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Levetiracetam Inhibits Both Ryanodine and IP3 Receptor Activated Calcium Induced Calcium Release in Hippocampal Neurons in Culture

Nisha Nagarkatti, Laxmikant S. Deshpande, and Robert J. DeLorenzo

Department of Neurology: NN, LSD, RJD Department of Pharmacology and Toxicology: NN, RJD Department of Molecular Biophysics and Biochemistry: RJD Virginia Commonwealth University, Richmond, VA 23298.

Abstract

Epilepsy affects approximately 1% of the population worldwide, and there is a pressing need to develop new anti-epileptic drugs (AEDs) and understand their mechanisms of action. Levetiracetam (LEV) is a novel AED and despite its increasingly widespread clinical use, its mechanism of action is as yet undetermined. Intracellular calcium ($[Ca^{2+}]_i$) regulation by both inositol 1,4,5-triphosphate receptors (IP3R) and ryanodine receptors (RyR) has been implicated in epileptogenesis and the maintenance of epilepsy. To this end, we investigated the effect of LEV on RyR and IP3R activated calcium-induced calcium release (CICR) in hippocampal neuronal cultures. RyR-mediated CICR was stimulated using the well-characterized RyR activator, caffeine. Caffeine (10mM) caused a significant increase in $[Ca^{2+}]_i$ in hippocampal neurons. Treatment with LEV (33 μ M) prior to stimulation of RyR-mediated CICR by caffeine led to a 61% decrease in the caffeine induced peak height of $[Ca^{2+}]_i$ when compared to the control. Bradykinin stimulates IP3R-activated CICR—to test the effect of LEV on IP3R-mediated CICR, bradykinin (1 μ M) was used to stimulate cells pre-treated with LEV (100 μ M). The data showed that LEV caused a 74% decrease in IP3R-mediated CICR compared to the control. In previous studies we have shown that altered Ca^{2+} homeostatic mechanisms play a role in seizure activity and the development of spontaneous recurrent epileptiform discharges (SREDs). Elevations in $[Ca^{2+}]_i$ mediated by CICR systems have been associated with neurotoxicity, changes in neuronal plasticity, and the development of AE. Thus, the ability of LEV to modulate the two major CICR systems demonstrates an important molecular effect of this agent on a major second messenger system in neurons.

Keywords

Levetiracetam; Ryanodine; Inositol 1,4,5-Trisphosphate; Hippocampal Culture

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To whom correspondence should be addressed: Robert J. DeLorenzo, M.D., Ph.D., M.P.H. Virginia Commonwealth University School of Medicine PO Box 980599 Richmond, VA 23298 Phone: 804-828-8969 Fax: 804-828-6432 rdeloren@hsc.vcu.edu.

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Introduction

Epilepsy is a major neurological condition that affects approximately 1% of the population worldwide and over 40% of all epilepsy cases arise following a previous brain insult [14], [2]. Progress has been made in the treatment of epilepsy during the past decade with the discovery of newer antiepileptic drugs (AEDs) and surgical treatments. Understanding the mechanisms of action of AEDs has helped in better characterizing neuronal excitability and may also aid in the development of more effective strategies to treat epilepsy. Levetiracetam (LEV) is a new AED that is currently in widespread clinical use. However, LEV appears to have different anticonvulsant properties from other AEDs [9] and little is known of its mechanism of action except that it acts in a manner and at sites distinct from other commonly used AEDs [22]. Understanding how this drug works as an AED and an examination of its ability to block certain cellular changes could provide insights into its powerful anticonvulsant effect and into the development of more effective AEDs that act in a similar manner.

LEV, the *S*-enantiomer of α -ethyl-2-oxo-1-pyrrolidine acetamide, is used to treat a variety of forms of epilepsy [32]. Unlike many AEDs, LEV does not appear to exhibit direct effects on GABAergic neurotransmission, fails to interact with the benzodiazepine binding site, and lacks a significant affinity for GABAergic or glutamatergic receptors [27],[23]. In addition, compared to other AEDs, LEV is not effective in a use dependent fashion on sodium channel inhibition, maximal electric shock or pentylenetetrazole tests for anticonvulsant agents [16]. Most recently, it has been demonstrated that LEV inhibits GABA-A current run-down [30] and that it binds to the synaptic vesicle protein 2A (SV2A) [22]. While studies have demonstrated that SV2A modulates exocytosis of neurotransmitter-containing vesicles [7], [8], the specific mechanistic relationship between LEV and SV2A remains to be elucidated. Thus, LEV represents a distinct class of AEDs.

Both changes in Ca^{2+} homeostatic mechanisms and persistent elevations in baseline intracellular calcium ($[\text{Ca}^{2+}]_i$) have been implicated in playing a key role in the induction and maintenance of post-injury spontaneous recurrent seizures, or acquired epilepsy (AE) [10]. Studies have demonstrated that inositol 1,4,5-triphosphate (IP3) and ryanodine receptor (RyR) mediated calcium-induced calcium release (CICR) play key roles in the changes in $[\text{Ca}^{2+}]_i$ observed in epileptic conditions [29],[26]. The effect of LEV on Ca^{2+} remains to be fully elucidated. However, it is known that LEV does not affect T-type voltage-gated Ca^{2+} and exhibits only a mild inhibition of high voltage activated Ca^{2+} channels [40]. LEV has been shown to inhibit caffeine-induced calcium transients in studies in rat pheochromocytoma PC12 cells [4], and bradykinin-induced calcium transients in hippocampal slices [1]. Since it has been shown that $[\text{Ca}^{2+}]_i$ regulation in hippocampal neurons plays an important role in modulating anticonvulsant and epileptogenic effects and in mediating injury during seizures [31],[12],[21] it is important to evaluate the effects of LEV on $[\text{Ca}^{2+}]_i$ in hippocampal neurons by regulating IP3R or RyR induced CICR.

This study was initiated to evaluate whether LEV was able to inhibit both IP3R- and RyR-induced CICR in primary hippocampal neurons in culture. The results demonstrate that LEV affects CICR from IP3 and Ry-sensitive stores, employing the use of single-cell Fura-2 imaging to record the response to caffeine and bradykinin, two agents that induce prototypic IP3 and Ry-mediated calcium release respectively [36],[28]. The exploration of whether LEV modulates CICR could prove a valuable addition to what is currently understood on the effects of LEV on hippocampal neurons. Moreover, the ability of a drug to reduce $[\text{Ca}^{2+}]_i$ may make it an ideal therapeutic candidate in neuroprotection following injury and the prevention of long-term plasticity changes leading to epilepsy.

Materials and Methods

Reagents

UCB Pharma supplied Levetiracetam. Caffeine and bradykinin were obtained from Sigma-Aldrich (St. Louis, MO). All agents were solubilized in physiologic bath-recording solution.

Hippocampal Neuronal Cultures

Preparation of primary mixed hippocampal neuronal culture was performed as previously described [33]. In summary, hippocampal cells were isolated from 2-day post-natal Sprague-Dawley rats (Harlan, Frederick, MD). Cells (7.5×10^5) were plated onto a glial support layer that was previously plated onto poly-L-lysine (0.05 mg/ml) coated Lab-Tex glass chambers (Nalge-Nunc International, Naperville, IL). Cultures were maintained at 37°C in 5% CO₂/95% air incubator and fed twice weekly with Neurobasal A medium (Invitrogen, Carlsbad, CA) with B-27 serum-free supplement (Invitrogen) and 0.5mM L-glutamine. All experiments were performed on neurons maintained for 14-17 days *in vitro* to ensure proper development. All procedures adhered strictly to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee.

Ca²⁺ Microfluorometry

Primary hippocampal cultures were loaded with fura-2 acetoxymethyl ester and then placed on the 37°C stage of an Olympus IX-70 inverted microscope (Olympus, Center Valley, PA) coupled to an ultra-high-speed fluorescence imaging system (Perkin-Elmer Life and Analytical Sciences, Boston, MA) as described previously [20]. Data were collected using the UltraVIEW Imaging System. Ratio images were acquired every 5 s using alternating excitation wavelengths (340/380 nm) with a filter wheel (Sutter Instruments, Novato, CA) and fura filter cube at 510/540 nm emissions with a dichroic mirror at 400 nm [20]. Image pairs were captured for 50 s before and 100 s post-addition of bradykinin or caffeine, and were corrected for background fluorescence by imaging a non-indicator loaded field [20].

Data Analysis

Data were statistically analyzed using StatView (Version 5.0.1). Experiments were repeated at least three times from different culture batches. The significance of the data was tested by one-way analysis of variance (ANOVA), or one-way repeated measures (RM) ANOVA followed by Fisher's post-hoc test when appropriate. $P < 0.05$ was considered significant for all data analysis.

Results

LEV does not affect baseline $[Ca^{2+}]_i$ in cultured hippocampal neurons

To determine the effect of LEV on basal $[Ca^{2+}]_i$, fura-2 340/380 ratios were measured in the presence and absence of clinically relevant concentrations [1] of LEV (1, 10, 33, and 100 μ M). Cells were pre-treated for 20 minutes with either vehicle (control) or LEV and fura-2 ratios were recorded. As shown in figure 1, no significant changes in 340/380 ratios were observed between control and LEV-treated cells during the 20-minute period following the addition of LEV to the hippocampal culture. These results demonstrate that at all concentrations tested, LEV does not affect baseline $[Ca^{2+}]_i$.

LEV inhibits caffeine induced CICR in hippocampal neurons

Caffeine-induced Ca^{2+} transients are mediated by stimulation of RyR [1],[24]. Thus, in order to investigate the effects of LEV on ryanodine induced Ca^{2+} release, caffeine-induced Ca^{2+}

transients were studied following pre-treatment with 33 μ M LEV in accordance with other studies [1]. Before the addition of caffeine, both control and LEV pre-treated cells exhibited similar baseline ratios of 0.294 ± 0.018 and 0.319 ± 0.120 respectively, consistent with ratios observed in figure 1. Stimulation with caffeine elicited a marked increase in $[Ca^{2+}]_i$ in control neurons as evidenced by increased 340/380 fura-2 ratios from the baseline value of 0.294 ± 0.018 to 0.653 ± 0.026 , as shown in representative trace (figure 2A). Pseudocolor ratio images prior and subsequent to caffeine stimulation demonstrate this change in calcium following caffeine challenge (figure 2B). Thus, the overall change from baseline 340/380 ratio following treatment with 10mM caffeine was 0.328 ± 0.018 (figure 2C).

In contrast, cells pre-treated with LEV displayed a significantly diminished response to caffeine-induced stimulation. In LEV-treated cells, the baseline 340/380 ratio of 0.319 ± 0.120 increased to a maximum value of 0.445 ± 0.045 following the caffeine challenge (figure 2A). Pseudocolor ratio images demonstrate the contrast in the ability of caffeine to mediate changes in calcium in LEV pre-treated cells when compared to control (figure 2B). Thus, as shown in figure 2C, following treatment with 10mM caffeine, the overall change from baseline 340/380 ratio was 0.126 ± 0.022 in cells pre-treated with 33 μ M LEV. Therefore, LEV pre-treatment inhibits the height of caffeine-induced Ca^{2+} transients by 61.50%.

LEV inhibits bradykinin induced Ca^{2+} transients in hippocampal neurons

To investigate how LEV affects IP3R activated Ca^{2+} release, the effect of LEV (100 μ M) pre-treatment on the inhibition of bradykinin-induced Ca^{2+} release was evaluated. Bradykinin-induced Ca^{2+} transients are mediated by activation of IP3R [28]. Before the addition of bradykinin, similar baseline ratio values of 0.304 ± 0.020 in control and 0.263 ± 0.015 in LEV (100 μ M) pre-treated cells were observed, as shown in figure 1. Control neurons demonstrated a marked increase in $[Ca^{2+}]_i$ when stimulated with bradykinin (1 μ M) from baseline to 0.574 ± 0.055 (figure 3A). This change in fura-2 ratio in control neurons is shown in the pseudocolor ratio image (figure 3B). Thus, treatment with 1 μ M bradykinin caused a change in 340/380 ratio of 0.269 ± 0.063 from the baseline.

LEV-treated neurons showed a markedly diminished response to bradykinin when compared to control neurons. Stimulation with bradykinin caused a small increase from baseline to 0.332 ± 0.037 (figure 3a), as shown also in the pseudocolor ratio image (figure 3B). Therefore, neurons pre-treated with 100 μ M LEV, bradykinin elicited a change from baseline of only 0.069 ± 0.032 (figure 3C). Thus, LEV pre-treatment inhibited the height of bradykinin-induced Ca^{2+} transients by 74.35%. We found at concentrations lower than 100 μ M LEV, there was no significant inhibition in bradykinin-induced Ca^{2+} transients.

Discussion

The results from this study provide direct evidence that LEV inhibits RyR and IP3R induced CICR in hippocampal neurons in culture. LEV was effective in decreasing both caffeine-mediated activation of RyR induced CICR and bradykinin activation of IP3R stimulated CICR. These results indicate that LEV is an effective inhibitor of $[Ca^{2+}]_i$ release mediated by two of the major CICR receptor activated systems. While the effect of LEV on Ca^{2+} dynamics in an in vitro model of acquired epilepsy remains to be determined, Ca^{2+} is a major second messenger and has been implicated in neurotoxicity, neuronal plasticity and the maintenance of epilepsy [25], [31] thus, the ability of LEV to inhibit CICR may prove to be an important property of this drug.

The experiments performed in this study were designed to test whether LEV was able to inhibit CICR and to evaluate the mechanisms mediating its effects on these systems, as dysregulation of RyR and IP3R has been associated with neurotoxicity, neuronal plasticity, and epilepsy

[25]. To study the ryanodine system, caffeine was employed because it has been demonstrated that it functions similarly on RyR as nanomolar concentrations of ryanodine with the added benefit of faster kinetics and the ability to quickly reverse its effects by washing [24]. Non-physiologic concentrations of caffeine (in the millimolar range) are utilized in studies evaluating RyR-mediated Ca^{2+} release and the Ca^{2+} response elicited has been well-characterized [35],[13],[1]. At sub-millimolar doses, the actions of caffeine are dependent on cytosolic concentrations of Ca^{2+} , whereas, at concentrations greater than 5 mM, caffeine causes a Ca^{2+} independent activation of the ryanodine receptor [18]. Thus, using caffeine is an established method to activate RyR-mediated Ca^{2+} release [35],[13],[1]. One study employed caffeine in a hippocampal slice model to suggest that LEV reduces caffeine-mediated Ca^{2+} transients [1]. In studying IP3R-mediated CICR, lack of commercially available membrane-permeable IP3 makes bradykinin an ideal choice to study this system because it has been demonstrated that it stimulates Ca^{2+} release through IP3 stores [28]. One study in PC12 cells demonstrated that LEV was able to reduce bradykinin-induced Ca^{2+} transients [4]. However, no studies have investigated the effect of LEV on both IP3 and RyR in the highly relevant hippocampal culture model. Conducting these studies in hippocampal cultures is of utility because networks of hippocampal neurons have been used as models to study AE [33],[10].

Our results supplement the current literature aimed at elucidating the mechanism of action of LEV [30],[40],[34]. Currently, little is known regarding the mechanism behind the anti-epileptic properties of LEV. Studies have demonstrated that while LEV does not exhibit any direct action on GABAergic transmission and does not show affinity for GABAergic or glutamatergic receptors, it does induce a change in GABA turnover in the striatum [27],[23],[9]. Other studies have focused on biochemical alterations induced by LEV and have found that it decreases levels of the amino acid taurine, a low affinity agonist for GABA_A receptors, in the hippocampus but does not elicit changes in other amino acids [34].

The most promising mechanistic hypothesis explaining the actions of LEV has come from experiments that suggest that the action of LEV is presynaptic, as it binds to the presynaptic membrane protein, SV2A [22]. SV2A is ubiquitously expressed in the brain [15], yet little is known regarding the effects of the interaction between SV2A and LEV. It appears that SV2A is involved in controlling exocytosis of neurotransmitter-containing vesicles [7],[39]. More recently, it has been demonstrated that LEV prevents GABA-A current run-down [30]; thus, increasing inhibitory tone. Interestingly, it has been observed that increases in cytoplasmic Ca^{2+} triggered by caffeine-induced Ca^{2+} release from intracellular pools depress the GABA-A response [11]. In light of our studies, it will be interesting to study whether the ability of LEV to inhibit RyR-mediated CICR contributes to the inhibition of GABA-A current run-down following treatment with LEV.

Studying the effect of LEV on CICR mechanisms is also important, since alterations in $[\text{Ca}^{2+}]_i$ have been associated with many of the second messenger effects of Ca^{2+} [3] and have been shown to play an important role in underlying neurotoxicity, neuronal plasticity, and the development of AE [25],[31]. Moreover, the neuroprotective properties of LEV have been observed in several different models [37],[17],[38]. Following neuronal injury, excitotoxic glutamate release has been associated with increased stimulation of NMDA receptors and the massive influx of Ca^{2+} into the cell [5],[10],[6]. This influx of Ca^{2+} triggers CICR and this irreversible Ca^{2+} overload has been linked to cell death [19],[26],[3]. Agents that can reduce these elevations in $[\text{Ca}^{2+}]_i$ have the potential to be neuroprotective [25] and thus, the findings in this study may shed light on a new application for LEV.

This study demonstrates that LEV is able to significantly inhibit Ca^{2+} release through both the RyR and IP3R systems. The ability of LEV to modulate the two major CICR systems demonstrates an important molecular effect of this agent on a major second messenger system

in neurons. Further studies on the effects of LEV may offer insight into novel therapeutic uses for this agent and may offer further opportunities to study Ca^{2+} -mediated epileptogenesis, neuronal plasticity and neurotoxicity.

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Fig. 1. LEV has no effect on baseline $[Ca^{2+}]_i$ in cultured hippocampal neurons

Hippocampal neurons loaded with Ca^{2+} indicator, Fura-2, were treated for 20 minutes with vehicle (control) or LEV (1, 10, 33, and 100 μ M). No significant difference in 340/380 ratio was observed between vehicle and drug treated cells at all concentrations tested. $n=9, 3, 3, 7, 5$ (Vehicle, 1, 10, 33, 100 μ M LEV, respectively) plates with ~15-20 cells imaged per plate.

Fig. 2. LEV inhibits caffeine induced CICR in cultured hippocampal neurons

A, representative time course of Ca^{2+} 340/380 ratios with baseline normalized to zero before and after 10 mM caffeine challenge in control ($n=74$) and LEV pre-treated ($n=88$) cells. 5 s intervals showing ratios 25 s before and 55 s following the addition of caffeine are displayed. Caffeine elicits an increase in basal Ca^{2+} , this increase is diminished in cells pre-treated with LEV (33 μM). **B**, 340/380 ratio images of representative cell before and after caffeine in both control and LEV-treated group. **C**, LEV inhibits height of caffeine-induced Ca^{2+} transients by 61.50%. An increase of 0.328 ± 0.0183 from the baseline was observed in control neurons post-caffeine; whereas, upon caffeine stimulation cells pre-treated with LEV showed a change from baseline of 0.126 ± 0.0229 . $n=5$, control and $n=6$, LEV group, with ~ 15 cells imaged per plate ($p=0.0002$).

Fig. 3. LEV inhibits bradykinin induced Ca^{2+} transients in hippocampal neuronal cultures

A, representative time course of Ca^{2+} 340/380 ratios with baseline normalized to zero before and after 1 μM bradykinin challenge in control (n=119) and LEV-treated (n=82) cells. 5 s intervals showing ratios 20 s before and 50 s following the addition of bradykinin are displayed. Bradykinin elicits an increase in basal Ca^{2+} , this increase is diminished in cells pre-treated with LEV (100 μM). **B**, 340/380 ratio images of representative cell before and after bradykinin in both control and LEV-treated group. **C**, LEV inhibits height of bradykinin-induced Ca^{2+} transients by 74.35%. An increase of 0.269 ± 0.063 from the baseline was observed in control neurons post-bradykinin; whereas, upon bradykinin stimulation cells pre-treated with LEV showed a change from baseline of 0.069 ± 0.032 . n=7 (control), n=5 (LEV group) with ~15 cells imaged per plate ($p < 0.05$).