Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 501 (2004) 71–78

www.elsevier.com/locate/ejphar

Toosendanin, a triterpenoid derivative, acts as a novel agonist of L-type Ca^{2+} channels in neonatal rat ventricular cells

Mu-Feng Li, Yu-Liang Shi*

Key Laboratory of Neurobiology, Institute of Physiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, P.R. China

Received 29 April 2004; received in revised form 5 August 2004; accepted 10 August 2004
Available online 12 September 2004

Abstract

Toosendanin, a triterpenoid derivative extracted from *Melia toosendan* Sieb et Zucc, was demonstrated to be potentially useful in medical and scientific researches. Here, we investigated the effects of toosendanin on L-type voltage-dependent Ca^{2+} channels in cultured neonatal rat ventricular cells, using whole-cell patch-clamp method. Toosendanin irreversibly increased L-type Ca^{2+} current ($I_{Ca(L)}$) in a concentration-dependent manner and shifted the maximum of the current/voltage relationship from 8.3±3.7 to 1.7±3.7 mV, without modifying the threshold potential of the current. Toosendanin shifted the steady-state activation and inactivation curves to the left. The deactivation kinetics of the $I_{Ca(L)}$ was significantly slowed by toosendanin while the activation kinetics was not affected. The cells pretreated with 100 nM 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (*S*(−)-BayK8644) still respond to further addition of 87 μM toosendanin, and vice versa. These results prove toosendanin to be a novel L-type Ca^{2+} channel agonist, which possesses a distinct binding site from BayK8644.

Keywords: Toosendanin; Neonatal rat ventricular cell; L-type Ca^{2+} channel; Whole-cell patch-clamp; Agonist

1. Introduction

Toosendanin ($C_{30}H_{38}O_{11}$, FW=574), a triterpenoid derivative (Chang et al., 1975; Shu and Liang, 1980), is an active ingredient extracted from the bark of *Melia toosendan* Sieb et Zucc, which is used in Chinese traditional medicine as an anthelmintic vermifuge against ascaris (Wang and Wen, 1959). Previous studies have demonstrated that toosendanin possessed extensively biological properties and might be potentially useful in medical and scientific researches. Firstly, toosendanin was indicated to be a selective presynaptic blocker acting on neurotransmitter release in both central and peripheral synapses (Shi et al., 1980, 1981a,b; Shi and Chen, 1999; Chen et al., 1999; Xu and Shi, 2003). Secondly, despite sharing some similar actions with botulinum neurotoxin, toosendanin was shown to have a dramatically antibotulismic effect in vivo and in vitro (Li et al., 1982; Shi and Xu, 1983a,b; Zhou et al., 2003). Thirdly, at low concentrations, toosendanin could induce PC12 cell differentiation and apoptosis (Tang et al., 2003). These actions of toosendanin seemed to be at least partially related to the action on ion channels.

In studying the effects of toosendanin on ion channels, we have already observed that toosendanin inhibited K^+ currents mediated by various kinds of K^+ channels, such as the fast K^+ channels in mouse motor nerve terminals (Xu and Shi, 1993), the inward rectifier K^+ channels in guinea pig papillary muscles (Gao et al., 1994), the delayed rectifier K^+ channels in the neuroblastoma×glioma NG108-15 cells (Hu et al., 1997), the inward rectifier K^+ channels, and large- and small-conductance Ca^{2+}-activated K^+ channels in rat hippocampal neurons (Wang and Shi, 2001a,b,c). In recent studies, we found that toosendanin increased intracellular Ca^{2+} concentration and L-type Ca^{2+} current in NG108-15 neuronal cells but did not affect the other types

* Corresponding author. Tel.: +86 21 54920270; fax: +86 21 54920276.
E-mail address: ylshi@server.shcnc.ac.cn (Y.-L. Shi).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.08.027

of Ca^{2+} channels (Ding et al., 2001; Xu et al., 2004; Li et al., 2004). However, the comprehensive effect of toosendanin on L-type Ca^{2+} channels is still unclear.

In cardiomyocytes, L-type Ca^{2+} current is the sole high-voltage-activated Ca^{2+} current (Cohen and Lederer, 1987). L-type Ca^{2+} channels are the major pathways for the entry of Ca^{2+} involved in the activation of contraction in the heart and smooth muscles. In working myocardial cells, they mediate excitation–concentration coupling and are responsible in part for the long plateau of the cardiac action potential (Callewaert, 1992; Katz, 1997). So, after a selective effect of toosendanin on L-type Ca^{2+} channels has been observed in NG108-15 cells, we thought it was worthy of investigating the effect of toosendanin on cardiomyocytes for full understanding the value of toosendanin in medical use.

In the present study, the cultured neonatal rat ventricular cells were used to elucidate the effects of toosendanin on voltage-dependent L-type Ca^{2+} channels in attempts to clarify whether toosendanin modulates cardiac Ca^{2+} currents and analyze the detailed action of toosendanin on Ca^{2+} channels.

2. Materials and methods

2.1. Neonatal cell culture

Cell cultures were prepared by using a modified protocol derived from the method used by Gomez et al. (1994). In brief, hearts (6–8) from 3- to 5-day-old Sprague–Dawley rats, previously anaesthetized by ether, were aseptically excised and finely minced. The pieces were immersed in filter-sterilized Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 0.25% trypsin (Sigma). Five successive incubations were carried out for 10 min each at 37 °C and the cell suspensions, except the first, were centrifuged ($1000\times g$ for 5 min). The cell pellets were resuspended in culture medium (see below) and preplated for 60 min. Then the suspended cells were subsequently collected and diluted to a final concentration of 1×10^5 cells/ml. They were plated (1 ml/dish) into 35-mm dishes and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Experiments were carried out between day 2 and day 3 in culture. All experiments conformed to the guidelines of the NIH on the ethical use of animals, and all experimental procedures were reviewed and approved by the Animal Care and Use Committee of Shanghai Institutes for Biological Sciences. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Solutions and drugs

For neonatal cell culture the Ca^{2+}- and Mg^{2+}-free Hanks' solution contained (in mM): NaCl, 137; KCl, 5.4; $NaHCO_3$, 4.2; Na_2HPO_4, 0.3; and glucose, 5.5, pH 7.3. The culture medium was composed of 85% Dulbecco's modified Eagle's medium (DMEM; Gibco), 15% fetal bovine serum (HyClone), 100 U/ml streptomycin and 100 U/ml penicillin.

Ca^{2+} currents were recorded in Na^+- and K^+-free solution. The external solution contained (in mM) *N*-methyl-D-glucamine (NMG), 130; tetraethylammonium chloride (TEACl), 20; $CaCl_2$, 1.8; $MgCl_2$, 2; HEPES, 10; and glucose, 10, pH 7.4 adjusted with HCl. The patch pipette solution was composed of (in mM): CsCl, 110; TEACl, 20; MgATP, 5; Na_2ATP, 2; HEPES, 5; EGTA, 3; and phosphate creatine, 5, pH 7.2 with Tris.

N^6, 2′-*O*-dibutyryl adenosine 3′, 5′-cyclic monophosphate (DB cAMP, 10 mM, Sigma) was prepared as stock solution in distilled water. Stock solutions of *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89, 1 mM, Sigma) and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (*S*(−)-BayK8644,1 mM, Sigma) were prepared in dimethylsulfoxide (DMSO). The drugs were diluted in incubation media or bath solution to a given concentration before experiments. Maximal concentration of DMSO (0.01%) in test solutions did not affect Ca^{2+} currents (data not shown). H-89 was applied by preincubation for at least 30 min while the cells were still in the humidified incubator. It was also added to the extracellular recording solution.

Toosendanin is a sample recrystallized in ethanol with a purity >98% (Hu et al., 1997; Shi and Chen, 1999). A concentration (87 μM) that produced obvious effects was used in most experiments. All other reagents were of analytical grade.

2.3. Electrophysiology experiments

Voltage-clamp recordings were performed in the whole-cell configuration of the patch-clamp method by using patch clamp amplifier (CEZ-2300, Nihon Kohden, Japan) and recording pipettes (Type 95, Shanghai Institute of Physiology) with resistance of 2–5 MΩ when filled with the pipette solution. The cells were continuously perfused in a 35-mm plastic dish with the external solution. The irrigation system was similar to the "Y tube" method (Akaike and Harata, 1994). Evocation and recording of the Ca^{2+} currents (I_{Ca}) were controlled by pClamp 5.7 running on a computer through an analog-to-digital interface (Labmaster TL-1, Tecmar, Solon, USA). The currents were filtered at 1 kHz, and the leakage and capacitative currents were digitally subtracted with a P/4 protocol. All experiments were carried out at room temperature (20–24 °C).

The L-type Ca^{2+} currents were elicited from a holding potential of −50 mV to a test potential of +10 mV for 200 ms every 15 s. The currents were abolished completely by 2 mM Co^{2+} consistent with the current being carried through Ca^{2+} channels.

The steady-state activation ($d\alpha$) curves were estimated from the relative membrane conductance as a function of the membrane potential, such that $G_{Ca}=I_{Ca}/(V_m-V_{rev})$ where G_{Ca} is the peak conductance, I_{Ca} the peak of Ca^{2+} current for

the test potential V_m and V_{rev} the apparent reversal potential of the Ca^{2+} current. The steady-state inactivation ($f\alpha$) curves were determined by using a double-pulse protocol: conditioning pulse duration was 5 s and test duration was 300 ms. The pulses were applied to test potential of +10 mV from various conditioning pulse levels (from −80 to +20 mV). There was a 5-ms interval to allow for resetting of the activation gate between the end of conditioning pulse and the beginning of the test pulse in which the membrane potential was returned to the holding potential of −80 mV. The data for the steady-state kinetic parameters were fitted to the following Boltzmann equations:

$$d\alpha = [1 + \exp(-(V_m - V_h)/k)]^{-1}$$

$$f\alpha = [1 + \exp((V_m - V_h)/k)]^{-1}$$

where V_m is the conditioning potential, V_h is the potential required for half-activation or half-inactivation of the current and k is the slope factor.

All data are presented as mean±S.E.M. Statistical analyses were performed using Student's unpaired and paired t tests and $P<0.05$ was defined as significant.

3. Results

3.1. Toosendanin irreversibly increased the $I_{Ca(L)}$ in a concentration-dependent manner without affecting $I_{Ca(T)}$

When a neonatal rat ventricular cell held at −50 mV was given 200 ms depolarizing test pulses from −40 to +30 mV, a series of evoked Ca^{2+} currents was recorded, and the current/voltage (I/V) relationship was obtained (Fig. 1A). The current was activated at −20 mV, reversed at +52 mV, and the average peak current density was 3.23±0.96 pA/pF (n=6). About 2 min after perfusion with 87 μM toosendanin-containing solution, the peak current density was increased significantly to 5.91±1.47 pA/pF ($P<0.05$) and the reversal potential was slightly shifted to +54 mV. The enhancement effect of toosendanin was irreversible; even with 8 min of washing in toosendanin-free solution, the increased currents were maintained (change <1%, n=4). An example of the time course of the change in the peak of $I_{Ca(L)}$ is shown in Fig. 1B.

Toosendanin increased the $I_{Ca(L)}$ in a concentration-dependent manner. Fig. 1C shows the dose–response relationship. Data points were fitted to the Hill equation, that is,

$$\text{enhancement}(\%) = \frac{[\text{drug}]^{n_H}}{[\text{drug}]^{n_H} + EC_{50}^{n_H}} \times E_{max}$$

where E_{max} is the maximum enhanced effect, n_H is the Hill coefficient, EC_{50} is the concentration for half-maximal effect of the drug. The E_{max} n_H and EC_{50} were 83%, 1.5 and 8.9 μM respectively.

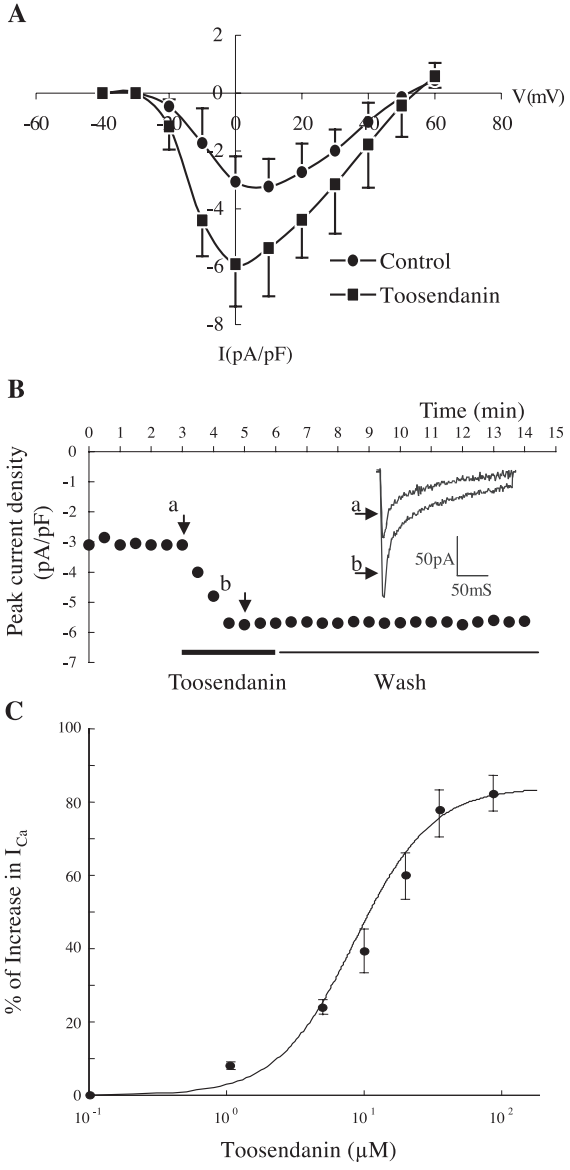

Fig. 1. The effect of toosendanin on Ca^{2+} current density in neonatal rat ventricular cells. (A) Current/voltage relationships taken before (●) and after (■) application of 87 μM toosendanin (n=6). The data were expressed as mean±S.E.M. (B) One example to show time course of the effect of toosendanin. $I_{Ca(L)}$ was measured as the peak current at a test potential of +10 mV by applying 200 ms duration pulse from a holding potential of −50 mV. After $I_{Ca(L)}$ was stabilized, the application of toosendanin increased the current from 83 pA (a) to 153 pA (b). After 8 min of washing, the current was 151 pA. (C) Concentration–response curve of toosendanin on $I_{Ca(L)}$ in neonatal rat ventricular cells. Data points represent the average percentage of current increase 2 min after toosendanin application. Points were curve-fit using the Hill equation. The data were expressed as mean±S.E.M. (n=5–7).

The neonatal rat ventricular cells used in this study expressed both T- and L-type Ca^{2+} channels. I_{Ca} recorded at a test potential of −20 mV from a holding potential of −90 mV, in the presence of 1 μM nifedipine, was T-type Ca^{2+} current. In fact, this current was not significantly modified by 87 μM toosendanin (n=4, data not shown).

3.2. Toosendanin shifted the steady-state activation and inactivation curves for $I_{Ca(L)}$ to more negative potentials

The *I*/*V* curves in Fig. 1 show that not only the current density was increased by toosendanin but also the potential giving maximal $I_{Ca(L)}$ was shifted to the left from 8.3±3.7 to 1.7±3.7 mV, without change in the activation threshold. The negative shift of $I_{Ca(L)}$ peak observed after addition of toosendanin was further studied with regards to the analysis of both steady-state activation and inactivation parameters.

The superimposed curves in Fig. 2 show that both activation and inactivation relationships are shifted to the left in the presence of 87 μM toosendanin. The data for activation and inactivation parameters, calculated from the Boltzmann function by fitting to the curves in Fig. 2, were summarized in Table 1. A comparison of these pooled data confirms that the half-maximum voltage for activation or inactivation, V_h is always significantly ($P<0.05$) shifted to the negative direction by the addition of toosendanin. This shift is achieved without change in the slope factor *k*, for inactivation curves but with a significant ($P<0.05$) decrease of *k* for activation curves.

Table 1
Effects of toosendanin on steady-state activation and inactivation parameters of $I_{Ca(L)}$

	Activation		Inactivation	
	V_h (mV)	*K* (mV)	V_h (mV)	*K* (mV)
Control (*n*=6)	1.5±1.4	7.4±1.0	−26.4±0.8	11.3±0.4
Toosendanin (87 μM, *n*=6)	−7.4±1.2**	4.3±1.0*	−32.1±1.0*	13.0±0.2

Values of V_h and *K* were obtained from data in Fig. 2 fitted by Boltzmann function.
* $P<0.05$ vs. control.
** $P<0.01$ vs. control.

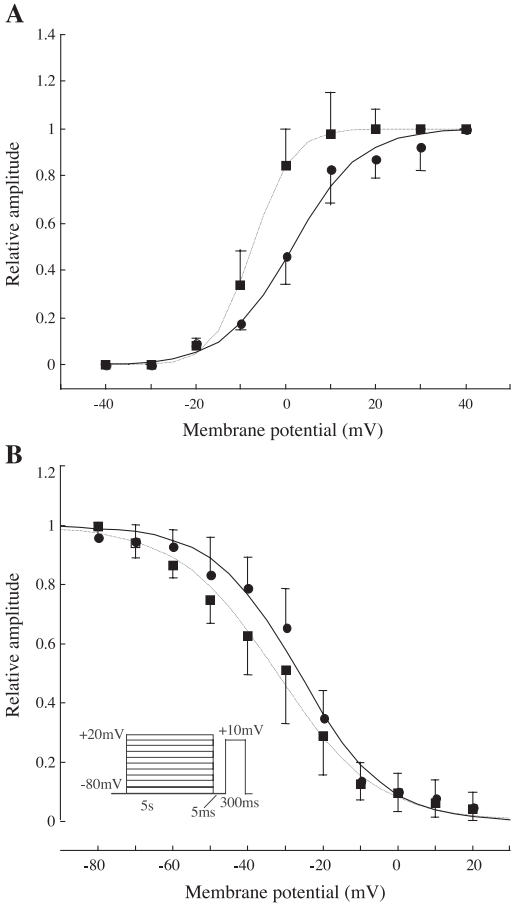

Fig. 2. The effects of toosendanin (87 μM) on both steady-state activation (d∝) and inactivation (f∝) curves in neonatal rat ventricular cells. (A) The d∝ curves of $I_{Ca(L)}$ before (●) and after (■) application of toosendanin (*n*=6). (B) The f∝ curves of $I_{Ca(L)}$ before (●) and after (■) application of toosendanin (*n*=6). Pulse protocol is given in the inset. The data for the steady-state kinetic parameters were both fitted to the Boltzmann equation. The data were expressed as mean±S.E.M.

3.3. Effects of toosendanin on activation and deactivation kinetics of the $I_{Ca(L)}$

To study the kinetics of current activation, the currents elicited from a holding potential of −50 mV to test potentials of −10 to +50 mV were recorded. Time constant (τ) for current activation was analyzed by fitting single exponential to the current traces. As shown in Fig. 3A, application of toosendanin induced no significant change in the τ for activation (*n*=5).

Tail currents were recorded between −80 and −10 mV following an 8-ms test pulse to +10 mV from a holding potential of −50 mV. The current traces could be fitted with

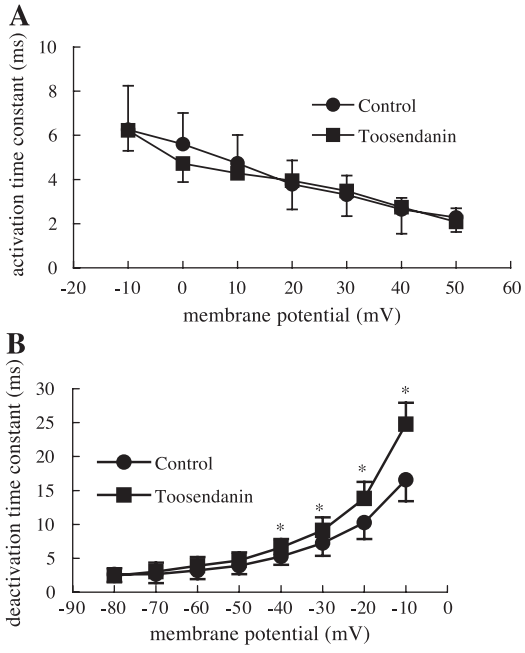

Fig. 3. The effects of toosendanin (87 μM) on both activation and deactivation kinetics. (A) The time constant for activation measured in the absence (●) or presence (■) of toosendanin is plotted against the membrane potentials (*n*=5). (B) The time constant for deactivation measured in the absence (●) or presence (■) of toosendanin is plotted against the membrane potentials. Each point represents the mean±S.E.M. (*n*=6), **P*<0.05.

single exponential curves (n=6). In the presence of toosendanin, the τ for deactivation was significantly prolonged at voltage more positive than −50 mV (Fig. 3B).

3.4. Toosendanin increased the $I_{Ca(L)}$ after the Ca^{2+} channels were phosphorylated

It has been well documented that the phosphorylation of L-type Ca^{2+} channel via cAMP-dependent protein kinase A (PKA) leads to an increase in channel open probability, mean open time and channel availability (Sperelakis et al., 1996). Hence, whether toosendanin increased the L-type Ca^{2+} current density by elevating the level of cAMP was tested. The cells were firstly phosphorylated by elevating intracellular cAMP following exposure to 0.2 mM DB cAMP, a permeant cAMP analogue, for 3 min before superfusing toosendanin (87 μM). Under these conditions, the $I_{Ca(L)}$, evoked by a depolarizing test pulse to +10 mV from a holding potential of −50 mV, was increased by 123±2% (n=4) in the presence of DB cAMP alone, similar to previous report (Gomez et al., 1996). However, in the presence of supplemented toosendanin, the current density was further increase by 70±2% of the control value (Fig. 4A).

3.5. Toosendanin increased the $I_{Ca(L)}$ in the presence of cAMP-dependent protein kinase inhibitor

To further confirm that the toosendanin-induced enhancement in $I_{Ca(L)}$ is not mediated by PKA, H-89, a selective inhibitor of PKA (Chijiwa et al., 1990), was used. After the cells preincubated with 1 μM H-89 for at least 30 min, application of 87 μM toosendanin increased the $I_{Ca(L)}$ by 80±11% (Fig. 4B, n=4). When a high concentration of H-89 (10 μM, 200 times the IC_{50} for inhibition of PKA) was used to completely inhibit the PKA, the $I_{Ca(L)}$ were generally of small amplitudes (<50 pA). However, addition of 87 μM toosendanin still increased the $I_{Ca(L)}$ by 79±2% (n=5). These results indicate that PKA is not involved in the toosendanin-induced enhancement in $I_{Ca(L)}$.

3.6. Interaction between the effects of toosendanin and S(−)-BayK8644 at the $I_{Ca(L)}$

Fig. 5 shows the interactions of S(−)-BayK8644 and toosendanin at L-type Ca^{2+} channels in neonatal rat ventricular cells. When the cells were treated with 100 nM S(−)-BayK8644 alone, the current density was increased by 135±7% and the current decay was accelerated (n=3). Further addition of 87 μM toosendanin caused a further increase of the current density by 41±5% (of the control value) with a slowing of the decay phase (Fig. 5A). When a saturated concentration (5 μM, 200 times the EC_{50} for activation of L-type Ca^{2+} channels) of S(−)-BayK8644 was used in the experiments instead of 100 nM (Hamilton et al., 1987), S(−)-BayK8644 alone increased the current density by 146±5% and further addition of 87 μM toosendanin caused a further increase of the current density by 40±4% (of the control value). In the experiments that the cells were treated with 87 μM toosendanin first and then 100 nM S(−)-BayK8644 (Fig. 5B), we found that the current density was firstly increased by 86±3% (by toosendanin) and then further increased by 67±9% of the control value (by further addition of S(−)-BayK8644). But under this condition

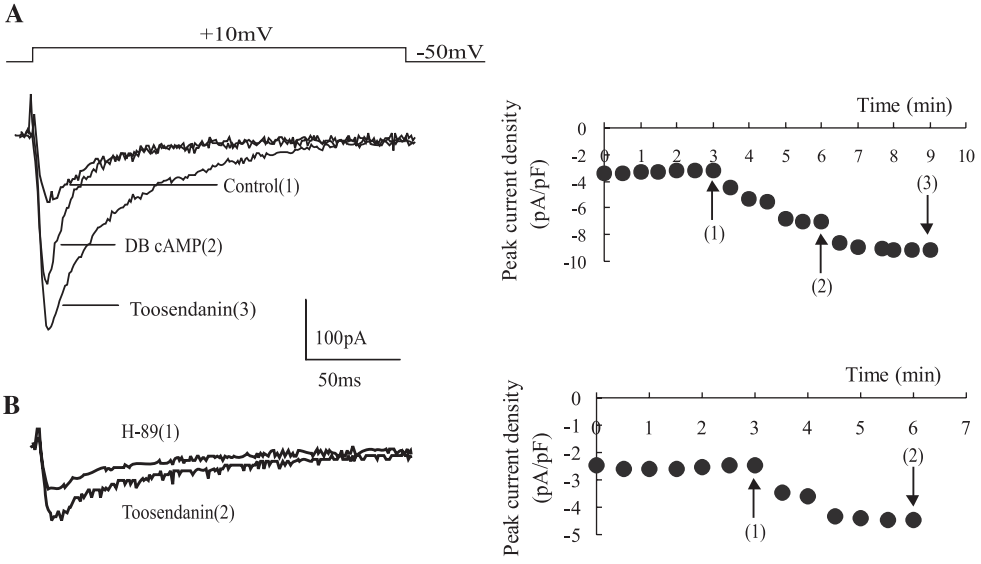

Fig. 4. PKA is not involved in the toosendanin-induced enhancement in $I_{Ca(L)}$. Superimposed traces were elicited by a depolarizing test pulse to +10 mV from a holding potential of −50 mV. Time courses of the effect of the drugs on Ca^{2+} channels were shown. (A) The effect of toosendanin on phosphorylated Ca^{2+} channels (by external application of permeant dibutyryl cyclic AMP). (B) The effect of toosendanin on $I_{Ca(L)}$ recorded in the presence of 1 μM H-89. Toosendanin increased the $I_{Ca(L)}$ recorded in the cells preincubated with H-89 for at least 30 min.

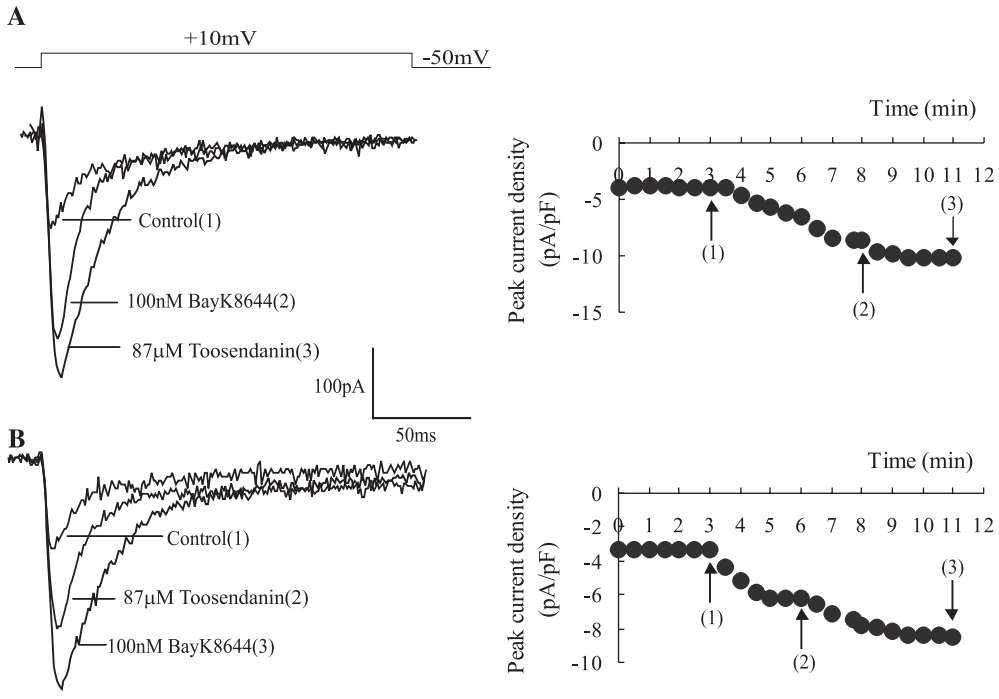

Fig. 5. Interaction of toosendanin and BayK 8644 at $I_{Ca(L)}$ in neonatal rat ventricular cells. Superimposed traces were elicited by a depolarizing test pulse to +10 mV from a holding potential of −50 mV. Time courses of the effect of the drugs on Ca^{2+} channels were shown. After the cells treated with BayK8644, toosendanin still caused an increase in the current (A), and vice versa (B). The number in brackets shows the sequence to take the trace.

S(−)-BayK8644 lost its ability to accelerate the current decay (n=3).

4. Discussion

In contrast to numerous drugs are known to block L-type Ca^{2+} channels, relatively few molecules predominantly activate them. Among the synthetic Ca^{2+} channel ligands, some dihydropyridine derivatives such as BayK8644 (Hess et al., 1984) and 4-[2-(Difluormethoxy)phenyl]-1,4,5,7-tetrahydro-2-methyl-5-oxo-furo[3,4-*b*]pyridine-3-carboxylic acid ethylester (CGP 28392, Kokubun and Reuter, 1984) as well as methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1*H*-pyrrole-3-carboxylate (FPL 64176), a benzoylpyrrole compound (Rampe and Lacerda, 1991), have been shown to possess predominantly agonist-like activity. Except for the synthesized molecules, some naturally occurring molecules (Knaus et al., 1990; Saponara et al., 2002) and certain animal toxins (Hamilton and Perez, 1987) have been shown to stimulate L-type Ca^{2+} channels in various mammalian tissues. Previously, it was demonstrated that toosendanin selectively increased the L-type Ca^{2+} current in neuroblastoma×glioma NG108-15 cells (Li et al., 2004). Here, we demonstrated that toosendanin concentration dependently increased the $I_{Ca(L)}$ in neonatal rat ventricular cells and the effect was irreversible.

In the present study, toosendanin was demonstrated to induce a current density enhancement and shift the maximum of the I/V relationship towards more negative potential without affecting activation threshold potential of the current. Thus, the effect of toosendanin on the I/V relationship might be the consequence of the hyperpolarizing shift in the activation curve. Toosendanin shifted the steady-state activation and inactivation curves to more negative potentials and caused a significant increase in the slope of activation, which reflects a yield of more current for a given potential. These results indicate that toosendanin may alter the voltage sensitivity of the channel. However, toosendanin did not affect Ca^{2+} channel activation kinetics over a wide range of membrane potentials. It indicates that toosendanin does not affect the transition from the closed to the open state of the channel. Additionally, the inward tail currents decayed more slowly in the presence of toosendanin, possibly reflecting a longer opening of the channel. All these elements indicate that toosendanin shares several basic features with the properties of some Ca^{2+} channel agonists such as BayK8644 and FPL 64176 (Wang et al., 1989; Rampe and Lacerda, 1991; Zheng et al., 1991).

It was reported that the myocardial L-type Ca^{2+} channels could be stimulated by cAMP and any agent that increased the cAMP level of the myocardial cell would potentiate $I_{Ca(L)}$ (Sumii and Sperelakis, 1995). However, just like BayK8644, it is unlikely that toosendanin-induced activation of $I_{Ca(L)}$ is the consequence of the increase in the intracellular levels of cAMP. As shown in Fig. 4, after the Ca^{2+} channels in neonatal rat ventricular cells were phosphorylated by DB cAMP, addition of toosendanin still induced a similar increase in $I_{Ca(L)}$. Pretreating the cells with H-89, a selective inhibitor of protein kinase A, also did not

affect the stimulatory action of toosendanin on $I_{Ca(L)}$. By the way, application of toosendanin also induced an increase in $I_{Ca(L)}$ in vascular smooth muscle (our unpublished data), in which both cAMP and its related kinase have been shown to inhibit L-type Ca^{2+} channels (Xiong and Sperelakis, 1995).

L-type Ca^{2+} channels, as well as other voltage-dependent ion channels, can be regarded as pharmacological receptors containing discrete drug binding sites. Previous study showed that *S*(−)-BayK8644 was a potent inhibitor of FPL 64176-induced responses and indicated a negative allosteric interaction between the 1,4-dihydropydine agonist binding site and the benzoylpyrrole binding site in intact tissue (Rampe and Dage, 1992). Here, we examined the interactions of *S*(−)-BayK8644 and toosendanin directly at L-type Ca^{2+} channels in neonatal rat ventricular cells. Unlike what occurred with FPL 64176, we found that the cells pretreated with *S*(−)-BayK8644 (either 100 nM or 5 μM) still preserved the ability to respond to further addition of 87 μM toosendanin, and vice versa. These results indicate that *S*(−)-BayK8644 and toosendanin possess different binding sites and there is no negative allosteric interaction between these two binding sites. However, owing to the structural complexity of the ion channels and their ability to exist in various closed, opened and inactivated states, interaction of drugs at ion channels are sometimes complicated.

The results in this study show that toosendanin is a potent activator of L-type Ca^{2+} channels in neonatal rat ventricular cells, and indicate that toosendanin possesses ability in regulating heart rate and contractility. This finding brings us more interesting in studying this compound. If this drug is valuable in medical use in the future, its effect on cardiomyocytes cannot be ignored.

Acknowledgements

This work was supported by the National Basic Research Program of China (G1999054000), the National Natural Science Foundation of China (30170302) and the Basic Research Program of Shanghai (025C14011).

References

Akaike, N., Harata, N., 1994. $GABA_A$ receptor–chloride channel complex. In: Narahashi, T. (Ed.), Methods in Neurosciences, Ion Channels of Excitable Cells. Academic Press, San Diego, pp. 148–156.

Callewaert, G., 1992. Excitation–contraction coupling in mammalian cardiac cells. Cardiovasc. Res. 26, 923–927.

Chang, C.C., Hsie, T.H., Chen, S.F., Liang, H.T., 1975. The structure of Chuanliansu. Acta Chim. Sin. 33, 35–47.

Chen, W.Y., Yin, P.B., Ye, W.L., Shi, Y.L., 1999. Toosendanin-induced change of dopamine level detected by microdialysis in vivo at rat striatum. Chin. Sci. Bull. 44, 502–506.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., Hidaka, H., 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J. Biol. Chem. 265, 5267–5272.

Cohen, N.M., Lederer, W.J., 1987. Calcium current in isolated neonatal rat ventricular myocytes. J. Biol. Chem. 391, 169–191.

Ding, J., Xu, T.H., Shi, Y.L., 2001. Different effects of toosendanin on perineurially recorded Ca^{2+} currents in mouse and frog motor nerve terminals. Neurosci. Res. 41, 243–249.

Gao, X.D., Tang, S.B., Lu, J., Shi, Y.L., 1994. Effects of toosendanin on electric and mechanical properties of guinea pig papillary muscles. Zhongguo Yaoli Xuebao 15, 147–151.

Gomez, J.P., Potreau, D., Branka, J.E., Raymond, G., 1994. Developmental changes in calcium currents from newborn rat cardiomyocytes in primary culture. Pflügers Arch. 428, 241–249.

Gomez, J.P., Fares, N., Potreau, D., 1996. Effects of BayK8644 on L-type calcium current from newborn rat cardiomyocytes in primary culture. J. Mol. Cell. Cardiol. 28, 2217–2229.

Hamilton, S.L., Perez, M., 1987. Toxins that affect voltage-dependent calcium channels. Biochem. Pharmacol. 36, 3325–3329.

Hamilton, S.L., Yatani, A., Brush, K., Schwartz, A., Brown, A.M., 1987. A comparison between the binding and electrophysiological effects of dihydropyridines on cardiac membranes. Mol. Pharmacol. 31 (3), 221–231.

Hess, P., Lansman, J.B., Tsien, R.W., 1984. Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. Nature 311, 538–544.

Hu, Q., Huang, F.S., Shi, Y.L., 1997. Inhibition of toosendanin on the delayed rectifier potassium current in neuroblastoma×glioma NG108-15 cells. Brain Res. 751, 47–53.

Katz, A.M., 1997. Molecular biology of calcium channels in the cardiovascular system. Am. J. Cardiol. 80 (9A), 171–221.

Knaus, H.G., Scheffauer, F., Romanin, C., Schindler, H.G., Glossmann, H., 1990. Heparin binds with high affinity to voltage-dependent L-type Ca^{2+} channels. Evidence for an agonistic action. J. Biol. Chem. 265, 1156–1166.

Kokubun, S., Reuter, H., 1984. Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells. Proc. Natl. Acad. Sci. U. S. A. 81, 4824–4827.

Li, P.Z., Zhou, J., Miao, W.Y., Ding, F.H., Meng, J.Y., Jia, G.R., Li, J.F., Ye, H.J., He, X.Y., Chen, M.Y., Huang, Z.M., 1982. Therapeutic effect of toosendanin on animal botulism. Chin. Tradit. Herb. Drugs 13, 28–30.

Li, M.F., Wu, Y., Wang, Z.F., Shi, Y.L., 2004. Toosendanin, a triterpenoid derivative, increases Ca^{2+} current in NG108-15 cells via L-type channels. Neurosci. Res. 49, 197–203.

Rampe, D., Dage, R.C., 1992. Functional interactions between two Ca^{2+} channel activators, (*S*)-Bay K 8644 and FPL 64176, in smooth muscle. Mol. Pharmacol. 41, 599–602.

Rampe, D., Lacerda, A.E., 1991. A new site for the activation of cardiac calcium channels defined by the nondihydropyridine FPL 64176. J. Pharmacol. Exp. Ther. 259, 982–987.

Saponara, S., Sgaragli, G., Fusi, F., 2002. Quercetin as a novel activator of L-type Ca^{2+} channels in rat tail artery smooth muscle cells. Br. J. Pharmacol. 135, 1819–1827.

Shi, Y.L., Chen, W.Y., 1999. Effect of toosendanin on acetylcholine level of rat brain, a microdialysis study. Brain Res. 850, 173–178.

Shi, Y.L., Xu, K., 1983a. Antibotulismic effect of toosendanin. Chin. Sci. Bull. 28, 885–887.

Shi, Y.L., Xu, K., 1983b. Anti-botulismic effect of toosendanin and its facilitatory action on miniature end-plate potentials. Jpn. J. Physiol. 33, 677–680.

Shi, Y.L., Wei, N.S., Yang, Y.Q., Wang, Z.X., 1980. Toosendanin—a presynaptic neuromuscular blocking agent. Acta Physiol. Sin. 32, 293–297.

Shi, Y.L., Wang, W.P., Xu, K., 1981a. Electrophysiological analysis on the presynaptic blocking effects of toosendanin on neuromuscular transmission. Acta Physiol. Sin. 33, 259–265.

Shi, Y.L., Yang, Y.Q., Wang, W.P., Xu, K., 1981b. Influence of stimulus frequency, temperature and Ca^{2+} on onset of toosendanin paralysis of isolated neuromuscular preparations. Acta Physiol. Sin. 33, 141–146.

Shu, G.X., Liang, X.T., 1980. A correction of the structure of Chuanliansu. Acta Chim. Sin. 38, 196–198.

Sperelakis, N., Katsube, Y., Yokoshiki, H., Sada, H., Sumii, K., 1996. Regulation of the slow Ca^{++} channels of myocardial cells. Mol. Cell. Biochem. 163/164, 85–98.

Sumii, K., Sperelakis, N., 1995. Cyclic GMP-dependent protein kinase regulation of the L-type calcium current in neonatal rat ventricular myocytes. Circ. Res. 77, 803–812.

Tang, M.Z., Wang, Z.F., Shi, Y.L., 2003. Toosendanin induces outgrowth of neuronal processes and apoptosis in PC12 cells. Neurosci. Res. 45, 225–231.

Wang, Z.F., Shi, Y.L., 2001a. Inhibition of large-conductance Ca^{2+}-activated K^+ channels in hippocampal neurons by toosendanin. Neuroscience 104, 41–47.

Wang, Z.F., Shi, Y.L., 2001b. Toosendanin-induced inhibition of small-conductance calcium-activated potassium channels in CA1 pyramidal neurons of rat hippocampus. Neurosci. Lett. 303, 13–16.

Wang, Z.F., Shi, Y.L., 2001c. Modulation of inward rectifier potassium channel by toosendanin, a presynaptic blocker. Neurosci. Res. 40, 211–215.

Wang, Y.B., Wen, Y.X., 1959. A comprehensive report on clinical ascaris anthelmintic therapeutic effect of Toosendanin pills. J. Tradit. Chin. Med. 262, 46–49.

Wang, R., Karpinski, E., Pang, P.K., 1989. Two types of calcium channels in isolated smooth muscle cells from rat tail artery. Am. J. Physiol. 256, H1361–H1368.

Xiong, Z., Sperelakis, N., 1995. Regulation of L-type calcium channels of vascular smooth muscle cells. J. Mol. Cell. Cardiol. 27, 75–91.

Xu, Y.F., Shi, Y.L., 1993. Action of Toosendanin on the membrane current of mouse motor nerve terminals. Brain Res. 631, 46–50.

Xu, T.H., Shi, Y.L., 2003. Inhibition of high K^+-evoked glutamate release from rat synaptosomes by toosendanin. 6th Chin Congr. Biol. Toxin, Changsha, p. P39.

Xu, T.H., Ding, J., Shi, Y.L., 2004. Toosendanin increases free Ca^{2+} concentration in NG108-15 cells via L-type Ca^{2+} channels. Acta Pharmacol. Sin. 25, 597–601.

Zheng, W., Rampe, D., Triggle, D.J., 1991. Pharmacological, radioligand binding, and electrophysiological characteristics of FPL 64176, a novel nondihydropyridine Ca^{2+} channel activator, in cardiac and vascular preparations. Mol. Pharmacol. 40, 734–741.

Zhou, J.Y., Wang, Z.F., Ren, X.M., Tang, M.Z., Shi, Y.L., 2003. Antagonism of botulism toxin type A-induced cleavage of SNAP-25 in rat cerebral synaptosome by toosendanin. FEBS Lett. 555, 375–379.