

Epilepsy-induced decrease of L-type Ca^{2+} channel activity and coordinate regulation of subunit mRNA in single neurons of rat hippocampal ‘zipper’ slices

Eric M. Blalock^{1 a,*}, Kuey-Chu Chen^{a,1}, Thomas C. Vanaman^b,
Philip W. Landfield^a, John T. Slevin^{a,c}

^a Department of Pharmacology, College of Medicine, University of Kentucky, MS-310 UKMC, Lexington, KY 40536, USA

^b Department of Biochemistry, College of Medicine, University of Kentucky, Lexington, KY 40536, USA

^c Department of Neurology, Lexington Veterans Administration, College of Medicine, University of Kentucky, Lexington, KY 40536, USA

Received 8 June 2000; received in revised form 6 September 2000; accepted 16 October 2000

Abstract

L-type voltage-sensitive Ca^{2+} channels (VSCCs) preferentially modulate several neuronal processes that are thought to be important in epileptogenesis, including the slow afterhyperpolarization (AHP), LTP, and trophic factor gene expression. However, little is yet known about the roles of L-type VSCCs in the epileptogenic process. Here, we used cell-attached patch recording techniques and single cell mRNA analyses to study L-type VSCCs in CA1 neurons from partially dissociated (zipper) hippocampal slices from entorhinally-kindled rats. L-type Ca^{2+} -channel activity was reduced by > 50% at 1.5–3 months after kindling. Following recording, the same single neurons were extracted and collected for mRNA analysis using a recently developed method that does not amputate major dendritic processes. Therefore, neurons contained essentially full complements of mRNA. For each collected neuron, mRNA contents for the L-type pore-forming α_{1D} / $\text{Ca}_v1.3$ -subunit and for calmodulin were then analyzed by semiquantitative kinetic RT-PCR. L-type α_{1D} -subunit mRNA was correlated with L-type Ca^{2+} -channel activity across single cells, whereas calmodulin mRNA was not. Thus, these results appear to provide the first direct evidence at the single channel and gene expression levels that chronic expression of an identified Ca^{2+} -channel type is modulated by epileptiform activity. Moreover, the present data suggest the hypothesis that down regulation of α_{1D} -gene expression by kindling may contribute to the long-term maintenance of epileptiform activity, possibly through reduced Ca^{2+} -dependent AHP and/or altered expression of other relevant genes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: α_{1D} subunit; Kindling; Calmodulin; Reverse transcriptase-polymerase chain reaction; Seizures; Gene expression

* Corresponding author. Tel.: +1-859-3236134; fax: +1-859-3231981.

E-mail address: emblal@uky.edu (E.M. Blalock¹).

¹ These authors contributed equally to the present work.

1. Introduction

Extensive study of epileptogenic processes over several decades has identified a number of potentially important underlying cellular and molecular mechanisms (cf. reviews, Dingledine et al., 1990; Dudek et al., 1999; McNamara, 1999; Stafstrom et al., 1999), including sprouting and reorganization of excitatory pathways (Sutula et al., 1996; Dudek et al., 1999; McNamara, 1999; Sloviter, 1999), altered inhibitory receptor/cell function (Sloviter, 1987; Farias, et al., 1992; Kapur and Macdonald, 1997; Brooks-Kayal et al., 1998; Schwartzkroin, 1998), intense activation of NMDA receptors (Dingledine et al., 1990; Sutula et al., 1996), changes in metabotropic receptors (Wong et al., 1999) and voltage-dependent ion channels (Wong and Prince, 1981; Chamberlin and Dingledine, 1989; Crill and Schwindt, 1999), and enhanced synaptic release (Jarvie et al., 1990; Kaura et al., 1995). In addition, there is increasing evidence that seizures activate expression of neurotrophin and immediate early genes (Gall and Isaackson, 1989; Isaackson et al., 1992; McNamara, 1999; Scharfman et al., 1999), which, in turn, can increase seizure severity (Croll et al., 1999).

Thus, multiple processes appear to contribute to epileptogenesis and the maintenance of epileptiform activity. Even in the context of this diversity of candidate mechanisms, however, the roles of specific types of voltage-sensitive calcium channels (VSCCs) in epilepsy seem notably puzzling and contradictory. Deleterious mutations in several VSCC subunits (primarily P/Q-type VSCCs) are associated with seizure-prone phenotypes (Puranam and McNamara, 1999), indirectly suggesting that at least some VSCCs can suppress aspects of epileptogenesis. Conversely, pharmacologic antagonism of the L-type VSCC can reduce seizure intensity, protect neurons from excitotoxic death, or inhibit the development of seizures in the kindling model of epileptogenesis (Palmer et al., 1993; Wurpel and Iyer, 1994; Stefani et al., 1997). In addition, several studies have found that 'non-inactivating' whole-cell Ca^{2+} currents which, depending on protocol and cell type, include the slowly inactivating L-, P-, and possibly

R- or N-types (Bean, 1989; Hille, 1992; Dolphin, 1995; Dunlap et al., 1995; Tsien, 1995; Catterall, 1998) are chronically enhanced by the epileptogenic process (Vreugdenhil and Wadman, 1992, 1994; Faas et al., 1996). In contrast, others have found that kindling induces either no change or a chronic decrease in slowly inactivating whole-cell Ca^{2+} currents (Mody et al., 1990; Köhr and Mody, 1991; Karst et al., 1997, 1999). However, it remains uncertain which specific types of VSCC are modified under the different experimental conditions.

Of the multiple types of defined high-threshold VSCC types (Bean, 1989; Dunlap et al., 1995; Tsien, 1995; Catterall, 1998; Ertel et al., 2000), the L-type appears of particular interest in relation to epileptogenesis. That is, the L-type VSCC preferentially regulates a number of neuronal processes that have been implicated in seizure susceptibility, including Ca^{2+} -dependent inhibitory K^{+} conductances (Mazzanti et al., 1991; Moyer et al., 1992; Marrion and Tavalin, 1998), postsynaptic plasticity (Kapur et al., 1998; Norris et al., 1998), and neurotrophin or CREB-dependent gene expression (Gallin and Greenberg, 1995; Bito et al., 1997).

The chronic activity of L-type VSCCs also appears to be readily responsive to a range of long-term regulatory influences, as might be expected for any inducible process that plays a role in the establishment or maintenance of epileptiform activity. That is, in hippocampal neurons, L-VSCC activity is up-regulated with age both *in vivo* (Landfield et al., 1989; Moyer et al., 1992; Disterhoft et al., 1994; Thibault and Landfield, 1996; Norris et al., 1998) and *in vitro* (Porter et al., 1997) in a type-selective manner (Blalock et al., 1999). Further, this regulation appears to occur in part at the genomic level. Brain L-VSCCs contain either the $\alpha_{1C}/\text{Ca}_v1.2$ or the $\alpha_{1D}/\text{Ca}_v1.3$ pore-forming subunit (Snutch et al., 1990; Catterall et al., 1993; Ertel et al., 2000), and $\text{Ca}_v1.3$ -mRNA expression has been found both to be upregulated in the same age-dependent models that exhibit increased L-type channel density (Porter et al., 1997; Herman et al., 1998), and to correlate with L-VSCC activity in single neurons (Chen et al., 2000). Moreover, tonic activation of D2 receptors

selectively down-regulates both L-type VSCC current and Ca_v1.3-mRNA in melanotropes (Fass et al., 1999).

Here, we tested the hypothesis that L-type VSCC activity is altered by the epileptogenic process, and that such alteration is correlated with gene/mRNA expression for L-type channel subunits. We used the rat entorhinal ‘kindling’ model, which has many properties in common with human epilepsy (Jarvie et al., 1990; McNamara, 1999), to establish epileptiform activity, and used the partially-dissociated hippocampal slice preparation (also termed the ‘zipper slice’ because it opens gradually, or ‘unzips’, along the pyramidal cell body layers) for recording and collection of single neurons. This preparation was initially developed by Gray, Johnston, and colleagues to facilitate single-channel recording in adult brain neurons (Gray et al., 1990), and later adapted for channel recording in brain cells of aged rats (Thibault and Landfield, 1996). In addition, we have used the ‘zipper slice’ preparation to develop a novel method for extraction and collection of largely-intact, physiologically characterized single neurons for subsequent molecular analysis (Chen et al., 2000). Because major dendritic processes are not amputated by this cell-collection procedure, essentially the full set of mRNA transcripts, including dendritic mRNA (Steward, 1997), can be recovered for quantitative analyses. In the present study, we used this enhanced quantitative accuracy to test the hypothesis of coordinate regulation of L-type VSCC expression and function in epilepsy.

2. Experimental methods

2.1. Kindling

Twelve Sprague–Dawley rats (14-weeks-old, Harlan Industries, Evanston, IL) were surgically implanted with stimulating electrodes in the right entorhinal cortex under pentobarbital anesthesia. After a one-week recovery period, rats were randomly assigned to either control ($n = 5$) or kindled ($n = 7$) groups. Kindling procedures were as described previously (Jarvie et al., 1990). Stimu-

lated (kindled), animals received a 1-s, 60-Hz train of biphasic, square-wave pulses daily. An average of 25 ± 4 ($n = 7$) stimuli were needed before full kindling was achieved. The amplitude of the kindling stimulus current was adjusted for each animal to the threshold that evoked an afterdischarge (mean \pm SEM: 914 ± 116 μ A; $n = 7$). An animal was considered fully kindled when a class V seizure was seen on two successive days. The establishment of chronic epilepsy in kindled animals was confirmed visually, and no animal was studied electrophysiologically within 48 h of an epileptic event.

2.2. Tissue preparation

Between 1.5 and 3 months after kindling criteria were met (mean \pm SEM: 65 ± 7 days; $n = 7$), control and kindled rats (5–6-months-old) were decapitated and their brains rapidly removed and immersed in ice-cold aCSF (in mM): 114 NaCl, 2.5 KCl, 2 MgCl₂, 30 NaHCO₃, 10 Glucose, and 2 CaCl₂. The right (ipsilateral) hippocampus was hand dissected and transverse slices (350 μ m thick) were cut with a tissue chopper (Brinkman, Westbury, CN). ‘Zipper slices’ were prepared using procedures developed by Gray, Johnston, and colleagues in guinea pigs (Gray et al., 1990), and modified slightly for rats (Thibault and Landfield, 1996). Hippocampal slices were immersed in oxygenated aCSF at 31.5°C in a covered, 35-mm plastic culture dish. Usually 12 slices were placed in the culture dish. A stream of 95% O₂/5% CO₂ gas, prehumidified by bubbling through water, was directed at the surface of the slice incubation media. Slices were treated with 0.7 mg/ml pronase for 30 min, followed by 0.5 mg/ml thermolysine for 15 min. Half of the thermolysine solution was removed afterwards and replaced with oxygenated aCSF, effectively halving the concentration of thermolysine.

Thirty minutes after the last solution change the first slice was removed from the incubator to begin the ‘unzipping’ procedure. The incubation medium was replaced with an oxygenated ‘shaking’ solution (as aCSF except 2 mM CaCl₂ was replaced with 2 mM EGTA). The slice was washed three times and left in shaking solution

for 2 min, then removed from the solution. The CA3 region was dissected away, and the CA1 layer was nicked with a scalpel to facilitate dissociation. The slice was then returned to shaking solution (~1 ml) in a clear plastic tube sealed with Parafilm M™ and gently shaken by hand. Periodically (every 1–3 agitations) the tube was inspected for increases in the length of the separation (unzipping) along *stratum pyramidale*, and for floating debris. Shaking solution was replaced periodically with fresh solution. Once the CA1 separation was clearly visible, the slice was placed in the recording perfusion chamber.

2.3. Recording

A continuous-flow perfusion chamber (Warner Instruments, Hamden, CT) was used to record from each slice. Ca²⁺-free aCSF was replaced with cell-attached ‘zeroing’ bath solution (Fox et al., 1987) containing (in mM): 140 K Gluconate, 3 MgCl₂, 10 Glucose, 10 EGTA, and 10 HEPES (pH adjusted to 7.35 with KOH, osmolarity adjusted to 300 mOsm by dilution with distilled water). The pipette solution contained (in mM): 20 BaCl₂, 90 Choline Cl, 10 TEA Cl, and 10 HEPES (pH adjusted to 7.35 by TEA OH, osmolarity adjusted to 290 mOsm by the addition of sucrose). Bay K 8644 (0.5 μM) was added to the pipette solution to facilitate L-type channel activity.

2.4. Recording pipettes

Drummond glass capillaries (Drummond Scientific, Broomall, PA; Cat. # 2-000-100) were pulled with a Flaming-Brown horizontal puller (Sutter Inst. Co., Novato, CA), coated with Sylgard (Dow Corning, Midland, MI), and fire-polished immediately prior to use (average electrode resistance was 4.2 ± 0.1 MΩs for the control group and 4.1 ± 0.1 MΩs for the kindled group). Seal resistances averaged 19.0 (± 1.4) GΩs for control cells and 20.6 (± 1.1) GΩs for kindled cells. There was no significant difference in either electrode or cell-attached patch resistance between control and kindled cells.

2.5. Voltage-clamp protocols

Patch current was recorded with an Axopatch 200A amplifier using Clampex (v.6.0.3) acquisition software (Axon Instruments, Foster City, CA). All current records were digitized at 5.32 kHz, and filtered at 2 kHz. Patches were held at a resting potential of –70 mV and 150 ms command steps were applied to elicit VSCC activity. Leak subtraction was performed offline by adding averaged waveforms of equivalent voltage but opposite polarity to the current record. In maximum current experiments (maximum current is typically generated at steps to 0 mV in our studies), the membrane was depolarized from rest to 0 mV 45 times (three repetitions of a 15 pulse protocol with a 30-s interpulse interval). The first 15 of these current records were then used to construct an average ensemble current for each cell in the study (seen below). In addition, current–voltage (*I–V*) relationships were generated by stepping the membrane in 10 mV increments between rest and +40 mV. Usually a single *I–V* experiment was performed per cell. In hippocampal neurons, Bay K 8644-treated total patch current amplitudes correspond closely to estimates of L-type channel number (*N*) and membrane density (Thibault and Landfield, 1996; Porter et al., 1997).

2.6. Electrophysiologic data analysis

Leak-subtracted activity was measured with the pClamp suite of analysis software (Axon Instruments, Foster City, CA). Leak-subtracted current records from maximum current steps were averaged together to generate average ensemble records. The amount of ensemble current within the voltage step was integrated for each patch, and divided by the length of the depolarization to yield mean current amplitude (pA).

In *I–V* experiments mean-current amplitude was normalized to maximum inward current for each cell, and the scatter of data from each group was fit by the Boltzmann equation:

$$y = \frac{1}{1 - e^{(V - V_{1/2})/k}}$$

where V is voltage, k is the slope, and $V_{1/2}$ is the voltage at which 50% of maximal activation occurred. Fitted parameters also yielded standard error of the fit (table inset in Fig. 2C).

2.7. Cell collection

It should be noted that the molecular experiments were performed ‘blind’ in that the investigators were aware of neither the current amplitude nor the treatment group (i.e. control or kindled) of individual, collected neurons. In the ‘zipper’ preparation, the neuronal soma and basal dendrites are exposed, while for the most part the apical dendritic tree remains embedded in the tissue of *stratum radiatum*. Extraction of the long dendritic tree from the slice was attained by maintaining slight negative pressure on the somatically attached recording electrode and gently pulling the CA1 neuron from the slice (Fig. 1). Once free of the slice, the cell was held near the inlet port of the perfusion chamber and perfused with normal bath solution to provide an uncontaminated neuron for collection. A second pipette (the collection pipette) was then placed in the bath. To prepare collection pipettes, glass capillaries were baked (180°C for 4 h) to destroy RNase, silinized (exposed to 1 ml volatile trimethylchlorosilane in a vacuum bell jar for 4 h) to prevent RNA-glass binding, and pulled on a Flaming–Brown horizontal puller (Sutter Inst., Novato, CA). The tips were broken back and fire polished to $\approx 10 \mu\text{m}$ bore diameter. Slight positive pressure was applied in order to prevent capillary action from drawing solution into the collection pipette before it was in position. Once in position the positive pressure was removed and capillary action drew the cell and $\approx 2 \mu\text{l}$ of bath solution into the collection pipette. The contents of the collection pipette were ejected into a pre-chilled solution of RT buffer (1 \times PCR buffer, 3.75 mM Mg^{2+} , 0.5 mM dNTPs, 0.25 μM random hexamer, 1 U/ μl RNase inhibitor, 0.5 $\mu\text{g}/\mu\text{l}$ gene32 protein and 2.5 U MuLV RT) and processed for cDNA synthesis. The RT reaction (20 μl total) was carried out at 42°C for 1 h and next at 95°C for 10 min. The reaction product was then stored at -20°C .

2.8. Single-cell PCR

To verify that successful cell collection and reverse transcription had occurred, the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was detected by PCR for each cell in the study. The sense and antisense primers were: sense [5'-CAAGGCTGTGGGCAAGG-3'] and antisense [3'-ATGGGAGTTGCTGTTGA-5'], and yielded a single 235bp PCR fragment (nt. 666–900, GenBank M17701, rat; Tso et al., 1985). For GAPDH, 1/20 of the RT solution was used as a template for the PCR reaction which ran at 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min per cycle for 35 cycles. Cells were considered positive for GAPDH if they showed a distinct 235 bp band on a polyacrylamide gel.

The L-type Ca^{2+} -channel $\text{Ca}_v1.3$ -subunit mRNA content in each cell was quantified by kinetic RT-PCR as described previously (Chen et al., 2000). The sense and anti-sense primers for $\text{Ca}_v1.3$ were: sense [5'-GTTACTATTGATGAC-TATCA-3'] and antisense [3'-GGATCGGGTTG-GTCTTGCTA-5'], which span a II–III linker region of the $\text{Ca}_v1.3$ sequence (nt. 2930–3149, GenBank M57682, rat brain) and yield a single, 202 basepair DNA fragment. Briefly, 1/4 of the RT solution was used as template for a two-step PCR. The first round of PCR was run in a 10 μl reaction mix (1 \times PCR buffer, 3.0 mM Mg^{2+} , 0.2 mM dNTPs, 0.2 μM each primer, and 0.25 U GoldTag) for 25 cycles (95°C for 45 s, 50°C for 45 s, and 72°C for 1 min), followed by a second 100 μl PCR for 30 cycles. Aliquots of the PCR products were collected during the second PCR period and analyzed on acrylamide gels to establish a kinetic curve of the PCR. In each preparation, approximately 2 μl of bath solution was collected as a control, and processed in parallel through the two-step PCR reaction. None of the controls gave a false-positive $\text{Ca}_v1.3$ signal.

For the CaM message, the PCR was performed using 1/10 of each single-cell RT cDNA added in a 100 μl reaction mixture. One round of PCR was performed for 45 cycles (94°C for 45 s, 60°C for 45 s and 72°C for 1 min). Aliquots were collected from cycles 40 to 45 and the PCR products were analyzed and quantified. The sense and antisense

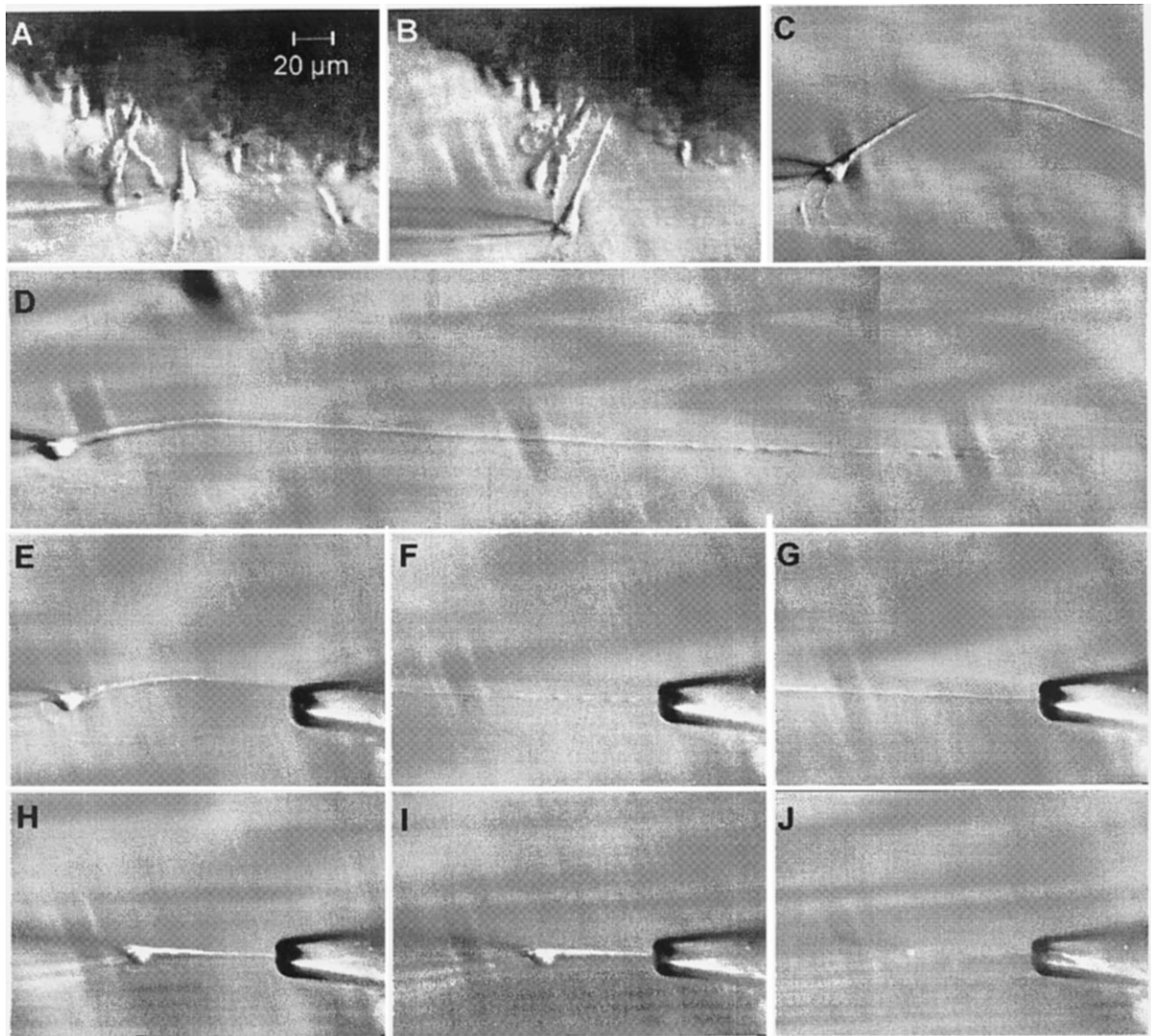


Fig. 1. Collection of a largely intact recorded CA1 neuron for gene/mRNA analysis. A–C. A series of sequential video images from a representative experiment. (A) A pyramidal cell soma is exposed by the hippocampal “zipper” slice technique, and the patch pipette (left) is lowered to form a high gigaohm seal for cell-attached patch recording. (B) While negative pressure is maintained on the recording pipette, the pipette is withdrawn and the apical dendritic tree is gently pulled free of the slice. (C) The cell is perfused/washed with uncontaminated bath solution immediately prior to, and during collection. (D) A composite image, constructed from sequential video captures, shows nearly the total length of this neuron’s apical dendrite after removal from the “zipper” slice. Small dendritic processes are present, but are not visible at this resolution. Occasional beading is seen in distal and smaller processes. (E) An empty collection pipette with positive pressure applied to counteract capillary force is lowered into the microscope’s field of view (right) on the same focal plane as the extracted neuron. (F–I) The positive pressure is removed, and the tip of the apical dendrite is threaded into the collection pipette. (J) The negative pressure on the recording electrode is released, and the cell is rapidly and completely drawn into the collection pipette by capillary action.

primers for CaM were: sense [5'-GGTTGTCT-GTTCTGGTCT-3'] and antisense [5'-AGGG-AAGTCGATTGTGCCATTA-3'], which span a non-coding region at the 5' end of the CaM sequence (nt. 30–272, GenBank M17069, rat brain) and yield a single 252 bp DNA fragment (Chen et al., 2000).

PCR fragments were gel purified and confirmed by DNA sequencing. The PCR products of the aliquots were analyzed by 9% acrylamide gel electrophoresis and quantified by fluorescence staining and imaging with a Storm PhosphorImager (Molecular Dynamics). To ensure that saturation did not occur during PCR, a kinetic analysis was performed on multiple aliquots for each cell to verify that all readings were taken in the exponential phase of amplification. A cycle number at which all cells were in the exponential phase was selected (cycle #45 for Ca_v1.3; cycle #40 for CaM) and the PCR products from the selected cycle for each mRNA species were then analyzed together for all cells.

2.9. Statistical analyses

Multiple neurons were analyzed from a single animal per day (averaging 3.65 patches/animal). Because one of the major goals in the study was to use the single cell method to study coordinate regulation of channel function and mRNA expression, it was important to determine whether the cells behaved independently or as non-independent samples. We used two tests to examine this. First, we tested the effect of individual animals on mean current amplitude within each treatment group (i.e. control and kindled) by one-way ANOVA. There was no significant effect of individual animal on current amplitude in either treatment group ($P > 0.5$). Second, we tested for correlated values in cells from the same animal. If cells from an individual animal behave similarly (i.e. are non-independent), then a correlation should be found between the channel activity of the first and second cells obtained from each animal. We used the Pearson Product Moment correlation analysis to test for covariance of the first and second cell in each animal in which multiple cells were recorded, and again found no

animal-based association (overall $n = 11$, $r = 0.426$, NS). This result indicated that animal-dependent variance contributed little to overall variance and consequently those individual neurons could be used as the sampling population. Nevertheless, since with sufficient sampling per animal, there seemingly should be some average effect across an animal's neurons, we also reexamined each neuron-based significant effect with tests based on an animal sampling population (i.e. averaging all cells per animal). Statistical tests were performed with SigmaStat 2.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Kindling effects on L-type voltage-sensitive Ca²⁺ channel activity

In hippocampal cell-attached, multichannel patches treated with Bay K 8644, nearly all of the recorded Ca²⁺ current is L-type (Thibault and Landfield, 1996). In the present study, peak patch current in Bay K 8644-treated multichannel patches from CA1 pyramidal cells varied from 5 to 70 pA. Despite this variability, analyses of 18 control and 26 kindled neurons, from 5 to 7 animals, respectively, showed that L-type VSSC patch current was markedly depressed 1.5–3 months after the last kindling session (Fig. 2). The decrease in current amplitude at maximally activating voltages was robust and highly significant ($P < 0.01$; t -test; $P < 0.01$, Mann–Whitney U-test; Fig. 3A). Current–voltage (I – V) analyses indicated clearly that these effects of kindling on current were not due to altered voltage dependence of L-type channel activation (Fig. 3B–C).

As noted (Section 2), we also analyzed the data using animals as individual observations (Fig. 4). The individual patch current amplitudes were plotted as a function of the animals (1–12) from which they were measured, and each animal's average value (\pm SEM) is plotted on a bar graph superimposed on the scatter graph of individual observations. The mean observations from each animal were then averaged, grouped according to treatment (control animals 1–5; kindled animals

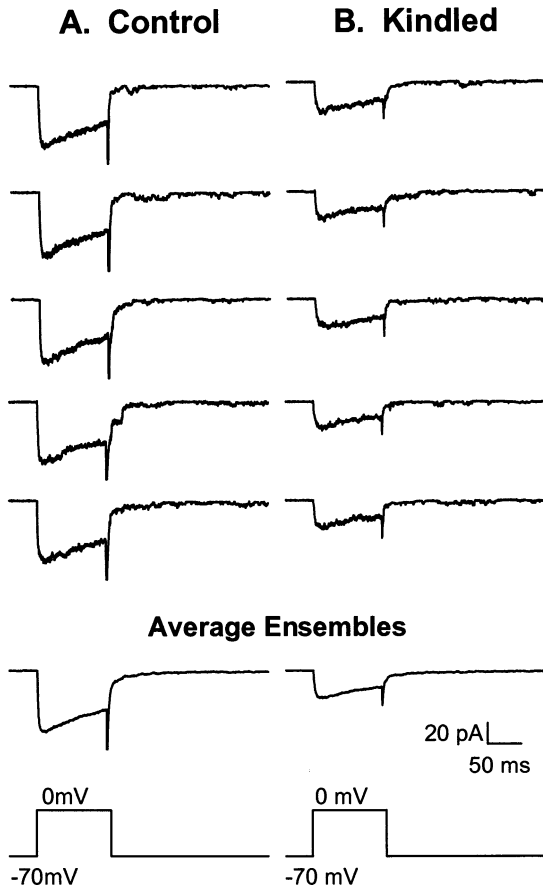


Fig. 2. Representative L-type Ca^{2+} -channel activity in control and kindled cells. A. Five representative traces are shown from a set of 45 cell-attached patch recording traces of Ca^{2+} -channel activity from a CA1 neuron of a control (A) and a kindled (B) animal. Beneath the individual traces are average ensemble current records, constructed by averaging the first 15 traces from a single experimental protocol. In these multichannel patches there were too many channels per patch at maximum activation to observe single-channel openings within the voltage step, although with 20 mM Ba^{2+} as the charge carrier, single openings are often seen in the repolarization phase after the pulse (Thibault et al., 1993; Thibault and Landfield, 1996).

Fig. 3. L-type Ca^{2+} -channel activity in CA1 neurons is reduced with kindling. (A) Mean ensemble current amplitude (Section 2) in the two groups (Control $n = 18$, Kindled $n = 26$) was obtained at maximally activating voltage steps. A highly significant reduction in current amplitude was seen with kindling ($P < 0.01$) (B) $I-V$ relationship protocols also revealed a significant decrease in patch current amplitude with kindling ($P < 0.01$, 2-way ANOVA) but no change in the voltage at

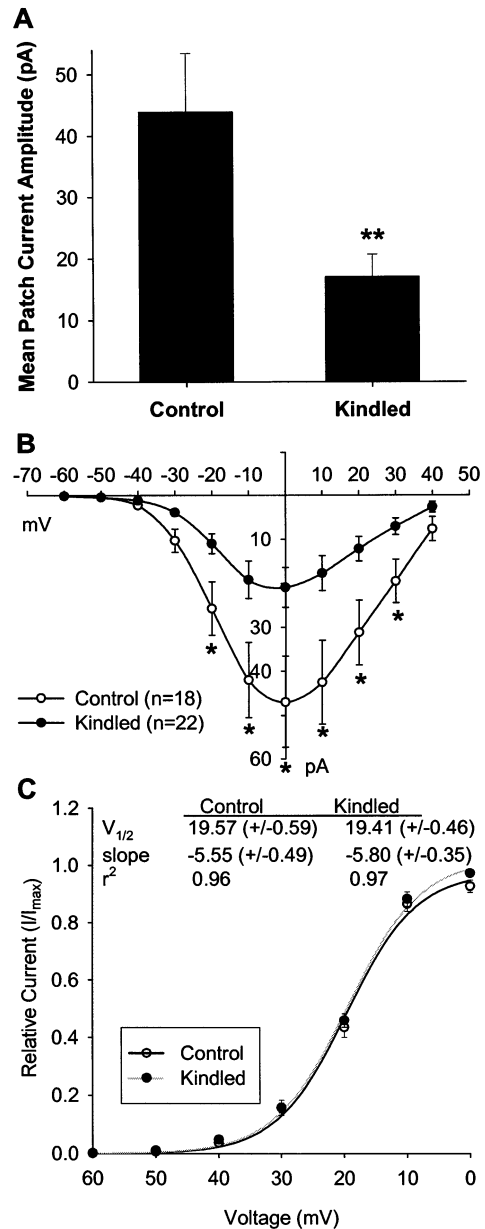


Fig. 3. (Continued)

which maximum inward current was generated. Patches were held at -70 mV and stepped from -60 to $+40$ in 10 mV increments. (C) The $I-V$ studies also showed that the decrease in Ca^{2+} current was not due to a shift in the voltage-dependence of activation. Current at each step was normalized to maximum inward current (I/I_{max}), and the current activation data for each group were fit using the Boltzmann equation (Section 2).

6–12), and statistically analyzed. Despite the lower n , the reduction of current with kindling using this animal-based analysis was similar in magnitude significance (Fig. 4 inset; $P < 0.05$, t -test) to that of the neuron-based analysis (Fig. 2A).

3.2. Collection of neurons for mRNA analysis

After recording, each neuron was extracted gently from the slice and collected for single-cell RT-PCR analysis. However, complete capture with the major processes intact could only be confirmed under the microscope for 10/18 control and 21/26 kindled neurons (Fig. 1). Each successfully collected cell was then tested by RT-PCR for

the presence of GAPDH mRNA signal. GAPDH is a relatively abundant mRNA, and its detection in a collected neuron was considered an indication of successful transfer to the RT tube and of mRNA integrity. Of collected neurons, however, only 5/10 control and 13/21 kindled neurons generated measurable mRNA signals for GAPDH. Thus, a subset of 18 of the original 44 electrophysiologically characterized single neurons was analyzed for mRNA expression of L-type VSCC subunit.

The $\text{Ca}_v1.3$ -mRNA signal was positive for 17/18 GAPDH positive cells ($n = 5$ cells from four control animals, 12 cells from six kindled animals). In addition, the CaM signal was positive

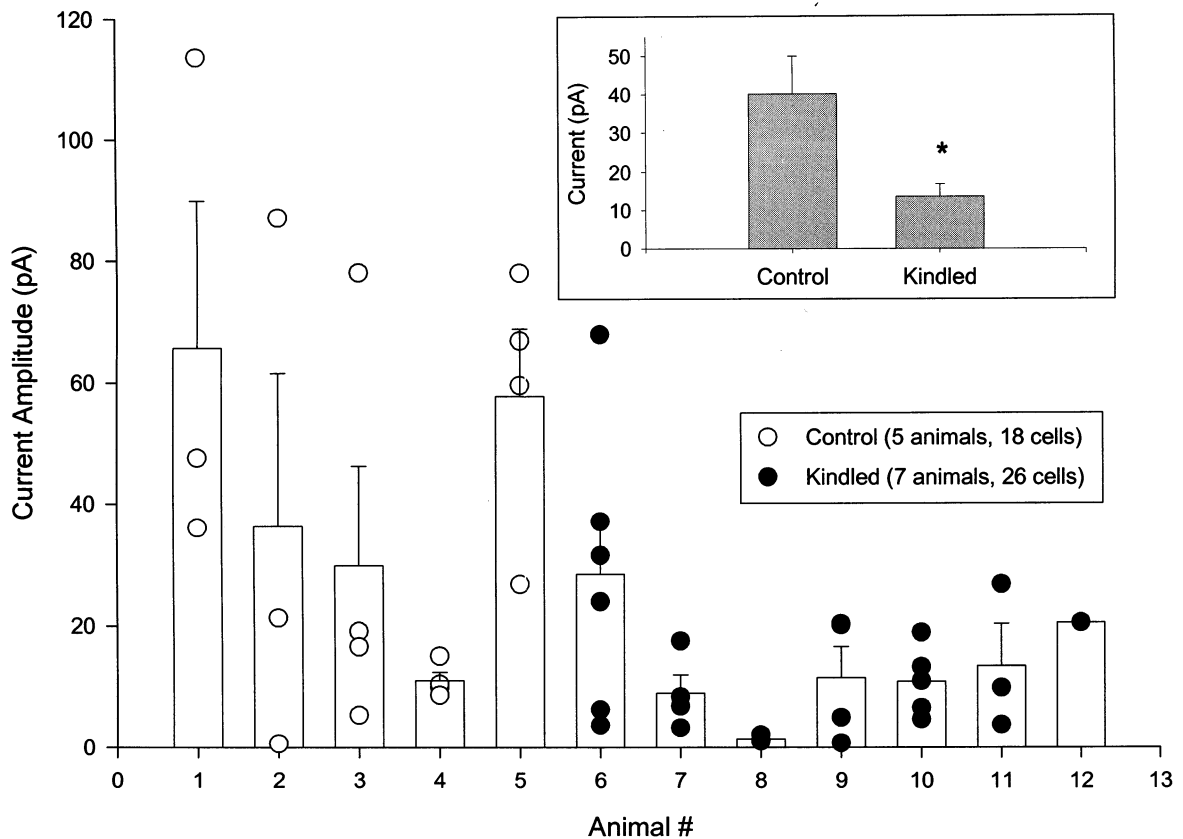


Fig. 4. Reduction in L-type Ca^{2+} current in animal-averaged analyses. Individual cell observations of channel activity are plotted as a function of the animals from which they were recorded. The bars represent the mean (\pm SEM) for observations from each animal. The inset shows that the mean values from each animal, when grouped according to treatment and averaged, also revealed a significant reduction in current amplitude with kindling ($P < 0.05$).

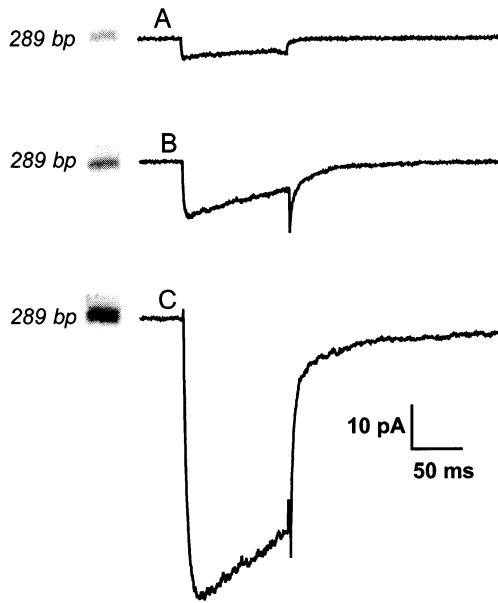


Fig. 5. Representative examples of correlated $\text{Ca}_v1.3$ -mRNA level and patch-current amplitude. Left: $\text{Ca}_v1.3$ PCR products at the 45th PCR cycle from 3 different cells. Right: Adjacently aligned average ensemble current records obtained from the same three neurons, respectively. Note the apparent correlation within cells between current amplitude and PCR signal intensity from the RT-PCR products of the largely intact neurons.

for 12/18 GAPDH positive neurons ($n = 4$ cells from two control animals, eight cells from four kindled animals). That the frequency of positive signals was lower for CaM than for $\text{Ca}_v1.3$ -mRNA may reflect differences in the temporal patterns of gene expression, mRNA stability, primer efficiencies, or, most likely, in the fractional volume of cell contents used for the two messages (Section 2). Because of the small sample sizes of the mRNA analysis subset, particularly for control neurons, no significant effect of treatment was detectable on group mean values.

Approximately the same direction and magnitude of electrophysiologic effect was seen in $\text{Ca}_v1.3$ - and CaM-positive cells as was seen in the larger data set (Fig. 3A), namely, a $> 50\%$ decrease in current amplitude in kindled neurons (e.g., $\text{Ca}_v1.3$ -mRNA subset: control = 60 ± 30 pA, kindled = 14.18 ± 3.48 pA, $P < 0.05$, t -test).

Therefore, the 'mRNA subset' appeared representative of the larger data set.

3.3. Correlation of mRNA expression with L-type VSCC activity

The small number and variability of collected control cells that were also positive for $\text{Ca}_v1.3$ -mRNA precluded statistical comparisons for group mean differences in mRNA. Nevertheless, correlating mRNA content with Ca^{2+} channel activity (for which group differences were highly significant) provides an alternative and more powerful approach for determining whether mRNA for $\text{Ca}_v1.3$ co-varies with L-type VSCC activity in epileptic and control neurons. That is, one of the major advantages of a single-neuron approach is that it allows statistical tests of association to be based on sufficient degrees of freedom to support a strong inference of correlation. In the present study we tested this correlation in the combined group of control and kindled $\text{Ca}_v1.3$ -positive neurons ($n = 17$). The $\text{Ca}_v1.3$ -mRNA RT-PCR products for all collected neurons from the same PCR cycle on the non-saturated, exponentially-rising phase of the reaction were quantified and the amount of PCR product for a given cell was then matched with patch current amplitude from the same neuron (e.g., Fig. 5). Neurons were rank-ordered from lowest (1) to highest (17) on both the electrophysiologic and mRNA expression variables, and a non-parametric analysis (Spearman's r test) was used to test for the predicted correlation. A highly significant correlation was found between L-type VSCC current and $\text{Ca}_v1.3$ -mRNA expression levels (Fig. 6A; $r_s = 0.64$, $P < 0.005$). However, a similar analysis found no trend to a correlation between L-type VSCC current and CaM-mRNA level (Fig. 6B). To ensure that these values were not affected by non-independence of samples (Section 2) we also studied the rank-order correlations between animal-averaged measures of current and $\text{Ca}_v1.3$ -mRNA levels. As for single neurons, we found a highly significant correlation between an individual animal's mean patch current amplitude and its mean $\text{Ca}_v1.3$ -mRNA level (Fig. 6A inset; $n = 10$; $r_s = 0.78$; $P < 0.01$).

4. Discussion

The present work indicates that induction of a state of apparently permanent epilepsy by entorhinal kindling is associated with substantial down-regulation of L-type VSCC single-channel activity

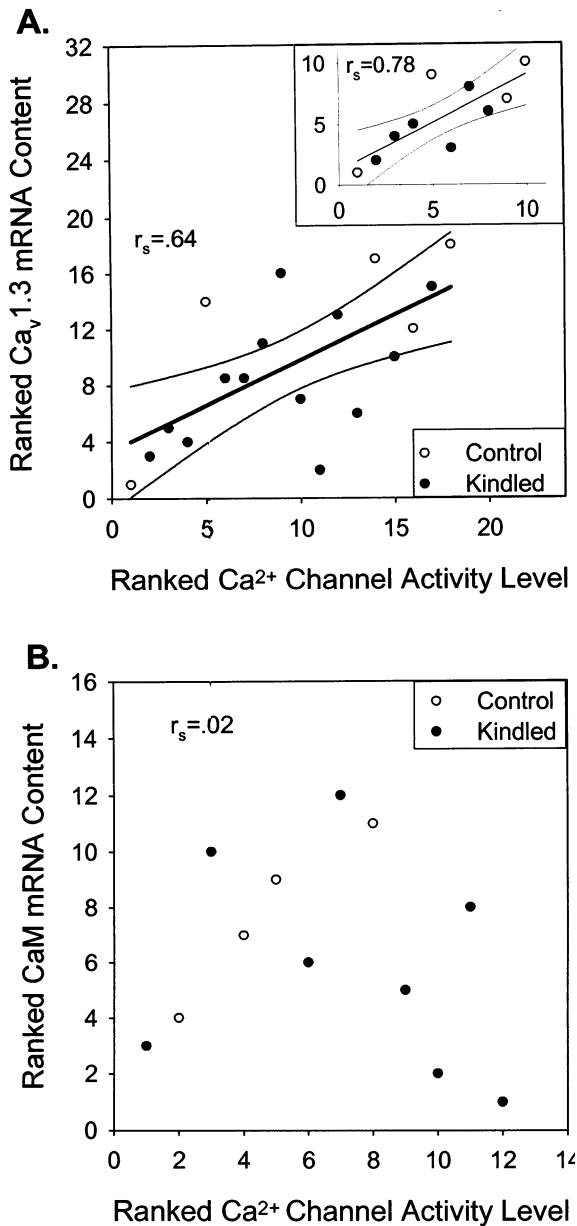


Fig. 6.

in rat CA1 hippocampal pyramidal neurons. In addition, extraction and collection of largely intact neurons from ‘zipper slices’ revealed a highly significant positive correlation between L-VSCC activity and semiquantitative RT-PCR estimates of $\text{Ca}_v1.3$ -subunit mRNA in the same single neurons. The finding of a positive correlation confirmed an important prediction of the hypothesis that L-VSCC subunit gene/mRNA expression is coordinately linked to the changes found in L-type channel activity after epileptogenesis. The correlation of 0.64 (r_s , Fig. 6A) indicates that approximately 40% of the relative L-VSCC current activity across individual neurons was accounted for by variance of $\text{Ca}_v1.3$ -subunit mRNA. Considering that L-type VSCC patch current is generated by both $\text{Ca}_v1.2$ - and $\text{Ca}_v1.3$ -subunit-containing L-type VSCCs, which overlap in many of the same neurons (Hell et al., 1994), and that there is considerable error variance in all present single-cell methodologies, this level of correlation suggests that a relatively large fraction of L-type VSCC activity may be directly regulated by changes in subunit gene expression.

As noted, prior results on Ca^{2+} currents in seizures have been inconsistent. Some studies have found a kindling-dependent increase in slowly-inactivating whole-cell Ca^{2+} current (Vreugdenhil and Wadman, 1994), whereas others have found a

Fig. 6. Correlation between L-type VSCC activity and $\text{Ca}_v1.3$ -mRNA content in single neurons from control and kindled animals. (A) Rank-ordered $\text{Ca}_v1.3$ -mRNA levels (ordinate) are plotted against rank-ordered Ca^{2+} -channel activity levels (abscissa) and fitted by linear regression (shown here with 95% confidence intervals). Values on both axes are ranked from lowest (1) to highest (17). The observed correlation ($r_s = 0.64$) between channel activity and $\text{Ca}_v1.3$ -message level was highly significant ($P < 0.005$, Spearman’s non-parametric test). Inset: The correlation between electrophysiologic function and molecular expression was also tested using animals as the statistical population. Averages of the single neuron measures of current amplitude and $\text{Ca}_v1.3$ mRNA content for each animal are rank ordered and plotted as in (A). Again, a highly significant correlation was found between an animal’s average $\text{Ca}_v1.3$ -mRNA expression level and its average L-type VSCC patch current amplitude ($r_s = 0.78$; $P = 0.005$, Spearman’s non-parametric test). (B) No trend to a correlation was found between ranked calmodulin (CaM) mRNA content and L-type VSCC activity.

seizure-induced decrease in similar currents, attributed in some cases to altered Ca^{2+} -dependent inactivation (Mody et al., 1990; Karst et al., 1999). In addition, *in situ* hybridization (ISH) studies have not found consistent evidence of lasting changes in VSCC expression during kindling (Hendriksen et al., 1997; Karst et al., 1999). However, earlier studies generally differed from the present one in terms of the subunits examined, the analytic methods used, the regions focused upon, or the kindling model employed (e.g. analysis of CA1 after Schaffer collateral stimulation [monosynaptic] versus the entorhinal [trisynaptic] stimulation used here). Moreover, experimentally isolated whole-cell or single channel L-type current has not to our knowledge, been examined in previous studies of epileptogenesis.

Thus, the present studies apparently provide the first direct electrophysiologic evidence that identified L-type Ca^{2+} channel activity is decreased in association with seizure development, and indicate that this reduction is in part mediated by down-regulation of L-VSCC subunit gene expression. These findings, therefore, extend the growing evidence that seizures induce widespread alterations in gene expression (Gall and Isaackson, 1989; Perlin et al., 1993; Brooks-Kayal, et al., 1998; McNamara, 1999) to include a long-lasting alteration in an identified Ca^{2+} channel.

The present studies also appear to have implications for optimal approaches to neuronal investigations of expression-function associations in general. Previous single neuron studies of relations between electrophysiologic function and subunit gene expression have generally collected cellular mRNA by cytoplasmic aspiration, often from neurons that were acutely dissociated (Eberwine et al., 1992; Monyer and Lambolez, 1995; Brooks-Kayal et al., 1998; Tkatch et al., 2000). With these procedures, however, major processes are often amputated and/or a highly variable fraction of mRNA content is collected from cell to cell (but see Baro et al., 1997). Consequently, semiquantitative estimates must be normalized to a control message, adding the measurement error, as well as potential regulatory variance, of that control. Further, much of the dendritic mRNA, which is increasingly recognized to be substantial

and relatively specific (Steward, 1997), is lost. Thus, collection of an unruptured cell with a near-complete dendritic tree (e.g. Fig. 4C), clearly seems likely to yield more accurate estimates of the mRNA content of a physiologically-characterized CNS neuron. This enhanced accuracy of single neuron molecular analysis appears to make quantitative correlations with function more feasible (e.g. Fig. 6A), and consequently, the 'zipper slice' could well become a preparation of choice for many kinds of function-expression correlation studies in single CNS neurons.

The Ca^{2+} dependence of several K^{+} and Cl^{-} inhibitory conductances (Storm, 1990; Hille, 1992; Scott et al., 1995), and the extensive postsynaptic localization of L-type VSCCs (Hell et al., 1994), suggest the hypothesis that down-regulation of L-type VSCC expression, as observed here, may contribute to epileptogenic pathogenesis and the establishment of long-term hyperexcitability. In particular, the Ca^{2+} -dependent, K^{+} -channel-mediated slow afterhyperpolarization (AHP) is a likely candidate mechanism through which an effect on excitability might occur. The AHP strongly and negatively regulates neuronal excitability (Madison and Nicoll, 1984; Storm, 1990; Hille, 1992), and is preferentially activated by Ca^{2+} influx through L-type channels (Mazzanti et al., 1991; Moyer et al., 1992; Marrion and Tavalin, 1998). Importantly, it has been shown that the AHP can directly inhibit epileptiform activity, and that its duration controls interburst interval (Chamberlin and Dingledine, 1989). Moreover, the AHP is responsive to altered activation patterns and is decreased during behavioral conditioning, which subsequently results in persistently elevated neural activity (Disterhoft et al., 1994). On the other hand, one study found no difference in slow AHP amplitude after kindling in amygdalar neurons (Asproдини et al., 1992). Thus, while the present data indicate that the AHP and other inhibitory conductances appear to deserve further study in relation to epileptogenesis, particularly in highly seizure-prone hippocampal pyramidal neurons, it is clear that the validity of the proposal that down-regulation of L-type VSCC influences excitability via the AHP, or through other pathways, remains to be tested in further studies.

Nevertheless, there are some indirect data in the literature that address this possibility. If decreased L-type current with kindling contributes to greater seizure susceptibility, as suggested here, then L-type VSCC channel blockers might be predicted to exacerbate seizure activity. However, the evidence is mixed in this regard. A number of studies have shown that L-type VSCC blockers have anticonvulsant and/or neuroprotective properties (Palmer et al., 1993; Wurlpel and Iyer, 1994; Stefani et al., 1997); similarly, Bay K 8644, the L-type VSCC agonist, can have convulsant actions (Palmer et al., 1993). Conversely, there are several reports indicating that, under some conditions, L-type VSCC antagonists can increase seizure activity. In studies of chronic treatment in rats, for example, L-channel blockers enhanced the intensity and duration of seizures (Yamada and Bilkey, 1991). A similar effect was seen in chronically-treated aging rats (van Luijtelar et al., 1995).

The effect seems particularly noteworthy in aged animals, since the density of L-type VSCC in CA1 neurons is increased (Thibault and Landfield, 1996), as is AHP amplitude (Landfield et al., 1989; Disterhoft et al., 1994). Thus, if the hypothesis suggested here is correct, aged subjects should be relatively resistant to seizure induction. Interestingly, aged rats are less susceptible to kindling induction (deToledo-Morrell et al., 1988). However, some measures of excitability are also increased in aged brain (Barnes 1994), again emphasizing the multiple mechanisms that govern excitability and possible epileptogenesis. Taken together then, there appear to be substantial data consistent with the view that conditions of reduced L-type VSCC activity can enhance general excitability, and vice versa.

In summary, the present results provide evidence that specifically identified L-type Ca^{2+} -channel activity is reduced following kindling-induced epilepsy. Overall, the data are consistent with the hypothesis that the kindling process induces down-regulation of L-type $\text{Ca}_v1.3$ -subunit mRNA expression (perhaps in response to initial excessive Ca^{2+} influx through L-type VSCCs), and consequently, decreases the density of available L-type VSCCs. Further, we

suggest that in turn, this process reduces one or more Ca^{2+} -dependent inhibitory conductances (e.g., the slow AHP) and thereby contributes to the development of a chronically lower seizure threshold and a permanent epileptic state. The view that L-type VSCCs are important in regulating thresholds might also account for the observations that seizure susceptibility is reduced with aging (deToledo-Morrell et al., 1988). Additional studies will clearly be required to determine conclusively whether the decline in L-type VSCC expression is functionally relevant to seizure susceptibility and, if so, whether it acts via an effect on the AHP. Nevertheless, if additional studies support aspects of this view, the regulation of L-type Ca^{2+} -channels may represent a new target for therapeutic approaches to epilepsy.

Acknowledgements

We thank Drs Tom Foster and Chris Norris for helpful comments on the manuscript. This work was supported in part by grants from the NIH (AG18228, AG04542, and AG10836) and funding from the Research Service, Department of Veterans Affairs.

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