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CALCIUM CURRENTS IN PYRAMIDAL CA1 NEURONS IN VITRO AFTER KINDLING EPILEPTOGENESIS IN THE HIPPOCAMPUS OF THE RAT

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Abstract—Calcium is an important second messenger which plays a role in the regulation of neuronal excitability and in many forms of synaptic plasticity. In kindling epileptogenesis, a model of focal epilepsy, calcium plays an important role. The *in situ* patch-clamp technique was used to record calcium currents in slices obtained from kindled rats and controls. We found that low-voltage-activated calcium currents, probably of dendritic origin, were larger after kindling (80%). The transient high-voltage-activated calcium currents were also enhanced after kindling (50% higher). The increase of the current is accompanied by a decrease in the time constant of inactivation. The change was still present six weeks after the kindling stimulations were stopped.

These data demonstrate that low-voltage-activated calcium currents are involved in epileptogenesis. Their enhancement in the dendrites will boost synaptic depolarization and result in enhanced calcium influx, which is critically dependent on the specific activation pattern. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Key words: epilepsy, high-voltage-activated calcium current, low-voltage-activated calcium current, *in situ* patch-clamp.

In the kindling model of focal epilepsy,^{15,30} daily repeated electrical stimulations (tetani, 2 s, 50 Hz) of limbic structures induce persistent enhancement of synaptic transmission and epileptiform activity. Ultimately, this leads to afterdischarges of increasing duration, severity and generalization.¹⁵ In the CA1 area of the rat, 20–30 tetanic stimulations to the Schaffer collateral/commissural fibres are sufficient to evoke generalized tonic clonic seizures.²⁰ The enhanced excitability may last for months, even after termination of the stimulations. Many properties of the neurons and the neuronal networks that they form can play a role in the establishment of this hyperexcitability. Epilepsy is not a disease of single neurons, because synchronization of a population of neurons is of great importance.^{17,20} However, this does not exclude an important role for cellular properties. Depending on the balance between inhibition and excitation, these properties will have a strong influence on the stability of the hippocampal CA1 network. Some of the most successful antiepileptic

drugs affect very specific properties of the sodium channel.²⁷

An important excitatory current which may be affected by kindling epileptogenesis is the calcium current. This, either directly by depolarization or indirectly through modulation of second messenger function, will contribute to changes in excitability such as synaptic efficacy,^{25,34} firing threshold and firing frequency.¹⁶ Indeed, it has been shown that calcium currents are involved in kindling epileptogenesis in the CA1 area of the rat hippocampus.^{46,47} Kindled animals, when compared to untreated animals, showed a marked enhancement in extracellular calcium decreases in the stratum radiatum of the CA1 area, induced by electrical stimulation or iontophoresis of excitatory amino acids.⁴⁹ In CA1 pyramidal cells, dissociated from kindled tissue, we found⁴⁶ a larger high-voltage-activated (HVA) calcium current than in control animals. This enhancement persisted for at least six weeks after the last seizure; it probably involved N- and L-type currents. In contrast to observations in the dentate gyrus,²⁴ it could not be explained by a reduced calcium buffering.⁴⁷ Potassium currents, measured under similar conditions, were not involved in kindling epileptogenesis.⁴⁸

An important calcium current with properties well suited to control excitability is the low-voltage-activated (LVA) current.^{5,16} Until now, this current could not be detected in dissociated neurons of adult

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Abbreviations: ACSF, artificial cerebrospinal fluid; EGTA, ethyleneglycolbis(β-aminoethyl ether)-N,N,N',N'-tetraacetate; GAERS, genetic absence epilepsy rats from Strasbourg; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid; HVA, high-voltage-activated; LVA, low-voltage-activated; TEA, tetraethylammonium; TTX, tetrodotoxin.

animals. We showed recently²¹ that the *in situ* patch-clamp technique in the slice allows the detection of LVA currents. The voltage-dependent and kinetic properties of the current suggested a dendritic location, which has recently been confirmed by single channel measurements.²⁸ Such a location could also explain why these currents are hard to detect in acutely dissociated neurons that have lost most of their dendrites.⁴⁶

The involvement of the LVA current in kindling epileptogenesis is not unique, because in a model for absence epilepsy (Genetic Absence Epilepsy Rats from Strasbourg or GAERS), the LVA calcium current in thalamic neurons is larger than in a non-epileptic control strain.⁴⁴ In the dentate area of the hippocampus, others³² have described a decrease of the amplitude of the HVA calcium current, mainly attributed to a loss of calcium binding proteins.²⁴ This implies that although calcium currents are often involved in kindling, their changes are not necessarily the same in different areas of the brain.

In this study, we investigated the effect of kindling epileptogenesis on calcium currents of hippocampal CA1 pyramidal cells in slices and focused particularly on the LVA current using the *in situ* patch-clamp technique. The calcium currents were separated in terms of their biophysical properties. They were investigated in fully kindled rats, 24 h after the last of at least nine generalized convulsions. A second group of animals (the long-term group) was treated in a similar way but investigated five to six weeks later than the fully kindled group, during which period no stimulations were given. In this manner the persistent changes induced by the kindling procedure could be separated from the ones immediately related to seizure activity.

EXPERIMENTAL PROCEDURES

Implantation

Twenty-five male Wistar rats (250–300 g; Harlan, Zeist, The Netherlands) were implanted under pentobarbital anaesthesia (65 mg/kg) with pairs of stimulation electrodes (stainless steel, 80 μ m diameter, straight cut tips, 500 μ m apart) and an identical pair of recording electrodes, as described in detail previously.²⁰ Briefly, the stimulation electrodes were placed in the stratum radiatum for bipolar stimulation of the Schaffer collaterals. The recording electrodes were placed so as to straddle the pyramidal cell layer; one electrode recorded from stratum alveus/oriens and the other one recorded from the stratum radiatum. All electrodes were placed under electrophysiological control. A screw placed on the skull served as a reference during recording. After one to three weeks recovery from surgery, 13 rats were randomly assigned for kindling and the remaining 12 rats were used as controls.

Kindling procedure

Kindling stimulations were given to the Schaffer collaterals twice daily, and consisted of a tetanus (200–300 μ A, 50 Hz, 1–2 s, biphasic, bipolar pulses of 0.1 ms). Afterdischarges were evoked and monitored to determine their duration. The length and severity of the behavioural seizures increased gradually over time. After 28 ± 3 kindling

stimuli, class V seizures³⁶ were evoked. The animals were kindled until they had at least nine class V seizures. These fully kindled animals were randomly divided into two groups. The animals in one group, the short-term animals ($n = 6$), were killed 24 h after the last class V seizure. The animals of the other group, the long-term animals ($n = 7$), were killed five to six weeks after their last class V seizure, during which period no stimuli were given. The latter group was used to test the persistence of the changes due to kindling and separate them from the immediate effects of seizures. The animals in the control groups (short-term, $n = 6$; long-term, $n = 6$) were handled in the same way as the kindled group, but did not receive the tetanic stimulations.

Preparation

Rats were decapitated after brief ether anaesthesia. The brain was quickly removed from the skull and put into ice-cold artificial cerebrospinal fluid (ACSF; in mM): 124 NaCl, 3.5 KCl, 1.3 MgSO₄, 1.25 NaH₂PO₄, 2 CaCl₂, 25 NaHCO₃ and 10 D-glucose, pH 7.4, saturated with carbogen (95% O₂, 5% CO₂). The hippocampus was dissected and transverse slices (275 μ m thick) were cut on a tissue chopper. The slices were kept in a holding chamber with ACSF at room temperature ($\sim 22^\circ\text{C}$), for at least 1 h after preparation. The experiments on kindled and control rats were done in a random order.

In situ patch-clamp

The slice was transferred to the recording chamber, which was continuously perfused with ACSF ($\sim 30^\circ\text{C}$). The potassium currents were blocked using 5 mM CsCl, 10 mM tetraethylammonium chloride (TEACl) and 5 mM 4-aminopyridine, and the sodium current was blocked by 0.5 μ M tetrodotoxin (TTX).

An upright microscope (Nikon Optiphot) with a $\times 40$ water immersion objective was used to locate pyramidal CA1 cells approximately 100 μ m under the surface of the slice. The overlying tissue was first cleared by applying pressure via a large pipette.¹⁰

Cells were approached with a patch pipette (impedance ~ 2 M Ω) filled with recording solution (in mM): 100 CsF, 0.5 CaCl₂, 2 MgCl₂, 10 HEPES, 10 EGTA, 2 MgATP, 0.1 Na₂GTP, 20 TEACl, 0.1 leupeptine, 20 phosphocreatine and 50 units/ml phosphocreatine kinase; pH was set to 7.2. Free Ca²⁺ concentration was estimated to be smaller than 10^{-8} M. At these calcium concentrations, the fluoride solution hardly affected the HVA currents in our preparation, as demonstrated previously.¹⁸ After establishing a gigaseal, the whole-cell configuration was entered by rupturing the membrane with gentle suction. The holding potential was set at -65 mV. Voltage-clamp experiments were made with an Axopatch 200 amplifier controlled by an Atari computer and custom-made interface and software. The lowpass filtered (10 kHz, 88 dB/oct) signal was sampled with a frequency of 1 kHz. Access resistance (2–4 M Ω) and cell capacitance were adjusted to cancel fast transients; access resistance compensation was always set higher than 50%. With the maximal evoked currents, the voltage error was estimated around 1 mV and never exceeded 5 mV. Capacitance readouts were taken for each cell and used as an indication for the membrane surface.

Data analyses

Linear non-specific leak was determined from the passive current evoked by voltage steps of -5 mV and $+5$ mV from a holding potential, where no active current was activated. All traces were corrected for linear leak. Transient artifacts are inevitably present in recordings from the superfused slice and were often removed:¹⁸ a trace without active current (e.g., the one from -130 to -90 mV) was fit with a double exponential function. After appropriate scaling to the amplitude of the voltage step, this fit result was subtracted from the current trace. This slightly more advanced

Fig. 1. (a) Currents evoked by 200-ms depolarizing voltage steps from resting membrane potential of -65 mV (protocol given as inset). (b) Currents evoked by depolarizing voltage steps from -130 mV. (c) Peak (squares) and sustained (circles) calcium currents plotted as a function of membrane potential. Open symbols represent currents evoked from -65 mV; closed symbols represent currents evoked from -130 mV. Note that peak currents evoked from -130 mV have a lower threshold potential and a higher amplitude than those evoked from -65 mV, while sustained currents show no difference.

procedure compensated the inevitably large artifacts in the superfused slice better than the classical P4 protocol.

The inactivation of the calcium current could be well described by a single (or double) exponential decay:

$$I(t) = I_{\text{sustained}} + I_{\text{transient}} \exp\left(\frac{t_0 - t}{\tau}\right), \quad (1)$$

with amplitudes $I_{\text{sustained}}$ and $I_{\text{transient}}$ and a time constant τ of inactivation after a voltage step at time t_0 . The fit to this function was done by a simplex iteration for the non-linear components and a least square fit for the amplitudes. The voltage dependency of removal of steady-state inactivation was determined from the amplitudes of currents evoked from different prepulse potentials. The steady-state distribution of the activated and inactivated states is determined by the membrane voltage. The current amplitudes were therefore well fit by a Boltzmann equation of the following form:

$$I(V) = \frac{I_{\text{max}}}{1 + \exp\left(\frac{V - V_h}{V_c}\right)}, \quad (2)$$

where V is the prepulse potential, V_h the potential of half-maximal inactivation, V_c is proportional to the slope at V_h and I_{max} is the maximal current which was used to

normalize the currents. All values are mean values $\pm$ S.E.M. Statistical analysis was performed with the Student t -test. $P < 0.05$ was considered a significant difference.

RESULTS

Calcium current characterization

Sodium currents were blocked by TTX and potassium currents by TEA, 4-aminopyridine and

Cs^+ (see Experimental Procedures for details). Under these conditions, at least two protocols were used to evoke LVA and HVA calcium currents in different proportions. The first protocol consisted of 200-ms depolarizations from a holding potential of -65 mV, while the second protocol used the same depolarizations preceded by a 3-s hyperpolarization to -130 mV (see the insets in Fig. 1a, b). The sweeps of both protocols were run alternately so that the data were acquired over the same period of time and comparisons could be made. All calcium currents could be characterized by their voltage-dependent peak amplitude and a sustained component, measured as the mean amplitude over the last 20 ms of the depolarization. The amplitude of the transient current component was calculated by subtracting the sustained amplitude from the peak amplitude. In addition, we determined the kinetics of the inactivation and their voltage dependence.

Depolarizations from the holding potential of -65 mV to levels up to 0 mV and higher evoked an HVA calcium current (Fig. 1a), with a threshold for activation around -40 mV and about equally sized transient and sustained components (voltage dependence of the amplitude is given in the I - V curve in Fig. 1c, open symbols). Depolarizations preceded by a 3-s hyperpolarizing pulse to -130 mV evoked calcium currents with a threshold around -70 mV (Fig. 1b and confirmed in the I - V curve given in Fig. 1c, closed boxes). In the voltage region from -70 to -40 mV, this current is by definition an LVA current and it only contained a transient component. Voltage steps to potentials higher than -40 mV evoked currents that contained a sustained component in

Fig. 2. Mean I - V curves of the calcium currents in cells from control (circles), short-term kindled (squares) and long-term kindled (triangles) animals. (a) The amplitudes of sustained currents evoked by depolarizations from -65 mV (open symbols) and from -130 mV (closed symbols) are the same for all groups. (b) The transient (peak – sustained) current components for the same groups (see legend for a) are larger and have a lower threshold when evoked from -130 mV (closed symbols). The transient current evoked from -130 mV has a larger amplitude after kindling in the -60 to -50 mV range, as well as in the -20 to $+20$ mV range. Error bars indicate S.E.M.

addition to the transient one. The sustained current evoked by the step from -130 mV was almost identical to the one evoked by the step from -65 mV (Fig. 1c, compare open and closed circles). The sustained and the peak currents reached their maximal amplitude around -20 to -10 mV. At depolarizations above 0 mV, a sustained outward current was often seen. This could represent a residual outward caesium current through calcium channels, or could be due to incomplete block of potassium channels in the deeper dendrites.

A separation of currents in terms of biophysical properties is incomplete. Extensive pharmacology is necessary to obtain more detailed information. The limited availability of kindled material and the ambiguity of most pharmacological tools made such pharmacology almost impossible in the comparisons made in this study. We decided to use the biophysical properties as a first indication of current types. Depolarizing from -130 mV to levels between -70 and -40 mV evokes a unique LVA current. The step from -130 to -40 mV and higher will activate both LVA and HVA currents, and will also activate a transient HVA current that needs removal of inactivation by hyperpolarization. We will restrict our comparison of currents in cells from kindled animals and controls to the LVA component, the sustained HVA current and the transient HVA current.

We investigated 13 cells from the short-term kindled group and 14 cells from the long-term kindled group. Controls from the short-term group ($n = 17$) and the long-term group ($n = 12$) were not different, so that these groups were eventually merged into one

control group ($n = 29$). We first used test statistics on a per cell basis; we did, however, only accept the conclusions if their significance also held when comparisons were made using the mean values of the animals as observations.

Comparison of the low-voltage-activated current

The mean I - V curve of the transient current (Fig. 2b) evoked by depolarizations from -130 mV demonstrate that an LVA current can be evoked in cells from kindled and control animals. The current had the same threshold potential of -70 mV in both groups. The LVA current evoked by the step from -130 to -50 mV was used for statistical comparison of cells from control and kindled animals. Typical examples of such currents are given in Fig. 3a. The amplitude of this LVA current was 0.25 ± 0.04 nA in the control group, 0.45 ± 0.11 nA in the short-term group ($P < 0.05$) and 0.46 ± 0.12 nA in the long-term group ($P < 0.05$). In both kindled groups, the peak current was approximately 80% larger (Fig. 4a) than in controls.

The LVA current evoked at -50 mV had a time constant of inactivation of 42 ± 4 ms in the control group. In cells from short-term kindled animals its value was reduced to 21 ± 2 ms ($P < 0.001$) and in the long-term group it was 29 ± 3 ms ($P < 0.05$). The enlarged LVA calcium current of the kindled animals was inactivated significantly faster than the current in control animals, but there was a partial recovery six weeks later (Fig. 4b).

Fig. 3. Typical examples of currents evoked by voltage steps from -130 to -50 mV (a), from -65 to -10 mV (b) and from -130 to -10 mV (c). The traces shown are from one cell (per group). The left column compares traces from controls (circle) with short-term kindled animals (square). The right column compares traces from controls (circle) with the long-term kindled group (square). Note the difference in amplitude scale between a and b and c, and the clear difference in time constant between control and kindled in a.

Fig. 4. Comparison between control animals and kindled animals, for the current amplitude (a) and the inactivation time constant for the transient components (b). LVA is the transient current evoked by a voltage step from -130 to -50 mV, HVA -65 is the current evoked by a voltage step from -65 to -10 mV and HVA -130 is the current evoked by a voltage step from -130 to -10 mV. Error bars are S.E.M. * $P < 0.05$, ** $P < 0.01$, Student's t -test.

An important parameter for cell function and a potential threat to the cell is the calcium influx, which for the LVA current would be enlarged due to amplitude enhancement but also reduced due to the faster time constant of inactivation. We determined the area under the LVA current over an arbitrary period of 195 ms, starting 5 ms after the voltage step. For the controls, the total charge carried by calcium was 23 ± 6 pC, for the short-term group it was 20 ± 8 pC and for the long-term group it was 23 ± 7 pC. For this long pulse duration, the influx of calcium due to the LVA current is not enhanced after kindling epileptogenesis; however, if we determine the influx for a pulse of 20 ms the LVA calcium current can carry 3.4 ± 0.8 pC in controls, 5.3 ± 1.5 pC in cells from short-term kindled animals and 4.8 ± 1.0 pC in the long-term kindled ones, which implies a substantial enhancement (see Discussion for more details).

Comparison of high-voltage-activated currents

High-voltage-activated currents evoked from a holding potential of -65 mV

Currents evoked from -65 mV can only consist of HVA components. The I - V curves show that the sustained component (Fig. 2a) and the transient component (Fig. 2b) have the same threshold potential of -40 mV in all groups of animals. The I - V curve of the sustained component showed a suspiciously low reversal potential for the calcium current (Fig. 2c), suggesting the presence of a sustained outward current. For statistical comparison we analysed the components evoked at -10 mV, as illustrated in Fig. 3b. The amplitude of the sustained component at -10 mV was 0.38 ± 0.05 nA in the control group, 0.37 ± 0.12 nA in the short-term kindled group and 0.38 ± 0.07 nA in the long-term kindled group. The control group was not different from the two kindled groups (Fig. 4a). This also holds for the outward component. The amplitude of the sustained component was identical for all groups; therefore, further comparing the transient component, instead of peak amplitude, has the advantage that the interference of the outward component is removed.

In the control group, the amplitude of the transient component was 0.45 ± 0.05 nA. The short-term kindled group had currents with an amplitude of 0.48 ± 0.10 nA and the long-term kindled group had an amplitude of 0.50 ± 0.11 nA (Fig. 4a). No significant difference could be found between control and kindled animals. The transient component inactivates with a time constant of 95 ± 5 ms in the control group; this time constant was decreased to 75 ± 5 ms ($P < 0.05$) in the short-term kindled group and to 73 ± 5 ms ($P < 0.02$) in the long-term kindled group (Fig. 4b). The difference in inactivation is small but significant, suggesting that the kinetics of this component may be affected by kindling epileptogenesis.

The calcium influx (total charge carried) was calculated for the total current evoked during 195 ms at -10 mV. For the controls we found 83 ± 13 pC, which was not significantly different from the short-term kindled animals (88 ± 24 pC) or from the long-term kindled animals (94 ± 18 pC).

High-voltage-activated currents evoked from a holding potential of -130 mV

Depolarizations from -130 mV evoked the LVA as well as the HVA currents analysed in the previous two paragraphs. In addition, this protocol also evoked an HVA component with a strong inactivation that needs to be removed by the deep hyperpolarizing prepulse to -130 mV. This additional component is clearly seen in the representative currents at -10 mV (Fig. 3c) and in the mean I - V curve when it is compared with the one evoked from -65 mV (Fig. 2b). The sustained component evoked from -130 mV is the same as the sustained component evoked by depolarizations from -65 mV (Fig. 2a). Statistical comparison of the HVA currents evoked by depolarizations from -130 mV in cells from control and kindled animals was made using the current at -10 mV (Fig. 3c).

The current was characterized by its transient and sustained components, as before. The sustained component is identical to the one evoked from -65 mV. At -10 mV it has an amplitude of 0.37 ± 0.06 nA for the control group, 0.35 ± 0.10 nA (n.s.) in the short-term kindled group and 0.40 ± 0.13 nA (n.s.) in the long-term kindled group (Fig. 4a). This confirmed the lack of change after kindling found for the sustained component evoked by steps from -65 mV.

The transient component at -10 mV has a large amplitude of 0.95 ± 0.08 nA in the control group. This amplitude was enhanced to 1.35 ± 0.14 nA ($P < 0.02$) in the short-term kindled group and to 1.61 ± 0.20 nA ($P < 0.001$) in cells from the long-term kindled group (Fig. 4a). The inactivation time constant for this transient component was reduced from 64 ± 3 ms in the control group to 53 ± 5 ms ($P < 0.01$) in the short-term group and 50 ± 3 ms ($P < 0.01$) in the long-term group.

In the range between -40 and -10 mV, it is likely that both LVA and HVA currents are evoked and therefore we should have fit the currents in this range with at least two exponentials. We investigated whether this approach lead to an improvement of the parametrization. At higher potentials, the HVA current is strongly dominant in amplitude. Moreover, exponentials are not orthogonal functions, so that the improvement in the actual fit did not outweigh the increased (correlated) variance in the time constants. We therefore did not try to separate the currents in greater detail.

The transient HVA current is about 50% larger and inactivates 16% faster in cells from kindled animals compared to cells from controls. The influx caused by this transient component over 195 ms is

Fig. 5. (a) Currents evoked by a depolarization to -30 mV from hyperpolarized levels between -150 and -60 mV to determine the removal of steady-state inactivation (voltage protocol given as an inset). (b) Normalized amplitude of the transient current evoked by a step to -30 mV is plotted as a function of the prepulse potential: control (circles), short-term kindled (squares) and long-term kindled (triangles). The curves represent the Boltzmann functions generated using the mean values of half-maximum inactivation (V_h) and the slope parameter (V_s). The heavy line is control and thin lines are kindled. Error bars indicate S.E.M.

123 ± 13 pC in controls versus 147 ± 30 pC in the short-term group and 156 ± 33 pC in the long-term group. The difference between control and kindled for this influx did not reach significance. The calcium influx for a pulse of 20 ms was enhanced from 22 ± 2 pC in controls to 30 ± 4 pC ($P < 0.05$) and 34 ± 5 pC ($P < 0.01$) in short-term and long-term kindled animals, respectively.

Steady-state inactivation

The voltage-dependent removal of steady-state inactivation was measured by depolarizing the cell to -30 mV from potentials varying between -150 and -60 mV (Fig. 5). Such a step evoked a calcium current that contained sustained and transient components (Fig. 5a). The amplitude of the transient component is an indication of the available calcium channels and therefore indicates the steady-state inactivation at the end of the long prepulse at the specified voltage. It was plotted as a function of the prepulse potential (Fig. 5b) and fit with a Boltzmann equation (Eqn 2). The potential where the removal of inactivation is half-maximal (V_h) in control animals was -107 ± 2 mV, while it was -108 ± 2 mV in the short-term kindled animals and -102 ± 2 mV in the long-term kindled animals. We observed a slight tendency for the inactivation to shift to positive levels in the long-term animals. The slope parameter (V_s) was the same for all groups, namely, 10.7 ± 0.5 mV for the controls, 9.5 ± 0.5 mV for the short-term kindled and 11.1 ± 0.6 mV for the long-term kindled animals.

Current density

In order to interpret the changes in absolute current one step further, we also determined the cell capacitance as the best available indication of the cell surface. In a previous study,⁴⁷ using acutely dissociated cells, we confirmed the relation between capacitance and surface estimation from photographs. Capacitance was identical in the controls (15.4 ± 0.7 pF) and the short-term kindled group (13.5 ± 0.8 pF), but it was significantly larger (20.5 ± 1.6 pF) in the long-term kindled group. We could not detect any kind of correlation within each group of animals between cell capacitance and current amplitude. Therefore, it is not surprising that our conclusions are not affected when we draw them on the basis of current density instead of absolute current amplitude. The means and S.E.M. were calculated from the densities of the individual cells. A small addition to the conclusions came from the fact that the LVA current (15 ± 2 pA/pF) in controls, which was still significantly enhanced in the short-term group (34 ± 7 pA/pF), showed a partial recovery in the long-term group (to 23 ± 6 pA/pF), which was neither significantly different from the controls nor from the kindled group.

DISCUSSION

Current separation

Using the *in situ* patch-clamp technique, we distinguished several calcium current components in

pyramidal CA1 cells. These components were separated on the basis of biophysical properties. There is no unambiguous link between them and the single channel level. Even the pharmacology necessary to be more precise is not absolute and was impossible to perform due to the limited amount of kindled material available. Nevertheless, we can distinguish three functionally distinct components: a transient LVA component, a transient HVA component and a sustained HVA component.

The LVA component, evoked between -70 and -40 mV from a holding level of -130 mV, is similar to the T-type,^{41,42,45} type I³³ or LVA^{1,4,5,6,8} described by others in several neuronal tissues. It has been shown that this current type is present predominantly in the dendrites.^{21,28} Since, in CA1 neurons, no other type of calcium current is known to activate at such low voltages (given the presence of TTX), we considered the LVA currents evoked at -50 mV to be a T-type calcium channel.

We often detected a residual sustained outward current at higher potentials, presumably carried by caesium through calcium channels or resulting from an incomplete block of dendritic potassium channels. The HVA calcium current can be separated into at least two components on the basis of voltage-dependent and kinetic properties.^{12,13,42,45} One of these components is the sustained HVA current, which could reflect an L-type calcium channel. For the L-type calcium current a removal of inactivation, if present at all,^{12,42} is suggested in the range between -60 and -10 mV.^{13,45} If inactivation could be found it was slow (time constant 500 ms) compared to the other HVA components. The sustained currents in our experiment were the same whether the depolarizing voltage steps were from -65 mV or from -130 mV. This suggests that the inactivation of the sustained HVA current is already removed at a potential of -65 mV and that the current is still present 200 ms after its onset; it has slow or no inactivation. Another sustained calcium channel is the P-type calcium channel.^{26,39} We did not expect our sustained current to resemble much of this channel type, based on recent single channel measurements.²⁸

The second component analysed in the HVA current was the transient current. Its distinction from the sustained component is based on kinetics. This class contains several types of calcium channels: N-type,^{12,16,42,45} Q-type³⁹ or R-type.^{11,38} To what extent each of the candidates is present could not be determined on the basis of voltage-dependent and kinetic properties alone.

Difference after kindling

In kindled animals, the transient HVA current was significantly larger than in controls. In dissociated cells a transient component with a similar time constant was also enhanced after kindling.^{46,47} The current shows a slightly faster inactivation. This

could mean that the properties of the channels underlying this current are changed, but because this current is probably a mixture of different current types, the faster inactivation may also be explained by a shift in the relative abundance of fast inactivating channel types.

A larger amplitude will cause a larger influx of calcium, although the faster inactivation of the transient HVA current may partly counteract the effect on total calcium accumulating in cells of kindled animals (details will be discussed below). As one of the important second messengers, calcium regulates:^{7,14} (i) the modulation of calcium-dependent enzymes; (ii) several calcium-dependent calcium and potassium channels;^{3,22,35} (iii) gene transcription like that initiated by immediate early genes;² (iv) calcium-dependent transmitter release; and (v) forms of synaptic plasticity induced by elevation of postsynaptic calcium levels.³⁴

The sustained component of the HVA current was the same in all experimental groups. Previous studies in dissociated cells found an enhancement of this component after kindling,^{46,47} but two aspects complicate a detailed comparison of these two experiments. Firstly, it is harder to separate, in the slice, the sustained calcium current from the leak or residual outward current. Secondly, differences in temperature,³³ calcium concentration⁴¹ and cell size predict the calcium current in the slice to be 10 times larger than in dissociated neurons, which is not the case. This discrepancy can be explained if we assume that current amplitude in the dissociated cells gives an indication of the maximum level of recruitment. This explanation would imply that the sustained current in the *in situ* patch is still modulated and that the regulatory mechanisms involved are not affected by kindling epileptogenesis.

The main emphasis in this study is on the LVA calcium current, which has not yet been investigated in kindling epileptogenesis.

The LVA current was larger in kindled animals and inactivated faster. An enhancement was found in short-term and in long-term kindled animals. This is the only conclusion that is slightly affected when current density is used instead of absolute current levels. The current density suggests at least partial recovery, in line with the observation of the changes in the time constant of inactivation. We tried to prevent a sampling bias in the cells used in the experiments and have no explanation for the significantly different cell size in the long-term kindled group.

The change in inactivation time constant could indicate a change in the properties of the channels underlying this current, but alternatively it could also result from a redistribution of channels over the dendrites.

The LVA current is present in the dendrites of hippocampal neurons^{21,28} and therefore errors in space-clamp have been of continuous concern. The measurement of LVA currents, however, is less

Fig. 6. Ratio of calcium influx of short-term kindled cells and controls as a function of depolarization time. Inset shows how calcium influx is calculated for a current of duration t . The heavy line shows the result for the LVA current, the thin line shows the result for the total current evoked by a step from -130 to -10 mV.

susceptible to errors than the measurements of HVA currents. During deep depolarizations, the cell is relatively tight and electrotonically small (but see below). Furthermore, the LVA currents are activated around -50 mV, which is close to the equipotential of the cell. The cable properties of the dendrites will give the LVA current recorded in the soma an apparently smaller amplitude, slower kinetics and a more hyperpolarized voltage dependence of inactivation.²¹ A relative increase in channels located electrotonically close to the soma could therefore explain our observations. The complexity of all dendritic factors that contribute makes a quantitative assessment extremely difficult. The slight tendency for V_h of inactivation to shift in the depolarizing direction observed in the long-term kindled animals is most likely not enough to explain the changes. Therefore, we conclude that redistribution of channel density along the dendrites, as well as changes in channel properties and total channel density, are involved.

An increase in amplitude leads to an increased calcium influx; however, the increase of inactivation will reduce the size of the effect for currents of long duration. This combination of change in amplitude and inactivation was observed for the transient HVA current and the LVA current. The combined effect of an enhanced amplitude and increased inactivation is critically dependent on the duration of the calcium current; short depolarizations will show an increased calcium influx dominated by the increased amplitude, while longer depolarizations will carry a charge that is proportional to the product of amplitude and inactivation time constant. The ratio of the influx in kindled cells over the influx in control cells as a function of activation duration is given in Fig. 6 for the LVA current at -50 mV and the HVA current evoked by a step from -130 to -10 mV. It can be seen that short depolarizations (<50 ms) give the highest increase in calcium influx in cells from

kindled animals, while long depolarization for the LVA current might even lead to a smaller influx. The change in calcium currents located at the dendrites is in accordance with the stimulus-induced enhancement in extracellular calcium decrease found in the stratum radiatum of kindled rats.⁴⁹

In hippocampal CA1 neurons, the synaptically activated increase in calcium concentration is primarily due to voltage-gated calcium channels.^{29,31,37} So, in kindled animals these short-lasting excitatory postsynaptic potentials might cause an increased calcium influx, as will other short depolarizations like back-propagating action potentials.⁴⁰ Without knowledge of the calcium buffering systems, it is impossible to predict the consequence of these changes for the actual calcium levels in the cell. The increased calcium influxes in the dendrites can change the regulation of local calcium-dependent channels, the modulation of local calcium-dependent enzymes and thereby influence the regulation of local synaptic plasticity.^{25,34}

Besides the enhanced calcium influx, the higher LVA current will also have a depolarizing effect. The dendritic location and the low activation threshold of this current make it suitable to enhance excitatory postsynaptic potentials. Small postsynaptic depolarizations can already activate the LVA channels to boost stronger depolarizations.^{29,31,40} The larger LVA current could lead to an amplification of the enhanced excitability.

Several modelling studies have shown the necessity of dendritic calcium spikes for somatic burst generation.⁴³ An increased LVA current can therefore increase the bursting probability of the CA1 pyramidal neuron. The bursting activity, as well as the rebound excitation, have been proposed as a cellular mechanism for the generation of prolonged epileptiform events.¹⁷

Kindling is not the only model of epilepsy where the LVA current is enhanced. In the model of absence epilepsy (GAERS),⁴⁴ the enhanced LVA calcium currents in the thalamus can drive rhythmic activities and underlie rebound activity.^{8,16,19} Anti-epileptic drugs like ethosuximide, trimethadione⁹ and valproic acid²³ have been reported to affect LVA calcium currents.

CONCLUSION

In this research we extended the observation of an increased transient HVA calcium current and showed that the LVA calcium current is enhanced after kindling. The LVA current contributes to the excitability by stimulus specifically increasing the calcium influx, by boosting the excitatory postsynaptic potentials and by shaping dendritic depolarizations. It may therefore play an important role in kindling epileptogenesis.

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