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The Inhibitory Effects of Dantrolene on Action Potential-Induced Calcium Transients in Cultured Rat Dorsal Root Ganglion Neurons

A. AYAR, H. KELESTIMUR[1]

Firat University, Faculty of Medicine, Departments of Pharmacology and [1]Physiology, Elazig, Turkey

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Summary

We investigated the actions of dantrolene Ca^{2+}-induced on Ca^{2+}-release (CICR) evoked by action potentials in cultured rat sensory neurons. The effect of dantrolene on action potential after-depolarization and voltage-activated calcium currents was studied in cultured neonatal rat dorsal root ganglion cells (DRG) using the whole-cell patch-clamp technique. Depolarizing current injection evoked action potentials and depolarizing after-potentials, which are activated as a result of CICR following a single action potential in some cells. The type of after-potentials was determined by inducing action potentials from the resting membrane potential. Extracellular application of dantrolene (10 μM) abolished after-depolarizations without affecting action potential properties. Furthermore, dantrolene significantly reduced repetitive action potentials after depolarizing current injection into these neurons, but had no significant effect on the steady-state current voltage relationship of calcium currents in these neurons. We conclude that dantrolene inhibits the induction of action potential after depolarizations by inhibiting CICR in cultured rat sensory neurons.

Key words

Dantrolene • CICR • Action potential after-potentials • Cultured sensory neurons • Patch clamp

Introduction

The peripherally acting muscle relaxant dantrolene is used in treating several diseases including malignant hyperthermia, malignant hyperpyrexia, the neuroleptic malignant syndrome and hypercatabolic syndrome and spasticity (Ward *et al.* 1986, Meredith *et al.* 1994).

Several studies on neuronal preparations have suggested the occurrence of Ca^{2+}-induced Ca^{2+}-release (CICR) in neurons (Mayer 1985, Smith *et al.* 1983), which was originally observed in muscles (Endo 1977). Additionally, we have previously shown that Ca^{2+} entry during a single action potential can activate Ca^{2+}-activated conductances, which are responsible for the action potential after-potentials in cultured rat DRG neurons (Ayar and Scott 1999).

Increased intracellular Ca^{2+} could be achieved either by entry of Ca^{2+} from the extracellular space through voltage and ligand-gated calcium channels or by release of Ca^{2+} to intracellular stores. The intracellular Ca^{2+} homeostasis mechanisms handle the raised $[Ca^{2+}]_i$ by taking it back to the internal stores or extruding it back into the extracellular space. Elevations in $[Ca^{2+}]_i$ due to

Fax+4202 24920590
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

enhanced action potentials activates a variety of cellular processes such as the control of neuronal excitability, neurotransmitter release and activation of Ca^{2+}-dependent ion channels (Morgan and Curran 1988, Mulkey and Zucker 1991).

Calcium-activated chloride currents, $I_{Cl(Ca),}$ have been shown to be responsible for action potential after-depolarizations (Mayer 1985, Ayar and Scott 1999). Previous studies have identified $I_{Cl(Ca)}$ in cultured rat DRG neurons using estimated reversal potential measurements, anion substitution and chloride channel blockers (Currie and Scott 1992, Currie *et al.* 1995). $I_{Cl(Ca)}$ can be activated by calcium entry through voltage-activated calcium channels as well as by the release of Ca^{2+} from intracellular stores by agonists including caffeine, ryanodine, cADP ribose and photorelease of dihydrosphingosine, as well as intracellular photorelease of calcium itself from DM-nitrophen (Currie *et al.* 1995, Scott *et al.* 1995, Crawford *et al.* 1997, Ayar *et al.* 1998, Ayar and Scott 1999). It has also been shown that $I_{K(Ca)}$, which is partly responsible for the after-hyperpolarization, is resistant to TEA (Belluzzi and Sacchi 1990) but is blocked by apamin (Kawai and Watanabe 1986). However, the effects of dantrolene on action potentials after depolarizations which is due to activation of CICR is not known in these neurons (Ayar and Scott 1999).

Because dantrolene is being increasingly used in neurons as a calcium release blocker from intracellular stores and even has been proposed as a potential neuroprotective agent for clinical use (Mody and MacDonald 1995), we aimed to investigate its effects on CICR using cultured rat DRG neurons. In the present study, we have used calcium-activated chloride ($I_{Cl(Ca)}$) conductance as a physiological index of raised free intracellular Ca^{2+} close to the cell membrane to investigate the action of dantrolene on CICR in cultured neonatal rat DRG neurons.

Methods

Cell cultures

Dorsal-root ganglion (DRG) neurons were grown in primary culture as previously described (Ayar and Scott 1999). Briefly, dorsal root ganglia from decapitated 1 to 2-day old Wistar rats were dissected out, and incubated at 37 ^{0}C in collagenase and trypsin (Sigma, 0.125 % for 13 min and 0.25 %, for 6 min, respectively). The ganglia were dissociated into single cells by trituration through a flame constricted Pasteur pipette. Cells were plated on poly-L-ornithine/laminin coated glass coverslips. The cells were grown in Ham's F14 (supplemented with glutamine, Imperial Laboratories, Andover, Hampshire UK) with 10 % heat inactivated horse serum (GIBCO, Grand Island, NY), penicillin/streptomycin (ICN, 5000 IU/ml and 5000 mg/ml respectively) and nerve growth factor (NGF, Sigma, 20 ng/ml), $NaHCO_3$ (14 mM, Sigma). Cultures were maintained at 37 ^{o}C in a humidified atmosphere of 5 % CO_2 and re-fed with a fresh culture medium before use between 2-20 days after plating.

Electrophysiology

Experiments were conducted at room temperature (18-20 ^{0}C). The whole cell variant of the patch clamp technique (Hamill *et al.* 1981) was used to record from cultured neonatal rat DRG neurons. The pipettes were made of Pyrex borosilicate glass tubing (1.4/1.6 mm outer diameter, 0.8/1.0 mm bore with a 0.15 mm fiber attached to the inside wall, World Precision Instruments Inc., Germany) using a two-stage vertical microelectrode puller (David Kopf Instruments, Tujunca, USA, Model 730 or P-30 Sutter Instruments, Novato, CA), with a resistance of 3-7 MΩ when filled with intracellular solutions. Recordings were made from cells with established GΩ seal resistance. The recording bath was connected with the electrode chamber through an agar-K^+ bridge and grounded with an Ag-AgCl electrode. Junction potentials were measured with the pipette tip in the bathing solution with appropriate correction. An Axopatch 2A switching amplifier (Axon Instruments, USA) was used for amplification of voltage signals at a sampling rate of 28-35 kHz.

Solutions and drug

For current clamp recordings the patch pipette was filled with the following solution (in mM): potassium chloride 140, calcium chloride 0.1, magnesium chloride 2.0, ATP 2.0, HEPES 10.0, ethyleneglycol bis-(-aminoethylether)-N,N-tetraacetic acid (EGTA) 1.1. The pH and osmolarity were adjusted to 7.4 and 310 mOsm with Tris and sucrose, respectively. The extracellular recording medium contained (in mM): sodium chloride 130, potassium chloride 3.0, magnesium chloride 0.6, calcium chloride 2.0, sodium bicarbonate 1.0, HEPES 10.0, glucose 5.0. The pH was adjusted with NaOH to 7.4 and the osmolarity to 310-320 mOsm with sucrose. Voltage clamp experiments were carried out a pipette solution containing (in mM): cesium chloride 140, calcium chloride 0.1, EGTA 1.1, magnesium chloride 2,

ATP 2, HEPES 10. The extracellular bathing solution for voltage clamp experiments contained (in mM): choline chloride 130, calcium chloride 2, potassium chloride 3, magnesium chloride 0.6, sodium bicarbonate 1, HEPES 10, tetraethylammonium chloride 25, tetrodotoxin 0.0025, glucose 5. All chemicals used preparing the solutions in the current experiments were obtained from Sigma (Deisenhofen, Germany) unless otherwise stated.

The drug used in this study was dantrolene (Sigma, Deisenhofen, Germany) and it was prepared in extracellular recording medium. Dantrolene was applied by low pressure ejection (World Precision Instruments, Inc, Germany) from a blunt pipette about 100 μm from the recorded cell.

Data analysis

Whole cell currents and action potentials were recorded on a digital tape using a digital audio tape recorder (DTR 1200, Biologic, France). Data analysis and acquisitions were performed by using the Cambridge Electronic Design and Clampex 7 (Axon Instruments, USA) computer software.

The data are presented as means ± standard error of the mean (S.E.M.) of the number of observations indicated. Student's *t* test was used for statistical comparison of paired and unpaired data (Microcal Origin, Northampton, USA). $P<0.05$ values were considered significantly different from control values.

Results

The effect of dantrolene on CICR in cultured DRG neurons cannot be attributed to inhibition of ionic currents through voltage-dependent Ca^{2+} channels. Calcium channels currents were activated by 100 ms depolarization of membrane from the holding potential of –90 mV to 0 mV. The voltage dependence of Ca^{2+} currents is shown during control conditions and in the presence of dantrolene (10 μM) at a holding potential of –90 mV. No significant change was observed (Fig. 1). Additionally, dantrolene failed to produce any significant effect on the peak or end of calcium currents activated from –90 mV (Fig. 1, inset). The mean peak and end of calcium currents was –0.86±0.12 nA (n=7), –0.42±0.07 nA (n=7) and –0.82±0.14 nA (n=7, $p>0.05$), –0.38±0.08 nA (n=7, $p>0.05$) before and after application of dantrolene.

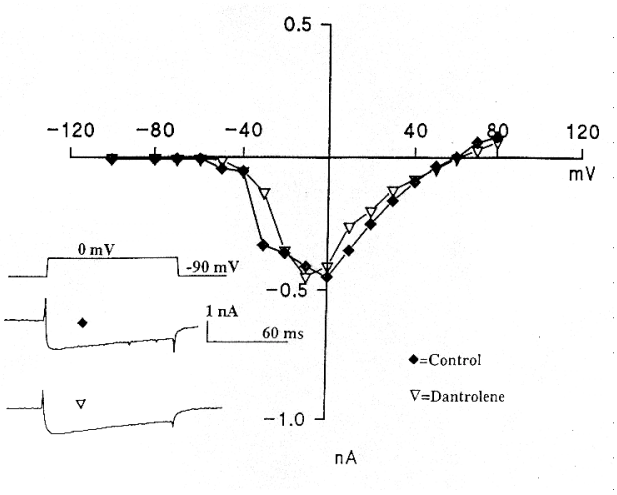

Fig. 1. *The voltage-dependent effect of dantrolene (10 μM) on the peak amplitude of calcium currents of rat DRG neurons. DRG neurons were held at –90 mV under voltage clamp mode of whole cell configuration and 100 ms depolarizing an hyperpolarizing voltage steps (-10 to +170 mV) were activated and resulting calcium channel currents were measured and plotted against the original membrane voltage under control conditions (◆) and after application of 10 μM dantrolene (▽). The figure at top is representative of data obtained in four other experiments concerning current voltage relations for peak calcium channel currents. The inset showing example of calcium channel currents elicited by depolarization from the holding potential of –90 mV to 0 mV under control conditions (◆, upper trace) and after application of 10 μM dantrolene (▽, lower trace). The inset is representative of data obtained in six other experiments.*

Some DRG cells fired multiple action potentials following 100 ms depolarization and application of dantrolene significantly reduced the number of spikes (Fig. 2). Dantrolene reduced the mean number of spikes from 8±3 to 3±1 (n=6, $p<0.05$).

In 7 out of the 21 cells studied, injection of depolarizing current from resting membrane potential elicited action potentials accompanied by after-depolarization. After determining the existence of the depolarizing after-potential following the action potential at resting membrane potential, cells were held at –75 mV by current injection and control values of action potential properties including the after-depolarization amplitude and decay time and effects of dantrolene were determined. The mean peak of the after-depolarization was 16±4 mV (n=7) and the mean time to decay to the baseline by 63% was 86±11 ms (n=7) under control conditions. Dantrolene completely and reversibly

abolished this after the depolarization (Fig. 3). The mean peak, threshold and duration of action potentials at 0 mV was 27±5 mV, –30±3 mV and 0.8±0.2 ms (n=7) before and 25±4 mV, –33±4 mV and 0.7±0.2 ms (n=7) after application of dantrolene, respectively. None of the values were significantly different from their respective control values (p>0.05).

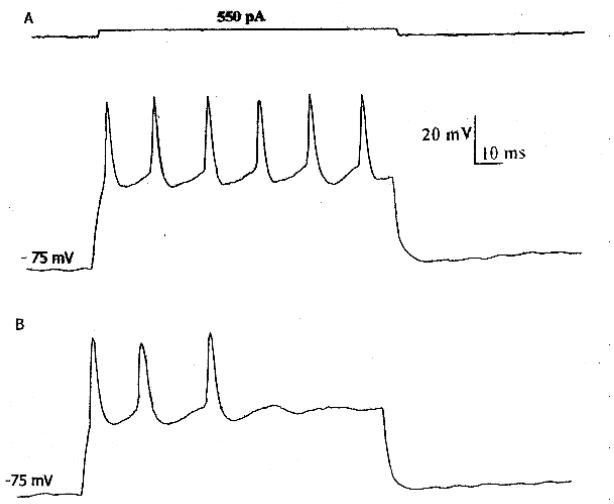

Fig. 2. *Dantrolene (10 μM) reduces multiple firing of action potentials in rat DRG neurons. DRG neurons were held at –75 mV under current clamp mode of whole cell configuration and 100 ms depolarizing current steps and the resulting action potentials are presented under control conditions (A) and after application of 10 μM dantrolene (B). Dantrolene reduced the number of action potentials fired during 100 ms step depolarization. Original recording are taken from one experiment representative of five others.*

Discussion

The results from the present study demonstrate that extracellular application of dantrolene inhibits the action potential after-depolarization without significantly affecting action potential properties when experiments are carried out under current clamp conditions; dantrolene has no significant effect on either the peak amplitude of calcium currents or the current voltage relationship.

Intracellular recordings and confocal imaging studies in bullfrog sympathetic neurons have shown the inhibitory effect of dantrolene on CICR (Hua *et al.* 2000). In the same study, a similar effect has been observed with ryanodine, which was also shown to attenuate the action potential after-depolarization in rat DRG neurons (Ayar

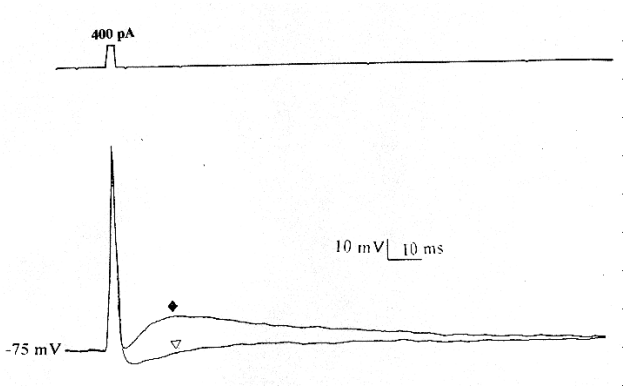

Fig. 3. *The inhibitory actions of dantrolene (10 μM) on action potentials after depolarizations in rat DRG neurons. DRG neurons were held at –75 mV under current clamp mode of the whole cell configuration and 5 ms depolarizing current steps used for activating the action potential. Representative action potentials with after depolarization from one DRG neuron are superimposed under control conditions (♦) and after application of 10 μM dantrolene (▽). Dantrolene abolished the after depolarization without significantly effecting the action potential peak amplitude, duration or threshold. These original recordings are from a single experiment representative of six others.*

and Scott 1999). Similarly, dantrolene was shown to inhibit Ca^{2+} release from internal stores and to depolarize after-potentials in the rat supraoptic nucleus magnocellular neurons (Li and Hatton 1997).

Most of DRG neurons fire a single action potential in response to depolarizing current injection, even if this current injection lasts several hundred milliseconds. However, some DRG neurons show multiple firing properties and this shows the heterogeneity of the population of cultured DRG neurones. Multiple firing in turn shows the excitatory influence of after-depolarizations. The multiple firing may indicate a decline in the efficacy of endogenous Ca^{2+} homeostatic mechanisms.

In this study, the primary cause of the $[Ca^{2+}]_i$ rise is considered to be due to Ca^{2+} entry from the extracellular space during the action potential. And this Ca^{2+} triggers the Ca^{2+} release from intracellular stores which cause further rise in free $[Ca^{2+}]_i$ levels and activating Ca^{2+}-dependent ion channels including $I_{Cl(Ca)}$. These ion channel activations are observed as activation of after-depolarizations following the action potentials. Dantrolene has been found ineffective on peak amplitude

of slow after-depolarizations in guinea pig olfactory neurons, where CICR from internal stores does not contribute significantly to the generation of these potentials (Postlethwaite *et al.* 2000).

The lack of the effect of dantrolene on the steady-state current-voltage relationship of Ca^{2+} currents (Fig. 1) suggests that dantrolene does not exert a direct effect on membrane voltage-gated Ca^{2+} channels and that its effect solely depends on the intracellular Ca^{2+} storage site. Additionally, dantrolene had no effect on action potential properties. These findings are in accordance with the literature (Mody and MacDonald 1995). In hippocampal cells, dantrolene was reported to inhibit intracellular Ca^{2+} increase induced by N-methyl-D-aspartate (Segal and Manor 1992, Mody and MacDonald 1995). Several studies have shown the blocking effects of dantrolene on ryanodine receptors following its intracellular or extracellular application (Van Winkle 1976, Usachev *et al.* 1993). Additionally, we have previously shown that the action of caffeine, ryanodine and caged sphingolipids can be effectively prevented by intracellular application of dantrolene in cultured rat DRG neurons (Ayar *et al.* 1998, Ayar and Scott 1999).

The findings in the present study supports the potential role of dantrolene in neuronal death associated with a variety of stimuli enhancing the free intracellular calcium concentration with subsequent damage (Frandsen and Schousboe 1993). Dantrolene was also found to exert beneficial effects in experimentally induced status epilepticus (Niebauer and Gruenthal 1999). It may not be long before dantrolene is used for treatment of excitotoxic injury in humans.

In conclusion, we have found that dantrolene inhibits action potentials after-depolarization by blocking CICR in rat sensory neurons independent of voltage dependent Ca^{2+} entry and solely interfering with intracellular Ca^{2+} stores.

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Reprint requests

Dr. Ahmet Ayar, Firat (Euphrates) University Faculty of Medicine (TIP FAK), Department of Pharmacology, TR-23200 Elazig, Turkey. Fax: + 90 424 237 91 38. E-mail: aayar@firat.edu.tr or a.ayar@mailexcite.com