respectively ($n=6$, $P>0.05$). The k value of $I_{Ca,L}$ under WCR and PPR was (7 ± 1) mV and (5 ± 2) mV, respectively ($n=6$, $P>0.05$).

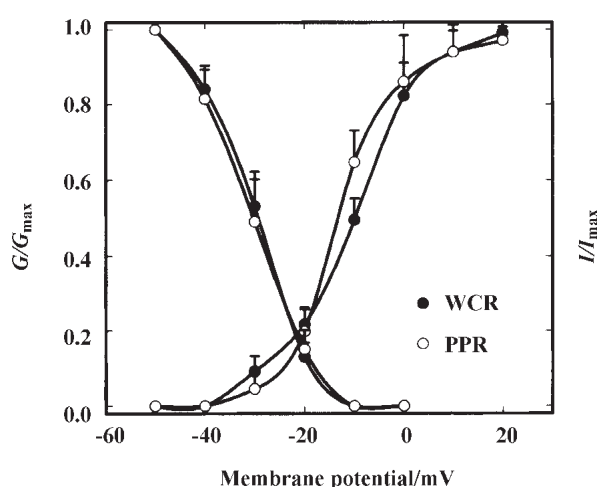


Fig 4. Steady-state activation and inactivation curves of $I_{Ca,L}$ under WCR and PPR condition. $n=6$. Mean \pm SD.

The steady-state inactivation of $I_{Ca,L}$ was evaluated using a conventional double pulse protocol. The inactivating prepulse was stepped from a holding po-

tential of -40 mV to membrane potentials of -50 mV-+20 mV in 10 mV steps for 1 second. The test pulse was stepped to 0 mV for 100 ms. The current elicited by the test pulse (I) was normalized as a fraction of the maximal current (I_{max}) obtained when the prepulse was -50 mV and plotted as a function of the prepulse membrane potential (Fig 4). The inactivation curves were also fitted by the Boltzmann function: $I/I_{max} = 1/[1+\exp(V-V_{1/2})/k]$, where $V_{1/2}$ was the membrane potential of half maximal inactivation and k was slope factor. Under WCR and PPR, $V_{1/2}$ was (-31 ± 2) mV and (-30 ± 4) mV and k value was (5 ± 1) mV and (6 ± 1) mV, respectively. No significant difference was observed between WCR and PPR ($n=6$, $P>0.05$).

Activation and inactivation kinetics of $I_{Ca,L}$

Under WCR and PPR, the activation course of $I_{Ca,L}$ at membrane potentials of -30 mV-+20 mV was fitted by single exponential function, the inactivation course of $I_{Ca,L}$ at membrane potentials of -30 mV-+20 mV was fitted by biexponential. There was no significant difference between the activation rate in WCR and PPR. The faster inactivation course had a time constant (τ_f) longer in PPR than WCR at membrane potentials of -20 mV-+10 mV ($n=6$, $P<0.05$). The slower time constant (τ_s) was also longer in PPR than in WCR at membrane potentials of -10 mV-+10 mV ($n=6$, $P<0.05$) (Tab 1).

DISCUSSION

Our results suggested that using β -escin 25 μ mol/L could obtain stable PPR in isolated guinea pig ventricular myocytes. Compared to conventional WCR, rundown of $I_{Ca,L}$ decreased effectively under this PPR mode.

Tab 1. Time constant (τ) for activation and inactivation course of L-type calcium currents ($I_{Ca,L}$) in WCR and PPR. $n=6$ cells. Mean \pm SD. ^b $P<0.05$ vs WCR.

V_m /mV	Activation τ /ms		Inactivation τ /ms					
	WCR	PPR	WCR	τ_f	PPR	WCR	τ_s	PPR
-30	1.6 \pm 0.4	2.0 \pm 0.4	5 \pm 2		8 \pm 4	165 \pm 47		206 \pm 97
-20	1.4 \pm 0.3	1.8 \pm 0.7	9 \pm 4		14 \pm 2 ^b	145 \pm 39		187 \pm 58
-10	1.5 \pm 0.4	1.7 \pm 0.7	8 \pm 3		17 \pm 6 ^b	88 \pm 44		152 \pm 32 ^b
0	1.2 \pm 0.3	1.4 \pm 0.4	8 \pm 2		19 \pm 6 ^b	80 \pm 40		142 \pm 35 ^b
10	1.5 \pm 0.5	1.4 \pm 0.4	8 \pm 3		14 \pm 5 ^b	78 \pm 36		149 \pm 63 ^b
20	1.3 \pm 0.5	1.4 \pm 0.4	14 \pm 5		12 \pm 3	106 \pm 35		145 \pm 40

V_m : membrane potential. τ_f : faster inactivation constant of $I_{Ca,L}$. τ_s : slower inactivation constant of $I_{Ca,L}$.

The amplitude of $I_{Ca,L}$ was relatively stable during the 30 min recording course after PPR was obtained. Using this method, it became possible to observe $I_{Ca,L}$ for a relatively long time. Although it was reported that high molecular weight substances could enter cells through β -escin pores and leave cells^[8], the run-down of $I_{Ca,L}$ was effectively slowed. This might be explained from two aspects, one was that a patch even with β -escin pores still represents more of a diffusion barrier to macromolecules than a ruptured patch, the other was that β -escin shows steep concentration dependence in pore forming and the concentration we used was very low. Compared to other reported PPR produced by nystatin and amphotericin B, PPR with β -escin 25 $\mu\text{mol/L}$ has several advantages, (1) the success rate was high (94 %), (2) permeability proceeded more rapidly (about 15 min), (3) the R_s could decrease to a relatively low level (20 $M\Omega$ -30 $M\Omega$), (4) in the concentration we used, β -escin did not affect the sealing course, (5) β -escin is water soluble and it is less expansive than nystatin and amphotericin B^[4,9].

The concentration of β -escin we used was lower than that was reported in isolated rat ventricular myocytes (30-50 $\mu\text{mol/L}$). The current-voltage relationship, activation and inactivation curve were not significantly changed in PPR compared to WCR, that is, β -escin 25 $\mu\text{mol/L}$ did not change the voltage-dependent characters of $I_{Ca,L}$.

The significant difference between WCR and PPR was the inactivation rate of $I_{Ca,L}$ in PPR. This might be because of the decrease in conductance. It was known that saponin interacts with cholesterol in the lipid bilayer, and it had been reported that variations in membrane cholesterol alter the kinetics of Ca-dependent K channel^[8,11]. So, we think that the alteration of the kinetics of $I_{Ca,L}$ might also be due to this variation.

The results of the present study indicated that using β -escin 25 $\mu\text{mol/L}$ could obtain stable PPR mode.

This method may be especially worthwhile when dealing with channels, which show run-down of activity under the normal WCR such as voltage-dependent Ca channels.

REFERENCES

- 1 Shuba YM, Hesslinger B, Trautwein W, McDonald TF, Pelzer D. Whole-cell calcium current in guinea-pig ventricular myocytes dialysed with guanine nucleotides. *J Physiol (Lond)* 1990; 424: 205-28.
- 2 Li Q, Yin JX, He RR. Effect of agmatine on L-type calcium current in rat ventricular myocytes. *Acta Pharmacol Sin* 2002; 23: 219-24.
- 3 Gillis KD, Pun RYK, Mislis S. Single cell assay of exocytosis from adrenal chromaffin cells using "perforated patch recording". *Pflügers Arch* 1991; 418: 611-3.
- 4 Horn R, Marty A. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol* 1988; 92: 145-59.
- 5 Pang ZP, Xu TL, Hu GY, Li JS. Dual effects of pentobarbital on rat sacral dorsal commissural neurons *in vitro*. *Acta Pharmacol Sin* 1998; 19: 327-31.
- 6 Kobayashi S, Kitazawa T, Somlyo AV, Somlyo AP. Cytosolic heparin inhibits muscarinic and α -adrenergic Ca^{2+} release in smooth muscle. *J Biol Chem* 1989; 264: 17997-8004.
- 7 Muraki N, Imaizumi Y, Watanabe M. Ca-dependent K channels in smooth muscle cells permeabilized by β -escin recorded using the cell-attached patch-clamp technique. *Pflügers Arch* 1992; 420: 461-9.
- 8 Konishi M, Watanabe M. Molecular size-dependent leakage of intracellular molecules from frog skeletal muscle fibers permeabilized with β -escin. *Pflügers Arch* 1995; 429: 598-600.
- 9 Palade P. Perforated patch recording with β -escin. *Pflügers Arch* 1998; 436: 1021-3.
- 10 Huang K, Dai GZ, Li XH, Fan Q, Chen L, Feng YB. Blocking L-calcium current by *l*-tetrahydropalmatine in single ventricular myocytes of guinea pig. *Acta Pharmacol Sin* 1999; 20: 907-11.
- 11 Mei YA, Huang LX, Wei H, Sun JT, Zhou HQ, Zhang ZH. Characterization of outward potassium current in embryonic chick heart cells. *Acta Pharmacol Sin* 1999; 20: 303-7.