*Original Research*

# Analysis of plasma membrane $Ca^{2+}$-ATPase gene expression during epileptogenesis employing single hippocampal CA1 neurons

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## Abstract

**Disruption of calcium homeostasis in epileptic cells is characterized by both short- and long-term perturbations of $Ca^{2+}$ buffering systems. Along with the $Na^{+}/Ca^{2+}$ exchanger, the plasma membrane $Ca^{2+}$-ATPase (PMCA) plays an important role in excitable cells. The involvement of PMCAs in epileptogenesis has primarily been studied in brief intervals after various stimuli; however, the specific contribution of this molecule to epileptogenesis is not yet fully understood. Our aim has been to investigate whether PMCA expression in the chronic stages of epilepsy is altered. Through an interdisciplinary approach, involving whole-cell recordings and real-time reverse transcriptase-polymerase chain reaction, we have shown that epileptic neurons in our preparation consistently show changes in electrical properties during the period of chronic epilepsy. These changes included increased spike frequency, altered resting membrane potential and changes in passive membrane properties. Following these observations, which indicate an altered excitability in the epileptic cells studied, PMCA mRNA transcripts were studied. It was found that while *PMCA1* transcripts are significantly increased one month following the pilocarpine epileptogenic stimulus, *PMCA3*, an isoform important in excitable tissues, was significantly, decreased. These findings suggest that, in the long-term, a slow PMCA (*PMCA1*) plays a role in the reestablishment of a new calcium homeostasis attained by epileptic cells. Overall, this phenomenon points out the fact that in seizure disorders, changes that take place in the balance of the different molecules and their isoforms in charge of maintaining neuronal calcium homeostasis, are fundamental in the survival of affected cells.**

**Keywords:** plasma membrane $Ca^{2+}$-ATPase, calcium homeostasis, epileptogenesis, hippocampal neurones, single-cell quantitative-polymerase chain reaction

*Experimental Biology and Medicine* 2011; **236**: 409–417. DOI: 10.1258/ebm.2011.010342

## Introduction

Intracellular $Ca^{2+}$ concentrations are regulated by a sophisticated battery of proteins. This degree of fine tuning is necessary for the maintenance of calcium homeostasis in eukaryotic cells. In the nervous system, diverse processes are dependent on the regulation of this critical cation. It has become clear that imbalances of calcium homeostasis in neurons underlie several neurodegenerative disorders.

Epilepsy can be a debilitating disorder that undoubtedly merits exhaustive examination of its underlying molecular mechanisms. It is known that the concentration of intracellular calcium is significantly elevated during *status epilepticus* in several models of epileptogenesis, and remains elevated in the chronic phase of epilepsy.[1–5] Control levels of $Ca^{2+}$ in hippocampal neurons correspond to 100–200 nmol/L, whereas after initialization of the *status epilepticus* in the pilocarpine model, the concentration reaches >800 nmol/L.[3] After 30 days, $Ca^{2+}$ levels are still high (>300 nmol/L) and sustained up to one year.[3]

In the initial phases of experimental epileptogenesis, increased intracellular levels of calcium are mediated via the entry of calcium through the *N*-methyl-D-aspartate (NMDA) receptor. In the chronic phase, when recurrent seizures are evident, this increase is likely due to an alteration in the equilibrium between several homeostatic mechanisms, including the aberrant extrusion of ions.[2]

In the process of epileptogenesis, the affected calcium machinery in the cell includes cytoplasmic buffering proteins,[6,7] the sarco/endoplasmic $Ca^{2+}$-ATPase (SERCA),[8,9] the $Na^{+}/Ca^{2+}$ exchanger (NCX)[10] and the plasma membrane

$Ca^{2+}$-ATPase (PMCA).[11] At the cell membrane, the PMCA is involved in the fine regulation of intracellular calcium via its high affinity for this cation, and therefore plays a critical role when sudden changes in intracellular calcium occur.[12] The PMCA family consists of four genes (PMCA1–4), which undergo complex alternative splicing to produce at least 20 different isoforms, each one displaying kinetic properties related to its cellular localization and function. Interestingly, the brain expresses a wide variety of these PMCA isoforms with varying patterns depending on the developmental stages examined.[13]

Knowledge gathered to date concerning the participation of the PMCAs in epileptogenesis is limited. We have recently reviewed the role of cell calcium extrusion systems in the development of epilepsy.[14] In the hippocampus, the basic isoforms of $PMCA^{1-4}$ have been detected with some variability depending on the specific technique employed.[15,16] In the rat, *PMCA1* and *PMCA3* are the most abundant transcripts,[11] whereas in the human, PMCA2 and PMCA4 mRNAs follow a differential distribution.[17] In early stages of kainic acid (KA)-induced epilepsy, a diminished expression of *PMCA1* and *PMCA2* mRNA, together with an unaltered expression of *PMCA3*, measured by *in situ* hybridization has been reported.[11] Other groups have reported no significant changes in PMCA mRNA expression when examined at a longer interval following KA treatment.[10] Changes in total PMCA protein have been detected 4–12 h after the epileptogenic stimulus.[11]

We have hypothesized that homeostatic calcium extrusion mechanisms may be altered in the chronic phase of epilepsy. Therefore, as an initial approach, we decided to study the expression of PMCA following pilocarpine-induced epileptogenesis. Considering the heterogeneity of cells forming the nervous tissue, we studied the expression of different *PMCA* isoforms in single epileptic neurons of the hippocampus through an approach involving both electrophysiological and quantitative polymerase chain reaction (qPCR) techniques. Upon isolating well-characterized epileptic neurons, as documented by electrophysiological analysis, we found that, while *PMCA1* expression increases significantly following epileptogenesis, *PMCA3* expression decreases and the expression of *PMCA4* presents minor changes. These data suggest that *PMCA1* plays a critical role in chronic stages of epileptogenesis.

Together, these results strongly support the hypothesis that, in neurons, an altered calcium extrusion process, which results in changes that modify intracellular calcium equilibrium, correlates with the onset of chronic epilepsy associated with an altered neuronal excitability. The newer calcium equilibrium achieved by the expression of different PMCAs allows for the management of chronically elevated calcium levels that would otherwise lead to cell death.

## Methods

### Materials

All reagents were of molecular biology grade; buffers, methylscopolamine and pilocarpine hydrochloride were obtained from Sigma-Aldrich (St Louis, MO, USA). Dimethyl-dichlorosilane was purchased from Supelco (Bellefonte, PA, USA). Primers and Taqman probes were from Primer Design Ltd (Southampton, UK). The SuperScript™ III Platinum® One-Step Quantitative RT-PCR System with ROX was from Invitrogen Corporation (Carlsbad, CA, USA). The water used for all the solutions was ultrapurified in a Milli-Q system (Millipore Corporation, Bedford, MA, USA), diethylpyrocarbonate (DEPC)-treated and sterilized. For qPCR experiments, an ABI Prism 7000 detection system from Applied Biosystems (Foster City, CA, USA) was used. Valium (Diazepam) was kindly provided by Hoffmann-La Roche Ltd.

### Epilepsy model

Experimental animals were handled according to Mexican Official Norm for use, care and reproduction of Laboratory Animals (NOM-062-ZOO-1999). Male Wistar rats of 20–25 days old were used, which were maintained under a standard 12:12 light/dark cycle with *ad libitum* access to food and water. All drugs were administered via intraperitoneal (i.p.) injection. Prior to pilocarpine treatment (30 min), experimental animals were administered methylscopolamine (1 mg/kg) and *status epilepticus* was then induced by a single dose of pilocarpine (350 mg/kg). Control animals were injected only with saline solution. Behavioral seizures were observed and the *status epilepticus* onset was defined as the time when seizure activity became sustained. Rats were allowed to seize for one hour and, in order to avoid high mortality, animals were treated with diazepam (4 mg/kg). Subsequent doses were administered as needed.[18] Rats were left in a nursing facility until spontaneous seizures occurred twice per week; seizures were evaluated according to Racine's scale.[19]

### Patch-clamp recordings

To document changes in the electrical behavior of cells from pilocarpine-treated animals, we carried out electrophysiological recordings one month after treatment. Only rats showing spontaneous seizures once or twice per week were used. Under ether anesthesia, rats were killed by decapitation and the brains were extracted. Slices of 400 $\mu$m were obtained with a vibratome and were incubated at room temperature (24–26°C) for one hour in artificial cerebrospinal fluid (ACSF) containing (in mmol/L): 126 NaCl, 3 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 1.25 $Na_2HPO_4$, 10 glucose (equilibrated with 95% $O_2$/5% $CO_2$, pH 7.4). Brain slices were transferred to a recording chamber and continuously superfused at 34°C with ACSF. Hippocampal CA1 pyramidal cells were visualized by infrared differential interference contrast (DIC) video microscopy using a 40 ×/1.0 objective in an upright microscope (FN1, Nikon Inc, Melville, NY, USA). Patch pipettes were pulled from borosilicate glass and filled with a solution containing (in mmol/L): 145 KCl, 5 HEPES, 5 EGTA, pH 7.3, with tip resistances of 4–5 MΩ. Recordings were made with current clamp in a whole-cell configuration with seal resistances over 1 GΩ,

using an EPC7 amplifier. To observe changes in the excitability of cells from control and pilocarpine-treated animals, a stimulation protocol was applied consisting of current pulses of 20 ms ranging from −800 to 800 pA (in 100 pA steps, Figure 2D). During a 100 ms pulse the membrane reaches the steady state before the end of the pulse. Under these conditions we evaluated membrane resistance. Cell potentials and the stimulus duration were used to plot every action potential as a function of its intensity.

### Single-cell quantitative reverse transcriptase-polymerase chain reaction

Prior to cell collection, patch electrodes and PCR tubes were soaked in a 5% (v/v) dimethyl-dichlorosilane/chloroform solution for 20 min, and then were sterilized.[20] To analyze *PMCA* expression after epileptogenic stimuli, only those neurons showing an increased excitability, a stable membrane potential and a high-resistant patch seal were considered. Following whole-cell recording, the contents of single cells were aspirated, placed in PCR tubes containing RNase inhibitors, frozen in liquid nitrogen and kept at −70°C until use.[21] Specific primers for rat $PMCA_s$ spliced at site C were as follows: *PMCA1* (NM_053311): Fwd 5′-ATGACATTGTGAAGACGGTGAT-3′, Rev 5′-TCGTTCT CGTTATCCCACTCT-3′; *PMCA2* (NM_012508): Fwd 5′-CATAGATACTGCTGACATACTGGAT-3′, Rev 5′-CACATC ATAAGGTTCATTCGGATC-3′; *PMCA3* (NM_133288): Fwd 5′-GCAGATGTTGTGGGTGAACT-3′, Rev 5′-CGTGA GATGAGAGGCTTGTC-3′; *PMCA4* (NM_001005871): Fwd 5′-AGCAGTTGCGTCAGTCAGA-3′, Rev 5′-TCTCGGAAA CTATGAAATACTTTGA-3′. Taqman probes sequences were: Atp2b1: ATGCCTAGCGTTCAGAGACTTCCCCG; Atp2b2: CCGAAAGCAGCAAGCCGTACCAACC; Atp2b3: CTTCC GCAGCAGCAGTGACTCAGTTG; Atp2b4: CTCCACCTC TTCTGCCGTTACACCTCC, for *PMCA1–PMCA4*, respectively. To avoid genomic DNA contamination, collected cell contents were incubated with deoxyribonuclease I. One-step reverse transcriptase-polymerase chain reaction reactions for control or experimental single cells were carried out in 20 $\mu$L final volumes according to the following protocol: 50°C/15 min; 95°C/2 min; 40 cycles: 95°C /15 s; 60°C/30 s.

### Statistical analyses

For all statistical and curve-fitting procedures, a Microcal Origin program 8.0 (Microcal Software) was employed. A no directional Student's *t*-test was used in comparisons of mean values between two populations of cells. The significance value was $\alpha = 0.05$ and the critic value was 95%. Values are expressed as mean ± SEM and $P < 0.05$ considered significant.

## Results

### Electrophysiology

Prior to employing single-cell quantitative-PCR, it was crucial to characterize the electrophysiological changes in cells isolated from pilocarpine-treated animals. As described in Methods, seven control rats were injected i.p. with saline solution, and 14 experimental rats treated with the pilocarpine–diazepam protocol. Recordings were made in current clamp in a whole-cell configuration employing pyramidal cells from the CA1 region of rat hippocampus.

One month following injection, recordings were made from a total of 127 cells; 52 control cells, of which 46 presented a stable resting membrane potential (RMP) between −60 and −70 mV and had action potentials with amplitudes between 90 and 100 mV. Rats treated with pilocarpine were sacrificed after they had exhibited one or two spontaneous seizures per week. Recordings were made from 75 experimental cells; 63 fulfilled the criteria of altered excitability, that had a more depolarized RMP (mean value = −55 mV; Figure 1C) than controls ($P < 0.05$). In control cells, each depolarizing stimulus produced only one or two action potentials, while cells from pilocarpine-treated animals exhibited two or three spikes per stimulus, suggesting an increased excitability (Figures 1A and B). This is in accordance with previous observations that mechanisms underlying epileptogenesis might alter neuronal excitability.[22]

To document the electrical properties of these cells, a region of 0–800 pA was screened under current-clamp conditions. Increased spike discharges in neurons from epileptic animals (Figure 2D) were accompanied by increased membrane resistance ($P < 0.05$; Figure 1B), suggesting changes in underlying ion currents or passive membrane properties. In agreement with the latter, the mean time constant of the tail response was significantly altered ($P < 0.05$) in neurons from epileptic rats (Figure 1D).

To evaluate possible differences in the excitability of neurons from control and pilocarpine-treated animals, we analyzed the neuronal response to increasing stimuli intensity. We define $I_R$ as the intensity of the stimulus at which we obtain an action potential in neurons from control or pilocarpine-treated animals exposed to equivalent stimulus duration. For example, at stimulus durations of 1 ms, $I_R = 600$ pA in control neurons and $I_R = 320$ pA in neurons from epileptic animals (Figure 2A). In general, neurons from epileptic animals required a minimal stimulus intensity to evoke action potentials in comparison with neurons from saline-injected animals ($P < 0.05$). We also measured $t_{min}$, the minimal duration (in ms) to obtain an action potential. We found $t_{min} = 0.1$ ms for neurons from epileptic animals, while $t_{min} = 1$ ms for controls (Figure 2A). Neurons from epileptic animals required a shorter stimulus duration to trigger an action potential, indicating an increased excitability in these neurons compared with controls ($P < 0.05$; Figure 2A). Therefore, enhanced excitability may account for the altered electrical phenotype of epileptic neurons.

It is known that the fast after hyperpolarization (fAHP) lasts 2–5 ms following the repolarization face of action potential and is carried by the calcium and voltage-dependent BK channels.[23] Its amplitude is dependent on the intracellular calcium levels. Figure 2C shows that epileptic neurons have a higher amplitude fAHP than control cells. These results can be taken as indirect evidence that

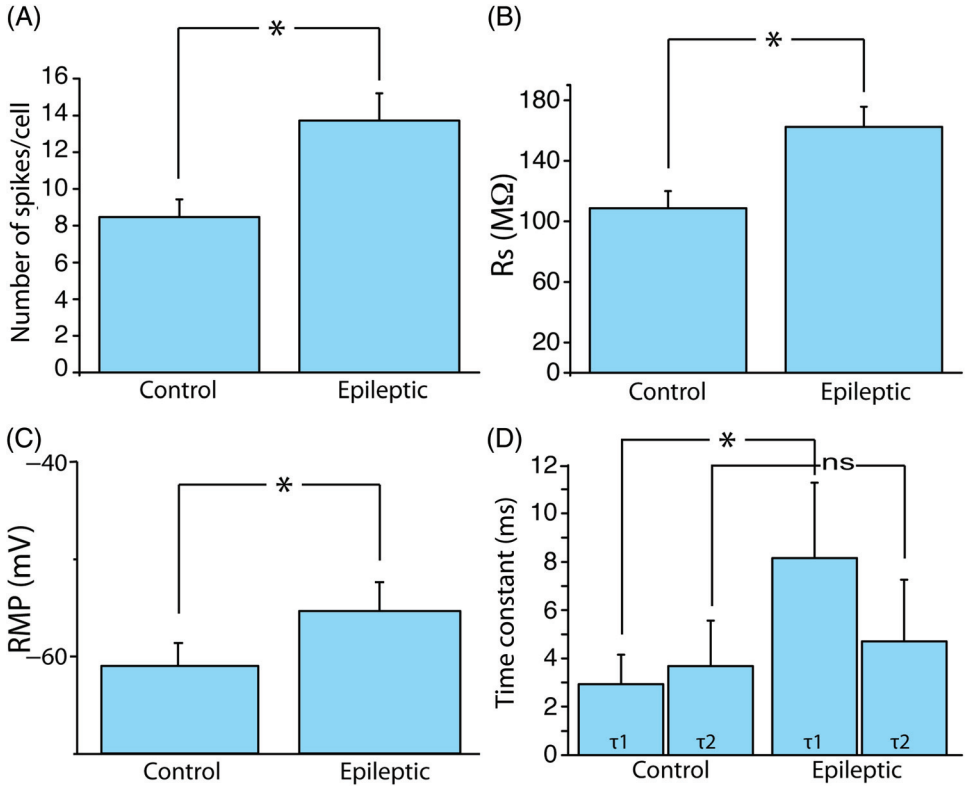


**Figure 1** Stimulus response properties of CA1 pyramidal neurons recorded in a whole-cell configuration. (A) Number of action potentials per cell at all depolarizing stimuli in control and epileptic cells. The mean number in control cells was 8 ± 0.96 ($n = 13$) and in epileptic cells was 13 ± 1.48 ($n = 11$). (B) Mean membrane series resistance during a 100 ms pulse in control and epileptic cells. Control and epileptic cells presented membrane resistance of 108 ± 16.7 MΩ and 162.48 ± 17.89 MΩ, respectively. (C) Mean resting membrane potential (RMP) of control and epileptic cells. The RMP in control cells was −61 ± 1.97 mV and in epileptic cells −55 ± 2.4 mV. (D) Tail currents for every stimulus were fitted to a double exponential function and time constants plotted ($\tau_1$ and $\tau_2$). Control cells ($n = 7$) show a $\tau_1$ of 2.99 ± 1.2 ms and a $\tau_2$ of 3.31 ± 1.95 ms. Epileptic cells show a $\tau_1$ of 8.16 ± 0.9 ms and a $\tau_2$ of 4.7 ± 3.0 ms. $\tau_1$ from epileptic cells is higher than control while $\tau_2$ do not show any differences. Average values ± SEM are indicated, $^*P < 0.05$

epileptic neurons maintain a chronic increased intracellular calcium level.

### Single-cell quantitative-PCR

Standard curves were constructed for each set of *PMCA* primers using the corresponding positive controls and Taqman probes.[24] A total of 109 neurons were analyzed; 46 of these cells were for control while 63 obtained from pilocarpine-treated animals fulfilled the criteria of altered excitability for qPCR analysis. Twelve of the 63 cells had poor expression of GAPDH probably because problems in the harvesting of these cells (7 cells) or had normal electrophysiological phenotype and normal expression of PMCAs and GAPDH (5 cells). Only 42 cells of the pilocarpine-treated rats were analyzed. In general, we noticed a high homogeneity in the expression levels of transcripts from the CA1 neurons analyzed, suggesting a strict transcriptional regulation (Figure 3).

We found the ubiquitous *PMCA1* isoform to be expressed at low levels (3.85 copies/cell) under control conditions in CA1 pyramidal neurons (Figure 3B). Interestingly, *PMCA1* was highly over-expressed (15-fold) in neurons from pilocarpine-treated animals ($P < 0.05$; Figure 3B). In contrast, under basal conditions, *PMCA3* shows only a slightly higher level of expression (~6 copies/cell) than *PMCA1* while, in neurons from epileptic animals, *PMCA3* expression is significantly decreased (>13-fold, $P < 0.05$; Figure 3C). Expression of *PMCA4* was relatively low (0.2 copies/cell) in CA1 neurons, and did not differ between neurons from control and epileptic neurons (Figure 3D). We observed that *GAPDH* levels show stable expression levels independent of pilocarpine treatment (Figure 3E).

Our ability to detect *PMCA2* transcripts at the single-cell level was limited, since this set of primers showed a low efficiency (1.93) and sensitivity (intercept 43.89) standard curve. Given the relevance of *PMCA2* to the nervous system, we then explored *PMCA2* expression in total hippocampal RNA isolated from control ($n = 3$) and pilocarpine-treated rats ($n = 3$). Hippocampal *PMCA2* expression levels were not significantly different ($P > 0.05$, data not shown) between control (3980082.2 mRNA copies/5 μL ± SEM 1876228.6) and pilocarpine-treated animals (2939450.4 mRNA copies/5 μL ± SEM 385395.1). However, considering the level of cell heterogeneity in the hippocampus, these measurements did not approach the sensitivity obtainable with single cells.

## Discussion

For the first time we have incorporated a novel approach that allows us to examine the electric behavior and the

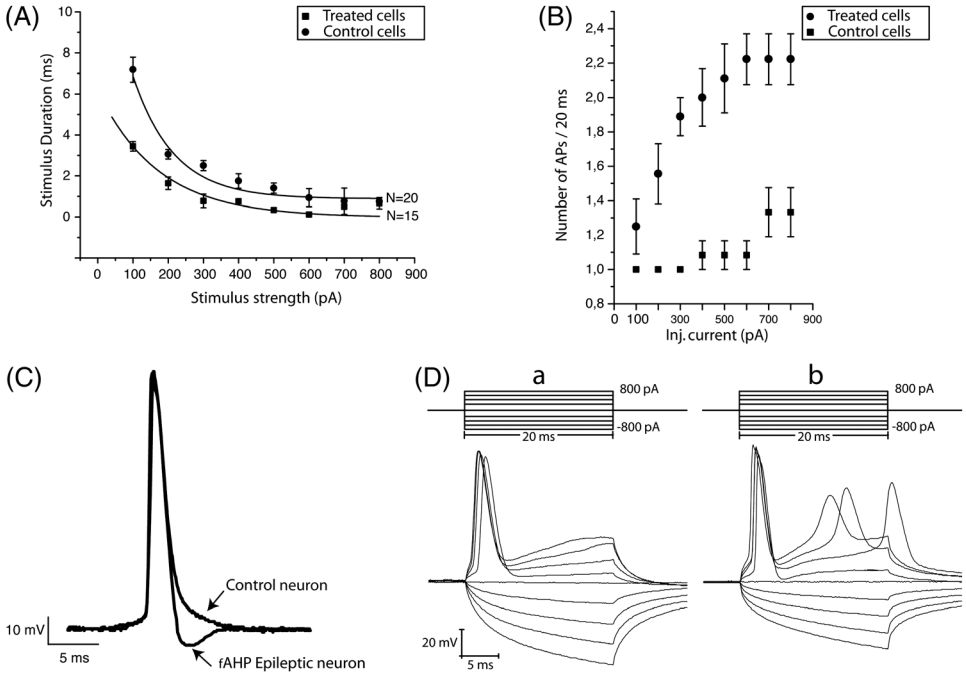


**Figure 2** (A) Responses of neurons obtained from control (full circles) and pilocarpine-treated animals (full squares) to increasing stimuli intensity. The stimulus duration is plotted as a function of its intensity at the action potential threshold for each cell. $I_R$ is defined as the intensity of the stimulus at which we obtained an action potential in neurons from control or pilocarpine-treated animals with equivalent stimulus duration. At a duration of 1 ms, control cells exhibited responses with an $I_R = 600$ pA, while cells from pilocarpine-treated animals with an $I_R = 320$ pA. We defined $t_{min}$ as the minimal duration to obtain an action potential. For neurons from pilocarpine-treated animals $t_{min} = 0.1$ ms, while for control cells $t_{min} = 1$ ms. (B) Number of action potentials as a function of the injected current stimulus from 100 to 800 pA in control ($n = 9$) and epileptic cells ($n = 12$). (C) Comparison of the fast after-hyperpolarization amplitude in control and epileptic CA1 neurons. (D) Stimulus protocol for control and epileptic cells is shown on top at left (a) and right (b) side of panel. Stimulus protocol consists of square current stimulus from −800 to 800 pA in steps of 100 pA. (a) control neurons produced one action potential for every depolarizing stimulus and (b) neurons from epileptic animals produced 2–3 action potentials for every depolarizing stimulus. For clarity we show only eight of the 16 traces

expression of *PMCA* isoforms in single hippocampal neurons during the chronic phase of experimental epilepsy. Using a combination of whole-cell patch recording and single-cell real-time PCR, we were able to correlate the electrical phenotypes of individual CA1 neurons with changes in the specific expression of different *PMCA* mRNAs during epileptogenesis.

A number of studies have suggested that altered electrical processes lead to changes in synchronic action potential discharges in epileptic neurons.[25,26] However, few studies have addressed the fundamental question of whether or not the basic electrical properties of the neuron are constitutively altered after long-term adaptive responses are established. In the current work, we have documented concomitant electrical and molecular changes in neurons that implicate the PMCA proteins in mechanisms underlying the adaptive response to epileptogenesis.

Increased action potential frequencies in epileptic neurons suggest an enhanced excitability, which can be characterized by the neuron's sub-threshold and passive responses in measures of stimulus–response properties.[27] The most striking, yet unsolved, question is whether increased epileptiform discharges are associated with changes in cell excitability. In the current study, an augmented spike frequency is accompanied by changes in sub-threshold responses (Figures 1, 2B and D), suggesting an increased basal excitability in the epileptic neuron. These results prompted us to examine the stimulus–response relationship following a classical protocol[28] that examined the behavior of the neuron following stimuli that varied in both intensity and duration. With this protocol, we observed that neurons from pilocarpine-treated animals responded to stimuli of a significantly shorter duration and lower intensity than control neurons (Figure 2A). Together, these results are consistent with underlying changes in the firing threshold, and thus excitability, of CA1 neurons from epileptic animals. Furthermore, the increased firing responses (Figure 1) can be readily related to the already known antiaccommodation effect observed in rat sympathetic neurons. This effect can be ascribed to changes in voltage-dependent, non-inactivating ionic conductance that are active at the action potential firing threshold.[29] Therefore, changes in both ionic and passive membrane conductances may underlie the electrical alterations observed in neurons from epileptic animals.

Previous studies of the expression patterns of the different PMCA isoforms demonstrate that this group of proteins plays a role not only as regulators of homeostatic intracellular calcium concentrations, but also as regulators of dynamic calcium signaling.[30] In the brain, where complex neural functions need a diverse range of highly specialized and regulated transduction signals, the PMCAs are notable for their regional and cellular heterogeneity. In the hippocampus, reports have shown PMCAs to upregulate extrusion in response to even minor calcium elevations to maintain calcium homeostasis, showing an important role for these proteins in calcium clearance.[31]

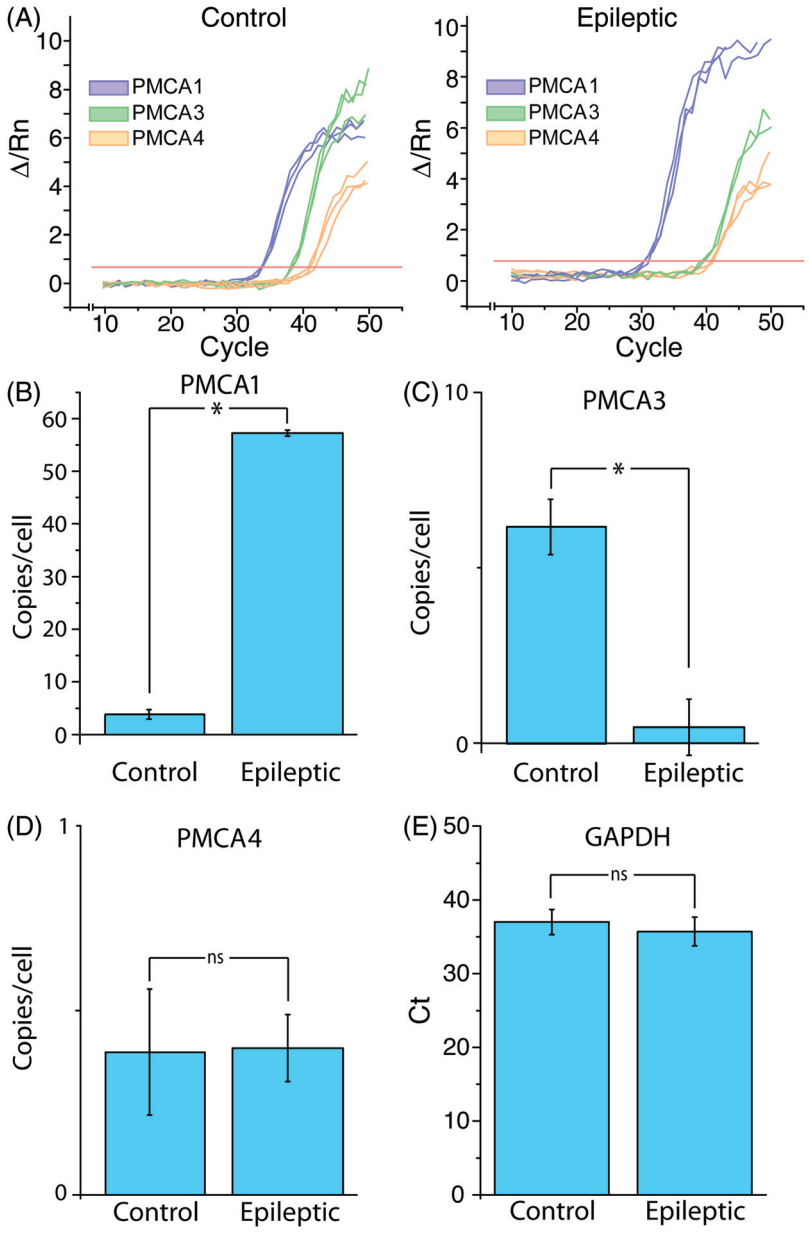


**Figure 3** Single-cell PCR quantification of *PMCA* transcripts expressed in single CA1 pyramidal neurons from control and pilocarpine-treated animals. (A) Amplification plots of PMCA1, 3 and 4 in control (left) and epileptic (right) neurons. The horizontal line represents the threshold. (B) *PMCA1*, control cells ($n = 13$, 3.85 copies/cell); epileptic cells ($n = 12$, 57 copies/cell). *PMCA1* was highly over-expressed (15-fold) in neurons from pilocarpine-treated animals. (C) *PMCA3*, control cells ($n = 11$, ~ 6 copies/cell); epileptic cells ($n = 11$, 0.6 copies/cell). *PMCA3* expression is significantly decreased (>13-fold). (D) *PMCA4* control cells ($n = 12$, 0.2 copies/cell); epileptic cells ($n = 10$, 0.18 copies/cell). Expression of *PMCA4* in CA1 neurons did not differ between neurons from control and epileptic rats. (E) *GAPDH* (control cells, $n = 10$; epileptic cells, $n = 9$) showing a similar expression in control and experimental groups. Ct average values ± SEM are plotted, $^{*}P < 0.05$

Previous studies on the early (1–4 h) stages of epileptogenesis following seizure induction with KA injections report no significant changes in *PMCA1* expression in the CA1 region of the rat hippocampus.[11] However, a significant decrease was observed from 12–72 h after seizure induction.[11] Therefore, the present study has focused on the chronic phase of epilepsy, at which time stable PMCA expression levels may reflect a newer calcium homeostasis.

Due to the complexity of the hippocampus, we quantified *PMCA* expression at the single-neuron level. Our results show that, one month after the epileptogenic stimulus, the expression of *PMCA1* in epileptic neurons increases nearly 15-fold over control levels (Figure 3B) while, in contrast, *PMCA3* shows a significant, but less pronounced decrease (Figure 3C). *PMCA4* expression remains relatively low and is unaffected by epileptogenesis (Figure 3D). Previous reports have shown that PMCA1 is the most abundant form in the hippocampus, followed by PMCA2 and PMCA3. These studies also showed PMCA4 to have the lowest expression of the hippocampal PMCAs.[15,16] In agreement with these findings, we confirmed low *PMCA4* expression in the pyramidal cell population of CA1. However, in this cell population, we found *PMCA3* to be more abundant than *PMCA1*. Based on available evidence,

PMCA1a is the most abundant isoform in human and rat in the CA1 region of hippocampus.[32,33] Since our *PMCA1* probes do not distinguish among the different splicing options, we are mainly detecting abundance of the *PMCA1a* isoform. Therefore, changes in *PMCA1* expression levels observed in epileptogenesis support a prominent housekeeping role for this isoform in CA1 neurons, in comparison to the role played by PMCA4.[34] Recent evidence found for the PMCA1a isoform, the most abundant in brain, shows a specialized role in neurones since it is located in the plasma membrane of somata, dendrites and spines.[33]

The physiological relevance of our findings can be analyzed in the context of the known kinetic properties of specific PMCA isoforms, previously reviewed by our group.[14,35] The majority of studies analyzing the calcium-extrusion properties of different PMCA isoforms have focused on PMCA isoforms that are spliced at site C, where the calmodulin (CaM)-binding domain is located. In spite of methodological limitations, it is now possible to distinguish fast isoforms, corresponding to 'a' variants, whose CaM affinity is low, from slow isoforms, or 'b' variants, which display a high affinity for CaM.[36,37] Previous studies using heterologous cells expressing different PMCAs have demonstrated a higher calcium extrusion efficiency for PMCA2 and PMCA3, which reduced cytoplasmic calcium levels 30 and 35%, respectively.[12] In contrast, the actions of PMCA4 lead to only a 15% decrease in intracellular calcium. In these studies, PMCA1 was unable to reduce the concentration of calcium.[12] Therefore, it appears that, although the calcium extrusion activity of the PMCA1 isoform is relatively low at baseline, the upregulation of this isoform in response to an insult such as epileptogenesis represents an efficient adaptive response to increases in cytoplasmic calcium, allowing epileptic cells to achieve a new equilibrium and calcium homeostasis.

Several studies analyzing the distribution of different PMCA isoforms have shown that apparently PMCA1 and PMCA4 follow a ubiquitous location in cells from different tissues.[38–40] However, in general the most abundant isoform in different cell types is precisely the PMCA1 isoform,[39] while in contrast PMCA2 and PMCA3 are enriched in cells from excitable tissues.[13,40–42] On the other hand, studies using *pmca1* and *pmca4*$^{-/-}$ deficient mice have strongly pointed to the fact that PMCA1 is indeed a housekeeping isoform, since *pmca1*$^{-/-}$ mice cannot survive.[34] *pmca4* deficient female mice seem to be normal, whereas male *pmca4*$^{-/-}$ mice are infertile.[34] Therefore, considering this body of evidence, PMCA1 might represent an indispensable housekeeping variant needed to extrude calcium in almost every developmental stage in mammals.[40,43] Although undoubtedly all PMCA variants influence the homeostasis of calcium, when cells are challenged by different stimuli, PMCA1 seems to adopt a relevant role (Figure 3). In order to preserve cell viability, once the intracellular calcium concentration reaches levels that PMCA3 is not able to handle, a slow isoform such as PMCA1 becomes critical to extrude calcium.

PMCA3 has been found to be a fast PMCA isoform, with a high affinity for calcium;[12] therefore we suggest that, under control conditions or in early stages of epileptogenesis with moderate calcium levels, PMCA3 has an important function in sensing small changes in cytoplasmic calcium concentrations and extruding calcium in short periods of time (Figure 3C). In contrast, PMCA1, which has a much lower affinity for calcium but can maintain the extrusion for longer periods, may be upregulated with a sustained increase in calcium concentration, such as occur following epileptogenesis. The over-expression of PMCA1 seems to be a physiological response attempting to counteract the important calcium increase through the sustained extrusion maintaining the viability of the epileptiform neuron.

Disturbances in the homeostasis of calcium in different models of animal epilepsy have been well described[1,2,44–46] and the resulting changes in the expression patterns of channels and membrane proteins involved in calcium mobilization have been documented.[47] Considering that previous studies have shown that specific transcripts of PMCA2 are induced as a result of depolarization,[48] it will be interesting to explore not only the specific temporal expression of different PMCA variants, but also the possibility of new isoforms being induced, or 'turned on,' during the process of epileptogenesis. In the near future, the study of temporal changes in the expression of the various PMCA isoforms, and their interactions with other calcium extrusion systems in hippocampal neurons, will be of critical importance.

In the context of the whole battery of proteins devoted to modulate cell $Ca^{2+}$ homeostasis, a large body of evidence points to the fact that every component of the system represents a physiological relevance on demand during a specific time and space. Accordingly, cells where calcium homeostasis has been disturbed, plasma membrane fast PMCAs sense and promptly extrude calcium followed by the activation of slow PMCAs. In parallel, the intervention of the sarco/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) and the $Na^{+}/Ca^{2+}$ exchanger present in the plasma membrane become also relevant for cell survival. Although these coordinated intracellular systems are known to be functioning in neurones, further experiments are being carried out in our laboratory in order to understand the specific contribution of each of the components that control all calcium homeostasis in the chronic phase of epilepsy at the single-neuron level.

**Author contributions:** All authors participated in the design of the experimental strategy. JB-M and BD-C carried out the experiments. JB-M and DEG analyzed electrophysiological data. All authors contributed in the preparation and review of the manuscript.

#### ACKNOWLEDGEMENTS

We thank the Molecular Biology Unit at IFC, Ascension Ortiz Espinosa and Ma. Isabel Arenas Reyes for excellent technical support, and also Héctor Malagón for care of experimental animals. We appreciate the excellent editorial work by Alison Munge. This research was supported by CONACyT (Grant 47333/A-1) and DGAPA-UNAM (Grant

IN228607/20) awarded to JM-O. This study was performed in partial fulfillment of the requirements for the PhD degree in Biomedical Sciences of JB-M at Universidad NacionalAutónoma de México.

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*(Received November 12, 2010, Accepted January 11, 2011)*