Neuroscience Letters 506 (2012) 307–311

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

# Amitriptyline modulates calcium currents and intracellular calcium concentration in mouse trigeminal ganglion neurons

Wenhui Wu[a], Qing Ye[b], Wenjuan Wang[c], LanYun Yan[a], Qiang Wang[c], Hang Xiao[c], Qi Wan[a,*]

[a] Department of Neurology, the First Affiliated Hospital of Nanjing Medical University, Guangzhou Street 300, Nanjing, Jiangsu Province, 210029, PR China
[b] Department of Neurology, Clinical Medical College, Yangzhou University, Nantongxi Street 98, Yangzhou, Jiangsu province, 225001, PR China
[c] Department of Toxicology, School of Public Health, Nanjing Medical University, Hanzhong Road 140, Nanjing, Jiangsu Province, 210029, PR China

ARTICLE INFO

*Article history:*
Received 30 July 2011
Received in revised form 9 November 2011
Accepted 18 November 2011

*Keywords:*
Amitriptyline
Migraine
Calcium channels
Patch-clamp recording
Intracellular $Ca^{2+}$
Trigeminal ganglion

ABSTRACT

Migraine is increasingly recognized as a channelopathy, and abnormalities of voltage-activated ionic channels could represent the molecular basis for the altered neuronal functioning. The high-voltage-activated (HVA) $Ca^{2+}$ channels in the trigeminovascular system play a role in the pathophysiology of migraine. In the present study, effects of amitriptyline (AMT), a commonly used migraine prophylactic drug, on the HVA calcium currents ($I_{Ca}$) were examined in mouse trigeminal ganglion neurons using whole-cell patch clamp technique. AMT produced concentration- and use-dependent inhibition of HVA $I_{Ca}$. Bath application of GÖ-6983 (a selective protein kinase C inhibitor) or H89 (a protein kinase A inhibitor) did not reduce the AMT-induced inhibition of HVA $I_{Ca}$. A similar inhibition was observed when calcium imaging was used to directly monitor the effects of AMT on KCl-induced increments of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). By blocking HVA $Ca^{2+}$ channels and $Ca^{2+}$ entry into cells, AMT could prevent the release of neurotransmitters and help restore the neuronal threshold for excitation. Our findings suggest interesting therapeutic mechanisms for AMT in migraine prevention.



## 1. Introduction

Amitriptyline (AMT), a tricyclic antidepressant (TCA), has been used for prevention of migraine for more than three decades. However, the underlying mechanisms for AMT to prevent migraine are not completely understood. It has been suggested that AMT works in migraine prophylaxis by altering serotonergic/noradrenergic synaptic transmission, as it does in depression [11]. Recent theories propose that AMT exerts its action by inhibiting ion channels, especially the voltage-activated $Na^+$ channels, based on the large body of experiments [3,19,28]. The evidence that AMT may also inhibit $Ca^{2+}$ channels is more limited. Experimental data only indicate that AMT blocks $Ca^{2+}$ channels in cardiomyocytes [12]; furthermore, its electrophysiological properties are shared with the L-type calcium channel antagonists [29].

The trigeminovascular system (TGVS) constituted by the trigeminal afferents innervating the meningeal blood vessels is a site in migraine pathophysiology with important significance [9]. Pain in migraine derives from activation of the TGVS. The neuronal changes that underlie TGVS activation involve processes where the high-voltage-activated (HVA) $Ca^{2+}$ channels play a role. HVA $Ca^{2+}$ channels enable calcium ions to enter neurons upon depolarization and thereby influence neurotransmitter release, cell membrane excitability, second and third messenger concentration and gene expression [2,23]. Because of these roles, regulation of $Ca^{2+}$ channels is vital in both the normal function and pathological states of the sensory systems.

The goal of this study was to investigate the effects of AMT on the HVA calcium currents ($I_{Ca}$) in trigeminal ganglion (TG) neurons and the intracellular calcium concentration ($[Ca^{2+}]_i$) that induced by 40 mM KCl. We also analyzed the influence of protein kinase C (PKC) as well as protein kinase A (PKA) in its action.

## 2. Material and methods

### 2.1. Preparation of TG neurons

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. TG neurons from male ICR (Institute of Cancer Research) mice (20–25 g) were prepared as previously described [25]. Briefly, mice were killed by decapitation under ethyl ether anesthesia. A pair of the TG were

*Abbreviations:* AMT, amitriptyline; TCA, tricyclic antidepressant; HVA $I_{Ca}$, high-voltage-activated calcium currents; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; TGVS, trigeminovascular system; TG, trigeminal ganglion; PKC, protein kinase C; PKA, protein kinase A; DMSO, dimethylsulfoxide; HBSS, Hank's balanced salt solution; TEA-Cl, tetraethylammonium-Cl.

* Corresponding author. Tel.: +86 02583714511; fax: +86 02583714511.
*E-mail address:* chinaqiwan@126.com (Q. Wan).

0304-3940/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2011.11.031

dissected and collected in Hank's balanced salt solution (HBSS), then incubated in HBSS for 15 min at 37 °C in 0.5 mg/mL trypsin and 1.5 mg/mL collagenase type I (both from Sigma–Aldrich, USA). After washing in standard extracellular solution, the tissues were triturated with a Pasteur pipette to dissociate individual cells. The cells were plated onto 35 mm dishes and maintained for 2–6 h at 37 °C in 95% $O_2$/5% $CO_2$ incubator. The components for HBSS contained (in mM): 130 NaCl, 5 KCl, 0.3 $KH_2PO_4$, 4 $NaHCO_3$, 0.3 $Na_2HPO_4$, 5.6 D-glucose and 10 HEPES, with pH adjusted to 7.3. The components for standard extracellular solution contained (in mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 10 D-glucose, with pH adjusted to 7.3. As previously described, TG neurons were classified into three groups, small-sized (0–23 μm), medium-sized (24–37 μm) and large-sized (38–60 μm) neurons [21].

### 2.2. Patch-clamp recordings

Conventional whole-cell patch-clamp recordings were performed at room temperature with EPC-9 amplifier and Pulse 8.02 software (both from HEKA, Germany). Patch pipettes were made from thin-walled borosilicate glass on a two-step vertical puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan; model PP-83) and had a resistance of 2–5 MΩ, when filled with the electrode internal solution. The internal solution contained (in mM): 125 CsCl, 2 $MgCl_2$, 20 HEPES, 11 EGTA, 1 $CaCl_2$, 4.5 Mg-ATP, 0.3 Li-GTP, and the pH was adjusted to 7.3 with CsOH. The extracellular solution for patch recording contained (in mM): 140 tetraethylammonium-Cl (TEA-Cl), 0.8 $MgCl_2$, 5 $CaCl_2$, 10 HEPES, 11 D-glucose, and the pH was adjusted to 7.3 with Tris-base. Data were low-pass filtered at 2 KHz, sampled at 10 KHz, and acquired using Pulse program (HEKA). Capacity transients were cancelled and series resistance compensated (>70%) by use of the internal circuitry of EPC-9. Leakage current was digitally subtracted by use of the p/n protocol, while liquid junction potential was corrected throughout all experiments.

### 2.3. Calcium image

$[Ca^{2+}]_i$ measurement was carried out according to the methods previously described [16]. Changes of $[Ca^{2+}]_i$ in TG neurons were detected with confocal laser scanning microscopy using fluo-4/AM (Invitrogen, USA) as a calcium fluorescent indicator that could monitor real-time alterations of $[Ca^{2+}]_i$. Prior to recording, the cells were plated onto special dishes for confocal laser scanning, loaded with Fluo-4/AM (2 μM) and further incubated at 37 °C for 30 min. After washing by standard extracellular solution three times, 1 mL such solution with or without AMT were added. $[Ca^{2+}]_i$ changes were measured by monitoring the alteration of cell fluorescence during application of KCl or AMT at room temperature. Point scans of several selected cells were performed at time resolutions of 2 s and at intervals of 2 s, the whole process of drug application were inspected for 5 min. To avoid the disturbance of osmotic pressure and pH, all drugs added to the dishes were dissolved in standard extracellular solution mentioned above. LSM 5 LIVE (Carl Zeiss, Germany) laser scanning confocal system was used with excitation at 488 nm and emission at 516 nm. These data were collected with LSM 5 LIVE Zen 2007 (Carl Zeiss, Germany). In all cases, fluorescence intensity was normalized to its initial value recorded in cells before drug application ($F_0$) and is expressed as the relative fluorescence ($F/F_0$).

### 2.4. Statistical analysis

Patch-clamp recording results obtained from different treatments of neurons were analyzed using PULSEFIT software (Heka Elektronik, Germany). The concentration-response curves were fitted to the Hill equation: $I/I_{max} = 1/[1 + (C/IC_{50})^H]$, where $I$ and $I_{max}$ are the observed and maximum blocking percentages of current, $C$ the concentration of the drug, and $IC_{50}$ the concentration producing a half-maximal current block. Data are expressed as means ± S.E.M. for all the experiments. Statistical analysis of results was performed with a paired $t$-test and ANOVA for the data of effects of AMT on $Ca^{2+}$ currents and the data of $[Ca^{2+}]_i$ changes, respectively. All tests of statistical significance were two-sided and the level of statistical significance was set at $P < 0.05$.

### 2.5. Drugs and chemicals

Amitriptyline, H-89, GÖ-6983, collagenase (type I) and trypsin (type I), DMSO, HEPES, EGTA, TEA-Cl, Mg-ATP, KCl, CsOH and CsCl were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All other chemical reagents used were of analytic grade. AMT was prepared as a 10 mM stock solution in DMSO and diluted in the extracellular solution for each experiment to give the desired final concentration just before use. The DMSO concentration in the perfusate was less than 1% (v/v), which had little effects on HVA $I_{Ca}$ (<2% in 5 min). Drugs were applied directly on to the cells by a tube. The distance from the tube mouth to the cell examined was around 200 μm. The flow rate (1 mL/min) was regulated by gravity to achieve a complete replacement of cell surroundings in less than 1 s.

## 3. Results

### 3.1. Effects of AMT on HVA $I_{Ca}$

Freshly isolated neurons from mouse TG in the range of 20–37 mm (small- to medium-size) were used in the present study. The HVA $I_{Ca}$ were elicited by a series of depolarizing pulses (from −50 to +30 mV for 80 ms in 10 mV increments every 20 s) from a holding potential of −60 mV (Fig. 1A). The current intensity (current amplitude divided by cell membrane capacitance) was plotted as a function of depolarizing potential. As shown in Fig. 1B, currents were activated at around −30 mV and peaked at −20 mV, which fit the features of HVA $I_{Ca}$ [24]. AMT (5 μM) reduced the current intensity over the voltage potentials higher than −30 mV. AMT had no effect on the threshold for activation and the potential at which HVA $I_{Ca}$ reached a maximum.

Effects of AMT on the peak amplitude of HVA $I_{Ca}$ were then investigated. HVA $I_{Ca}$ were elicited by 80 ms depolarizing pulses to −10 mV applied from a holding potential of −60 mV, at 30 s intervals. AMT reduced the peak currents by 19.0 ± 5.8% ($n = 5$, $P < 0.05$), 42.5 ± 9.9% ($n = 5$, $P < 0.05$), 62.3 ± 4.1% ($n = 7$, $P < 0.05$), 75.4 ± 7.8% ($n = 7$, $P < 0.05$) and 87.3 ± 6.9% ($n = 5$, $P < 0.05$) at 1, 5, 10, 50 and 100 μM, respectively (Fig. 1C and D). The concentration of AMT that induced a half-maximal currents block ($IC_{50}$) was 5.1 μM. The inhibition of AMT in 1–10 μM started 30 s after application and it took up to 240 s until a steady state was reached. In contrast, concentrations of AMT higher than 10 μM led to more rapid effects.

To examine the use-dependent block of HVA $I_{Ca}$ by AMT, currents were evoked at 0.1 Hz, 0.5 Hz and 1 Hz by 10 consecutive 20 ms pulses to −10 mV from the holding potential. The ratio, $I_{10}/I_1$ (current amplitude at 10th pulse/current amplitude at 1st pulse) was used to calculate the decrease of HVA $I_{Ca}$ (Fig. 2A). At 0.1 Hz frequency, accumulation of inactivated channels resulted in a reduction in peak current amplitude ($I_{10}/I_1 = 96.0 \pm 3.2\%$) that was not statistically different from that obtained during AMT (5 μM) application ($I_{10}/I_1 = 95.1 \pm 3.6\%$, $n = 6$, $P > 0.05$). In contrast, at higher frequency the reduction in peak current amplitude during drug application ($I_{10}/I_1 = 87.5 \pm 4.2\%$ at 0.5 Hz and 82.4 ± 5.3%