CHAPTER 7

RICH DYNAMICS IN A SIMPLIFIED EXCITABLE SYSTEM

Shimon Marom,[1] Amir Toib[1] and Erez Braun[2]

ABSTRACT

The study aims at exploring effects of microscopic channel fluctuations on macroscopic dynamics of excitable systems. Molecular biology techniques are used in order to construct a minimal excitable system that is built of cloned channels embedded in a small ($\sim$1 μm^2) isolated patch of membrane. This simple synthetic "point" system exhibits dynamics in time scales that are several orders of magnitude longer then a single spike.

INTRODUCTION

Excitability stands in the basis of many physiological control systems. In most cases, the action potential (the excitation event itself) operates on a relatively fast time scale, whereas the system is modulated at time scales several orders of magnitude slower. The search for mechanisms to bridge this time gap, fuels an immense number of detailed studies which usually lead to additional intra- and inter-cellular complexities. Recent experiments [1-3] and theoretical considerations [4-5] suggest that intrinsic gating mechanisms of voltage-sensitive ion channels (the molecules of excitability) might contribute significantly to slow modulations in simple excitable systems. The study presented here aims at experimentally uncovering basic principles of modulations that are inherent to the process of excitation itself. For this purpose, a biological minimal excitable system is constructed using molecular biology techniques. The system is built of a binary mixture of cloned sodium and potassium voltage-gated ion channels embedded in a small ($\sim$1 μm^2 diameter) patch of an otherwise naive membrane. The dynamics of this "point" excitable system is studied under well controlled physico-chemical conditions, free of intracellular bio-chemical complications. This simplified excitable unit shows traces of what is usually ascribed to more complex systems, such as activity dependent excitability potentiation,

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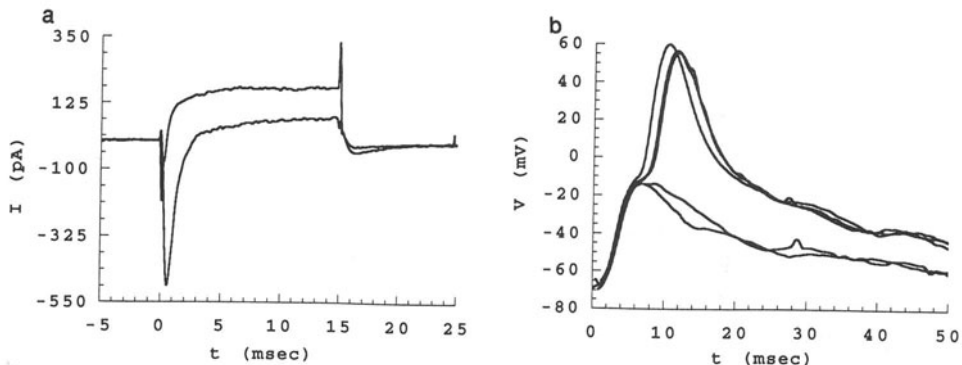Edited by S. Sideman and R. Beyar, Plenum Press, New York, 1995

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temporal integration and bistability. The challenge is to understand these sophisticated input–output functions. The hope is that the principles underlaying dynamics of the simplified unit can be carried up the scale to help us in the study of more complex ensembles.

METHODS

A mixture of *in vitro* made mRNA coding for mammalian sodium and potassium channels was injected to a frog oocyte which serves as a protein production line, so that the desired channels are expressed at the outer membrane of the oocyte. This membrane is otherwise non–excitable and for all practical purposes does not exhibit any voltage-dependent conductances in comparison to the ones carried by the expressed channels. mRNA coding for potassium channels is prepared from sequences of the Shaker related Kv1.3 gene [6]. mRNA coding for sodium channel gene type II [7] was used. Standard protocols for the preparation of DNA, RNA, and RNA injection to Xenopus oocytes are described elsewhere [8]. Recordings were made in the detached patch configuration. The pipet solution contains 96 mM NaCl, 2mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes. The bath solution contained 100 mM KCl, 1 mM EGTA and 10 mM Hepes. Both solutions were at pH of 7.5. Generally, the resting potential ranges from –70 to –50 mV. An Axopatch 200A amplifier (Axon Instruments) was used.
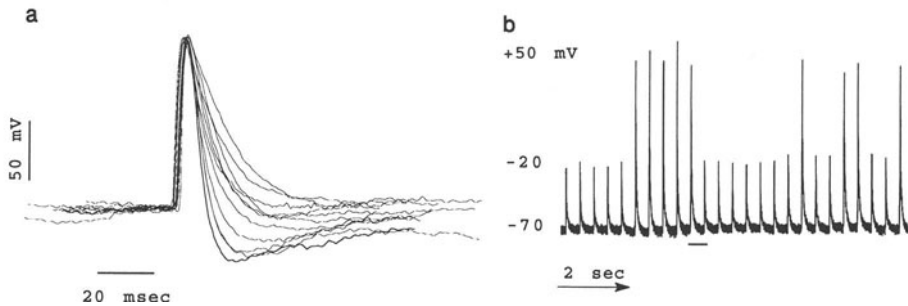

RESULTS

The hallmarks of an excitable system containing a mixture of sodium and potassium channels in a detached patch are demonstrated in Fig. 1. The different traces of Fig. 1a are current responses to pulses of voltage. Figure 1b shows action potentials and aborted action potentials (i.e. below threshold reactions) recorded under current clamp conditions in which the current is held at zero except for a short stimulation period. The control parameter which determines the dynamics is the ratio of sodium to potassium peak conductances, measured in voltage clamp experiments as recorded in Fig. 1a. This parameter is coarsely controlled at the preparation phase by injection of different ratios of mRNA mixtures.
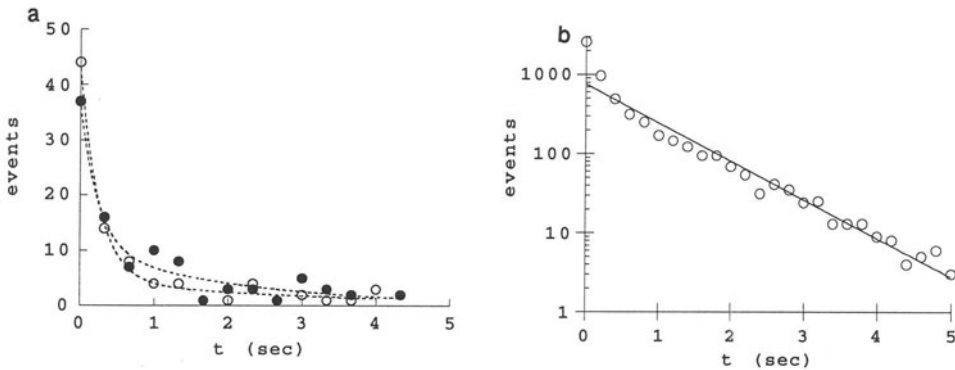

Figure 1. Current and voltage recordings from a detached patch of a membrane containing an embedded mixture of sodium and potassium ion channel proteins. **a:** Two typical voltage clamp traces. **b:** Action potentials and aborted action potentials recorded under current clamp conditions in which the current is held at zero, except for a short stimulation period.

To explore the *long term dynamics* of the system, we have studied its response to repetitive, constant amplitude, current stimulations. As shown in Fig. 2 these patches of membrane, which contain only a mixture of sodium and potassium channels, demonstrate dynamics at time scales much beyond the msec duration of a single spike. Figure 2a demonstrates a fairly trivial transient response of spike broadening and after–hyper-polarization modulation at a time scale of several seconds. This response reflects activity-dependent potassium conductance reduction (i.e. inactivation). About 30 sec of quiescent period, where no stimulation is applied, are required for full recovery of the channels and the reappearance of original profile spikes. Figure 2b demonstrates a less trivial long–term response, where the patch alternates between regions of low amplitude, passive responses to the current stimulus, and high amplitude, active responses in the form of action potentials. These clusters of responses have a wide range of durations, as shown in the binned data of Fig. 3. Apparently, there is no single characteristic time scale. As this simplified excitable system is devoid of any cellular complications the dynamical response must be intrinsic to the channels. The simplest interpretation of this behavior is that the threshold of the membrane for the generation of an action potential is not fixed by the

Figure 2. a: Modulation of after hyperpolarization and widening of action potential profiles in response to a series of short pulses every 400 msec. **b:** Clustering of active responses and aborted action potentials evoked by a long series of identical near threshold current stimulations.

Figure 3. a: Binned clustered durations of action potentials (black circles) and sub–threshold responses (open circles) measured from data as in Fig. 2b, with interpulse intervals ranging from 100 to 400 msec. The broken lines are best fits to the data by sums of two exponents with time constants of approx. 250 msec and 2 sec. **b:** A summary of a Monte–Carlo simulation of 50 channels based on the three–state model above, where binned durations of responses, corresponding to the experimental results of Fig. 3a, are shown. The best fit of the results is by an exponent with a time constant of 1.3 sec, fairly similar to the data of Fig. 3a.

initial conditions of the sample (i.e. by the number of different channels), but itself becomes a slowly varying entity which fluctuates due to the stochastic activity of the ion channels.

To understand the source of the long term dynamics it is imperative to look into the microscopic operation of the ion channels. The reactions of the sodium channel are all within the msec time scales [9–10]. Therefore, we assume that slow modulation of activity in the excitable system arises from slow transitions of the potassium channel. The actual states of the potassium channel alone, and the transition rates between them, have been studied in detail under the same working conditions [3,8]. As shown in [3], the potassium channel can be simplified to a three–state system (Fig. 4) where v is the transmembrane voltage (in millivolts).
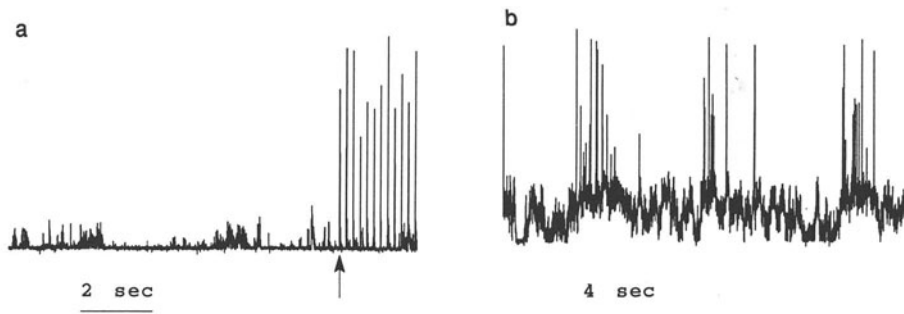
$$\text{Closed} \xrightleftharpoons[\beta(v)]{\alpha(v)} \text{Open} \xrightleftharpoons[\delta]{\gamma} \text{Inactivated}$$

Figure 4. Schematic of a three–state system of the potassium channel. The transition rates, in $msec^{-1}$ are: $\alpha(v) = -0.021 \ (v + 8.3) \ / \ (\exp(- \ (v + 8.3) \ / \ 9.8) \ -1)$; $\beta(v) = 0.0008 \ \exp(- \ (v + 23.6) \ / \ 20.7)$; $\gamma = 0.0074$; $\delta = 0.0002$.

The channel is conducting only when in the open state. The closed and inactivated states, both non–conducting, are different physical states of the channel and a direct transition between them is not possible. The transitions between the open and closed states are voltage dependent, and are fast (down to msec). The transitions between the inactive and open states (γ, δ) are voltage independent, and are relatively slow (up to sec). The coupling, of slow voltage–independent to fast voltage–dependent processes, is the source of the long–term dynamics. Recovery from inactivation is the slowest rate in our system so that any channel that made a transition into that state is trapped for a long time before being able to return to the pool of active channels available for conductance [3]. In the experiment, the number of available potassium channels becomes a dynamical parameter which affects strongly the excitable modes of the membrane. The activity dependent decrement of potassium channel availability leads to an increased excitability because the potassium current serves as a restoring force which acts against the sodium current exciting force. The passive restoring due to the leakage current is not significant during excitation – only a dynamical restoring can relax the membrane from an action potential. Therefore, the voltage threshold for action potential generation can be translated into a threshold in the number of available potassium channels. When this number is below the threshold, the patch responds to the stimulus by firing an action potential, and the system is highly excitable due to the weak restoring force.

The process described above is entirely based upon the stochastic nature of the channels. A typical patch, exhibiting fluctuations in its mode of firing action potentials as in Fig. 2, contains around 50 potassium and 500 sodium channels. This number of channels, when normalized to the capacitance of the experimental setup (1–2 pF, mostly contributed by the glass pipet) is a realistic representation of excitable systems [10]. To test if a simple three–state model is enough to determine the observed bursting of activity in Fig. 2, we ran a Monte–Carlo simulation for 50 channels, in which the probability of transition from one state to the other is given by: $f = \exp[-k \ \Delta t \ ]$, where k is the actual transition rate and Δt is a small time step. Experimental values for the resting potential and

for the membrane voltage during sub–threshold responses and at the peak of an action potential, were used. We have arbitrarily chosen a critical number of active channels, Kc = 4, as the threshold below which the system starts to fire action potentials. The entire range of fluctuations which determines the value of Kc is very narrow, making the above choice (= 4) not unreasonable. The binned durations of the active–inactive states, chosen in such a way, are shown in Fig. 3b. The distribution is exponential with a mean time of 1.3 sec, very similar to the experimental data.

So far we have looked at the excitable system near the threshold for firing an action potential. What happens if we start with a large number of potassium channels so that the initial restoring force is large compared with the exciting force? If we repeatedly stimulate the membrane with an interpulse intervals smaller than the time scale of recovery from inactivation, a successively large amount of potassium channels will be trapped in the inactivated state. A continuous reduction of the restoring force is expected ("cumulative inactivation" [3, 4, 8, 11]). After some stimulation period the threshold for firing an action potential will start to be sensitive to the past history of stimulations. In fact, such temporal integration behavior can be observed in neurons [1]. Recently, a prediction was made [5] that in the limit where the small availability of potassium channels eliminates the threshold for firing action potential the system becomes an oscillator. Such a transition is observed in our experiment shown in Fig. 5, where an initially quiescent patch is stimulated to trap most of the potassium channels in the inactive state and becomes a spontaneous oscillator after the stimulations cease. The spontaneous firing of the patch is stable over the experimental life–time (many minutes) and seems to exhibit random bursts which originate from the fluctuations in the number of available channels. Note that there are large fluctuations also in the resting potential which presumably come due to weak, although still significant, leakage conductance. The strength of the sodium and leakage current components together with the capacitance of the membrane determine the minimal stimulation period needed to make this transition.

Figure 5. a: A silent patch is stimulated to fire action potentials, starting at the point marked by an arrow. The stimulation is ceased after ~0.5 min and, as shown in b, the patch becomes a free–running oscillator.

CONCLUSION

A conceptually simple construction – a point biological excitable system – enables us to study the temporal dynamics devoid of intra– and inter–cellular uncontrolled parameters. We have shown that, under realistic conditions, the microscopic fluctuations of the channels activity are imprinted on the macroscopic behavior of the system. These results raise the possibility that extended excitable systems can be treated as a continuous

mesh of excitable elements each of which has the ability to generate complex dynamics due to its internal structure.

DISCUSSION

Dr. H. Fozzard: I was interested in the calcium binding and its action. Where the histidine residue is responsible, then that should make the process quite pH sensitive. Have you looked at that.

Dr. S. Marom: Inactivation is sensitive to pH. Histidine is protonated at low pH and the rate of inactivation decreases as pH is reduced. This was shown by others as well, by *Busch et al.* two years ago [*Biochem & Biophys Res Comm.* 1991;179(3):1384–1390].

Dr. H. Fozzard: I trust you have experiments where you remove all of the calcium. Does the process occur?

Dr. S. Marom: The problem then is that we replace calcium by magnesium and we get a slight left over of activation that we do not know how to explain yet. It definitely removes the fast phases of inactivation that are seen with calcium. It fits 30 μM or so for calcium. Note that the calcium Kd is not in the physiological range. The potassium Kd is within a physiological range because we are talking abut 2–5 mM to prevent 50% of the channels to go into inactivation.

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