The Antiepileptic Drug Levetiracetam Decreases the Inositol 1,4,5-Trisphosphate-Dependent [Ca\(^{2+}\)]\(_i\) Increase Induced by ATP and Bradykinin in PC12 Cells

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ABSTRACT

The present study explores the hypothesis that the new antiepileptic drug levetiracetam (LEV) could interfere with the inositol 1,4,5-trisphosphate (IP\(_3\))-dependent release of intracellular Ca\(^{2+}\) initiated by G\(_\text{q}\)-coupled receptor activation, a process that plays a role in triggering and maintaining seizures. We assessed the effect of LEV on the amplitude of [Ca\(^{2+}\)]\(_i\) response to bradykinin (BK) and ATP in single Fura-2/acetoxymethyl ester-loaded PC12 rat pheochromocytoma cells, which express very high levels of LEV binding sites. LEV dose-dependently reduced the [Ca\(^{2+}\)]\(_i\) increase, elicited either by 1 \(\mu\)M BK or by 100 \(\mu\)M ATP (IC\(_{50}\), 0.39 ± 0.01 \(\mu\)M for BK and 0.20 ± 0.01 \(\mu\)M for ATP; Hill coefficients, 1.33 ± 0.04 for BK and 1.38 ± 0.06 for ATP). Interestingly, although the discharge of ryano dine stores by a process of calcium-induced calcium release also took place as part of the [Ca\(^{2+}\)]\(_i\) response to BK, LEV inhibitory effect was mainly exerted on the IP\(_3\) dependent stores. In fact, the drug was still effective after the pharmacological blockade of ryodine receptors. Furthermore, LEV did not affect Ca\(^{2+}\) stored in the intracellular deposits since it did not reduce the amplitude of [Ca\(^{2+}\)]\(_i\) response either to thapsigargin or to ionomycin. In conclusion, LEV inhibits Ca\(^{2+}\) release from the IP\(_3\) sensitive stores without reducing Ca\(^{2+}\) storage into these deposits. Because of the relevant implications of IP\(_3\) dependent Ca\(^{2+}\) release in neuron excitability and epileptogenesis, this novel effect of LEV could provide a useful insight into the mechanisms underlying its antiepileptic properties.

Levetiracetam (LEV), the S-enantiomer of \(\alpha\)-ethyl-2-oxo-1-pyrroolidine acetamide, is a new antiepileptic drug (AED) with a broad-spectrum antiepileptic potential, a good tolerability, and a low propensity for pharmacokinetic drug interactions (Dooley and Plusker, 2000). Because of these pharmacological properties, LEV is becoming more and more popular among clinical neurologists. Noticeably, since its approval in 1999 for the add-on therapy of drug-resistant partial seizures in adults, almost 500,000 patients have been treated with this drug (http://www.Keppra.com).

Despite its widespread use in the treatment of epilepsy, the mechanisms underlying the antiseizure effect of LEV still remain to be fully elucidated (Margineanu and Klitgaard, 2002). However, it is well established that the drug binds in a reversible and stereoselective way to specific binding sites found in the brain and in neuronal cell lines, including rat pheochromocytoma PC12 cells (Fuks et al., 2003), and recently identified as the synaptic vesicle protein SV\(_{2A}\) (Lynch et al., 2004). The functional consequences of this binding remain, however, currently unclear. LEV, in fact, does not seem to interfere with the AED classical targets in any significant way. It does not affect the activity of voltage-dependent Na\(^+\) channels or T-type voltage-gated Ca\(^{2+}\) channels (Zona et al., 2001) while exerting a moderate inhibition of high voltage-activated Ca\(^{2+}\) channels (Lukyanetz et al., 2002). Furthermore, LEV merely decreases GABA\(_A\) receptor sensitivity to Zn\(^{2+}\) and \(\beta\)-carbolines (Rigo et al., 2002), without displaying any conventional GABA-enhancing activity (Margineanu and Klitgaard, 2003).

ABBREVIATIONS: LEV, levetiracetam; AED, antiepileptic drug; IP\(_3\), inositol 1,4,5-trisphosphate; BK, bradykinin; AM, acetoxymethyl ester; TG, thapsigargin; AUC, area under the curve; ANOVA, analysis of variance; Rya, ryano dine; Iono, ionomycin; CICR, calcium-induced calcium release; RyR, ryano dine receptor.
These findings suggest that the mechanisms of action underlying LEV’s antiseizure effect are unrelated to those of classical AEDs. Therefore, given the considerable interest accruing around the identification of new pharmacological targets exploitable for the treatment of seizures unresponsive to conventional AEDs, a better understanding of the pharmacodynamic properties of LEV has become of significant importance for clinical neurology.

Actually, a number of molecular factors involved in epileptogenesis but currently orphan of specific drug approaches have already been identified. This is the case of the signaling cascade triggered by Gq-coupled receptor activation involving the IP3-dependent Ca2+ release from intracellular Ca2+ stores. Indeed, the intracellular injection of IP3 triggers epileptiform discharges in hippocampal neurons (Jin et al., 2000), and IP3-dependent release of Ca2+ ions from the intracellular stores is regarded as a major factor responsible for the consistent elevation of [Ca2+]i observed in epileptic neurons (Pal et al., 2001). Furthermore, since Honchar et al. (1983) first reported that the systemic administration of cholinergic agents induces seizures in lithium-treated rats, it has been firmly established that Gq-coupled M1 muscarinic receptor activation is responsible for the convulsant effects of centrally acting muscarinic drugs (Bymaster et al., 2003). Similarly, glutamate proconvulsant activity can be in part ascribed to the activation of Gq-coupled group I metabotropic receptors because their specific agonist (R,S)-3,5-dihydroxyphenylglycine induces limbic seizures attenuated by dantrolene, an inhibitor of calcium release from intracellular stores (Tizzano et al., 1995).

Ca2+ ion release from the IP3-sensitive intracellular stores is also triggered by neuropeptides involved in seizure generation. This is the case of bradykinin (BK), which, as suggested by the proconvulsant activity of its agonists (Bregola et al., 1999) and by the changes in its receptor density occurring in epilepsy (Arganaraz et al., 2004), could be involved in epileptogenesis.

The therapeutic potential of blocking intracellular Ca2+ release in epilepsy is exemplified by the suppression of bicuculline-induced epileptic bursting occurring after the deple-
tion of intracellular Ca$^{2+}$ stores with the Ca$^{2+}$-ATPase inhibitor thapsigargin (TG) (Wulfert and Margineanu, 1998), and by the marked reduction of pilocarpine-induced ictal discharges induced by dantrolene and TG (Hadar et al., 2002).

The present study explores the possibility that LEV could inhibit Ca$^{2+}$ ion release from the IP$_{3}$-sensitive stores. To address this hypothesis, we studied, by means of single-cell Fura-2 video imaging, the effect of LEV on the [Ca$^{2+}$], response to BK challenge are shown in the panel. B, concentration dependence curve of LEV-induced reduction in [Ca$^{2+}$], response to BK expressed as $\Delta \%$ increase above baseline [Ca$^{2+}$]. The curve reports, as a function of LEV concentration, LEV-induced reduction in [Ca$^{2+}$], increase elicited by 1 $\mu$M BK. The data were calculated as $\Delta \%$ increase above baseline [Ca$^{2+}$]. The values, obtained for each LEV concentration, were expressed as percentage of mean $\Delta \%$ in control cells ($n = 149$). Each data point is the mean $\pm$ S.E.M. of the data obtained in all the cells treated with each LEV concentration tested in four different experimental sessions. The number of the tested cells was: 148, 108, 108, 152, and 78 in control, 0.1, 0.3, 1, and 10 $\mu$M LEV groups. C, concentration dependence curve of LEV-induced reduction in [Ca$^{2+}$], response to BK expressed as [Ca$^{2+}$], AUC. The curve reports, as a function of LEV concentration, LEV-induced reduction in [Ca$^{2+}$], response to 1 $\mu$M BK expressed as AUC. AUC was calculated by integrating [Ca$^{2+}$], over a 100-s time period, after the BK challenge, and expressed as [Ca$^{2+}$], (nanomolar) per second. For each LEV concentration, AUCs were expressed as percentage of mean AUCs in control cells ($n = 151$). The number of tested cells was: 151, 106, 108, 149, and 76 in control, 0.1, 0.3, 1, and 10 $\mu$M LEV groups. Each data point is the mean $\pm$ S.E.M. of the data obtained in all the cells treated with each LEV concentration tested in four different experimental sessions. Concentration dependence curves were fitted as reported under Materials and Methods.

Materials and Methods

Cell Culture. Undifferentiated PC12 rat pheochromocytoma cells (courtesy of Dr. L. Colucci d’Amato, National Research Council (CNR) Institute of Endocrinology and Experimental Oncology, Naples, Italy) were grown in plastic dishes in RPMI medium supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. NIH-3T3 cells (courtesy of Dr. C. Miele, Department of Biology and Cellular and Molecular Pathology, Federico II University of Naples, Naples, Italy) were cultured in plastic dishes of Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin.

Cells were cultured in a humidified 5% CO$_2$ atmosphere; the culture medium was changed every 2 days. For microfluorometric studies, the cells were plated on glass coverslips (Fisher Scientific Co., Pittsburgh, PA) coated with poly-l-lysine (30 $\mu$g/ml) (Sigma-Aldrich, St. Louis, MO) and were used at least 12 h after seeding. All PC12 cell experiments were performed at a cell culture passage between 10 and 25.

[Ca$^{2+}$], Measurements. [Ca$^{2+}$], was measured by single-cell computer-assisted videoimaging (Cataldi et al., 1996). Briefly, PC12 or NIH-3T3 cells, grown on glass coverslips, were loaded with 5 $\mu$M Fura-2/AM for 1 h at room temperature in Krebs-Ringer saline solution containing the following: 5.5 mM KCl, 160 mM NaCl, 1.2 mM MgCl$_2$, 1.5 mM CaCl$_2$, 10 mM glucose, and 10 mM Hepes-NaOH, pH 7.4. At the end of the Fura-2/AM loading period, the coverslips were placed into a perfusion chamber (Medical System, Co., Greenvale, NY) mounted onto the stage of an inverted Nikon Diaphot fluorescence microscope (Nikon, Melville, NY). A 100-W xenon lamp
Levetiracetam Effect on IP<sub>3</sub>-Dependent Ca<sup>2+</sup> Release

**Fig. 3.** LEV effect on [3H]BK binding to PC12 cells. A, [3H]BK binding to PC12 cells. The panel shows the mean ± S.E.M. values of total and nonspecific radioactivity bound to PC12 cell after a 90-min incubation on ice with different concentrations of [3H]BK. All the points reported have been obtained in duplicate, and the curve shown relates to one of two different experimental sessions performed with similar results. As detailed under Materials and Methods, curve fitting of total radioactivity was performed using the equation \( B = x \cdot B_{\text{max}}/(K_d + x) + x \cdot N \), where \( N \) is the nonspecific binding and \( x \) the concentration of free ligand whereas linear fitting was used in the case of nonspecific binding. B, effect of LEV on [3H]BK binding on PC12 cells. The bar plot reports the mean ± S.E.M. of total radioactivity bound on PC12 cells after a 90-min incubation on ice with 6 nM [3H]BK in the presence or in the absence of 1 μM LEV expressed as percentage of control values. LEV was added to the assay buffer simultaneously with [3H]BK. The data shown have been obtained in duplicate and relate to one experimental session of three performed with similar results.

(Osram, Berlin, Germany) with a computer-operated filter wheel, bearing two different interference filters (340 and 380 nm), illuminated the microscopic field with UV light every 3 s, alternating the wavelengths at an interval of 500 ms. The light emitted by Fura-2-loaded cells was passed through a 400-nm dichroic mirror filtered at 510 nm and finally collected with an intensified camera (Photonic Science, Robertsbridge, UK). Images were digitized and analyzed with a Magiscan image processor (Applied Imaging Ltd., Dukesway, UK) driven by the AUTOLAB software (RBR Altair, Florence, Italy).

Ratiometric values were automatically converted by the software into [Ca<sup>2+</sup>], using a preloaded calibration curve obtained in preliminary experiments (Cataldi et al., 1996). No significant overlap with Fura-2 absorption or emission was observed when the spectra of LEV were obtained using a PerkinElmer LS 50B (PerkinElmer Life and Analytical Sciences, Boston, MA) spectrofluorometer.

To test the effect of LEV on [Ca<sup>2+</sup>], increase elicited by the different drugs used in this study, the cells were incubated for 5 min with increasing concentrations of LEV or vehicle dissolved in a 1.5 mM Ca<sup>2+</sup>-containing Krebs’ solution. Then, 5 ml of a 1.5 mM EGTA-containing Ca<sup>2+</sup>-free Krebs’ solution with LEV (or vehicle) and the appropriate drug were quickly injected into the recording chamber. The time required for injection ranged around 5 s.

**Data Calculation.** [Ca<sup>2+</sup>], responses to the different pharmacological challenges used in the study were measured both as change in percentage increase above baseline [Ca<sup>2+</sup>], and as area under the curve (AUC).

For [Ca<sup>2+</sup>], we used the maximal value of [Ca<sup>2+</sup>] attained after stimulation and, as baseline [Ca<sup>2+</sup>], the mean of [Ca<sup>2+</sup>], recorded during the 200 s preceding the challenge.

AUC was calculated by integrating [Ca<sup>2+</sup>], after the pharmacological challenge and was expressed as [Ca<sup>2+</sup>], (nanomolar) per second. Given the different duration of the [Ca<sup>2+</sup>] responses to the different challenges, different times of integration (reported in the figure legends) were used to assess the responsiveness to different drugs. AUCs were calculated using the area below the curve macro of the Sigma plot 8.0 software, which performs integrations using the trapezoidal rule. Both 3% and AUCs were normalized to the mean of the respective controls and expressed as percent values.

Dose-effect curves were obtained by fitting the data to the four parameter logistic equation \( y = \min + (\max - \min)/(1 + (x/IC_{50})^n) \), where \( y \) is the normalized value of 3% increase in [Ca<sup>2+</sup>], or [Ca<sup>2+</sup>], AUC, \( x \) is the LEV concentration in micromoles per liter, and \( n \) is the Hill coefficient. Data fitting was performed using the fitting routines of the Sigma Plot 8.0 software (SPSS Inc., Chicago, IL).

[3H]Bradykinin Binding and Displacement Assays. [3H]BK binding to BK plasma membrane receptors in PC12 cells and the potential interference of LEV with this binding reaction were studied using the experimental approach reported by Nardone et al. (1994). Briefly, PC12 cells were plated onto 12-well plastic dishes at the density of 1 × 10<sup>6</sup> cells/well. Twenty four hours after seeding, the culture medium was removed and replaced with 2 ml of an ice-cold assay buffer containing the following: 137.5 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM glucose, 10 mM Hepes, 1 mM bacitracin, and 0.01 mM captopril. The binding reaction was started replacing this solution with 500 μl of the same buffer supplemented with different concentrations of [3H]BK (PerkinElmer Life and Analytical Sciences; specific activity 90 Ci/mM) with or without cold BK or LEV as potential interference of LEV with this binding reaction were studied using the experimental approach reported by Nardone et al. (1994).

Binding assays were obtained using a Magiscan image processor (Applied Imaging Ltd., Dukesway, UK) driven by the AUTOLAB software (RBR Altair, Florence, Italy).

**Statistical Analysis of the Data.** All data are reported as mean ± S.E.M. When comparing two data sets, the Student’s t test for unpaired data was used. To compare multiple groups, we assessed the normal distribution of the data with the Bartlett test. Statistical comparisons were performed, for normally distributed data, with ANOVA followed by the Tukey-Kramer post hoc test and, for non-normally distributed data, with the Kruskal-Wallis nonparametric ANOVA followed by the Dunn’s multiple comparison test. The
threshold for statistical significance was set at $P < 0.01$. Statistical comparisons were carried out using the Graph-PAD 2.04 software suite (GraphPad Software Inc., San Diego, CA).

**Drugs and Chemicals.** LEV was kindly provided by UCB Pharma (Braine-l’Alleud, Belgium). It was dissolved in water as a 100 mM stock solution and kept frozen in 50-μl aliquots at −20°C until use. Culture media, horse and fetal calf sera, and antibiotics were purchased from Invitrogen (Carlsbad, CA). Fura-2/AM, iodoacetate acid, and bacitracin were obtained from Calbiochem (San Diego, CA). BK, ATP, Ryanodine (Rya), TG, Ionomycin (Iono), and all other chemicals were from Sigma-Aldrich.

**Results**

**LEV Does Not Modify Basal $[Ca^{2+}]_{i}$ in Undifferentiated PC12 Cells.** To assess whether LEV acutely affects basal $[Ca^{2+}]_{i}$, we continuously monitored $[Ca^{2+}]_{i}$ in single Fura-2-loaded PC12 cells before and after the addition of different concentrations of this drug to the extracellular solution. In particular, after 300 s of basal $[Ca^{2+}]_{i}$ monitoring, LEV (0.1–10 μM) was added to the recording chamber, and $[Ca^{2+}]_{i}$ was fairly stable all over the observation period both before and after the addition of LEV, and at all the concentrations tested, the drug failed to induce any change in $[Ca^{2+}]_{i}$ (control versus LEV mean basal $[Ca^{2+}]_{i}$, 114.9 ± 4.1 versus 117.3 ± 3.6 nM in the 0.1 μM LEV group, $n = 79$; 115.1 ± 1.7 versus 116.9 ± 1.6 nM in the 0.3 μM LEV group, $n = 79$; 114.7 ± 3.5 versus 115.6 ± 2.6 nM in the 1 μM LEV group, $n = 83$; 113.5 ± 4.5 versus 113.9 ± 2.6 nM in the 10 μM LEV group, $n = 72$) (Fig. 1, A–D).
LEV Decreases the [Ca\(^{2+}\)], Response to BK and ATP in PC12 Cells But Not in NIH-3T3 Fibroblasts. To determine whether LEV affects the [Ca\(^{2+}\)], response to IP\(_3\)-linked agonists, we examined the effect of this drug on the [Ca\(^{2+}\)], response evoked in PC12 cells by BK and ATP, two neurotransmitters whose IP\(_3\)-coupled receptors are expressed in this cell line (Nardone et al., 1994; Moskvina et al., 2003). ATP and BK were delivered to PC12 cells in a nominally Ca\(^{2+}\)-free Krebs' solution obtained by omitting Ca\(^{2+}\) ions and by adding 1.5 mM EGTA. This approach prevented the confounding effect of the influx of extracellular Ca\(^{2+}\) ions. Such phenomenon could be due to two different mechanisms: the first could entail the activation of the so-called plasma membrane refilling channels, which open when the intracellular calcium stores are depleted; the second, in the case of ATP, could involve the opening of the ionotropic P2X receptors, which are also expressed in PC12 cells. A potential drawback of exposing cells to a Ca\(^{2+}\)-free medium is the slow emptying of intracellular Ca\(^{2+}\) stores determined by the lack of Ca\(^{2+}\) ions in the extracellular solution and by the presence of strong Ca\(^{2+}\) ion chelators. To prevent this undesired effect, the cells were kept in a Ca\(^{2+}\)-containing Krebs' solution until they were challenged with BK or ATP in the Ca\(^{2+}\)-free Krebs' solution.

In control cells, 1 μM BK induced a rapid increase in [Ca\(^{2+}\)]\(_i\) that reached a peak of 231 ± 11 nM (n = 149) in approximately 10 s and returned to baseline in approximately 100 s (Fig. 2A).

When the cells were exposed to increasing concentrations of LEV (ranging from 0.1–10 μM), a concentration-dependent inhibition of this response was observed (Fig. 2, B and C). LEV-induced decrease in [Ca\(^{2+}\)]\(_i\) response was fitted with the four-parameter logistic Hill equation as described under Materials and Methods. When the inhibition was quantified as percentage decrease in the Δ%, an IC\(_{50}\) of 0.39 ± 0.01 μM and a Hill coefficient of 1.33 ± 0.04 were estimated (Fig. 2B). This Hill coefficient value suggests that LEV interacts with a single binding site. Similar results were obtained when we considered the drug-induced percentage decrease in [Ca\(^{2+}\)]\(_i\), AUC. The IC\(_{50}\) was 0.46 ± 0.01 μM, and the Hill coefficient was 1.14 ± 0.01 (Fig. 2C).

To determine whether this LEV-induced decrease in [Ca\(^{2+}\)]\(_i\), response to BK was determined by an interference with the binding of this peptide to its plasma membrane receptors, we performed displacement studies aiming to assess the ability of LEV to compete with [\(^{3}\)H]BK for its binding sites on PC12 cells. As already reported by Nardone et al. (1994), [\(^{3}\)H]BK displayed a specific binding to PC12 cells, and the [\(^{3}\)H]BK binding curve was adequately fitted under the assumption of a single homogeneous population of plasma membrane receptors with a K\(_d\) of 5.1 nM (Fig. 3A). When LEV was added to the assay buffer in a concentrations of 1 μM that is effective in submaximally reducing the BK-induced [Ca\(^{2+}\)]\(_i\), response, no change in the binding of [\(^{3}\)H]BK (6 nM) to PC12 cells was observed (Fig. 3B).

These results suggested that LEV effect on BK-induced intracellular Ca\(^{2+}\) release was not the mere consequence of a drug-induced displacement of this G\(_{q}\)-coupled ligand from its receptors. To further substantiate the idea that the ability of LEV to interfere with [Ca\(^{2+}\)]\(_i\), homeostasis was a more general phenomenon not restricted to BK receptor pharmacology, we explored the effect of this drug on [Ca\(^{2+}\)]\(_i\), responsiveness to another G\(_{q}\)-coupled agonist with a completely different chemical structure, the nucleotide ATP. To this aim, we used an experimental approach similar to that used in the case of BK. In control PC12 cells, ATP (100 μM) markedly increased the [Ca\(^{2+}\)]\(_i\), that peaked at 241.7 ± 3.7 nM (n = 300) approximately 10 s after the challenge (Fig. 4A). Interestingly, although this [Ca\(^{2+}\)]\(_i\), peak response was not different from that evoked by BK, the duration of [Ca\(^{2+}\)]\(_i\), increase...
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It was reported that LEV was effective in inhibiting Ca2+
educed calcium release (CICR) process (Reber et al., 1993). Therefore, to determine the extent of their involvement in
our experimental system, we compared the amplitude of the
[Ca2+]i response elicited by 1 μM BK in PC12 cells whose ryanodine
receptors (RyRs) had been blocked by an overnight exposure
of Rya stores in neurons (Angehagen et al., 2003),
response triggered by the activation of G rodz-coupled receptors
because they are activated in the context of a calcium-
derived inhibition of ATP-evoked [Ca2+]i response
in PC12 cells whose ryanodine
receptors (RyRs) had been blocked by an overnight exposure
to 100 μM Rya with that of control cells treated only with
vehicle. As expected, the treatment with Rya significantly
decreased cell responsiveness to BK. The Δ% increase in
[Ca2+]i elicited by this peptide dropped, indeed, from 121.5
+ 5.8% in control cells (n = 90) to 62.1 + 3.9% in cells exposed
overnight to 100 μM Rya (n = 84) (P < 0.01; unpaired
Student’s t test). Noticeably, Rya not only reduced the amplitude of BK-elicited [Ca2+]i response but also significantly
shortened its duration (Fig. 6A).

Since LEV was reported to inhibit Ca2+
educed calcium release from the intracellular Rya stores in neurons (Angehagen et al., 2003),
we tested whether its effect on the intracellular Ca2+
educed calcium release induced by IP3-generating agonists was due to an inhibition of the CICR that accompanies IP3-sensitive deposit dis-
charges. To this aim, we studied LEV (1 μM) effect on the
[Ca2+]i response elicited by 1 μM BK after blocking RyR with
an overnight exposure to Rya (100 μM) reasoning that, if
LEV acted exclusively at the level of the Rya stores then, it
should have been ineffective after the pharmacological block-
ad of RyR. Contrary to this hypothesis, LEV was effective in
reducing the [Ca2+]i response to BK in Rya-treated cells [Δ% 41.4 + 3.4 versus 62.1 + 3.9% (P < 0.01); AUC, 30.5 + 3.3

panel, report the mean [Ca2+]i response to 50 mM caffeine in all the cells of the control (n = 106) and LEV (n = 106) groups. The top of the panel expresses Δ% increase above baseline [Ca2+]i, whereas the bottom expresses the AUC. AUC and Δ% data are expressed as percentage of the respective mean values obtained in the control group. For AUC calculation, an integration time of 30 s was used because of the short duration of caffeine-elicited [Ca2+]i response.
versus 46.7 ± 3.5 nM/s (P < 0.01) in cells treated with LEV (n = 100) versus controls (n = 84)) (Fig. 6A). These data suggest that, under our experimental conditions, LEV at low micromolar concentrations affects the IP₃-dependent stores but not the Rya-sensitive deposits. To verify the inefficacy of LEV, we directly examined the effect of 1 μM LEV on a kind of [Ca²⁺], response that is mediated exclusively by these deposits, using the Rya store depletor caffeine (50 mM). As expected, LEV was unable to modify the [Ca²⁺], response to 50 mM caffeine [Δ%, 121.5 ± 3.9 versus 131.2 ± 3.2%, N.S.; AUC, 58.5 ± 1.6 versus 54.2 ± 2.2 nM/s, N.S., respectively, in control (n = 84) and in 1 μM LEV-treated (n = 100) cells] (Fig. 6B).

LEV Increases [Ca²⁺], Response to the Two Ca²⁺-free Krebs' solution, the intracellular Ca²⁺ stores were markedly depleted, as revealed by the consistent increase in [Ca²⁺], that peaked at 185 ± 2.7 nM (n = 94) in response to TG and at 228.9 ± 1.9 nM (n = 171) in response to Iono (Fig. 7, A and B). As expected, the duration of [Ca²⁺], response was significantly longer in response to TG ranging around 200 s than to Iono ranging around 30 s.

In cells treated with 1 μM LEV for 5 min, before the TG or the Iono challenge and exposed to LEV throughout the entire stimulation, the [Ca²⁺], response to these store-depleting agents was significantly enhanced. Indeed, in the case of TG, the Δ% increases were 46.38 ± 1.2 versus 36.56 ± 1.3% in cells exposed to LEV (n = 71) and in control cells (n = 93), respectively (P < 0.01; unpaired Student's t test), whereas in the same groups, the AUCs were 25.7 ± 0.9 versus 36.56 ± 1.3 nM/s (P < 0.01; unpaired Student's t test) (Fig. 7A). When the cells were challenged with 1 μM Iono with or without 1 μM LEV, the Δ% increases in [Ca²⁺], above baseline were 65.95 ± 1.3% versus 55.8 ± 1.2% in cells exposed to LEV (n = 191) and in control cells (n = 171) (P < 0.01; unpaired
The major finding of the present study is that LEV reduces the IP₃-dependent [Ca²⁺]ᵢ increase elicited by the activation of Gₛ-coupled neuropeptide and neurotransmitter receptors. This conclusion is supported by the evidence that in PC12 pheochromocytoma cells, LEV reduced, in a concentration-dependent manner, the [Ca²⁺]ᵢ increase elicited by BK and ATP, two neurotransmitters that, in these cells, trigger IP₃ generation via the Gₛ-coupled B₂ (Nardone et al., 1994) and P₂Y (Moskvina et al., 2003) receptors. Actually, several arguments sustain the hypothesis that, under our experimental conditions, these responses depended on IP₃-triggered Ca²⁺ store depletion rather than on Ca²⁺ influx from the extracellular space or Ca²⁺ release from the Rya stores. Specifically, BK and ATP challenges were purposely delivered in a Ca²⁺-free solution containing EGTA (1.5 mM) so as to render extracellular Ca²⁺ unavailable for influx. By using this approach, we excluded that Ca²⁺ could enter into the cells either through the ionotropic P₂X ATP receptors, which are also expressed in PC12 cells (Moskvina et al., 2003), or through the so-called refilling channels, which are expected to open in response to the store depletion induced by BK or ATP.

Because Rya channels open during the CICR process whenever [Ca²⁺]ᵢ significantly increases (Verkhratsky and Shmigol, 1996), it was crucial to establish whether in our system these intracellular Ca²⁺ deposits played any role in determining the intracellular Ca²⁺ response to IP₃-generating agonists. Accordingly, to obtain a pharmacological ablation of the intracellular Rya stores, we used the plant alkaloid Rya, for it irreversibly blocks RyRs whenever used at high micromolar concentrations (Verkhratsky and Shmigol, 1996). Since RyR blockade induced a consistent decrease in [Ca²⁺]ᵢ response to BK, we evinced that CICR played a relevant role in the intracellular Ca²⁺ increase elicited by BK as already reported by Reber et al. (1993). Because of the important contribution of Rya stores in the [Ca²⁺]ᵢ response to BK in PC12 cells, it was essential to determine whether LEV was acting directly on the IP₃-dependent Ca²⁺ release or whether it was, instead, reducing CICR. In this regard, we should consider that, in hippocampal neurons, LEV significantly reduces Ca²⁺ release from the Rya stores in response to caffeine (Angehagen et al., 2003). However, under our experimental conditions, LEV did not act at the Rya store level. In fact, its inhibitory effect persisted in PC12 cells whose RyR had been blocked with high micromolar concentrations of Rya. Furthermore, low micromolar LEV concentrations proved to be ineffective in reducing the [Ca²⁺]ᵢ response to caffeine, whereas at high micromolar concentrations, the drug determined a small but significant decrease in this response (data not shown). This is in accordance with the data reported by Angehagen et al. (2003) who found that in hippocampal neurons, LEV did interfere with Ca²⁺ release from the Rya stores only at concentrations higher than 10 μM being ineffective in the low micromolar range.

LEV is not the first antiepileptic drug to be proposed as an inhibitor of IP₃-dependent intracellular Ca²⁺ release. In fact, Imazawa et al. (1989) demonstrated that phenytoin, phenobarbital, and carbamazepine displayed a modest ability to block IP₃-induced calcium release from microsomal fractions in vitro. However, the inhibitory effect resulting from these drugs is smaller than that exerted by LEV. Most likely, the contribution of this effect on the antiseizure efficacy of these drugs is only marginal. Indeed, these drugs, contrary to LEV, potently affect voltage-dependent channels and GABAₐ receptors, and this accounts for their antiepileptic properties.

The mechanism of action through which LEV reduces IP₃-dependent intracellular Ca²⁺ release is still unclear. A direct competitive blockade of Gₛ-coupled receptors by LEV seems an unlikely explanation of our data for at least two different reasons. First, LEV was effective in reducing the intracellular Ca²⁺ response elicited by two structurally unrelated Gq-agonists, the peptide BK and the cyclic nucleotide ATP; second, it was not able to displace [³H]BK from its binding sites on PC12 cells. This points to an action on Ca²⁺ discharge from IP₃ stores exerted somewhere downstream the receptors. Several studies suggest that IP₃-dependent Ca²⁺ stores are composed of different compartments selectively mobilized by specific agonists (Den Hertog et al., 1992). In particular, BK and ATP mobilize two different and functionally independent IP₃-sensitive intracellular Ca²⁺ compartments (Suh et al., 1995). Because both these compartments were affected by LEV, we may suggest that the drug's effect was not compartment-specific, but it was affecting a more fundamental step of the IP₃ cascade. For example, LEV could be acting by decreasing Ca²⁺ ion accumulation in the intracellular deposits. The study results excluded this possibility. In fact, when we determined the amount of Ca²⁺ stored in the intracellular deposits by measuring the [Ca²⁺]ᵢ response to TG and Iono, two compounds that deplete intracellular Ca²⁺ stores by acting directly on them, we found that LEV treatment did not decrease the amount of Ca²⁺ available for release from the intracellular deposits, but, on the contrary, it slightly increased it. This finding could be explained accordingly to the existing evidence on the constitutive activity of G protein-coupled plasma membrane receptors (Milligan, 2003). It can be assumed, in fact, that spontaneously active plasma membrane Gₛ-coupled receptors continuously release small amounts of Ca²⁺ ions from the IP₃-dependent stores in PC12 cells and that LEV could increase the amount of Ca²⁺ retained in the intracellular deposits by inhibiting this Ca²⁺ leak. Clearly, the increased availability of Ca²⁺ for release will not be evident if the cells are challenged with a Gₛ-coupled receptor agonist, as LEV would inhibit this response. Conversely, it will be manifest if the cells are treated with compounds directly acting on the stores, such as TG or Iono. Future studies will be necessary to establish whether LEV action on Ca²⁺ release from IP₃-sensitive stores is exerted at the level of Gₛ phospholipase C, or IP₃ receptors.

LEV inhibition of IP₃-dependent intracellular Ca²⁺ release can be observed at drug concentrations in the low micromolar range that are easily reached in plasma of patients treated with LEV as mean LEV Cmax at equilibrium ranges between 30 and 300 μM (Dooley and Plosker, 2000). Hence, the effect of LEV on intracellular Ca²⁺ homeostasis reported in the present study could be important in explaining the therapeutic efficacy of this drug. Indeed, the activation of several Gₛ-coupled receptors affects neuron excitability at central synapses and probably plays a role in epileptogenesis. For
instance, presynaptic metabotropic Gα-coupled receptors depress GABA release in the hippocampus CA1 field (Gereau and Conn, 1995) and in cultured hippocampal neurons (Fitzsimonds and Dichter, 1996). Similarly, presynaptic M1 Gα-coupled receptors negatively regulate GABA release in cerebral cortex slices (Hashimoto et al., 1994). Consistent evidence also reports that metabotropic group I glutamate receptors increase cell excitability by a postsynaptic action in different brain areas, including the cortex (Libri et al., 1997) and the hippocampus (Davies et al., 1995). Analogously, muscarinic M2 and M3 receptors have a definite role in promoting cell excitability by a postsynaptic mechanism in the neocortex (Cox et al., 1994). These effects on cell excitability can account for the well known proconvulsant ability of group I metabotropic glutamate receptor (Tizzano and Schoepp, 1995) and M1 muscarinic receptor (Crickshank et al., 1994) agonists. Accordingly, Gα-coupled receptors are very likely involved in epileptogenesis so that the antiseizure effect of LEV could be ascribed, in part, to its ability to interfere with excitability increase that might contribute to epileptogenesis.

Interestingly, LEV is highly effective in preventing the kindling response in rats (Loscher et al., 1998), a process involving the activation of Gα-coupled receptors, such as group I metabotropic glutamate receptors (Greenwood et al., 2000) and M1 and M3 muscarinic receptors (Mingo et al., 1998). Furthermore, it is worth noticing that BK is also involved in this model of epilepsy (Bregola et al., 1999).

The recent identification of LEV binding sites with the presynaptic protein SV2A (Lynch et al., 2004) raises the important question of establishing whether LEV might affect [Ca2+]i homeostasis by binding to this protein. An argument supporting this idea comes from the observation that LEV was ineffective in NIH-3T3 fibroblasts, a cell type that lacks SV2A. Although a functional connection between binding to SV2A and [Ca2+]i homeostasis has not been established, the results reported by Janz et al. (1999) suggest that the binding of LEV to SV2A and [Ca2+]i homeostasis might be involved in this model of epilepsy (Bregola et al., 1999).

In conclusion, the present study provides evidence for a new pharmacological effect of the antiepileptic drug LEV: the inhibition of Ca2+ release from the IP3-sensitive stores. Because of the relevant implications of IP3-dependent Ca2+ release in neuron excitability and epileptogenesis, this effect could provide promising insights into the antiepileptic properties of LEV. More important, LEV could represent a prototype example of a new pharmacological approach to treat epilepsy by countering Ca2+ release from the intracellular stores.

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