Dantrolene Inhibits Ca^{2+}-Induced Ca^{2+}-Release in Cultured Rat Dorsal Root Ganglion Neurones[11]

DANTROLEN SIÇAN DORSAL KÖK GANGLİYON SİNİR HÜCRE KÜLTÜRLERİNDE KALSİYUM-TETİKLEMELİ KALSİYUM SALIVERİLMESİNİ İNHİBE EDER

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Summary

Purpose: The aim of this study was to investigate the effects of dantrolene on Ca^{2+}-induced Ca^{2+}-releasc (CICR) in cultured rat sensory neurones.

Materials and Methods: We have investigated the effects of dantrolene on action potential after-depolarisation and voltage-activated calcium currents in cultured neonatal rat dorsal root ganglion (DRG) neurones using the whole-cell variant of the patch clamp technique.

Results: Depolarising current injection evoked action potentials and depolarising after potentials, which are shown to be activated as a result of CICR following a single action potential, were followed action potentials in certain cells. The types of after potential were determined by activation of action potentials from resting membrane potentials. Extracellular application of dantrolene (lOmM) abolished after-depolarisations without affecting action potential properties. Additionally, dantrolene significantly reduced multiple action potential firing observed after depolarising current injection to these neurones. Dantrolene had no significant effect on steady-state current voltage relationship of calcium currents in these neurones.

Conclusion: We concluded that dantrolene inhibits CICR in cultured rat sensory neurones.

Key Words: Dantolene, CICR,
Action potential-after-potentials,
Cultured sensory neurones,
Patch clamp

T Klin J Med Res 2001, 19:88-93

Özet

Amaç: Bu çalışmanın amacı duyusal nöronlarda dantrolenin kalsiyum-tetiklemeli-kalsiyum salıverilmesine (KTKS) etkilerini incelemekti.

Materyal ve Metod: Patch kenetleme tekniğinin tüm hücre diyaliz yöntemi kullanılarak yeni doğan sıçanlardan kültüre edilen dorsal kök gangliyon (DKG) sinirlerinde dantrolenin aksiyon potansiyeli depolarize edici ard potansiyelleri ve voltaj bağımlı kalsiyum kanallarına etkilerini inceledik.

Bulgular: Depolarize edici akım enjeksiyonları, aksiyon potansiyelleri ve belli hücrelerde KTKS aktivasyonu sonucu ortaya çıkan aksiyon potansiyeli depolarize edici ard potansiyellerini aktive etti. Aksiyon potansiyelleri istirahat membran potansiyelinden aktive edilerek ard potansiyellerin tipi belirlendi. Dantrolenin (lOmM) ekstraselüler uygulanması aksiyon potansiyeli parametrelerini etkilemeksizin depolarize edici ard potansiyelleri tamamen ortadan kaldırdı. İlave olarak dantrolen, bu nöronlara depolarize edici akım uygulaması ile aktive edilen multipl aksiyon potansiyeli ateşlemesini anlamlı derecede inhibe etti. Dantrolen bu nöronlarda kalsiyum kanal akımlarının sabit durum akım-voltaj eğrisi ilişkisi üzerine anlamlı etki oluşturmadı.

Sonuç: Sonuç olarak bu nöronlarda dantrolenin kalsiyum tetiklemeli kalsiyum salıverilmesini inhibe ettiğini tespit ettik.

Anahtar Kelimeler: Dantrolen, Kalsiyum tetiklemeli
kalsiyum saliverilmesi (KTKS),
Aksiyon potansiyeli ard potansiyelleri,
Duyusal sinir hücre kültürü,
Patch kenetleme

T Klin Araştırma 2001, 19:88-93

Received: Dec. 18, 2000

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part of this manuscript previously reported in abstract form in 25th Turkish Physiological Society Meeting, Elazig, Turkey

The peripherally acting muscle relaxant dantrolene is used in treatment of several diseases including malignant hyperthermia, malignant hyperpyrexia, neuroleptic malignant syndrome and hypercatabolic syndrome and spasticity (1,2).

Several studies on neuronal preparations have suggested the occurrence of Ca^{2+}-induced Ca^{2+}-re-

lease (CICR) in neurones (3, 4), which was originally observed in muscles (5). Additionally, we have previously shown that Ca^{2+} entry during a single action potential can activate Ca^{2+}-activated conductances, which are responsible from action potential after-potentials in cultured rat DRG neurones (6).

Increases in intracellular Ca^{2+} could be achieved either by entry of Ca^{2+} from extracellular space through voltage and ligand-gated calcium channels or by release of Ca^{2+} from intracellular stores. The intracellular Ca^{2+} homeostasis mechanisms handles the raised $[Ca^{2+}]i$ by taking up to the internal stores or extruding back to the extracellular space. Elevations in $[Ca^{2+}]i$ due to activation of action potentials activates variety of cellular processes such as control of neuronal excitability, neurotransmitter release and activation of Ca^{2+}-dependent ion channels (7, 8).

Calcium-activated chloride currents, ICl(Ca), have been shown to be responsible for action potential after-depolarisations (3, 6). Previous studies have identified ICl(Ca) in cultured rat DRG neurones using estimated reversal potential measurements, anion substitution and chloride channel blockers (9, 10). ICl(Ca) can be activated by calcium entry through voltage-activated calcium channels as well as by 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 (6, 10-13). It has also been shown that IK(Ca), which is in part responsible for the after-hyperpolarisation, is resistant to TEA (14) but blocked by apamin (15).

Because dantrolene has been increasingly used in neurones as calcium release blocker from intracellular stores and even has a proposed potential neuroprotective agent for human use (16) we aimed to investigate its effects on CICR using cultured rat DRG neurones. In the present study we have used calcium-activated chloride (ICl(Ca)) conductance as physiological index of raised free intracellular Ca2+ close to the cell membrane to investigate the actions of dantrolene on CICR in cultured neonatal rat DRG neurones.

Materials and Methods:

Cell Culture: Dorsal-root ganglion (DRG) neurons were grown in primary culture as previously described (9). Briefly, ganglia from spinal column of decapitated 1-to 2- day old Wistar rats dissected out, and incubated at 37°C in collagenase and tyripsin (Sigma, 0.125%, 13min., 0.25%>, 6"min. respectively). 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. 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, 5.000 IU/ml and 5.000 g/ml respectively) and nerve growth factor (NGF) (Sigma, 10 mg/ml). Cultures were maintained at 37°C in a humidified atmosphere of 5%> $C0_2$ and re-fed with fresh culture medium before used between 2-20 days after plating.

Electrophysiology: Experiments were conducted at room temperature (18-20 °C). The whole cell variant of the patch clamp technique (17) was used to record from cultured neonatal rat DRG neurones. The patch pipettes were made from Pyrex borosilicate glass tubing (1.4/1.6mm outer diameter, 0.8/1.0mm bore with 0.15mm fibre 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), giving a resistance of 3-7 MW when filled with intracellular solutions. Recordings were made from cells with established GW seal resistances. 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 of the recording chamber and corrected for. An Axopatch 2A switching amplifier (Axon Instruments, USA) used for amplification of voltage signals, at a sampling rate of 28-35 kHz.

Solutions and Drugs: For current clamp recordings the patch pipette was filled with solution (in mM): KC1, 140; $CaCl_2$, 0.1; $MgCl_2$, 2.0; ATP, 2.0; HEPES, 10.0; EGTA, 1.1. The pH and osmo-

larity were adjusted to 7.4 and 310 mOsm with Tris and sucrose, respectively. The extracellular recording medium contained (in mM): NaCl, 130; KC1, 3.0; MgC12,0.6; $CaCl_2$,2.0; $NaHC0_3$,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 using pipette solution containing (in mM): CsCl, 140; CaC_2, 0.1; EGTA, 1.1; $MgCl_2$, 2; ATP, 2; HEPES, 10. The extracellular bathing solution for voltage clamp experiments contained (in mM): ChCl, 130; $CaCl_2$, 2; KC1, 3; $MgCl_2$, 0.6; $NaHC0_3$,1; HEPES, 10, glucose, 5.

The drug used in this study was dantrolene (Sigma) 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 mM away from the cell being recorded.

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

Data are presented as means ± standard error of 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). P values <0.05 considered statistically significant.

Results

The effect of dantrolene on CICR in cultured DRG neurones cannot be attributed to inhibition of ionic currents through voltage-dependent Ca^{2+} currents. Calcium channels currents were activated by 100 ms depolarisation of membrane from the holding potential of -90 mV to 0 mV. Voltage dependencies of Ca^{2+} currents are shown during control conditions and in presence of dantrolene (lOmM) at a holding potential of -90 mV and no significant change was observed (Fig 1). Additionally dantrolene was failed to produce any significant effect on peak or end of calcium currents activated from -90 mV (Figure 1, insert). The mean peak and end of

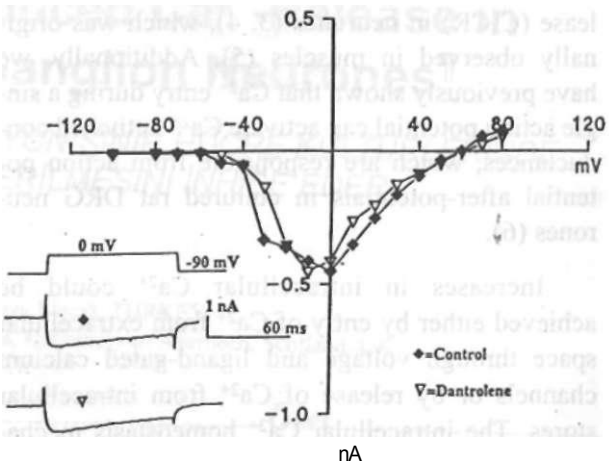

Figure 1. The voltage-dependent effect of dantrolene (10 uM) on the peak amplitude of calcium currents of rat DRG neurones.

DRG neurones were held at -90 mV under voltage clamp mode of whole cell configuration of the patch clamp technique and 100 ms depolarising an hyperpolarizing voltage steps (-90 to +170 mV) were activated and resulting calcium channel currents were measured and plotted against to the original membrane voltage under control conditions (•) and after application of 1 OmM dantrolene (V).

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

Some DRG cells fired multiple action potentials following a 100 ms depolarisation and application of dantrolene significantly reduced the number of spikes (Figure 2). Dantrolene reduced the mean number of spikes from 8±3 to 3±1 (n=6, PO.05).

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

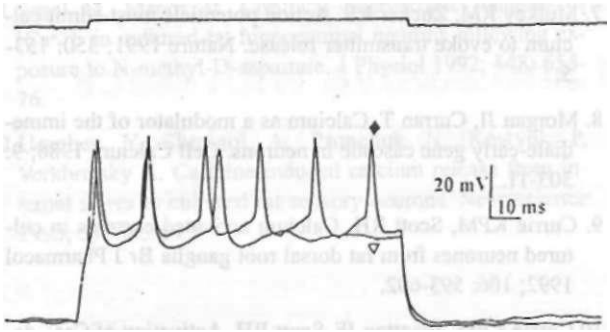

Figure 2. Dantrolen (10 pM) reduces multiple firing of action potentials in rat DRG neurones.

DRG neurones were held at -75 mV under current clamp mode of whole cell configuration of patch clamp technique and 100 ms depolarising current steps resulting action potential are presented under control conditions (•) and after application of lOmM dantrolene (V). Dantrolen reduced the number of action potential fired after 100 ms step depolarisation. A representative data recorded from a DRG neurone presented.

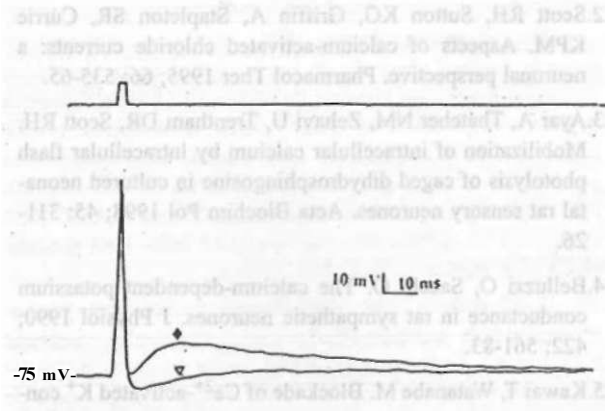

Figure 3. The inhibitory actions of dantrolen (10 uM) on after depolarisations in rat DRG neurones.

DRG neurones were held at -75 mV under current clamp mode of the whole cell configuration of the patch clamp technique and 5 ms depolarising current step used to activate action potential. Representative action potentials with after depolarisation from one DRG neurone are superimposed under control conditions (•) and after application of lOmM dantrolene (V). Dantrolene abolished the after depolarisation without significantly effecting the action potential peak amplitude, duration or threshold.

sations (Figure 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 ($p>0.05$).

Discussion

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

Intracellular recording and confocal imaging studies in bullfrog sympathetic neurones has shown the inhibitory effect of dantrole on CICR (18). In the same study similar effect has been observed with ryanodine, which also shown to attenuate action potential after depolarisations in rat DRG neurones (6). Similarly dantrolene has shown to inhibit Ca^{2+} release from internal stores and depolarizing after-potentials in rat supraoptic nucleus magnocellular neurones (19).

Most of DRG neurones fire a single action potential in response to a depolarizing current injection, even if this current injection commands lasts several hundred milliseconds. However, some DRG neurones 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 efficiency of endogenous Ca^{2+} homeostatic mechanisms.

In this study the primary cause of $[Ca^{2+}]i$ raise is considered to be Ca2+ entry from extracellular space during the action potential. And this Ca^{2+} is triggering Ca^{2+} release from intracellular stores which causing further raise in free $[Ca^{2+}]i$ levels and activating Ca^{2+} dependent ion channels including ICl(Ca). These ion channel activations are observed as activation of after-depolarizations following action potentials. Dantrolen have been found ineffective on peak amplitude of slow after-depolarisations in guinea pig olfactory neurones where CICR from internal stores does not contribute significantly to generation of these potentials (20).

The lack of effect of dantrolene on steady-state current-voltage relationship of Ca^{2+} currents (Fig 1) suggests that dantrolene does not exert a direct ef-

feet on membrane voltage-gated Ca^{2+} channels and its effect solely depends on intracellular Ca^{2+} storage site. Additionally, dantrolene had no effect on action potential properties. These finding are in accordance with the literature (16). In hippocampal cells dantrolene inhibits intracellular Ca^{2+} increase by N-methyl-D-aspartate (16, 21). Several studies have shown the blocking effects of dantrolene on ryanodine receptors following its intracellular or extracellular application (22, 23). Additionally we have previously shown that actions of caffeine, ryanodine and caged sphingolipids can effectively be prevented by intracellular application of dantrolene in cultured rat DRG neurones (6, 13).

The finding of this study supports the potential role of dantrolene in neuronal death associated with a variety of stimuli causing increase in free intracellular calcium concentration and subsequent damage (24). Dantrolene also found to have beneficial effects in experimentally induced status epilepticus (25). It may not be long before dantrolene would be used for treatment of excitotoxic injury in humans.

In conclusion we have found that dantrolene blocks CICR in rat sensory neurones independent of voltage dependent Ca^{2+} entry and solely interfering with intracellular Ca^{2+} stores.

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