Potential Antiarrhythmic Effect of Methyl 3,4,5-Trimethoxycinnamate, a Bioactive Substance from Roots of Polygala Radix: Suppression of Triggered Activities in Rabbit Myocytes

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3,4,5-Trimethoxycinnamic acid (TMCA), methyl 3,4,5-trimethoxycinnamate (M-TMCA) and p-methoxycinnamic acid (PMCA) have been identified as the major bioactive components in the serum collected from rats treated with oral administration of Polygala Radix ("YuanZhi," the roots of Polygala tenufolia Willd.), a traditional Chinese medicine used to relieve insomnia, anxiety and heart palpitations. The present study was designed to investigate its direct electrophysiological effects on isolated ventricular myocytes from rabbits. Whole-cell configuration of the patch-clamp technique was used to measure action potential (AP) and membrane currents in single ventricular myocytes enzymatically isolated from adult rabbit hearts. Ca2+ transients were recorded in myocytes loaded with the Ca2+ indicator Fluo-4AM. Among three bioactive substances of Polygala metabolites, only M-TMCA (15–30 µm) significantly shortened action potential duration at 50% and 90% repolarization (APD50 and APD90) in cardiomyocytes in a concentration-dependent and a reversible manner. M-TMCA also inhibited L-type calcium current (I\textsubscript{Ca,L}) and the suppression of intracellular Ca2+ transients, which consequently abolish the generation of transient inward current (I\textsubscript{to}). These findings suggest that M-TMCA may protect the heart from arrhythmias via its inhibitory effect on calcium channel.

Key words methyl 3,4,5-trimethoxycinnamate; L-type calcium current; cardiomyocyte; action potential; Ca2+ transient; Polygala Radix

Polygala Radix (Yuan Zhi, Polygala tenufolia Willd.) is a well known Chinese traditional medicine used as a sedative, expectorant and tonic agent. It has been widely used to treat insomnia, anxiety, restlessness and disorientation.1,2) The studies on the chemical components of this crude drug have demonstrated the presence of polygalitol, N-acetyl-D-glucosamine, onjisaponins A–G,3) various xanthones,4) tenuifolioses A–P, 3,4,5-trimethoxycinnamic acid (TMCA), tenuifolisides A–D and sucrose derivatives.5) However, the main constituents in blood and bile sample of rats after oral administration of the extracts of Polygala Radix were analyzed and showed that 3,4,5-trimethoxycinnamic acid (TMCA), methyl 3,4,5-trimethoxycinnamate (MTMCA) and p-methoxycinnamic acid (PMCA) were its bioactive substances.6–9) For example, these compounds were reported to prolong sleeping time induced with hexobarbital in mice7); TMCA exhibits anti-stress actions via the suppression of norepinephrine content in rat locus coeruleus induced by corticotropin-releasing hormone.10)

In addition, Polygala Radix was also reported to be effective in the treatment for heart palpitations.11) Palpitations are described as unpleasant sensations of irregular and/or forceful beating of the heart, most of which are of cardiac origin although psychiatric disorders, such as anxiety, are also common causes.12,13) Therefore, any type of tachyarrhythmias, regardless of whether or not there is an underlying structural or arrhythmogenic heart disease, can give rise to palpitations. Particularly, the adrenergic hyperactivation connected with intense emotions and anxiety can, in itself, predispose the patient to supraventricular and/or ventricular arrhythmias.14–16) Thus it is apparently interesting to test whether the bioactive components (such as M-TMCA) exhibit any antiarrhythmic effects. The present study was aimed to investigate the basic electrophysiological effects of these compounds at the cellular level.

MATERIALS AND METHODS

This investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All animal experimental procedure were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School and by the Ethical Committee of Xi’an Jiaotong University. All experiments in single myocyte were performed at 35–37°C.

Cell Isolation Single ventricular myocytes were enzymatically isolated from adult rabbit hearts as previously described.17,18) Briefly, New Zealand white rabbits (male, 2 to 3 kg) were anesthetized with intravenous sodium pentobarbital (50 mg/kg) after injecting heparin (200 U/kg, intravenous). The hearts were excised and perfused retrogradely in Langendorff

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fashion at 37°C with nominally Ca$^{2+}$-free Tyrode’s solution containing ca. 1.4 mg/mL collagenase (type II; Worthington, Biochemical Corp., Lakewood, NJ, U.S.A.) and 0.1 mg/mL protease (type XIV, Sigma, St. Louis, MO, U.S.A.) for 25–30 min. After the enzyme solution was washed out, the hearts were removed from the perfusion apparatus and swirled in a culture dish. The Ca$^{2+}$ concentration was slowly increased to 1.8 mM, and the cells were stored at room temperature and used within 8 h.

**Patch-Clamp Methods** Myocytes were patch clamped using the whole cell configuration of the patch-clamp technique in the current-clamp or voltage-clamp mode. Voltage or current signals were measured with a MultiClamp 700A patch-clamp amplifier controlled by a personal computer using Digidata 1322 acquisition board driven by pCLAMP 10 software ( Molecular Devices, Sunnyvale, CA, U.S.A.).

To record action potential (AP), patch pipettes (resistance 2 to 4 MΩ) were filled with internal solution containing (in mM): 110 K-aspartate, 30 KC1, 5 NaCl, 10 N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 0.1 ethylene glycol-bis(2-aminoethyl ether)-N,N',N',N'-tetraacetic acid (EGTA), 5 MgATP, 5 Na$_2$-phosphocreatine, 0.05 cAMP (pH 7.2, adjusted with KOH). Myocytes were superfused with Tyrode’s solution containing (in mM): 136 NaCl, 4.0 KCl, 0.33 Na$_2$PO$_4$, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 glucose and 10 HEPES (pH 7.4, adjusted with NaOH). Action potentials (APs) were elicited with 2-ms, 2- to 4-nA square pulses at a pacing cycle length (PCL) of 2 s.

To record the $I_{Ca,L}$, patch pipettes were filled with internal solution containing (in mM): 110 Cs-aspartate, 30 CsCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 Na$_2$-phosphocreatine, 0.05 cAMP (pH 7.2, adjusted with CsOH), and myocytes were perfused with a modified Tyrode’s solution in which KCl was replaced with CsCl. The cells were stimulated at a PCL of 2 s with a double-pulse protocol. Following a 100-ms prepulse to −40 mV from the holding potential of −80 mV (to inactivate Na$^+$ current and T-type Ca$^{2+}$ current), the amplitude of peak $I_{Ca,L}$ was elicited by a subsequent test depolarization step to 0 mV for 500 ms, and the current–voltage curves were elicited by voltage pulses of 500-ms duration from −40 to 50 mV in 10-mV steps.

To record outward K$^+$ currents, the pipette and superfusion solutions were the same as those for AP recording. Tetrodotoxin (TTX, 10 µM) and CdCl$_2$ (0.5 mM) were added into the Tyrode’s solution to inhibit $I_{Na}$ and $I_{Ca,L}$. Total outward K$^+$ currents were elicited by 3-s voltage pulses from −40 to 50 mV in 10-mV steps at a PCL of 10 s. The amplitudes of peak current ($I_{K,peak}$) at the beginning, and the steady state current ($I_{K,SS}$) at the end of the 3-sep depolarizing pulse were evaluated.

**Ca$^{2+}$ Transient Measurements** Myocytes were loaded with the Ca$^{2+}$ indicator Fluo-4AM (Molecular Probes, Eugene, OR, U.S.A.) by incubating them for ca. 30 min in bath solution containing 4 µM Fluo-4 AM and 0.016% pluronic (wt/wt; Molecular Probes, Sigma), after which the cells were washed and placed in a heated chamber on an inverted microscope. Ca$^{2+}$ fluorescence was excited at 485 nm and the emission measured at 515 nm. The fluorescence was monitored using a Nikon Eclipse TE200 inverted microscope with a Fluor x40 oil objective lens (numerical aperture 1.3) and recorded using an Andor Ixon Charge-Coupled Device (CCD) camera (Andor Technology) operating with Imaging Workbench software (INDEC BioSystems) at ca. 100 frames per second with a spatial resolution of 500×400 pixels. Fluorescence intensity was recorded as the ratio $F/F_0$ of the fluorescence ($F$) over the basal diastolic fluorescence ($F_0$).

**Chemicals** TMCA, PMCA and M-TMCA were obtained from Meryer Chemical Technology Co., Ltd. (Shanghai, China). They were dissolved in dimethylsulfoxide (DMSO) as stock solution (50 mM) before adding to the bath Tyrode’s solution at the final concentration. The maximum DMSO concentration was <0.2% (vol/vol). Other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless indicated. All experiments on single myocyte in the present study were carried out at 35–37°C.

**Statistical Analysis** Data are shown as mean±S.D. Statistical differences were evaluated using Student’s paired or unpaired t-tests or Fisher’s exact test, with $p<0.05$ considered as statistically significant.

**RESULTS**

The Effect of TMCA, PMCA and M-TMCA on APs in Rabbit Ventricular Myocytes We first investigated the effects of TMCA, PMCA and M-TMCA on APs in isolated rabbit ventricular myocytes. As shown in Fig. 1, only M-TMCA significantly attenuated action potential duration (APD) in a reversible and concentration-dependent manner (at 15, 30 µM (Figs. 1C, Dc), while TMCA and PMCA did not show any effect (Figs. 1A, B) at either the same concentrations or a higher concentration (50 µM). The decrease of APD appeared at 30 s–1 min after the application of M-TMCA and reached a steady state at 3–4 min. M-TMCA (30 µM) significantly reduced APD at 50% and 90% repolarization (APD$_{50}$-APD$_{90}$) from 219.1±15.7 and 292.4±17.3 ms to 184.5±16.2 and 240.6±18.5 ms, respectively, (Figs. 1Db, Dc; n=6, p<0.01), while the resting membrane potential (RMP), action potential amplitude (APA) and APD at 20% repolarization (APD$_{20}$) were not changed (Figs. 1C, Da).

The Inhibitory Effect of M-TMCA on L-Type Calcium Current Since M-TMCA reduced APD$_{50}$, but not APD$_{20}$ in rabbit myocytes, we assumed this effect was most likely due to its inhibition on $I_{Ca,L}$. In the following experiments, we recorded whole-cell $I_{Ca,L}$ from rabbit ventricular myocytes. As shown in Fig. 2, M-TMCA (30 µM) reduced the amplitude of $I_{Ca,L}$ at 0.5–1 min after exposure and reached the steady state at 2–3 min (Fig. 2A). Similarly, the inhibitory effect of M-TMCA on $I_{Ca,L}$ was reversible and concentration-dependent (Figs. 2A, Ba). The mean peak amplitude of $I_{Ca,L}$ (at 0 mV) was pronouncedly decreased by 15.2±3.6% from 11.2±1.1 to 9.2±0.9 pA/pF at 15 µM (n=8, p<0.05) and by 30.3±5.6% to 7.8±1.3 pA/pF at 30 µM (n=5, p<0.01). The current–voltage relations for the peak current (Fig. 2Bb) showed that the $I_{Ca,L}$ amplitude was significantly attenuated at testing potentials −10 to +40 mV, without any significant voltage shift by M-TMCA.

The Effect of M-TMCA on Ca$^{2+}$ Transients Since the alteration of $I_{Ca,L}$ would affect Ca$^{2+}$ transient in myocytes, we further investigated the effect of M-TMCA on Ca$^{2+}$ transient. Cells were first paced at a PCL of 2 s under field stimulation. After Ca$^{2+}$ transient reached steady state, cells were perfused with M-TMCA at 5, 15 and 30 µM, respectively, for ca. 5 min. M-TMCA concentration-dependently decreased the magnitudes of Ca$^{2+}$ transient (Figs. 3Aa, b). The mean amplitude of
Ca\(^{2+}\) transient was significantly reduced from \(F/F_0=1.50\pm0.27\) (Control) to 1.31\(\pm\)0.15 (15 \(\mu\)M M-TMCA, \(n=6\), \(p<0.05\)) and to 1.24\(\pm\)0.08 (30 \(\mu\)M M-TMCA, \(n=6\), \(p<0.01\)), respectively.

Furthermore, we also observed the effect of M-TMCA on the staircase of Ca\(^{2+}\) transient in rabbit myocytes. After Ca\(^{2+}\) transient reached steady state, cells were paced with field
stimulation at consecutive PCLs of 5 s, 2 s, 1 s, and 0.8 s. As shown in Figs. 3Ba–c, a positive staircase was observed under control condition, whereas in the presence of 30 µM M-TMCA the Ca^{2+} transients were reduced and the positive staircase phenomenon was suppressed.

The Inhibitory Effect of M-TMCA on Afterdepolarizations. The early afterdepolarization (EAD), delayed afterdepolarization (DAD) and triggered activity (TA) are major causes for arrhythmias. The increase or reactivation of \( I_{\text{Ca,L}} \) has been identified to contribute to the generation of afterdepolarizations, while the inhibition of \( I_{\text{Ca,L}} \) suppresses the incidence of EADS and TAs in isolated myocytes. Next, we further investigated the effect of M-TMCA on afterdepolarizations induced under Ca^{2+} overloaded condition (by Isoprenaline and BayK8644) in ventricular myocytes as we previously reported.\(^{19}\) APs were recorded at a PCL of 2 s. As shown in Fig. 4A, there is no EAD, DAD or TA observed under control condition (Fig. 4Aa). Exposure of isoprenaline (100 nmol) plus BayK8644 (50 nmol) (Iso/BayK) for 2–3 min consistently caused EADS, DADS or TAs (Fig. 4Ab). Treatment of 30 µM M-TMCA for 3 min in myocytes significantly shortened Iso/BayK–induced prolongation of APD, completely abolished EADs and TAs (Figs. 4Ac, d). In the presence of M-TMCA (30 µM), the incidence of EAD by counting the number of EADs within 20 consecutive APs was lowered from 95 ± 4.5 to 0% (\( n = 5, p < 0.01 \)), the number of DADS or TAs was suppressed from 48 ± 5.9 to 1 ± 1.2% (\( n = 6, p < 0.01 \)). In addition, M-TMCA (30 µM) markedly attenuated the increases of \( I_{\text{ca,L}} \) amplitude (at 0 mV) induced by Iso/BayK from 28.3 ± 5.9 to 18.9 ± 3.8 pA/pF (\( n = 5, p < 0.01 \)). The transient inward currents \( (I_{\text{L}}) \) induced by Iso/BayK were also eliminated from 0.94 ± 0.08 to 0.02 ± 0.01 pA/pF (\( n = 5, p < 0.01 \)) (Figs. 5A, Ba, b).

Little Effect of M-TMCA on Outward K+ Currents. Since the reduction of APD may be also attributed to an increase in outward K+ currents, we next evaluated the effects of M-TMCA on total outward K+ currents. As shown in Fig. 6, the current–voltage curves were elicited by voltage pulses of 3-s duration from −40 to 50 mV in 10-mV steps. Neither peak \( (I_{\text{K,peak}}) \) (Control: 12.5 ± 1.7 vs. M-TMCA: 11.7 ± 1.4 pA/pF, \( n = 7, p > 0.05 \)) nor steady state \( (I_{\text{K,SS}}) \) (Control: 2.4 ± 0.3 vs. M-TMCA: 2.6 ± 0.4 pA/pF, \( n = 7, p > 0.05 \)) outward potassium currents were significantly altered by M-TMCA at the concentration of 30 µM that significantly reduced APD under current clamp condition.

**DISSUSSION**

TMCA, PMCA and M-TMCA have been shown to produce sedative effects and prolong sleeping time in mice,\(^{1,8}\) but their cardiovascular effects remain unclear. In the present study, we have investigated the electrophysiological effects of the bioactive substances of traditional Chinese medicine Polygalae Radix on rabbit ventricular myocytes. The major findings are: 1) Among the three bioactive substances of Polygala metabolites, (TMCA, M-TMCA, and PMCA), only M-TMCA exhibits significantly electrophysiological effects on isolated rabbit ventricular myocytes; 2) M-TMCA significantly shortens APD of cardiomyocytes in a concentration-dependent and a reversible manner, which are mainly mediated by the inhibition of \( I_{\text{Ca,L}} \); 3) M-TMCA inhibits the intracellular Ca^{2+} transient and suppresses \( I_{\text{L}} \) induced by Iso/BayK. 4) M-TMCA suppresses EADs, DADs and TAs induced by Iso/BayK and thus may have antiarrhythmic potentials.

Our results showed that M-TMCA concentration-dependently shortened APD_{50} and APD_{90}. APD_{20} represents the early phase of action potential repolarization in cardiomyocytes and the fast \( I_{\text{L}} \) predominately contributes to this repolarizing process. Our results showed that M-TMCA had no significant effect on APD_{20} (Fig. 1), consistent with the result that M-TMCA did not affect fast \( I_{\text{L}} \) (Fig. 6A). The shortenings of APD_{90} and APD_{99} may implicate a decrease in inward currents (e.g. \( I_{\text{Ca,L}} \)) or an increase in outward currents (e.g. \( I_{\text{K}} \)). The present study demonstrated that M-TMCA rapidly inhibited \( I_{\text{Ca,L}} \) in ventricular myocytes, consistent with the time course for APD_{99} shortening (Fig. 1A). However, M-TMCA at 30 µM, the concentration which dramatically shortened APD and suppressed \( I_{\text{Ca,L}} \), did not show any alteration in \( I_{\text{K,SS}} \) (Fig. 6). We further showed that M-TMCA inhibited Ca^{2+} transient in a concentration-dependent manner and abolished the positive staircase effect of Ca^{2+} transient in single isolated cardiac myocytes (Fig. 3). It has been suggested that frequency-dependent
potentiation of $I_{Ca,L}$ might be an important mechanism underlying positive staircase in cardiomyocytes. It has been evidenced that positive staircase is characterized mainly by an increase in peak current amplitude or slowing inactivation of $I_{Ca,L}$ kinetics. Blocking $I_{Ca,L}$ (e.g., verapamil, nifedipine) eliminated the positive staircase of contraction in cardiomyocytes. Our results revealed that M-TMCA significantly suppressed the amplitude of $I_{Ca,L}$ and Ca$^{2+}$ transient, suggesting that the inhibitory effect of M-TMCA on peak $I_{Ca,L}$ plays a critical role in the elimination of the positive staircase of Ca$^{2+}$ transient in rabbit ventricular myocytes.

It was reported that onjisaponin E, F and G in water extract of Radix Polygalae inhibited cAMP phosphodiesterase using an in vitro screening method, which might cause alterations of cAMP metabolism. Although onjisaponin E, F and G, sucrose derivatives and others containing 3,4,5-trimethoxycinnamoyl moiety within their chemical structures in Polygalae Radix were metabolized to TMCA and M-TMCA in rat gastrointestinal tract and liver, it is not known whether M-TMCA itself has the same effect in cardiac myocytes so far. Since our results show that M-TMCA suppresses $I_{Ca,L}$ and decreases Ca$^{2+}$ transients, we assume that M-TMCA should play less effect on phosphodiesterase inhibition in cardiomyocytes. Further studies are needed to clarify this point.

Polygala, often used as a sedative agent, is also applied in the treatment for heart palpitations in traditional Chinese medicine.
medical practice. Strictly, a palpitation is an abnormality of heartbeat that ranges from often unnoticed skipped beats or accelerated heart rates to very noticeable changes. Based on our previous studies, therefore, we investigated the effects of M-TMCA on Iso/BayK-induced EADs, DADs, and TAs, which are triggers for arrhythmias at single cell level. Both Iso and BayK potentiate \( I_{\text{Ca,L}} \). It has been shown that reactivation of \( I_{\text{Ca,L}} \) is implicated in EAD generation. Furthermore, enhanced \( I_{\text{Ca,L}} \) may lead to Ca\(^{2+}\) overload, which consequently results in DADs. In addition, BayK also causes SR Ca\(^{2+}\) leaking. These effects further induce spontaneous SR Ca\(^{2+}\) waves, and subsequently cause DADs and TAs by activating \( I_{\text{ti}} \) due to electrogenic Na\(^{+}\)–Ca\(^{2+}\) exchange current. We found that 30 \( \mu \)M M-TMCA abolished Iso/BayK-induced EADs and significantly reduced the occurrence of DADs and TAs (Fig. 4). Previous studies by our group and others have documented that selective calcium channel blockers (e.g. nifedipine) effectively suppress EADs and DADs in various models. Consistent with these findings, the suppression of EADs, DADs, and TAs by M-TMCA is most likely mediated by its inhibitory effect on \( I_{\text{Ca,L}} \). Since the synergistic interactions between \( I_{\text{Ca,L}} \) and \( I_{\text{ti}} \) play important roles in EAD generation in the Iso/BayK model, M-TMCA significantly inhibited the augmentation of \( I_{\text{Ca,L}} \) induced by Iso/BayK, and thereby reduced Ca\(^{2+}\) influx and SR Ca\(^{2+}\) overload, which account for the suppression of subsequent generation of \( I_{\text{ti}} \) and EADs, DADs and TAs. It should be noted that we cannot exclude the possibility that M-TMCA may antagonize the \( \beta \)-adrenergic effects via the direct blockade of \( \beta \)-adrenergic receptors, which may also suppress the EADs, DADs and TAs induced by Iso/BayK.

To our knowledge, there is no report on the plasma concentrations of M-TMCA in human subjects who take *Polygala Radix*. However, it has been reported that the plasma concentration of M-TMCA is ca. 1–2 \( \mu \)g/mL (equivalent to ca. 4–8 \( \mu \)m) in rats with single oral administration of water extract of *Polygala Radix* (5–10 g/kg), and is ca. 7–8 \( \mu \)g/mL (equivalent to ca. 28–32 \( \mu \)m) in rats with intravenous administration of M-TMCA (10 mg/kg). We assume that the steady-state plasma concentration of M-TMCA after repetitive administrations would reach the level that is close to its effective concentration range (>10 \( \mu \)m) for the inhibition of \( I_{\text{Ca,L}} \) and potential suppression of arrhythmias.

Calcium channel blockers (e.g. verapamil) are used as class IV antiarrhythmic drugs in clinic. Since they preferentially affect the rate of slow response myocardial tissue (i.e. the sinoatrial and atrioventricular nodes), calcium channel blockers have been primarily used in the management of certain arrhythmias such as supraventricular tachyarrhythmias associated with atrioventricular nodal reentrant tachycardia, as well as in the rate control for atrial fibrillation. It has also been demonstrated that calcium channel blockers suppress EADs, DADs, and TAs, suggesting class IV drugs may be effective in treating afterdepolarization-induced triggered arrhythmias. For example, verapamil is capable of preventing ischemic and reperfusion arrhythmias. M-TMCA, which exhibits L-type calcium channel inhibitory effect, should also be effective in treating these types of arrhythmias (i.e. supraventricular tachyarrhythmias and triggered ventricular arrhythmias) in clinical setting. This notion was supported by the result showing M-TMCA suppressed Iso/BayK-induced EAD, DAD and TAs in isolated myocytes (Fig. 4). Further experiments are needed to fully demonstrate the antiarrhythmic properties of M-TMCA in intact animal models.

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