

Modulation by muscarinic receptor antagonists on negative chronotropic effects of tetrandrine¹

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KEY WORDS muscarinic receptors; tetrandrine; action potentials; patch-clamp techniques

ABSTRACT

AIM: To investigate the influence of selective antagonist for muscarinic (M) receptor subtype on tetrandrine (Tet) reducing heart rate, inhibiting sinoatrial node (SAN) function, and its ionic mechanism. **METHODS:** Effects of reducing heart rate of Tet were maintained in isolated right atrium and pithed rats. Modification on action potentials (AP) of SAN cells and L-type calcium current (I_{Ca-L}) by Tet were recorded by means of standard microelectrode and patch-clamp whole cell recording techniques. **RESULTS:** Tet inhibited spontaneous beating rate of isolated right atrium (EC_{50} , $23.7 \mu\text{mol} \cdot \text{L}^{-1}$) and reduced heart rates in pithed rats in a concentration-dependent manner (EC_{50} , $18.6 \text{ mg} \cdot \text{kg}^{-1}$). Automaticity of SAN was inhibited by Tet. AP upstroke velocity (V_{max}), spontaneous depolarization rates in phase 4 (SP_4) were decreased and sinus cycle length (SCL) was prolonged when treated with Tet. Tet ($30 \mu\text{mol} \cdot \text{L}^{-1}$) caused a reduction in peak value of I_{Ca-L} from $(1275 \pm 190) \text{ pA}$ to $(498 \pm 94) \text{ pA}$ in isolated single cardiomyocyte. Atropine and AF-DX 116 (M_2 subtype selective antagonist) could attenuate such effects of Tet in a competitive mode. **CONCLUSION:** Negative chronotropic effects of Tet are due to

its inhibition of I_{Ca-L} . Modification on I_{Ca-L} is the major mechanism of M receptor modulating Tet effects.

INTRODUCTION

Tetrandrine (Tet), a calcium antagonist obtained from Chinese herb (*Stephania tetrandra* S Moore), can interact with cardiovascular α -adrenergic and muscarinic receptor^[1]. In anesthetized cat, its hypotensive effect was inhibited by atropine and enhanced by acetylcholine. With radioligand binding assay in rat brain, Tet had high affinity with muscarinic (M) receptor^[2]. The present experiment was to observe the modulation by cardiac selective M receptor antagonists on chronotropic effects of Tet *in vitro* and *in vivo*, and to determine its possible ionic mechanism.

MATERIALS AND METHODS

Materials Tet obtained from Zhejiang Jinhua Pharmaceutical Company. It was dissolved in HCl $1 \text{ mol} \cdot \text{L}^{-1}$, then its pH value was adjusted to 5-6 by NaOH $1 \text{ mol} \cdot \text{L}^{-1}$. Dr van Meel, Karacl Thomae GmbH Pharmaceutical Company, provided AF-DX 116 and 4-DAMP. Atropine was purchased from Sigma. All other reagents were of AR.

Guinea pigs (250-300 g, Grade II, Certificate No 19-023), Wistar rats ($\hat{\delta}$, 300-350 g, Grade II, Certificate No 19-050), and white rabbit (1.5-2 kg, Certificate No 19-025) were purchased from Laboratory Animal Center, Tongji Medical University.

Spontaneously beating rate of isolated right atrium Isolated guinea pig right atrium with intact sinus nodes were suspended in Tyrode's solution 10 mL (NaCl 146.9, KCl 2.68, CaCl_2 1.80, MgCl_2 1.05, NaH_2PO_4 0.62, NaHCO_3 11.9, glucose 5.55 mmol ·

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L⁻¹, pH 7.4, bubbled with 95 % O₂ + 5 % CO₂). The spontaneously beating rate was measured with a force transducer connected with a double-pen recorder (LMS-2A type, Chengdu Equipment Factory). The resting potential (0.5 g) was kept constant throughout the experiment. All preparations were allowed to equilibrate for 1 h before any intervention. Tet was added in a cumulative concentration (0.1 – 300 μmol·L⁻¹) with or without atropine (0.03 μmol·L⁻¹) or AF-DX 116 (1 μmol·L⁻¹).

Heart rates of pithed rats Rats were anesthetized by sodium pentobarbital (45 mg·kg⁻¹, ip) and subjected to artificial respiration via tracheal canal with positive pressure. The rats were pithed by introducing a blunt needle into the spinal canal via orbit. Rectal temperature was maintained at about 37 °C. Right femoral vein was cannalized for administration of drugs. Heart rate was measured from ECG-1, Nihon Kohden. Pretreatment with selective mAChR subtype antagonists or saline was carried out 15 min before administration of Tet (5 – 25 mg·kg⁻¹).

Transmembrane action potential Rabbit sinus node preparations were made^[3]. Transmembrane action potentials of primary pacemaking cells were led to microelectrode amplifier (MEZ7101, Nihon Kohden) by a standard intracellular electrode technique. The signal was displayed and fed to computer (IBM/PC 386) via A/D, D/A converter. Parameters were analyzed by software-AP6 (designed by Huazhong Institute of Science and Technology). The preparations were superfused with saline or antagonists (atropine 0.03 μmol·L⁻¹, AF-DX 116 1 μmol·L⁻¹) 15 min before tetrandrine was used.

Patch clamp experiment Single ventricular cells of guinea pigs were isolated by collagenase digestion. Transmembrane currents were recorded using the whole cell recording techniques. The currents were measured with an Axo 200A amplifier via a D/A converter (Digital 1200). Voltage clamping, signal acquisition, and analysis of membrane currents were achieved by computer program pCLAMP 6.01. All experiments were carried out at room temperature (25 – 28 °C).

Extracellular solution: Choline Cl 136, KCl 15.4, MgCl₂ 1.0, HEPES 5.0, CaCl₂ 2.0, glucose 10, TEACl 20 mmol·L⁻¹, pH was adjusted to 7.2 by CsOH.

Pipette solution: CsCl 20, CsAspartate 110, HEPES 5.0, MgCl₂ 1.0, egtazic acid 10, MgATP 5.0 mmol·L⁻¹, pH was adjusted to 7.4 by CsOH.

Data analysis All data were expressed as $\bar{x} \pm s$. Statistical significance was determined by *t*-test. EC₅₀ values were calculated using computer software "Sigmaplot 3.0".

RESULTS

Spontaneous beating rate Tet inhibited spontaneously beating rate in a concentration-dependent manner with EC₅₀ of 23.7 (21.0 – 29.5) μmol·L⁻¹ (*n* = 6, control). Atropine and AF-DX 116 inhibited the effects of Tet while the maximal effects of Tet were unchanged. The EC₅₀ changed to 169 (126 – 198 μmol·L⁻¹, atropine, *n* = 6, *P* < 0.01 vs control) and 115 (107 – 131 μmol·L⁻¹, AF-DX 116, *n* = 6, *P* < 0.01 vs control) (Fig 1).

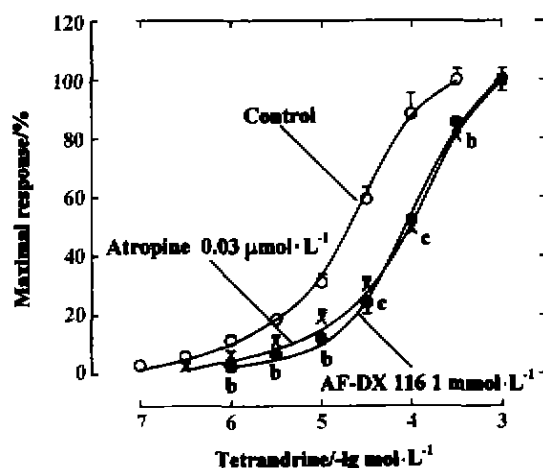


Fig 1. Effects of different antagonists on negative chronotropic effects of Tet. *n* = 6 guinea pigs. $\bar{x} \pm s$. ^b*P* < 0.05, ^c*P* < 0.01 vs control.

Heart rates in pithed rats Tet (5 – 25 mg·kg⁻¹) caused a dose-dependent decrease in heart rates. Heart rates were decreased from (300 ± 40, control) to (295 ± 40), (263 ± 34), (198 ± 40), (130 ± 22), (74 ± 15) beats/min by Tet 5, 10, 15, 20, 25 mg·kg⁻¹, respectively. Control EC₅₀ was 18.6 (17.8 – 19.4) mg·kg⁻¹ (*n* = 7). The heart rates were changed to (31 ± 3, *n* = 6, *P* < 0.05 vs control), (19.8 ± 0.6) beats/min, (*n* = 5), after pretreatment with AF-DX 116 and 4-DAMP (M₃ selective

antagonist), respectively (Fig 2).

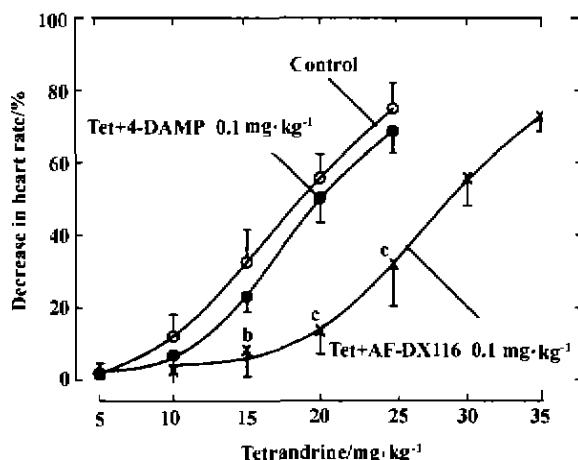


Fig 2. Influence of M receptor antagonists on bradycardic effects of Tet in pithed rats. $n = 7$ rats. $\bar{x} \pm s$. ^b $P < 0.05$, ^c $P < 0.01$ vs control.

Action potential Tet decreased V_{max} , SP_4 , and APA, also prolonged APD_{50} , APD_{90} , and SCL in rabbit sinus node primary pacemaking cells. In the presence of atropine and AF-DX 116, such effects were no longer significant except that of increasing APD_{90} (Tab 1).

L-type calcium current In voltage-clamp

mode for measurement of I_{Ca-L} , the holding potential was kept at -40 mV for 4 ms to inactivate I_{Na} and I_{Ca-T} . The command potential was 0 mV and the duration was 300 ms. The current was steady in 30 min after forming whole cell state, so it avoided "run-down" during this period. When the bath solution was changed to Tet ($30 \mu\text{mol} \cdot \text{L}^{-1}$), the Ca^{2+} current was inhibited. The peak current of I_{Ca-L} was reduced from (1275 ± 190) pA to (498 ± 94) pA ($n = 5$, $P < 0.01$). While pretreatment with atropine ($0.03 \mu\text{mol} \cdot \text{L}^{-1}$), the inhibitory effects of Tet ($30 \mu\text{mol} \cdot \text{L}^{-1}$) were antagonized. The peak current was reduced to (790 ± 82) pA from (1318 ± 206) pA. Increasing the concentration of Tet to $100 \mu\text{mol} \cdot \text{L}^{-1}$, the antagonistic modification of atropine was reversed, and I_{Ca-L} was further reduced to (315 ± 65) pA (Fig 3).

Keeping holding potential at -40 mV, depolarizing voltage pulses were applied from command potential of -30 mV to $+60$ mV, with duration of 300 ms in 10 mV increments. The $I-V$ relation of I_{Ca-L} was obtained. Similar results were found (Fig 4).

DISCUSSION

Negative chronotropic effects of Tet have been well studied, and concentrated on its calcium channel

Tab 1. Effects of Tet on action potential of SAN primary pacemaker cells. $\bar{x} \pm s$. ^b $P < 0.05$, ^c $P < 0.01$ vs control. SP_4 = Spontaneous depolarization rates in phase 4; SCL = sinus cycle length.

Tet/ $\mu\text{mol} \cdot \text{L}^{-1}$	$V_{max}/\text{V} \cdot \text{s}^{-1}$	APA/mV	APD_{50}/ms	APD_{90}/ms	$SP_4/\text{mV} \cdot \text{s}^{-1}$	SCL/ms
0	3.9 ± 0.9	67 ± 10	100 ± 9	192 ± 26	44 ± 6	525 ± 43
1.0	3.6 ± 1.0	64 ± 8	102 ± 9	195 ± 27^b	41 ± 4	533 ± 45^c
3.0	3.2 ± 0.9^b	59 ± 8^b	105 ± 7^b	200 ± 27^b	40 ± 4^b	555 ± 53^c
10	2.7 ± 0.9^b	54 ± 6^b	107 ± 6^b	204 ± 28^c	34 ± 5^c	598 ± 72^c
30	2.1 ± 0.7^c	50 ± 8^c	111 ± 5^c	212 ± 26^c	28 ± 5^c	634 ± 58^c
100	1.3 ± 0.4^c	42 ± 7^c	116 ± 5^c	226 ± 27^c	23 ± 3^c	683 ± 34^c
Tet + AF-DX 116 ($1 \mu\text{mol} \cdot \text{L}^{-1}$)						
0	4.0 ± 0.8	64 ± 5	98 ± 9	200 ± 16	40 ± 3	501 ± 62
1	3.7 ± 0.8	66 ± 5	98 ± 8	202 ± 8	41 ± 5	508 ± 64
10	3.5 ± 1.1^b	63 ± 5^b	99 ± 8	205 ± 19^b	37.2 ± 2.3^c	516 ± 66
100	2.6 ± 0.7^b	58 ± 7^b	103 ± 8^c	219 ± 19^c	31 ± 5^c	573 ± 63^c
300	1.4 ± 1.0^c	41 ± 8^c	109 ± 4^c	241 ± 20^c	20 ± 4^c	618 ± 75^c
Tet + atropine ($0.03 \mu\text{mol} \cdot \text{L}^{-1}$)						
0	3.9 ± 0.4	58 ± 6	99 ± 5	202 ± 15	39 ± 6	521 ± 29
1	3.7 ± 0.6	57 ± 7	100 ± 5	204 ± 16	38.4 ± 2.2	523 ± 29
10	3.3 ± 0.9^b	56 ± 7	102 ± 4^b	205 ± 16^b	37 ± 7^c	526 ± 29
100	2.6 ± 0.9^b	47 ± 7^b	103 ± 4^b	218 ± 19^c	27 ± 6^c	549 ± 34^c
300	1.7 ± 0.6^c	40 ± 8^b	106 ± 7^b	235 ± 18^c	20 ± 7^c	620 ± 43^c

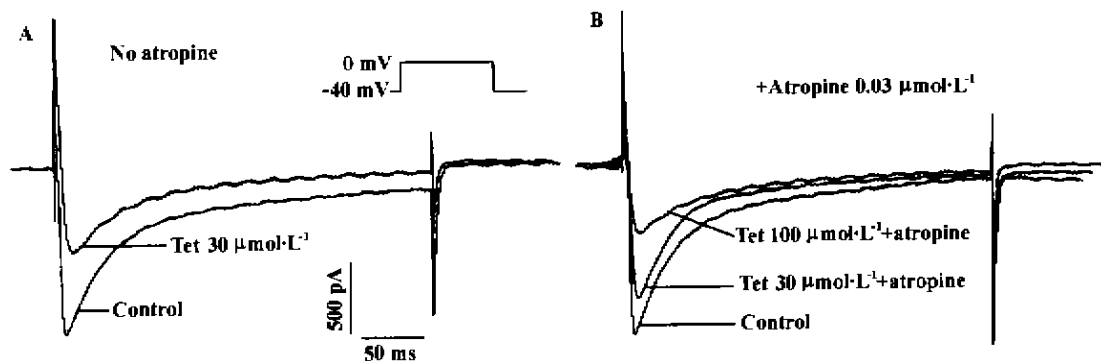


Fig 3. Effects of Tet on I_{Ca-L} in single ventricle cell of guinea pig.

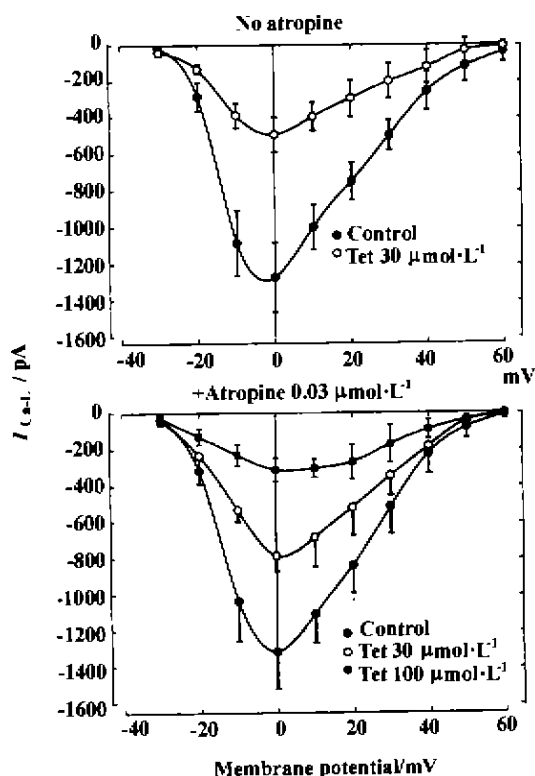


Fig 4. Effects of tetrandrine on $I-V$ relation of I_{Ca-L} in isolated guinea pig single ventricle cell. $n = 5$ cells of 5 guinea pigs. $\bar{x} \pm s$.

blocking effects. But Tet is not a selective calcium antagonist in vascular smooth muscle cells and could interact with α -adrenergic receptor^[4]. Recently with radio-ligand binding assay it was found that Tet acted as an allosteric modulator for M receptor, and showed high affinity with M_1 , M_2 subtype^[5]. Our results showed that selective blockade of cardiac M_2 receptor

could antagonize negative chronotropic effects of Tet both *in vitro* and *in vivo*.

Among ionic mechanisms of pace making, I_{Ca-L} plays an important role. It is the major current for 0-phase upstroke and 4-phase spontaneously depolarization in SA node primary pace-making cells. Stimulation of vagal nerve or activation of M receptor in heart contributes to regulation of normal cardiac rhythm and SA node function^[6]. Though opening acetylcholine activated potassium channel ($I_{K(Ach)}$) is a main mechanism for M receptor to modify the cardiac electrophysiological properties, it is now believed that inhibition of I_{Ca-L} and hyperpolarization-activated current (I_f) also play important roles in M receptor modulating SAN pace-making^[7,8]. Brown reported that low dose Ach reduces calcium current in isolated sinoatrial node cells of rabbit^[8]. Moreover, without the presence of agonists, atropine and AF-DX 116 showed stimulatory effects on calcium channel^[9]. Our results from action potential and whole cell patch-clamp recording suggested that atropine and AF-DX 116 could counteract the inhibitory effects of Tet on I_{Ca-L} . More and more results show that vagal nerve and M receptor play an important role in regulating cardiac function in physiological and pathophysiological state, and Ach is believed to be an endogenous cardiac protective agent^[10]. Besides Tet, classical calcium antagonist verapamil interacts with cardiac muscarinic receptor as an allosteric antagonist^[11]. Though the signal transduction of M receptor modulating calcium channel has not been clearly defined, our results suggests that M receptor antagonists could directly modify the effect of Tet on I_{Ca-L} , and it may be the main ionic mechanism

of M-receptor modulating negative chronotropic effects of Tet.

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M受体阻断剂对粉防己碱负性频率作用的调节¹

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关键词 毒蕈碱受体; 粉防己碱; 动作电位; 膜片钳技术

目的: 研究 M 受体亚型阻断剂对粉防己碱(Tet)减慢心率, 抑制窦房结功能的影响并探讨其离子机制. **方法:** 在离体右心房标本及毁脊髓大鼠模型观察粉防己碱减慢心率的作用, 用标准微电极及膜片钳全细胞记录技术观察 Tet 对窦房结动作电位及 L 型钙电流(I_{Ca-L})的影响. **结果:** Tet 浓度依赖性抑制离体右心房自发收缩频率并减慢毁脊髓大鼠心率. Tet 抑制窦房结自律性, 降低 V_{max} 、 SP_4 、并延长 SCL. 在分离单个心肌细胞 Tet 抑制 I_{Ca-L} 的峰值电流. 阿托品和 M_2 受体亚型阻断剂 AF-DX 116 能竞争性抑制 Tet 的上述作用. **结论:** 粉防己碱的心肌负性变时作用是由于其对 I_{Ca-L} 的抑制, 而 M 受体阻断剂对 Tet 阻滞钙通道作用的调节是其影响 Tet 作用的离子机制.

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