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INHIBITION OF SODIUM CURRENT BY CARBAMAZEPINE IN DORSAL ROOT GANGLION NEURONS *IN VITRO*

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Abstract : Carbamazepine (CBZ), one of the most commonly prescribed antiepileptic drug, is proposed to inhibit Na^+ channel. In this study, we have investigated the effects of CBZ on Na^+ current, evoked in cultured dorsal root ganglion (DRG) neurons from neonatal rats using whole cell patch clamp technique. In small DRG neurons (20–25 µm), Na^+ current was obtained by blocking K^+ and Ca^{2+} currents with appropriate ion replacement and channel blockers. Separation of the Na^+ current components was achieved on the basis of response to the conditioning voltage. The CBZ depressed Na^+ current in a dose-dependent manner. The maximal Na^+ current was depressed at 300 µM of CBZ, where 94±5.1% of depression was observed. The depression of normalized current amplitude was found to be 72±13.2%, 84±10%, 85±7.1% and 95±5.2% at 10, 30, 100 and 300 µM of CBZ concentrations, respectively, at –20 mV test pulse, when compared with control. The depression of current amplitude was observed as 48±12.3%, 42±15.2%, 71±17.7% and 90±5.8% at 10, 30, 100 and 300 µM of CBZ concentration, respectively, at 0 mV voltage pulse. The depression of Na^+ currents was found to be dose-dependant at –20 and –10 mV but not at 0 mV. It is concluded that the depression of Na^+ currents by CBZ may be responsible for inhibiting the neurotransmitter release.

Key words : sodium channel, carbamazepine, sensory neurons, whole cell patch clamp

INTRODUCTION

Carbamazepine (CBZ) is a tricyclic anticonvulsant drug used for treatment of epilepsy, neuralgia, psychiatric diseases and to control epileptogenic discharge (1). More recently it has been found to be useful for treating neuropathic pain, possibly due to their action on Na^+ channels in sensory neurons (1). Currently, various mechanisms of action of CBZ are proposed (2). One of them is depression of voltage-dependent Na^+ channels (2–4). Local anesthetic compounds that have been used in the clinic (i.e. lidocaine, mexiletine and CBZ) are known to have Na^+ channel blocking properties (5, 6).

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Phenytoin, lamotrigine and carbamazepine attach to a common binding site or "receptor" on Na^+ channels in hippocampal neurons (7). It appears that this receptor is associated with the inactivation state of Na^+ channel that operates via the "hinged-lid" mechanism (7). Furthermore, interactions with voltage-activated Ca^{2+} channels have also been described indicating calcium antagonistic properties (8). Besides these, CBZ has been shown to potentiate γ-aminobutyric acid-induced Cl^- currents in cultured rat cortical neurons (9). It has been also shown in rat neocortical cells that outward voltage-dependent K^+ currents were enhanced by CBZ (10). The slow K_A current, which was also termed the K_D current by Everill et al. (11), was preferentially expressed in small-sized DRG neurons that had tetrodotoxin-resistant action potentials with inflections and responded to capsaicin (12–14). Thus, it seems that K_{DR} and slow K_A currents are likely to be involved in modulating excitability in small-sized, nociceptive DRG neurons. CBZ was not effective in blocking low-voltage–activated Ca^{2+} current in adult rat DRG neurons (15). These actions contribute to the CNS effects of CBZ. Further, the progressive reduction of both motor and sensory conduction velocity by CBZ in long-term therapy has been reported in adult rat (16). The reduction of conduction velocity and sensory loss may be due to actions of CBZ on peripheral nerves and sensory conduction pathway including sensory neurons in adult animals involving Na^+ channels. Therefore, to obtain better insight into the mechanism of CBZ action on Na^+ channels the present investigation was carried out on cultured neonatal rat DRG neurons using whole cell patch-clamp technique.

MATERIALS AND METHODS

Cell preparation

Four day old Sprague-Dawley rat pups were anesthetized and the vertebral column was removed. The spinal cord with DRG was dissected and placed in a petri dish containing Mg^{2+}- and Ca^{2+}-free oxygenated phosphate buffered saline with 30 mM glucose. DRG were harvested from spinal cord, freed from their connective sheaths in sterile Mg^{2+}- and Ca^{2+}-free oxygenated phosphate buffer solution. DRG were then incubated with trypsin (2.0 mg/ml) for 30 min at 37°C. The trypsin inhibitor (1.5 mg/ml) was added to stop the enzymatic action of trypsin. The cells were gently triturated with fire-polished pasteur pipettes and then placed in poly-D-lysine coated 35 mm culture plates. The cells were allowed to adhere to the culture plates for 50–60 min prior to the electrophysiological recordings. Cell diameter was determined using a calibrated reticule in the light path of the microscope during recording, using an approximate average of the longest and shortest dimensions of cells that deviated slightly from spherical shape. Neurons from 20–25 µm diameters were used for this study. All the experiments were approved by the Institutional Animal Ethics Committee, National Institute of Pharmaceutical Education and Research, SAS Nagar, India for conducting animal experiments. Care was taken to restrict the number of animals to the minimum possible.

Electrophysiological recording

Whole cell patch clamp recordings were

performed using Axopatch–200B amplifier (Axon Instruments, Foster City, CA, USA). Pipettes were fabricated from borosilicate glass with filament (Clark Electromedical Instrument, UK) and pulled by using a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument Co., USA). The pipettes were polished by using microforge (Model MF-900, Narishige, Japan) to give resistances of 1–2 MΩ. Data acquisition and pulse protocols were controlled with the pCLAMP software (Axon Instruments, Foster City, CA, USA) and digitized using analog/digital converter (Digidata 1322 A, Axon Instruments, Foster City, CA, USA). The recordings were done at 20±0.5°C using temperature controller (Harvard Apparatus, Model TC-202A, USA). Currents were sampled at 20 kHz and filtered at 5 kHz. Cell capacitance and series resistance were read from the dials of the patch clamp amplifier after correcting the capacitive transient current obtained during a small depolarizing test pulse.

For isolation of Na^+ currents extracellular solution contained (mM): NaCl, 65; Choline chloride, 50; Tetraethylammonium chloride (TEA), 20; KCl, 5; $CaCl_2$, 0.01; $MgCl_2$, 5; glucose, 10 and HEPES, 10. The pH was adjusted to 7.4 by addition of NaOH. The intracellular (pipette) solution contained (mM): CsF, 110; $MgCl_2$, 5; EGTA, 11; NaCl, 10; HEPES, 10. The pH was adjusted to 7.2 by the addition of CsOH (total Cs^+ concentration; 140 mM). The osmolarity of these solutions was kept in the range of 310–325 mOsm/kg.

Isolation of Na^+ current in DRG neurons

Na^+ currents were recorded in small DRG neurons (n = 13) at holding potential at –70 mV with a pre-pulse of –120 mV. This pre-pulse served to remove inactivation of voltage-gated Na^+ channels. The Na^+ currents were isolated by blocking the K^+ and Ca^{2+} currents with appropriate ion replacement and channel blockers. K^+ channels were blocked using Cs^+ in internal solution and externally by TEA. Ca^{2+} channels are difficult to block selectively and rat DRG cells possess several different types of calcium channels, each being sensitive to different class of blocking agent. Ca^{2+} channels were blocked by using Ca^{2+} 10 µM concentration externally. 10 µM concentration Ca^{2+} being thought to be sufficiently low to block the channels by binding to a high affinity site within the channel, while being at the same time sufficiently high to prevent them becoming Na^+ conducting. Internal EGTA was used to buffer internal Ca^{2+} and Mg^{2+}, a non-permeant Ca^{2+} competitor was present in both the external and internal solution. The major internal anion, F^- has also been shown to inhibit I_{Ca} in immature rat DRG cells. The isolated Na^+ current showing the inward and outward current is showing the bell-shaped current and voltage (I-V) relationship. As reported Na^+ channels open with the stepwise depolarization pulse of -50 mV to 40 mV with 10 mV step (17–18). The Na^+ channel closes with the depolarization pulses above 20 mV. The holding potential was maintained at –120 mV, currents were recorded at voltages between –50 and + 40 mV in 10 mV steps after 50 ms conditioning pre-pulse to –120 mV (Fig. 1).

Drugs and solutions

CBZ (Amoli Organic Pvt. Ltd. Baroda, India) was dissolved in 2% dimethyl sulfoxide

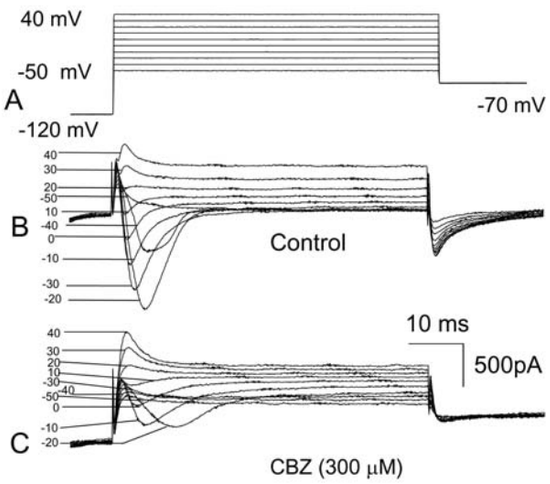

Fig. 1 : Whole cell Na^+ currents recorded from dorsal root ganglion (DRG) neurons and their depression by CBZ. (A) Voltage pulse protocol illustrated for the current traces obtained from the DRG neurons. In control (B) or in the presence of CBZ, 300 μM (C), the cells were held at –70 mV and then pre-pulsed to –120 mV for 10 ms followed by test pulse from –50 mV to 40 mV in 10 mV steps for 50 ms. CBZ depressed the sodium current significantly.

(DMSO, Sigma). CBZ was prepared in the external medium to the desired concentration just before use. Drug was applied in the external bath, to achieve the desired concentrations. All the chemicals used in the intracellular and extracellular solution were from Sigma-Aldrich, CA, USA.

Statistics

All results are presented as mean±SEM. Student's t-test and ANOVA were used for comparing the different groups. $P<0.05$ was considered as statistically significant.

RESULTS

CBZ Depressed the Na^+ current

CBZ blocked the Na^+ current in a concentration-dependent manner (Fig. 2 and 3). The maximal Na^+ current is depressed at 300 μM of CBZ, where 94±5.1% of depression was observed. The maximal peak current was observed at –20 mV which was depressed by 72±13% at 10 μM; 83±9.9% at 30 μM; 84±7.12% at 100 μM and 94±5.1% at 300 μM (Fig. 2 and 3). Normalized current at depolarization step to –20 mV was significantly depressed at all the concentration of CBZ (paired Student's t-test, $P<0.05$ for 10 μM, 30 μM and 100, 300 μM; Fig. 2). At 0 mV test pulse, the depression of current amplitude was observed as 48±12.3%, 42±15.2%, 71±17.7% and 90±5.8% at 10, 30, 100 and 300 mM of CBZ concentrations, respectively. Moreover, at –10 mV test-pulse, the depression of current amplitude was 40±16.5%, 49±14.3%, 61±13.9% and 88±10.0% at 10, 30, 100 and 300 mM of CBZ

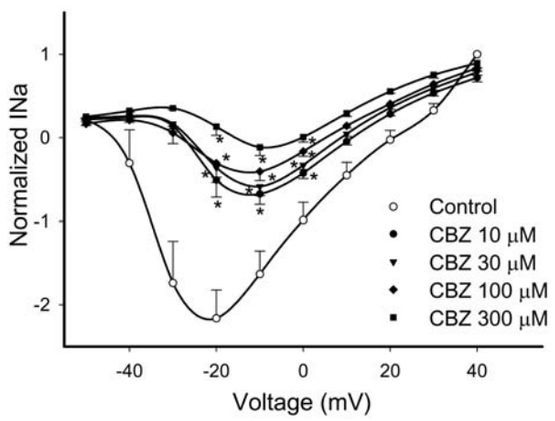

Fig. 2 : Whole-cell Na^+ currents in DRG and their dose-dependent depression by CBZ. The I-V relationship of the normalized Na^+ current, against the test potential (control) and after 15 min exposure to CBZ (10, 30, 100, 300 mM) in the bath. Data are presented as mean±SEM (n=13). Normalized current at depolarization step was significantly depressed at all the concentration of CBZ in comparison with control (two-way ANOVA, F-value is 17.045 for 0 mM, 19.481 for 30 and 100; 12.003 for 300 mM; $P<0.05$).

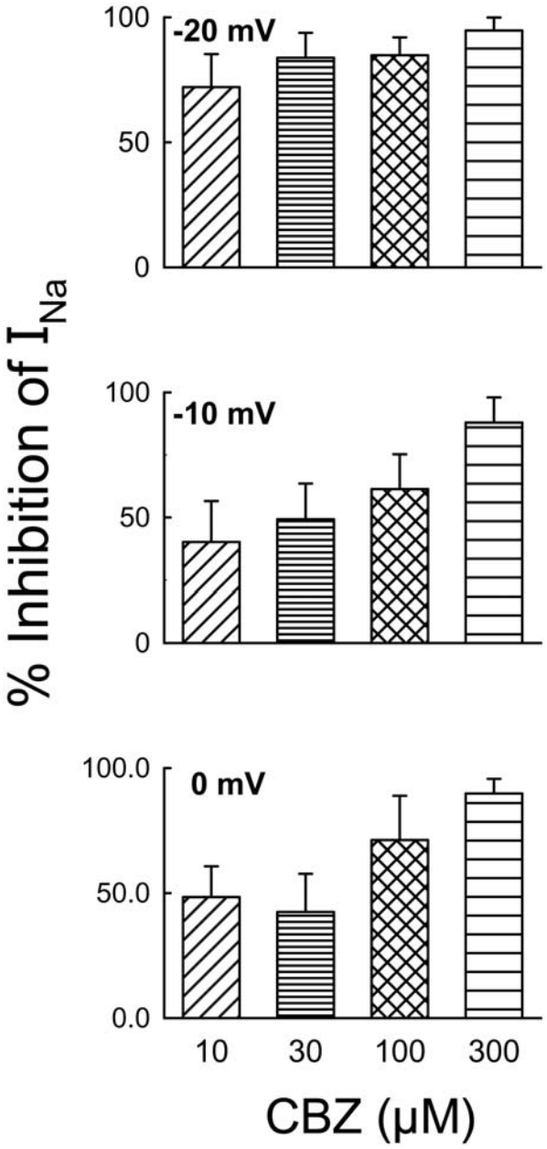

Fig-3 Voltage-dependent inhibition of Na^+ currents by CBZ (10-300 μM) in DRG neurons. The graph shows percentage inhibition by different concentration of CBZ at test-potentials –20, –10 and 0 mV (Fig. 2). Bar represents mean ± SEM values of 13 neurons. Depression at all the concentration of CBZ was significantly different in comparison with control (paired Student's t-test, t-value- 4.882; df = 24; $P<0.05$ between the control and CBZ concentration).

concentrations, respectively. The depression is statistically significant at all the concentrations of CBZ compared to control at –20 mV, –10 mV and 0 mV test pulse (one-way ANOVA; paired Student's t-test, $P<0.05$). The results suggest that the blockade of Na^+ current by CBZ is concentration and voltage-dependent manner.

The peak current conductance (g_{Na}) at each potential was calculated as a chord conductance, from the corresponding peak current :

$$g_{Na} = I_{Na}/(V-V_{Na})$$

where V_{Na} is the Na^+ current reversal potential. The relationship between the peak I_{Na} amplitude and the test potential (V) was found to be described by the change in conductance with potential. The mean maximum value of Na^+ conductance, g_{Na}/g_{max}, calculated from the peak current elicited by the step depolarization from pre-pulse level of –120 mV was 15.05±0.74 ns for I_{Na} and after CBZ (300 μM) the g_{Na}/g_{max} was reduced to –1.15±3.5.

DISCUSSION

Our results demonstrate that CBZ depressed the Na^+ currents in DRG neurons (Fig. 1). CBZ has different effects at different holding potentials. At –100 mV holding potential CBZ has negligible effect on the Na^+ currents and even 10 times higher concentration of CBZ produces no more than 10% inhibition of the Na^+ currents. However, at a holding potential of –70 mV CBZ significantly inhibited the Na^+ current. We have observed the effect at –70 mV with a pre-pulse of –120 mV.

DRG neurons are heterogeneous in size as well as in electrophysiological properties and represent more than 25 different populations that encode a variety of sensory information (19–21). Interpretations of electrophysiological data from isolated cells require that the same groups of neurons are isolated with their "current signature" for a physiological or pharmacological study (20, 21). In a study reported elsewhere, consideration of the size of neurons was avoided for the study of Na^+ and K^+ currents. In their study CBZ (50 mM) did not depress the Na^+ currents or K^+ currents (22). However, in our study DRG neurons with diameters 20–25 mm, which are associated with C-fibers and nociceptive sensation as reported elsewhere (19), a concentration-dependent depression of Na^+ current was observed (Fig. 2 and 3). Although cell size is an approximation of functional class of DRG neurons, size alone is not the absolute predictor of functional type of neurons. This presents a difficulty in biophysical studies when the neurons are acutely dissociated for *in vitro* patch clamp study because they are disconnected from their targets. Depression of sodium current by CBZ was observed in a concentration- and voltage-dependent manner in the present study (Fig. 2). Failure to observe a CBZ effect could be related to the fact that size dependent selection of DRG neurons and restriction of the DRGs to the lumbar region of the spinal cord for the electrophysiological studies was not used (22).

Several mechanisms could explain the CBZ inhibition in a sub population of DRG neurons. Although their principle action is usually explained through a suppression of voltage-gated Na^+ conductance as observed in this study. There is a continuously increasing number of reports showing that the block of other ionic channels (Ca^{2+}, K^+ or transmitter-activated) may also be involved (23–24). Among them, Na^+ channels represent the most diverse family of ionic channels with multiple physiological functions. CBZ increased the fast and slow time constants for recovery from inactivation and the fraction of the fit attributed to the slow time constant (25).

The Na^+ channels are mostly involved in the spike generation and propagation in peripheral axons, central neurons and their suppression by local anesthetics directly results in a conduction block. We have observed significant depression of Na^+ current in a concentration- and voltage-dependent manner. Some reports describe that there is no significant depression of Na^+ current after exposure of CBZ. But, in these studies size of the DRG neurons was not taken into consideration (22). The Na^+ channel inhibition was more potent at –20 mV depolarized potentials. CBZ exhibited a significant depression in the normalized peak current between 10 –300 µM.

In conclusion, we have shown that a population of DRG neurons are sensitive to CBZ and depress Na^+ currents. These findings contribute towards the possible mechanism of action of CBZ so as to inhibit the neurotransmitter release. Our study also indicates that the depression of the non-inactivating current component would affect the action potential waveform in the small DRG neurons. This will be confirmed experimentally using current clamp measurements of action potential waveforms.

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