Activation of a Calcium-Activated Cation Current During Epileptiform Discharges and Its Possible Role in Sustaining Seizure-Like Events in Neocortical Slices

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Schiller, Yitzhak. Activation of a calcium-activated cation current during epileptiform discharges and its possible role in sustaining seizure-like events in neocortical slices. J Neurophysiol 92: 862–872, 2004; 10.1152/jn.00972.2003. Epileptic seizures are composed of recurrent bursts of intense firing separated by periods of electrical quiescence. The mechanisms responsible for sustaining seizures and generating recurrent bursts are yet unclear. Using whole cell voltage recordings combined with intracellular calcium fluorescence imaging from bicuculline (BCC)-treated neocortical brain slices, I showed isolated paroxysmal depolarization shift (PDS) discharges were followed by a sustained afterdepolarization waveform (SADW) with an average peak amplitude of 3.3 ± 0.9 mV and average half-width of 6.2 ± 0.6 s. The SADW was mediated by the calcium-activated nonspecific cation current (I_{ca}) as it had a reversal potential of −33.1 ± 6.8 mV, was unaffected by changing the intracellular chloride concentrations, was markedly diminished by buffering [Ca^{2+}], with intracellular bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), and was reversibly abolished by the I_{ca} blocker flufenamic acid (FFA). The Ca^{2+} influx responsible for activation of I_{ca} was mediated by both N-methyl-D-aspartate-receptor channels, voltage-gated calcium channels and, to a lesser extent, internal calcium stores. In addition to isolated PDS discharges, BCC-treated brain slices also produced seizure-like events, which were accompanied by a prolonged depolarizing waveform underlying individual ictal bursts. The similarities between the initial part of this waveform and the SADW and the fact it was markedly reduced by buffering [Ca^{2+}], with BAPTA strongly suggested it was mediated, at least in part, by I_{ca}. Addition of FFA reversibly eliminated recurrent bursting, and transformed seizure-like events into isolated PDS responses. These results indicated I_{ca} was activated during epileptiform discharges and probably participated in sustaining seizure-like events.

INTRODUCTION

Epilepsy is a common disease effecting ~1% of the population and manifesting clinically as recurrent unprovoked seizures (Wyllie 2001). Seizures are typically short-lived events, lasting 10–90 s. The clinical manifestations of seizures vary widely (Wyllie 2001). Transient hypersynchronous activity within the cortical network lies at the pathophysiological heart of epilepsy. When this hypersynchrony is sufficiently long and widespread, clinical symptoms occur (Dichter et al. 1998; Neidermeyer and Da Silva 1999). However, it is still unclear what mechanisms sustain electrographic seizures and gap over the periods of electrical quiescence in between ictal bursts.

Recurrent bursting during seizures can be mediated by two fundamentally different mechanisms. The first requires the neurons to undergo a prolonged state of activation that extends over the quiescent inter-burst periods. This extended state of activation can result from activation of a prolonged depolarizing current or from spontaneous initiation of axonal action potentials (Traub et al. 1996). The second mechanism that can mediate recurrent bursting is a cyclic interplay between a hyperpolarizing current, such as the calcium-activated potassium current and a hyperpolarization activated depolarizing current, such as the I_{h} current or the T-type voltage-gated calcium current.

Neocortical brain slices exposed to BCC in vitro produce both isolated PDS discharges and seizure-like events (Hablitz 1987). In this study, I used whole cell voltage recordings combined with calcium imaging measurements to investigate the cellular mechanisms involved in the sustaining seizure-like events in BCC treated neocortical brain slices.

METHODS

Slice preparation and electrophysiological recordings

Parasagittal neocortical brain slices (350–400 μm in thickness) were prepared from 13- to 35-day-old Wistar rats as previously described (Schiller 2002; Traub and Jeffreys 1994; Traub et al. 1996). Neocortical brain slices were initially exposed to BCC. All data in this study were obtained from BCC-treated brain slices.

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described (Schiller et al. 1997). Neurons were visualized using infrared illumination and differential interference contrast optics (IR-DIC) video microscopy. The microscope used was a fixed stage BX-51WI (Olympus) equipped with a ×60 water-immersion objective (NA 0.9). Whole cell voltage recordings from the soma were obtained as previously described (Schiller et al. 1997) using the Axoclamp 2B or the multi-clamp 700A amplifiers (Axon Instruments, Foster City, CA). Somatic (3–6 MΩ resistance) recording pipettes were filled with (in mM) 115 K-glucionate, 20 KCl, 2 Mg-ATP, 2 Na₂-ATP, 10 Na₃-phosphocreatine, 0.3 GTP, 10 HEPES, pH 7.2, and either 0.1 Calcium Green-1 or 0.1 bis-(o-aminophenoxy)-N,N',N'-tetraacetic acid (BAPTA). The extracellular solution contained (in mM) 125 NaCl, 25 NaHCO₃, 25 glucose, 4 KCl, 1.25 NaH₂PO₄, 1.5 CaCl₂, 1 MgCl₂, 0.01 bicuculline (BCC), pH 7.4. All experiments were performed in 35 ± 0.5°C (mean ± SD). Synaptic stimulation was performed using patch pipettes filled with artificial cerebrospinal fluid. All chemicals were purchased from Sigma aside from Calcium Green-1 (Molecular Probes, Portland, OR), α-methylserine-Ο-phosphate (MSOP), α-methyl-4-carboxyphenylglycine (MCPG), and BCC (Tocris, Bristol, UK). Flufenamic acid (FFA) was first dissolved in DMSO and added to the extracellular solution with the final concentration of DMSO being 0.1%.

**Fluorescence measurements**

High-speed [Ca²⁺], fluorescence imaging measurements were performed with the calcium-sensitive fluorescence dyes Calcium Green-1 (100 μM) or Fluo-5F (400 μM). The experiments were performed using an FEV-500 confocal scan head (Olympus, Tokyo, Japan). Calcium Green-1 and Fluo-5F were excited at 488 nm with an argon laser. Fluorescence measurements were collected from different dendritic regions in the line scan mode with a temporal resolution of 512 Hz. Background subtraction and bleach correction were performed, and the fluorescence values were converted to delta F/F in percent values as previously described (Schiller et al. 2000).

**Data analysis**

The electrophysiological and imaging data were transferred to a separate PC workstation and analyzed using the Igor software (WaveMetrics, Lake Oswego, Oregon). The data were presented in the form of average and SD values. Statistical analysis was performed using the Student’s t-test.

**RESULTS**

**PDS discharges were accompanied by a sustained afterdepolarization waveform (SADW)**

Neocortical brain slices exposed to the GABAₐ receptor blocker BCC (10 μM) produced two types of epileptiform discharges. All slices examined (104 slices from 61 animals) produced isolated PDS discharges, which mimicked inter-ictal spikes. In addition brain slices from 12- to 17-day-old rats (67 slices from 44 animals) produced much longer seizure-like events consisting of recurrent bursts of intense activity separated by quiescent inter-burst periods. The initial bursts of seizure-like events consisted of PDS discharges. As the seizures evolved, ictal bursts gradually attenuated (see also Haßbitz 1987; Schiller 2002).

Close inspection of isolated PDS discharges revealed they are followed by a SADW lasting several seconds (Fig. 1A). This SADW had an average peak amplitude of 3.3 ± 0.9 mV (n = 83), average half-width of 6.2 ± 0.6 s (n = 83), and average time to peak of 1.3 ± 0.3 s (n = 62, time to peak measured for a 10–90% voltage change). In most cases, the SADW had an ascending phase, peak, and descending phase (for example see Fig. 1). However, in some neurons, the SADW had the appearance of a plateau potential or was devoid of an ascending limb and only gradually descended after the PDS terminated (for example see Figs. 3A and 4A). In both these cases, the time to peak could not be determined, and in the later case the peak amplitude was measured 100 ms after the PDS terminated.

**FIG. 1.** A sustained afterdepolarizing waveform (SADW) develops after isolated paroxysmal depolarization shift (PDS) discharges. A: a whole cell voltage recording of an isolated PDS discharge evoked by extracellular synaptic stimulation in the presence of 10 μM extracellular bicuculline (BCC). The 3 traces show the same waveform at different voltage and time scales. Note the large SADW developing after the isolated PDS discharge. The resting membrane potential was -62 mV. B: simultaneous whole cell voltage recordings from a pair of pyramidal neurons located in neocortical layers 2/3 and 5 during an isolated PDS response. The arrow heads mark 2 isolated action potentials occurring only in the layer-5 and not in the simultaneously recorded layer-2/3 neuron. The resting membrane potential of the layer-5 and layer-2/3 neurons were -59 and -71 mV, respectively.
In most trials, fast action potentials initiated exclusively during the PDS discharge. However, in 16 of 83 recorded layer-5 pyramidal neurons (19%), fast axonal action potentials were also observed at a later stage, overriding the SADW. To investigate whether these action potentials occurred synchronously throughout the network, I performed simultaneous double recordings from pairs of pyramidal neurons (12 pairs of 2 layer-5 pyramidal neurons and 10 pairs of a layer-5 and layer-2/3 pyramidal neurons). In 5 of the 22 pairs recorded (23%, 3 pairs of layer-5 pyramidal neurons and 2 pairs of a layer-5 and a layer-2/3 pyramidal neurons), isolated axonal action potentials over-rode the SADW response in only one of the recorded neurons (Fig. 1B). In the two experiments in which this phenomenon occurred in a pair of layer-5 and layer-2/3 pyramidal neurons, the isolated action potentials were observed in the layer-5 neuron.

**Calcium-activated cation current mediated the SADW**

Isolated PDS discharges evoked large \( \text{Ca}^{2+} \) influx into dendrites of cortical pyramidal neurons (Albowitz et al. 1997; Schiller 2002). This finding is demonstrated in Fig. 2A, where PDS-evoked \([\text{Ca}^{2+}] \), transients were measured from the main apical trunk of a layer-5 pyramidal neuron 250 \( \mu \text{m} \) from the soma. Similar results were obtained from five additional neurons filled with 400 \( \mu \text{M} \) Fluo-5F, and nine additional neurons filled with 100 \( \mu \text{M} \) Calcium Green-1 (for a complete quantified profile of PDS-evoked \([\text{Ca}^{2+}] \), transients in the various dendritic regions of BCC-treated layer-5 pyramidal neurons, see Schiller 2002).

Intracellular calcium serves as a common second-messenger activating several types of ionic channels. I raised the possibility that the SADW was mediated by one such calcium-activated channel. To investigate this possibility, I used the calcium buffer BAPTA applied intracellularly via the recording pipette. In these experiments, isolated PDS discharges were recorded at different time intervals after establishing a whole cell recording with a pipette containing 10 mM BAPTA. The results of these experiments showed the SADW progressively disappeared as neurons were gradually loaded with BAPTA (Fig. 2B). On average, 40 min after establishing the whole cell recording with a BAPTA-containing pipette, the peak and area under curve of the SADW declined to 11.7 ± 5.4 and 8.1 ± 2.1% of their original value recorded 1–2 min after establishing the whole cell recording (n = 8, P < 0.001). In addition to the effect on the SADW, BAPTA also shortened the duration of the PDS without effecting its peak in four of the eight neurons examined (for example, see Fig. 2B).

In a set of control experiments, neurons were loaded with the control intracellular solution containing no BAPTA. In these experiments, no change was observed in the SADW during 60 min of whole cell recording (n = 6, data not shown). These findings confirmed that elimination of the SADW resulted from buffering of the intracellular calcium with BAPTA rather than washout of intracellular substances.

Previous studies have described several types of calcium-activated ion channels. These included calcium-activated potassium, sodium, chloride, and nonspecific cation (CAN) channels (for review, see Partridge et al. 1994; Scott et al. 1995; Vergara et al. 1998). Increasing the intracellular chloride concentration via the recording pipette (pipette solution contained 75 mM K-glucosate and 60 mM KCl) had no effect on the amplitude or shape of the SADW (n = 5, data not shown), indicating the current mediating the SADW was impermeable to chloride ions.

To further characterize the current mediating the SADW, I measured its reversal potential. Isolated PDS discharges were recorded at different resting membrane potentials, and the peak amplitude of the SADW was plotted as a function of the resting membrane potential. The reversal potential was obtained by fitting a linear curve to the experimental data and calculating

![Image](http://jn.physiology.org/)

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**FIG. 2.** The SADW evoked by isolated PDS discharges is calcium-dependent. **A:** concomitant calcium fluorescence imaging (top gray trace) and voltage recordings (bottom black trace) during an isolated PDS discharge. The voltage recording was performed using a somatic whole cell recording. The calcium fluorescence imaging presented was obtained from the main apical dendrite 250 \( \mu \text{m} \) from the soma using 400 \( \mu \text{M} \) Fluo-5F and expressed as delta \( \text{F/F}_0 \) in percent values. The resting membrane potential was ~64 mV. **B:** intracellular voltage recordings during an isolated PDS discharge recorded 1 (black trace) and 40 min (gray trace) after obtaining a whole cell recording with a somatic pipette containing 10 mM bis-(o-aminophenoxy)-X,N,N,N′-tetraacetic acid (BAPTA). The trace in the bottom panel was obtained by subtracting the 2 traces. Note that buffering [\( \text{Ca}^{2+} \)], with intracellular BAPTA eliminated the SADW. The resting membrane potential was ~58 mV and was unaffected by BAPTA.

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the interception of this linear curve with the x axis (Fig. 3A). To prevent initiation of axonal action potentials, the resting membrane potentials examined were kept below (more hyperpolarized than) the threshold for action potential initiation. The average reversal potential of the SADW calculated in these experiments was $-33.1 \pm 6.8$ mV ($n = 5$).

Measuring the reversal potential of a waveform in an intact neuron has several inherent inaccuracies, especially the inability to control voltage throughout the neuron, and the modifications imposed by the voltage change on other unrelated voltage-gated channels. Moreover, in the case of a calcium-dependent current, reversal potential measurements are further complicated by a possible dependence of calcium influx on resting membrane potential. In the case of PDS-evoked $[Ca^{2+}]_i$ transients, changing the resting membrane potential may in fact have two opposing effects. On the one hand, hyperpolarizing the membrane potential may reduce activation of voltage-gated calcium channels and $N$-methyl-$d$-aspartate (NMDA)-receptor channels. On the other hand, if fully activated by a power activator such as epileptiform discharges, $Ca^{2+}$ influx may increase when the resting membrane potential is hyperpolarized due to the larger driving force. To investigate these possibilities, I measured PDS-evoked $[Ca^{2+}]_i$ transients at different resting membrane potentials. Figure 3A demonstrates a reduction in PDS-evoked $[Ca^{2+}]_i$ transients as the resting membrane potential was held at more depolarized values. Again the resting membrane potentials examined were kept below (more hyperpolarized than) the threshold for action potential initiation. Similar results were obtained in one additional neuron. The dependence of the PDS-evoked $[Ca^{2+}]_i$ transients on the resting membrane potential probably resulted in a shift of the measured SADW reversal potential to the left. It is interesting to note that despite the dependence of PDS-evoked $[Ca^{2+}]_i$ transients on the resting membrane potential, no notable rectification of the I-V curve was observed at the resting membrane potential range examined experimentally. One explanation for this behavior is that the channels mediating the SADW are fully activated at these $[Ca^{2+}]_i$ values.

The SADW was a depolarizing waveform and hence was mediated by a calcium, sodium, or mixed cation current. Despite the inherent limitations of reversal potential measurements, the fact the measured reversal potential was far removed from the equilibrium potential of sodium, potassium, and calcium ions indicated the SADW was mediated by a mixed cation current. A cation current with equal permeability to sodium and potassium ions was expected to have a reversal potential of $\sim 0$ mV. In contrast, the reversal potential measured for the SADW was $-33.1$ mV. This difference can be explained in one of three ways: a shift of the reversal potential due to the dependence of calcium influx on the resting membrane potential, a cation current more permeable to sodium than potassium ions, and co-activation of several different currents. It is important to note that the accurate method of assessing the relative permeability of a current/channel is to perform measurements at different internal and external concentrations of the involved ions. However, these experiments

**FIG. 3.** The SADW was mediated by activation of $I_{NaP}$. A: measurement of the reversal potential of the SADW. Inter-ictal-like PDS discharges were recorded at different resting membrane potentials. Top: the traces recorded in the same neuron at 4 different resting membrane potentials. Middle: the peak amplitude of the SADW was plotted as a function of resting membrane potential. The line represents a linear fit of the experimental values, and the reversal potential of the SADW was obtained by the interception of this line with the x axis. The calculated reversal potential in this experiment was $-31.8$ mV. Bottom: the effect of the resting membrane potential on PDS-evoked $[Ca^{2+}]_i$ transients. The calcium imaging measurements were performed from the apical dendrite 250 $\mu$m from the soma of a layer-5 neuron filled with 400 $\mu$M Fluo-5F. The results are presented as delta $F/F$. All results shown in A are from the same neuron. B: the effect of flufenamic acid (FFA) on the SADW developing after isolated PDS discharges. Recordings were obtained under control conditions (upper black, control), after the addition of 200 $\mu$M FFA to the bath solution (lower gray, FFA), and after washing out FFA (bottom). The resting membrane potential in all conditions was $-60$ mV.
Moreover, in fi
bursting and abolished seizure-like events

DMSO (0.1%) had no effect on PDS discharges (n = Flufenamic acid was dissolved in 0.1% DMSO. However, solution restored the SADW (1002; Ghamri-Langroudi and Bourque 2002; Morisset and
decreased from 3.2 B

TABLE 1. Effect of the I_{can} blocker FFA on inter-ictal like PDS discharges and seizure-like events in BCC treated neocortical brain slices

<table>
<thead>
<tr>
<th>Frequency of PDS Discharges, PDS/min</th>
<th>SADW Peak Amplitude, mV</th>
<th>Frequency of Seizure-Like Events, Seizures/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.9</td>
<td>3.2 ± 1.3</td>
</tr>
<tr>
<td>FFA</td>
<td>4.6 ± 0.7</td>
<td>−0.5 ± 0.3 mV*</td>
</tr>
<tr>
<td>Wash</td>
<td>5.1 ± 1.3</td>
<td>2.9 ± 1.4</td>
</tr>
</tbody>
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* P < 0.001. The effect of flufenamic acid (FFA) on the frequency of isolated paroxysmal depolarization shift (PDS) discharges and seizure-like events was examined on altogether 7 slices, and FFA was washed out in 4 of the 7 experiments. The effect of FFA on the peak amplitude of the sustained after-depolarization waveform (SADW) during isolated PDS discharges was examined on altogether 9 neurons, and FFA was washed out in 5 of the 9 experiments.

could not be performed in our preparation due to secondary effects on the excitability and wellbeing of intact neurons and on the epileptiform discharges in the slice.

Previous studies have reported the existence of a calcium-activated nonspecific cation current (I_{can}) in multiple cell types, including cortical pyramidal neurons (for review, see Partridge et al. 1994). The experimental findings described in the previous sections suggested the SADW was mediated by I_{can}. To further investigate this possibility, I used the I_{can} blocker flufenamic acid (FFA) (Di Prisco et al. 2000; Egorov et al. 2002; Ghamri-Langroudi and Bourque 2002; Morisset and Nagy 1999; Partridge and Valenzuela 2000). The addition of 100–200 μM FFA to the bath solution abolished altogether the SADW (Fig. 3B, Table 1). The peak amplitude of the SADW decreased from 3.2 ± 1.3 mV under control conditions to −0.5 ± 0.3 mV after the addition of FFA (n = 9). The effect of FFA was reversible, and washing out FFA from the bath solution restored the SADW (n = 5, Fig. 3B and Table 1). Flufenamic acid was dissolved in 0.1% DMSO. However, DMSO (0.1%) had no effect on PDS discharges (n = 4).

Moreover, in five of the experiments the control solution contained 0.1% DMSO (for the effect of FFA on resting membrane potential and excitatory postsynaptic potential (EPSP) amplitude, see I_{can} blocker FFA eliminated recurrent bursting and abolished seizure-like events).

Taken together the findings indicated that the SADW was mediated by I_{can}. The I_{can} current was probably activated by the large calcium influx evoked by PDS discharges. However, I cannot rule out the possibility that the SADW was in fact mediated by a ligand-gated cation channel, which is activated only when both a and yet unknown neurotransmitter is bound to it and [Ca^{2+}]_i is elevated.

Sources of calcium influx responsible for I_{can} activation

In the previous sections, I have shown that [Ca^{2+}]_i, transients evoked by PDS discharges activated I_{can}, which in turn resulted in the development of the SADW. I next wanted to investigate what were the routes of Ca^{2+} influx responsible for I_{can} activation. Calcium ions can enter the cytoplasm via several routes including the NMDA-receptor channels, voltage-gated calcium channels (VGCC) and internal calcium stores. I used different pharmacological blockers combined with voltage recordings and calcium imaging measurements to dissect the sources of Ca^{2+} influx responsible for I_{can} activation.

Blockade of NMDA-receptor channels had the greatest effect on both the SADW and PDS-evoked [Ca^{2+}]_i, transients. Addition of APV (100 μM) to the bath solution resulted in amplitude reduction of both PDS-evoked [Ca^{2+}]_i, transients and SADW (Fig. 4, A and E). On average, APV decreased the peak amplitude of the PDS-evoked [Ca^{2+}]_i, transients by 74 ± 14% and the peak amplitude of the SADW by 67 ± 8% (n = 7, P < 0.01 in both cases, Fig. 4). In addition to its effects on PDS-evoked [Ca^{2+}]_i, transients and SADW, APV caused narrowing of the PDS response (for example, see Fig. 4A).

The contribution of VGCC to I_{can} activation was more difficult to assess, as extracellular application of VGCC blockers was expected to reduce transmitter release. To overcome this problem, I applied the L-type VGCC blocker d-600 intracellularly via the recording pipette and examined its effect on the SADW. Similar to APV, intracellular d-600 decreased the amplitude of the SADW (Fig. 4B). Forty minutes after establishing the whole cell recording with a pipette containing 0.5 mM d-600 the average peak of the SADW declined by 49 ± 12% of its original value recorded 1–2 min after establishing the whole cell recording (n = 6, P < 0.05, Fig. 4E). As pointed out earlier the SADW did not change during 60 min of whole

FIG. 4. The sources of Ca^{2+} influx contributing to activation of I_{can} by isolated PDS discharges. A: concomitant voltage (top) and calcium imaging (bottom) recordings during and isolated PDS discharge under control conditions (black, control) and after the addition of 100 μM APV to the extracellular bath solution. The isolated PDS discharge was evoked by extracellular synaptic stimulation, and calcium imaging was performed using the calcium sensitive dye Fluo-5F (400 μM), and expressed as delta F/F in percent values. Note blockade of N-methyl-D-aspartate (NMDA)-receptor channels markedly reduced both the SADW and the [Ca^{2+}]_i, transient evoked by the isolated PDS discharge. The resting membrane potential was −62 mV. B: intracellular voltage recordings during an isolated PDS discharge recorded 1 (black trace) and 40 min (gray trace) after obtaining a whole cell recording with a somatic pipette containing 0.5 mM d-600. Note blockade of the L-type VGCC markedly reduced the SADW developing after an isolated PDS discharge. The resting membrane potential was −59 mV. C: concomitant voltage (top) and calcium imaging (bottom) recordings during and isolated PDS discharge under control conditions (black, control) and 35 min after the addition of 4 μM thapsigargin to the extracellular bath solution (gray, thapsigargin). The recordings were performed as described in A. Note depletion of the intracellular calcium stores via thapsigargin decreased both the SADW and the [Ca^{2+}]_i, transient evoked by the isolated PDS discharge. The resting membrane potential was −57 mV. D: intracellular voltage recordings of an isolated PDS discharge during control conditions (black trace) and after blockade of both voltage-gated calcium channels (VGCC) and NMDA-receptor channels (gray trace). The control recording was performed 1 min after obtaining a whole cell recording with a somatic pipette containing 0.5 mM d-600. Blockade of NMDA-receptor channels was obtained by extracellular application of 100 μM APV, and VGCC blockade was obtained after 40 min of whole cell recording with a somatic pipette containing 0.5 mM d-600. Note that the SADW disappeared almost altogether after Ca^{2+} influx via both VGCC and NMDA-receptor channels was eliminated. The resting membrane potential was −64 mV. E: the average effect (in percent) on the peak amplitude of the SADW (left bars) and PDS-evoked [Ca^{2+}]_i, transients (right bars) after application of APV, n=600, thapsigargin, APV and n=600, and APV together with n=600 and thapsigargin. F: the average effect (in percent) on the peak amplitude of the SADW of sequential application of d-600 and thapsigargin, APV and thapsigargin, and APV together with n=600 and thapsigargin.

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cell recordings with pipettes containing the control intracellular solution ($n = 6$). This finding ruled out the possibility that the reduction of the SADW amplitude resulted from washout of an unknown cytoplasmatic molecule. In the D-600 experiments it was impossible to measure the effect of on $[\text{Ca}^{2+}]_i$ transients, as calcium imaging could only be performed 10–15 min after whole cell recording was established and adequate concentrations of the calcium-sensitive dye reached dendrites.

To investigate the contribution of internal calcium stores to the activation of $I_{\text{can}}$, I used two pharmacological agents, ryanodine and thapsigargin. Ryanodine, which at the concentrations of 10 μM blocked calcium-induced calcium release
from internal calcium stores (Berridge 1998; Meissner 1986; Sitsapesan et al. 1995), had no effect on the SADW and only slightly reduced (<10%) the PDS-evoked [Ca\(^{2+}\)]\(_{\text{i}}\) transients (n = 3, data not shown). In contrast, thapsigargin (4 \(\mu\)M), which depleted internal calcium stores by blocking Ca\(^{2+}\) uptake into the stores (Treiman et al. 1998), decreased both PDS-evoked [Ca\(^{2+}\)]\(_{\text{i}}\) transients and the SADW (Fig. 4C). On average thapsigargin decreased the peak amplitude of PDS-evoked [Ca\(^{2+}\)]\(_{\text{i}}\) transients by 21 ± 9% and reduced the SADW peak amplitude by 17 ± 7% (n = 4, P < 0.01 in both cases, Fig. 4E). In two of four experiments, the amplitude of the SADW transiently increased for 15–20 min after application of thapsigargin and only then decreased below the control value.

Taken together the experimental findings indicated that several routes of Ca\(^{2+}\) influx contributed to \(I_{\text{can}}\) activation by PDS discharges. These routes included NMDA-receptor channels, VGCCs, and to a lesser extent internal calcium stores.

The sum effects of the three separate blockers on the SADW exceeded 100%, hence suggesting interactions between the three sources of calcium influx. To investigate this possibility, several pharmacologically blockers were applied jointly in the same experiment. When intracellular d-600 was combined with extracellular APV, the SADW almost disappeared altogether, leaving only 8 ± 5% of it’s control value (n = 3, Fig. 4, D and E). The results of simultaneous application of intracellular d-600 and extracellular APV and TPG on the SADW were not significantly different, leaving 5 ± 4% of it’s original peak amplitude (n = 3, P > 0.1, Fig. 4E). These findings indicated that the combined Ca\(^{2+}\) influx through NMDA-receptor channels, VGCC and internal calcium stores was responsible for all, or almost all, calcium influx mediating \(I_{\text{can}}\) activation. Moreover, these findings suggested that Ca\(^{2+}\) release from internal calcium stores was dependent, at least in part, on calcium influx via NMDA-receptor channels and/or VGCC. To further investigate this possibility, I added TPG subsequent to either APV, d-600 or both blockers. When TPG was applied alone, the peak amplitude of the SADW decreased by 17 ± 7%. In contrast when TPG was added subsequent to d-600, APV or both APV and d-600, the peak amplitude of the SADW decreased by 12 ± 9, 4 ± 7, and 3 ± 4%, respectively (n = 3 for all cases, Fig. 4, E and F). These findings indicated, Ca\(^{2+}\) release from internal calcium stores was dependent on calcium influx via NMDA-receptor channels and possibly VGCC as well.

Development of a prolonged depolarizing waveform during seizure-like events

In addition to isolated PDS discharges, brain slices from 12- to 17-day-old rats produced seizure-like events. Seizure-like events were composed of recurrent bursts of intense activity separated by quiescent inter-burst periods, and typically lasted 6–40 s (Fig. 5) (see also Hablitz 1987; Schiller 2002). A prominent feature of seizure-like events was a prolonged depolarizing waveform, which followed the entire course of the seizure, and laid beneath the recurrent ictal bursts (Fig. 5A). The amplitude and half-width of this prolonged depolarizing waveform could not be measured accurately, due to the overriding bursts. When these measurements were performed in between individual bursts, the prolonged depolarizing waveform had an average peak amplitude of 7.3 ± 1.4 mV and average half-width of 11.5 ± 1.9 s (n = 45). In ~5–10% of traces, the peak amplitude of the prolonged depolarizing waveform could not be measured with this method due to the high-frequency of individual ictal bursts and the short inter-burst quiescent periods. Moreover, it should be noted that these measurements probably underestimated the true value of the prolonged depolarizing waveform during seizure-like events.

Comparison of isolated PDS discharges and seizure-like events in the same neuron revealed the SADW developing after isolated PDS discharges was comparable to the initial part of the prolonged depolarizing waveform during seizure-like events. When the second burst of the seizure occurred the amplitude of the prolonged depolarizing waveform exceeded that of the SADW (Fig. 5B). Similar observations occurred in 21 additional neurons examined. These findings suggested \(I_{\text{can}}\) contributed to the prolonged depolarizing waveform developing during seizure-like events. I attempted to measure the reversal potential of the prolonged depolarizing waveform during seizure-like events; however, I was unable to accurately do so, as these experiments yielded unreliable results with large inter-trial variability.

FIG. 5. A prolonged depolarizing waveform developing during seizure-like events. A: a whole cell voltage recording performed during an electrographic seizure evoked by extracellular synaptic stimulation in the presence of 10 \(\mu\)M extracellular BCC. Bottom and top: the same waveform at different voltage scaling. Note the prolonged depolarizing waveform that follows the entire course of the seizure-like event, and lies beneath the individual ictal bursts. The resting membrane potential was −58 mV. B: an isolated PDS discharge and a seizure-like event recorded from the same neuron. Both the PDS discharge and the seizure-like event were evoked by extracellular synaptic stimulation in the presence of 10 \(\mu\)M extracellular BCC. Note the similarity between the SADW developing after the isolated PDS discharge and the initial part of the prolonged depolarizing waveform accompanying the seizure-like event. The resting membrane potential was −63 mV.
Prolonged depolarizing waveform during seizure-like events was Ca\(^{2+}\) dependent

Similar to isolated PDS discharges, seizure-like events were also associated with a large Ca\(^{2+}\) influx into the dendritic tree. Figure 6A demonstrates this finding in a layer-5 pyramidal neuron filled with the dye Calcium Green-1 (see also Schiller 2002). Similar results were obtained in six additional neurons.

To investigate the contribution of seizure-evoked [Ca\(^{2+}\)]\(_t\) transients to the formation of the prolonged depolarizing waveform during seizures, I applied BAPTA intracellularly via the recording pipette. Seizure-like events were recorded at different time intervals after establishing a whole cell recording with a pipette containing 10 mM BAPTA. Similar to the results obtained for isolated PDS discharges, the prolonged depolarizing waveform associated with seizure-like events progressively declined as the neuron gradually loaded with BAPTA (Fig. 6B). As pointed out earlier, accurate measurements of the prolonged depolarizing waveform were limited technically and performed in between individual bursts. With these measurements, the average peak amplitude of the seizure-associated prolonged depolarizing waveform measured 40 min after establishing the whole cell recording with a BAPTA-containing pipette declined to 38.7 ± 5.5% of its original value, recorded 1–2 min after the recording was initiated (n = 8, P < 0.001). Control experiments performed with a pipette containing a regular intracellular solution revealed the seizure associated prolonged depolarizing waveform was unchanged during 60 min of whole cell recording (n = 4). Hence, the BAPTA-containing pipettes affected the prolonged depolarizing waveform by buffering [Ca\(^{2+}\)]\(_t\) rather than by washing out essential unknown cytoplasmic molecules.

Previous studies have shown that metabotropic glutamate receptor (mGluR) agonists can evoke seizure-like events in hippocampal brain slices (for review, see Lee et al. 2002; Wong et al. 1999), possibly by activating mGluR activation. To investigate this possibility, I examined the effect of the group 1 and 2 mGluR antagonist MCPG (1 mM, n = 5) and the group 3 mGluR antagonist MSOP (250 μM, n = 3) on seizure-like events. In both cases, application of the mGluR blocker did not change the amplitude or shape of the prolonged depolarizing waveform nor did it eliminate recurrent bursting during seizure-like events (data not shown) (see also Karnup and Stelzer 2001).

Similar to mGluR, I\(_{can}\) has also been shown previously to be activated by muscarinic receptor agonists (Fraser and MacVicar 1996). However, in my preparation, bath application of the muscarinic antagonist atropine (0.5 μM) did not change the amplitude or shape of the prolonged depolarizing waveform nor did it eliminate seizure-like events (n = 4).

**FFA eliminated recurrent bursting and abolished seizure-like events**

A previous modeling study suggested seizures are sustained by activation of a prolonged depolarizing dendritic current (Traub et al. 1996), and I\(_{can}\) serves as a good candidate for such a current. To further investigate this possibility, I examined the effect of the pharmacological I\(_{can}\) blocker FFA on seizure-like events. Under control conditions, extracellular stimulation produced alternately isolated PDS discharges and seizure-like events. The addition of 100–200 μM FFA to the bath solution eliminated altogether seizure-like events, leaving only isolated PDS discharges devoid of an SADW (n = 7, Fig. 7). Similarly, FFA eliminated altogether spontaneous seizure-like events without effecting the frequency of spontaneous PDS discharges (Table 1). The effect of FFA was reversible. Washing out FFA restored the stimulus-evoked seizure-like events (Fig. 7, n = 4) and partially restored spontaneous seizure-like events (Table 1). These findings indicated that FFA specifically targeted the mechanisms involved in sustaining seizure-like events.

FFA was dissolved in DMSO (0.1%). However, 0.1% DMSO had no effect on seizure-like events (n = 3). Moreover, in four of the seven experiments, the control solution contained 0.1% DMSO. I examined the effect of FFA on the resting membrane potential and the amplitude of excitatory postsynaptic potentials (EPSP). Both these parameters were unaffected by FFA. The resting membrane potential was −64.1 ± 1.4 and −64.3 ± 1.7 mV before and after the addition of FFA (n = 7). The amplitude of EPSPs evoked by extracellular synaptic...
Hence, elimination of seizure-like events by FFA was not complete and stimulus-evoked seizure-like events and abolished action potentials in BCC (10 mM FFA). The recordings were performed from a layer-5 pyramidal neuron in BCC (10 μM). The resting membrane potential was ~63 mV and was unaffected by the addition of FFA.

The main findings of this study are that the large [Ca^{2+}]_i transients evoked by isolated PDS discharges and seizure-like events activate $I_{\text{can}}$. In turn, activation of $I_{\text{can}}$ depolarizes the membrane potential and probably participates in sustaining seizure-like events.

Epileptic seizures typically last 10–90 s and are composed of recurrent bursts of intense firing separated by periods of electrical quiescence. The importance of these mechanisms lies in the fact they are probably responsible for sustaining seizure activity for many seconds and hence serve as a potential target for new antiepileptic drugs. One of the mechanisms by which recurrent bursts can be generated is activation of prolonged depolarizing dendritic currents (Traub et al. 1996). In this study, I showed that $I_{\text{can}}$ serves as a good candidate for such a prolonged depolarizing current.

Previous studies have shown that prolonged depolarization waveforms accompany seizures and seize-like events in virtually all models of neocortical and mesial temporal epilepsy in vivo and in vitro. Moreover, in some of these studies, prolonged depolarizing waveforms have been implicated in seizure initiation and maintenance (Avoli et al. 1991, 1999; Ayala et al. 1970; Demir et al. 1999; Gean and Shinnick-Gallagher 1988; Hablitz 1987; Matsumoto and Ajmone-Marsan 1964; Speckmann and Erwin 1999; Traub et al. 1996; Trayanovs and Dingleine 1988; Wong et al. 1999). Several types of voltage- and ligand-gated channels have been reported to mediate these prolonged depolarization waveforms in the various models of epilepsy. These include GABA-A receptor channels, metabotropic glutamate receptors, muscarinic receptors, NMDA-receptor channels, and possibly voltage-gated calcium channels (Demir et al. 1999; Hablitz 1987; Lee et al. 2002; Perreault and Avoli 1992; Traub and Jefferys 1996; Wong et al. 1999). These findings suggest that various experimental models of epilepsy possess different mechanisms for generating prolonged depolarizing waveforms. However, it is also possible that these seemingly different mechanisms have in fact a common final pathway in the form secondary activation of $I_{\text{can}}$. Some of the above-described currents, such as metabotropic glutamate receptors and muscarinic receptor channels, probably exert their action by directly activating CAN channels (Colino and Halliwell 1993; Congar et al. 1997; Fraser and MacVicar 1996), whereas other channels, such as voltage-gated calcium channels and NMDA-receptor channels, may secondarily activate the CAN channels by increasing the [Ca^{2+}]_i (for example see Hall and Delaney 2002). Further in vitro and in vivo studies are required to investigate this possibility.

In addition to prolonged depolarizing currents, other mechanisms can also generate recurrent bursts and sustain seizures. These mechanisms include electrical coupling via gap junctions between neurons (Carlen et al. 2000; Kohling et al. 2001; Valiante et al. 1995), changes in the extracellular ionic concentration and especially of potassium and calcium (Heinemann et al. 1977), and spontaneous initiation of axonal action potentials (Stasheff et al. 1993; Traub et al. 1996).

In this study, I investigated the sources of Ca^{2+} influx responsible for $I_{\text{can}}$ activation using various pharmacological blockers. The largest component of PDS-evoked Ca^{2+} influx contributing to $I_{\text{can}}$ activation was dependent on activation of NMDA receptor channels. This result is consistent with the results of a previous studies that reported NMDA receptor channels play a critical role in activation of $I_{\text{can}}$ in granular frog olfactory bulb cells (Hall and Delaney 2002) and brain stem lamprey motor neurons (Di Prisco et al. 2000). In addition to the NMDA dependent component, I identified two additional sources of Ca^{2+} influx contributing to $I_{\text{can}}$ activation by PDS discharges, a VGCC-dependent component and, to a lesser extent, a calcium-dependent component.
extent, internal calcium stores. It is interesting to note that the different sources of Ca\(^{2+}\) influx interacted with one another to further amplify elevation [Ca\(^{2+}\)]. Ca\(^{2+}\) influx via NMDA-receptor channels, and to a lesser extent VGCC, enabled Ca\(^{2+}\) release from internal stores. Moreover, when Ca\(^{2+}\) influx via both NMDA receptor channels and VGCCs was eliminated, Ca\(^{2+}\) release from internal stores was virtually eliminated (see also Emptage et al. 1999).

In a previous study, Partridge and Valenzuela (1999) have shown Ca\(^{2+}\) release from internal calcium stores potentiated \(I_{\text{can}}\) activation by high-frequency stimulation in CA1 neurons. Consistent with this report, the findings of this study indicated calcium release from internal calcium stores contributed to \(I_{\text{can}}\) activation. However, despite the similarities in the conclusions of the two studies, the experimental results in this study differed from those reported by Partridge and Valenzuela (1999). In that study, ryanodine and thapsigargin potentiated \(I_{\text{can}}\)-dependent depolarization evoked by high-frequency stimulation in CA1 neurons. In contrast, in this study, ryanodine had no effect on the \(I_{\text{can}}\)-mediated SADW, whereas thapsigargin decreased it. These differences may result from differences in the preparations used (CA1 versus layer-5 neurons) or more likely by manner by which \(I_{\text{can}}\) has been activated (high-frequency stimulation in the presence of APV, 6-nitro-7-sulphamoylbenzo quinoxaline-2,3-dione (NBQX) vs. PDS discharges). More specifically, as PDS-evoked [Ca\(^{2+}\)] transient values reached micromolar concentrations (Cho et al. 2003; Guinamard et al. 2002; Miyashita et al. 2001). Inter-ictal-like discharges and seizure-like events evoke very large dendritic [Ca\(^{2+}\)], transients, which reach values of 13.3–71.0 μM in different dendritic regions (Schiller 2002) and far exceed the [Ca\(^{2+}\)] values reached under physiological activation (compare with Schiller et al. 1995 and Schiller 2002).

In conclusion, in this study, I showed \(I_{\text{can}}\) is activated by epileptiform discharges, and probably participates in generation of recurrent bursts and sustaining seizure-like events in BCC-treated neocortical brain slices. Further studies are needed to investigate whether \(I_{\text{can}}\) also participates in sustaining seizure-like events in chronic animal models of epilepsy in vivo, and whether \(I_{\text{can}}\) blockers can serve as novel antiepileptic drugs in patients with epilepsy.

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**References**


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