Single neuron dynamics during experimentally induced anoxic depolarization

Bas-Jan Zandt, Tyler Stigen, Bennie ten Haken, Theoden Netoff, and Michel J. A. M. van Putten

1MIRA–Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands; 2Department of Biomedical Engineering, University of Minnesota, Minneapolis, Minnesota; and 3Department of Clinical Neurophysiology, Medisch Spectrum Twente, Enschede, The Netherlands

Submitted 9 April 2013; accepted in final form 2 July 2013

Zandt BJ, Stigen T, ten Haken B, Netoff T, van Putten MJ. Single neuron dynamics during experimentally induced anoxic depolarization. J Neurophysiol 110: 1469–1475, 2013. First published July 3, 2013; doi:10.1152/jn.00250.2013.—We studied single neuron dynamics during anoxic depolarizations, which are often observed in cases of neuronal energy depletion. Anoxic and similar depolarizations play an important role in several pathologies, notably stroke, migraine, and epilepsy. One of the effects of energy depletion was experimentally simulated in slices of rat cortex by blocking the sodium-potassium pumps with ouabain. The membrane voltage of pyramidal cells was measured. Five different kinds of dynamical behavior of the membrane voltage were observed during the resulting depolarizations. Using bifurcation analysis of a single cell model, we show that these voltage dynamics all are responses of the same cell, with normally functioning ion channels, to particular courses of the intra- and extracellular concentrations of sodium and potassium.

anoxia; bifurcation; depolarization; dynamics; ion concentration

Address for reprint requests and other correspondence: B.-J. Zandt, Univ. of Twente, Faculty of Science and Technology, MIRA–Institute for Biomedical Technology and Technical Medicine, P.O. Box 217, 7500 AE Enschede, The Netherlands (e-mail: b.zandt@utwente.nl).

PHYSIOLOGICAL FUNCTIONING of neurons is critically dependent on the ion concentration gradients across the cell membranes. Molecular pumps and transporters, notably the sodium-potassium pump, in the membranes of neurons and glia cells work to maintain the physiological intra- and extracellular concentrations of ions and neurotransmitters.

These pumps require a constant supply of energy in the form of ATP. After an ischemic stroke, or during anoxia, the supply of glucose and oxygen from the blood is insufficient for the metabolic needs of the tissue. This leads to failure of ion homeostasis. A characteristic phenomenon resulting from this failure is anoxic depolarization, an initial slow increase of the membrane voltage ($V_m$), followed by a sudden, large depolarization, accompanied by a loss of membrane resistance and large shifts in the intra- and extracellular ion concentrations (Somjen 2004). Modeling studies (Zandt et al. 2011; Cressman et al. 2009, 2011; Kager et al. 2000) show that during anoxia/ischemia potassium ions leak into the extracellular space, which causes a slow, gradual increase of $V_m$. When the extracellular potassium concentration reaches a certain threshold, the neurons start spiking, causing a large efflux of potassium, resulting in a fast depolarization.

Also, during epileptic seizures, the intense neuronal activity can cause the extracellular potassium concentration to rise out of its physiological range. This initially excites neurons but may subsequently result in depolarization and cessation of electrical activity. This is a possible mechanism for spontaneous termination of seizures (Bragin et al. 1997; Krishnan and Bazhenov 2011; Cressman et al. 2009, 2011; Kramer et al. 2012). A phenomenon similar to anoxic depolarization, spreading depression, is the physiological substrate of a migraine aura. Spreading depression is a wave that slowly propagates through the neural tissue, during which there is a large shift in intra- and extracellular ion concentrations, glutamate is released, cells depolarize, and electrical activity is depressed (Dreier 2011; Zandt et al. 2013). When blood flow is sufficient, these depolarizations are relatively harmless and cells recover within minutes (Lindquist and Shuttleworth 2012). However, when blood flow is restricted, as during ischemia, these depolarizations induce massive cell death (Nakamura et al. 2010) and are therefore a potential target for therapy.

Despite the importance of anoxic depolarizations in several neurological disorders, the underlying physiology is not yet fully known (Lauritzen et al. 2011). This is probably due to the complex interplay of neuronal membrane dynamics, synaptic activity, glial and ion-pump activity, composition of the extracellular space, and blood flow during these depolarizations. Although the membrane voltage dynamics have a central role in the initiation of the rapid depolarization, there is little experimental literature available on these dynamics during anoxic depolarization. Here we present such measurements. We experimentally simulated one of the effects of ischemia by applying ouabain (Hauger et al. 1985; Schlue 1991; Sandtner et al. 2011) to slices of rat brain. This blocks the sodium-potassium pump activity and induces anoxic depolarizations.

First, we will discuss the rich variety of membrane voltage dynamics that was observed during this halt of the Na-K pumps. Then, we analyze these dynamics in terms of a dynamical system and compare them with the behavior predicted by single neuron models with dynamic ion concentrations (Bazhenov 2011; Cressman et al. 2009, 2011; Kramer et al. 2012). When blood flow is restricted, as during ischemia, these depolarizations induce massive cell death (Nakamura et al. 2010) and are therefore a potential target for therapy.

MATERIALS AND METHODS

Experimental procedure. Long Evans rats aged 14–21 days were anesthetized with isoflurane and decapitated, and the brain was removed. The brain was then blocked to prepare transverse slices of entorhinal cortex and ventral hippocampus. With the use of a micromtome, 400-μm thick slices were prepared. The slices were then placed in a submersion chamber containing artificial cerebrospinal fluid (in mM: 125 NaCl, 25 NaHCO3, 11 d-glucose, 3 KCl, 1.25 NaH2PO4, 2 CaCl2, and 1 MgCl2) and allowed to recover for 1 h at 37°C. Brain slices were then transferred to a submerged recording chamber. Neurons were visualized using differential interference contrast optics. An extracellular electrode, Ag/AgCl microelectrode
inside a borosilicate micropipette, was placed in layer 2/3 of entorhinal cortex. Based on its morphology, a pyramidal cell was selected for patching, located ~40 μm adjacent to the extracellular electrode. Intracellular recording electrodes were filled with intracellular recording fluid (in mM: 120 K-gluconate, 20 KCl, 10 HEPES, 7 Na₂-phosphocreatine, 4 Na₂-ATP, 2 MgCl₂, 0.3 Tris-GTP, and 0.2 EGTA) and used to whole cell patch the neuron.

After stable intracellular and extracellular recordings were established, the perfusion fluid was switched for 30 s to artificial cerebral spinal fluid containing 200 μM ouabain. This corresponds approximately to t = 0–30 s in the recordings. The flow rate of the recording chamber was ~4 ml/min, and its volume was 0.5 ml. The applied ouabain concentration, 200 μM, is two orders of magnitude above the binding constant Kᵣ \( \approx \) 50 nM (Maki et al. 1992), resulting in a complete block of the Na-K pumps. A relatively high concentration was chosen to ensure rapid diffusion of the substance into the slice.

The washout time of the ouabain was determined to be much longer than the duration of the experiments, on the order of 4–6 h.

Recordings were amplified using an Axon 700B amplifier (Molecular Devices, Sunnyvale, CA) with a 2.4-kHz low pass filter. Extracellular signals were amplified 500 times before digitization. Analog-to-digital conversion was performed using a National Instruments (Austin, TX) M-6259 16-bit data acquisition card. Data was recorded using the Real-Time eXperiment Interface (RTXI, www.rtxi.org).

Data were analyzed using MATLAB (Natick, MA). The transmembrane voltage signals were obtained by subtracting the extracellular voltage from the intracellular signal. Of the 12 obtained recordings, 2 were discarded. In one measurement we suspected a problem with the patch, while the other was halted after 180 s, before the cell fully depolarized. This resulted in 10 measurements for analysis. Experiments were conducted in accordance with Institutional Animal Care and Use Committee–approved protocols at the University of Minnesota.

**Analytical methods.** To analyze the observed dynamics, we will use the results of Barreto and Cressman (2011), who characterized a Hodgkin-Huxley (HH) neuron model for different ion concentration gradients. Their analysis was performed to qualitatively study how changes in sodium and potassium Nernst potentials can induce periodic bursting behavior of neurons.

The Nernst potential is a function of the intra- and extracellular ion concentrations, which are not constant in our case. However, since the dynamics of these concentrations are slow compared with the membrane dynamics, the behavior of the membrane voltage can be investigated as though the concentrations are constant. The bifurcation diagram of Barreto and Cressman (2011) depicts the different behaviors of the HH neuron, e.g., resting, tonically spiking, as a function of both the intracellular sodium concentration and extracellular potassium concentration. In the HH model, these concentrations affect only the corresponding Nernst potentials, \( E_{Na} \) and \( E_{K} \), which are direct parameters in many single cell models. Therefore, the diagram was redrawn with \( E_{K} \) and \( E_{Na} \) as parameters, calculated from the concentrations stated in their paper as

\[
E_{x} = 27mV \cdot \ln \frac{[C_{x}]}{[C_{x}^0]}. \quad (1)
\]

Here \([C_{x}^0]\), denote, respectively, the intracellular and extracellular concentrations of ion species \( x = Na, K \).

The measurements were grouped in categories based on the dynamic states and transitions that were observed. For example: “from resting state, to tonic spiking starting with zero frequency, to a depolarized resting state through depolarization block,” or “depolarization without spiking”.

### RESULTS

**Membrane voltage.** After the addition of ouabain, the majority of cell membranes depolarized to a resting voltage close to zero, as for example can be seen in Fig. 1A1. This is expected, because the main mechanism for maintaining the ion gradients, the sodium-potassium pump, is halted. Interestingly, the cells showed different kinds of dynamical behavior during this process. Five different types of behavior were observed.

Figure 1A shows an example of the first type, recorded in 2 out of 10 measurements. After the application of ouabain, the cell membrane potential increases from about −65 to −55 mV (\( t = 20 \text{ to } 60 \text{ s} \)). In the next 30 s, \( V_m \) shows large subthreshold variations and a few action potentials. This suggests that the cell receives substantial synaptic input. The height of the voltage plateau does not change significantly during this period. Then, from 90 to 120 s, the voltage plateau depolarizes and the spiking frequency increases. The neuron achieves a maximal firing rate of 50 Hz, just before going into depolarization block. In the case shown, the neuron goes into block and back to spiking several times. After the cell goes into block the last time, it has a membrane voltage around −20 mV. Then, in ~30 s, it depolarizes and finally reaches a membrane potential of ~0 mV.

In four recordings, labeled as type 2, we observed a spiking epoch with a similar onset, but with a different ending, as shown in Fig. 1B. Here, the fastest firing rate occurs in the middle of the spiking period. The cell does not go into depolarization block but decreases its spiking frequency to zero. Then, the membrane depolarizes from about −40 mV up to its final potential in ~30 s.

Furthermore, Fig. 1B3 shows the occurrence of two-spike bursts, with an interspike interval <10 ms (>100 Hz). The bursts are followed by after-spike hyperpolarizations, which are deeper than those following single spikes. These bursts were observed in several of the recordings labeled as type 1 or 2.

Labeled as type 3, shown in Fig. 1C, one recording displayed a spiking epoch with a frequency decreasing towards zero, as the second type, but the onset of spiking was not at almost zero frequency but had an initial spike rate of ~7 Hz. The amplitude of the spikes is small, and the minimum voltage during spiking is above the resting state voltage. Two recordings, that were labeled as the fourth type, did not exhibit a spiking epoch. No, or only a few, spikes are observed before the neuron depolarizes in a similar way as the second type. Figure 2D shows one recording as an example. The second recording was similar but did not exhibit any action potentials.

A fifth type was observed in one recording, shown in Fig. 2E: a single spike followed by a relaxation oscillation to a depolarized membrane voltage.

**Analysis of the membrane voltage dynamics.** We will use bifurcation analysis to explain the different observed dynamics. Bifurcations are the points in parameter space where the system changes behavior, for example, from resting to tonic spiking. The bifurcation analysis performed by Barreto and Cressman (2011) of a HH model, determines the dynamics of a neuron for different ion concentrations. Figure 3 shows this bifurcation diagram.
The saddle-node-on-invariant-circle (SNIC) bifurcation line depicts the points where the neuron changes its behavior from resting to tonic spiking. When either $E_K$ or $E_{Na}$ increases (moving right or down in Fig. 3), the net inward current is increased, depolarizing the neuron. Upon crossing the bifurcation line the resting state vanishes, which causes the neuron to tonically spike, starting at zero frequency. To the left of this curve the neuron is excitable, the closer to the line, the more excitable. To the right of this line, the neuron tonically spikes, with increasing frequency as the system moves further from the bifurcation line. This is known as class 1 excitability (Izhikevich 2006).

The Hopf bifurcation line corresponds to the points where the neuron goes into depolarization block. Such a block occurs when the spiking frequency becomes so high that the sodium gates have no time to de-inactivate completely after an action potential. This reduces the peak amplitude of the action potential, which in turn decreases the opening of the potassium gates and the resulting repolarization. Upon crossing the line, the amplitude of the action potential becomes zero (Izhikevich 2006). Hence the membrane voltage oscillates towards a stable depolarized state. The sodium and the potassium gates are then half open, and the electrical membrane resistance is low (Bianchi et al. 2012).

A saddle-homoclinic orbit (HC) and two saddle-node (SN) bifurcations connect the SNIC and Hopf lines. Upon crossing the HC bifurcation, the potassium current has become too small to fully repolarize the membrane voltage to the resting state once an action potential is induced, and the cell keeps spiking. The neuron has become bistable, and input can switch it between spiking and resting. The bifurcation can be identified from the lack of hyperpolarization below the resting voltage, when the cell is generating action potentials.

When instead the left SN bifurcation line is crossed, a stable depolarized equilibrium is formed and the cell can be switched between the polarized resting state and the depolarized resting state. When the system subsequently crosses the right SN line, the polarized resting state loses stability.

We now show that a single cell, with the same electrophysiological membrane properties, can exhibit any of the five types of the experimentally observed dynamics, depending on the trajectory that is followed in parameter space by the sodium and potassium Nernst potentials, $E_{Na}$ and $E_K$. Under physiological circumstances these potentials are stable at $\pm 50$ and $\pm 90$ mV respectively. When all ion pumps stop functioning, the ion gradients diminish and these voltages will go towards the so-called Donnan equilibrium (Somjen 2004). The ion
gradients do not completely vanish, due to charged, large molecules in the intracellular space and gradients may even reverse. In our experiments, the chloride pumps are still functioning, which also keeps the equilibrium potentials from zero.

In Fig. 4, hypothetical trajectories are drawn that correspond to the experimentally observed behaviors. Both trajectories 1 and 2 cross the SNIC bifurcation, transitioning from resting to regularly spiking. Trajectories 4 and 5 do not cross this bifurcation. In trajectory 1, the potassium reversal potential is lost faster than that of sodium and the cell goes through the Hopf bifurcation into depolarization block. In trajectories 2, 4, and 5, the sodium reversal potential is lost faster than that of potassium and the cell exits the spiking regime via the SNIC bifurcation and firing rate slows down to zero (trajectory 2), or the trajectory stays above the SNIC bifurcation and the cell does not spike at all (trajectories 4 and 5).

For types 2 and 4, no further bifurcations occur and the membrane depolarizes due to loss of the potassium gradient. For type 3, the trajectory crosses two SN bifurcations. This causes a depolarized equilibrium to stabilize, followed by the loss of stability of the physiological resting equilibrium. This leads to a fast relaxation oscillation to the depolarized equilibrium. The trajectory of type 3 (not shown in Fig. 4) lies slightly below trajectory 5 and crosses the homoclinic orbit bifurcation. This leads to a short period of spiking that starts at a nonzero frequency and shows small action potentials that are not followed by a hyperpolarization. Because the spiking frequency decreases to zero, it can be concluded that, rather than moving through the Hopf bifurcation, the trajectory crossed the HC bifurcation again before the membrane completely depolarized.

These five trajectories produce all dynamic transitions that can occur when the sodium and potassium gradients change monotonically in this model. As comparison, Fig. 5 shows the behavior of the HH model in which the Nernst potentials were set to follow these trajectories. The behavior largely agrees with that experimentally observed.

**DISCUSSION AND CONCLUSION**

We conducted this study to improve our understanding of the dynamics of a neural unit during anoxic depolarizations. Here, we focused on the membrane voltage. The membrane voltage of pyramidal cells from the rat neocortex was measured...
after blocking the sodium-potassium pumps with ouabain. This induced anoxic-like depolarizations. Five different kinds of dynamic behavior were observed. Bifurcation analysis shows that these correspond to different paths through the state space of the sodium and potassium Nernst potentials of the same neuron model.

To our knowledge, this is the first time this variety in voltage dynamics of depolarizing neurons is experimentally observed. The observed types are characterized by the bifurcations through which the system goes, which are shown to be the bifurcations that occur in the HH model. All the bifurcations predicted by the HH model, but not all possible combinations, were observed in the measurements. The bifurcation diagram suggests that other types of dynamics can occur that are combinations of the observed dynamics. For example, the spiking epoch of type 2 could be followed by the relaxation oscillation of type 5, and the epoch of low amplitude spikes of type 3 could end with the Hopf bifurcation shown by type 1, instead of slowing down in spike rate (c.f. Figs. 1C and 3–5).

From the limited amount of measurements (10), however, we cannot exclude the existence of behavior not predicted by the HH model. Furthermore, neurons with spiking dynamics that are not described by this HH model, such as intrinsically bursting cells, will certainly show different membrane dynamics during depolarization.

Some of the voltage dynamics, for example, those in Fig. 1C, may appear atypical. They do not, however, indicate a diseased state of the cell but are rather the normal response of the membrane voltage to particular courses of the intra- and extracellular ion concentrations. When performing experimental measurements, classifying the membrane dynamics during the depolarizations can yield additional information on the changes in the sodium and potassium gradients.

The dynamics of the ion concentrations are a complex function of various parameters, including cell volume, membrane area and extracellular volume, local glial cell activity, the effective distance for diffusion between the cell and the bath fluid, and the ion channel densities of the cells (Cressman et al. 2009, 2011). Furthermore, the bifurcation diagram itself depends on the cell channel conductances and the synaptic input it receives, which is different from cell to cell. This may account for the differences between the measurements.

Since the synapses were fully functional during the measurements, they affect the membrane dynamics. Synaptic input can shift the positions of the bifurcations, but does not qualitatively change the structure of the bifurcation diagram. Therefore, only the fluctuations in synaptic input can be observed from the membrane voltage trace, showing as subthreshold movements, variations in the spike intervals or the occurrence of a few spikes (e.g., Figs. 1A1 and 2D2). When the neuron is

---

**Fig. 4.** Trajectories through the bifurcation diagram of a HH type model. Physiological state is denoted R. Solid lines denote 4 hypothetical trajectories of the ion concentrations, leading to the experimentally observed dynamics. Trajectory 3 (not shown for clarity) lies slightly below trajectory 5 and crosses the HC bifurcation. Bifurcation lines are calculated from the diagram depicted in Barreto and Cressman (2011).

**Fig. 5.** Membrane voltage dynamics of the HH model for 5 different trajectories of the Nernst potentials. Inset: spiking frequency. Time courses of \( E_{K0} \) and \( E_{Na0} \) were set to follow approximately the trajectories in Fig. 4. Since the HH model shows a much higher firing rate than the measured neurons, the Nernst potentials were changed on a much shorter time scale (4 s) than experimentally observed (minutes), to show the depolarization and the spiking behavior in the same plot: \( E_{K} = -90 + 65 \times \tanh(t-2) + 1 \) and \( E_{Na} = 53 + \Delta E_{Na} \times \tanh((E_{K} - \mu)/w + 1)/2 \), using \( \Delta E_{Na,\mu,w} = [103, -40, 10; 103, -60, 5; 68, -70, 10; 103, -70, 10; 82, -70, 10] \) mM, respectively. Parameters of the voltage-gated channels were chosen as in Barreto and Cressman (2011).
close to a bifurcation, these fluctuations can even cause transitions back and forth between states, as, for example, observed in Fig. 1A2, where the dynamics fluctuate between spiking and depolarization block.

During our analysis of the single neuron dynamics, we considered the courses of the potassium and sodium Nernst potentials as given. Also, to replicate the dynamics observed in the measurements with the HH model, trajectories in the state space of the Nernst potentials were artificially chosen. In reality, however, these trajectories are determined by the closed-loop interaction between the transmembrane ion fluxes resulting from neuronal activity and the dynamics of the ion concentrations. This interaction determines the critical conditions for triggering a rapid depolarization, such as a potassium concentration threshold or minimum ion pump activity.

Further efforts are needed to experimentally verify this interaction, as modeled in (Barreto and Cressman 2011; Zandt et al. 2011), as well as the influence of synaptic coupling between the cells. This requires simultaneous measurement of the ion concentrations, membrane voltage, and neural activity. This may also allow a deeper understanding of clinically relevant phenomena, i.e., the occurrence of (spreading) depolarizations in migraine or ischemia/anoxia. Indeed, to identify the conditions responsible for these mesoscopic events, the dynamics of the ion concentrations must be considered as well.

Trajectories in the bifurcation diagram derived from the calculations of Barreto and Cressman (2011) must reach a sodium Nernst potential less than −20 mV to circumvent the Hopf bifurcation line and hence produce a depolarization without a depolarization block (trajectories 2–5). This is not likely, as the intracellular sodium concentration would have to become almost twice as large as the extracellular sodium concentration. However, the positions of the bifurcations depend heavily on the values for the conductances and the specific model used. Krishnan and Bazhenov (2011), for example, constructed a similar, more elaborate model, using values of mammalian cortical cells. Their bifurcation diagram is qualitatively similar, but they find the highest point of the spiking regime at a sodium Nernst potential of 47 mV, rather than −20 mV. This sodium potential can certainly be reached during anoxic depolarization. Therefore, although a mammalian neuron is more complex than the HH model and the values at which the bifurcations occur in the model will not be commensurate with the experimental findings, the model is able to explain the bifurcations. Therefore, our observations agree very well with the model’s qualitative behavior.

We studied membrane dynamics after failure of the Na/K-pumps by blocking them with ouabain. The results may therefore be slightly different from in vivo pathophysiology, where other processes play a role as well. During oxygen and glucose deprivation (OGD), for example, synaptic transmission is one of the first processes to fail (Hofmeijer and van Putten 2012). Furthermore, the chloride pumps stop functioning, which results in a hyperpolarizing chloride current (Müller 2000). Also, ATP-sensitive potassium channels can open (Müller et al. 2002). Due to these effects, neurons tend to temporarily hyperpolarize during OGD. Therefore, it is expected that during OGD the onset of depolarization will be later than application of ouabain. However, since the gated currents are larger than the currents generated by these effects, the dynamic behavior during the depolarization is expected to be similar.

Our results may also be relevant to epilepsy and seizure generation. It is known that an increase of extracellular potassium, in the range observed in vivo during seizures, depolarizes cortical pyramidal neurons resulting in increased excitability. Failure in maintaining homeostasis of the extracellular potassium concentrations has therefore been proposed to play a significant role in seizure generation and termination (Fröhlich 2008). Our results help identify different “ionic routes” to depolarization block in seizures. Different rates of accumulation of extracellular potassium and intracellular sodium may determine different seizure dynamics. This may be useful in classifying seizure types and determining which drug targets may be more effective depending on the seizure dynamics.

To conclude, we report on experimental observations on single neurons during anoxic-like depolarizations. In these measurements, five different types of dynamics were observed. These are explained with a bifurcation diagram of a single cell model and were shown to correspond to different courses of the sodium and potassium gradients. All dynamical states and transitions predicted by the model were observed. This shows that bifurcation analysis of single cell models for the sodium and potassium Nernst potentials is indeed a successful approach to understand the membrane voltage dynamics during anoxic and anoxic-like depolarizations, that can occur during epilepsy, migraine, and stroke.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


