Chapter 1

Introduction

When in 1665 Robert Hooke was looking through one of the first light microscopes, he had an idea that would prevail for centuries. He was investigating the structure of cork and drew images of what he saw. It seemed that cork consisted of small chambers that were tightly attached to one another. He coined the expression *cell* for one of these tiny compartments christening an object that has attracted vivid attention since then.

In the following decades cells were found in other plant and animal preparations. Eventually this led to the conclusion that all living matter consisted of cells. Nowadays we are not limited any more to state the pure existence of cells. We have access to any part of it. We may investigate the outer membrane, i.e. the plasma membrane, or look at compartments in the cell like the nucleus or the mitochondria. We can probe mechanically properties or study chemical reactions in the cytosol. It is even possible to tell a cell what molecules to produce. They can be used to control inter- and intracellular responses.

A major family of intracellular reactions are initiated by the second messenger Ca^{2+}. This is a chemical substance that enters the stage after another chemical process has taken place. As a versatile second messenger, Ca^{2+} fulfills many different tasks. It controlls muscle contraction or communicates the fertilization through an egg cell. It is involved in secretion in the pancreas or in the programmed cell death, i.e. apoptosis (Berridge et al. 2000). Therefore Ca^{2+} is truly characterized as an intracellular companion from the very beginning of cells to their deaths.

Ca^{2+} performs its signaling tasks by a transient rise in the cytosolic Ca^{2+} concentration. It increases for a short time and drops again. This may be a single event or may result in periodic patterns which are shown in figure 1.1. It depicts oscillations of the Ca^{2+} concentrations in hepatocytes, i.e. liver cells. The first train of

oscillations are caused by treating the cells with adenosine triphosphate (ATP). As soon as the cells are exposed to PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid) the pattern of the oscillations changes. The amplitude decreases, whereas the frequency increases. This is a typical example for a cellular response. A cell transcribes changes at the plasma membrane into a varying signal in the cytosol.

The plethora of distinguished processes that are controlled by Ca^{2+} demands a sophisticated encoding. It cannot be achieved by binding of Ca^{2+} to proteins, enzymes or receptors. The cell utilizes a broad spectrum of spatio-temporal patterns for signaling. There are strongly localized events that last for a few milliseconds only. They are termed sparks or blips. The former is mostly used for heart muscle cells. A cell can employ these spatially restricted Ca^{2+} elevations to supervise reactions that have to take place in a specific part of the cell. On the other hand, some information need broadcasting, like fertilization. This is achieved by Ca^{2+} waves. They form spirals (Lechleiter et al. 1991b), target patterns (Lechleiter et al. 1998) or fronts (Nuccitelli et al. 1993). These patterns differ in amplitude, frequency and duration. Some of them last for hours.

Binding of agonists like ATP or PPADS is not the only way to induce Ca^{2+} oscillations. They can arise spontaneously as well without any outer stimulation. Figure 1.2 illustrates such a behavior. It shows spontaneous Ca^{2+} spikes monitored from peripheral cell layers. Each trace is recorded from one cell within the same preparation. The period as well as the amplitude differ considerably. No synchronization between the cells has been found, although they are relatively closely positioned in the sample.
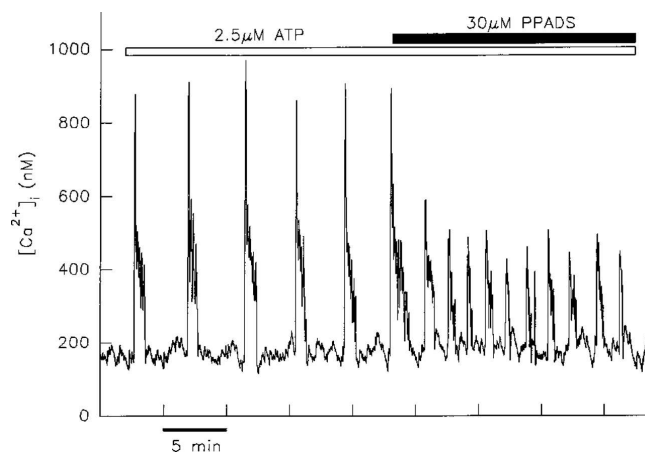

Figure 1.1: ATP induced Ca^{2+} oscillations in rat hepatocytes. Figure from (Dixon et al. 2000).

A property that almost all oscillations in figures 1.1 and 1.2 share is that elevated values of the Ca^{2+} concentration occur for short time intervals only. The cells return to the resting level very quickly. This prevents the lethal impact that exposing a cell to a high Ca^{2+} concentration for a longer time has. Therefore the concentration of free Ca^{2+} in the cytosol is usually very low. It ranges between 40nM to 100nM. This leads to the question where the Ca^{2+} for the oscillations comes from. Peak concentrations in the oscillations are orders of magnitude higher than the resting level.

In general two predominant mechanisms have to be considered. On the one hand, Ca^{2+} may flow from the extracellular space through ion channels in the plasma membrane into the cytosol. For instance, this has been reported for voltage gated Ca^{2+} channels in excitable cells (Hille 2001). Applying a voltage to a cell opens an ion channel and allows the entry of Ca^{2+} into the cell. On the other hand, Ca^{2+} oscillations have been observed when cells were bathed in Ca^{2+} free solutions. Hence, cells are able to store Ca^{2+} in their interior. A more detailed picture of a cell reveals additional compartments that reside in the cytosol. These so called organelles are membrane bound chambers that fulfill a variety of tasks. They fold proteins or are responsible for the production of ATP which serves as fuel for a cell. Additionally, some of them accumulate huge amounts of Ca^{2+}. This holds in particular for the endoplasmic reticulum (ER), the sarcoplasmic reticulum (SR) and the mitochondria. Measurements have shown that they release Ca^{2+} into the cytosol during Ca^{2+} oscillations (Alberts et al. 1994). Both, the ER and SR employ ion channels for this task. The ion channel that is most abundant on the membrane of the SR is the ryanodine receptor (RyR) release channel. The name stems from its high affinity to ryanodine and not so much from the impact of ryanodine on the receptor's dynamics. It is Ca^{2+} that controlls the opening of the release channel. The probability for an open channel grows with increasing cytosolic Ca^{2+} concentration. This autocatalytic behavior is pivotal for intracellular Ca^{2+} dynamics (Berridge et al. 2000).

Focusing on the endoplasmic reticulum we find the inositol-1,4,5-trisphosphate (IP_3) receptor (IP_3R). It is the prevailing receptor on the membrane of the ER that controlls Ca^{2+} liberation from the ER to the cytosol. Contrary to the ryanodine receptor, IP_3 essentially determines the response of the IP_3 receptor. The higher the IP_3 concentration, the higher the probability for an IP_3 release channel to open. The production of IP_3 can proceed along different ways. The inositol phospholipid pathway ranks among the most important. After the stimulation of an receptor on the plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) is cleaved into diacylglycerol (DAG) and IP_3. DAG remains in the plasma membrane, but IP_3 diffuses through the cytosol and can reach the IP_3 receptor on the membrane of the ER.
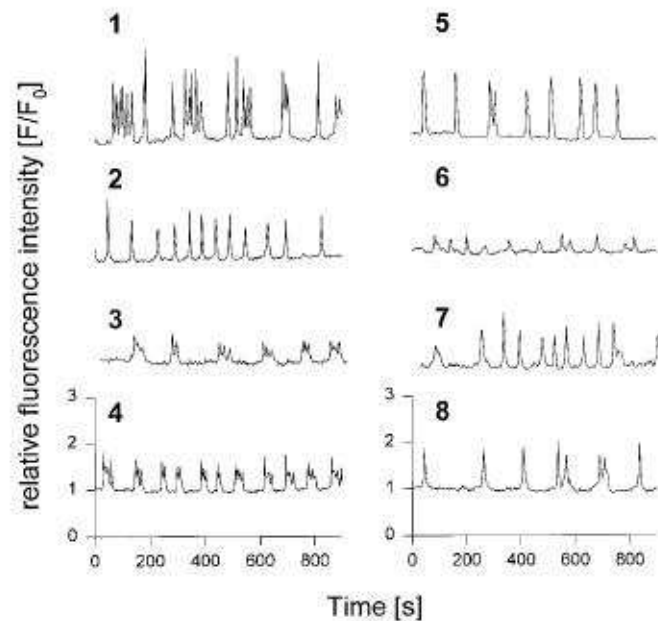
Figure 1.2: Spontaneous Ca^{2+} oscillations in peripheral cell layers. Each trace corresponds to a single cell. Figure from (Sauer et al. 1998).

In this thesis, the IP_3 receptor plays a central role. Up to now three different types have been found (Taylor and Swatton 2003, Falcke 2004). All of them share basically the same response with respect to the Ca^{2+} concentration. As for the RyR, the open probability increases with growing Ca^{2+} concentration. However, too high a Ca^{2+} concentration inhibits the IP_3Rs. The positive feedback for low Ca^{2+} concentrations and the negative feedback for high Ca^{2+} concentrations causes a biphasic open probability. This bell shaped dependence of the open probability on the cytosolic Ca^{2+} concentration (see figure 3.16) leads to fascinating spatio temporal patterns of the kind mentioned earlier.

Although some IP_3Rs are found isolated on the membrane of the ER, most of them build clusters. The exact number of channels per cluster is still unknown today, but estimates yield 5-40 release channels (Swillens et al. 1999, Sun et al. 1998, Callamaras et al. 1998). These clusters are randomly scattered with a distance that is much larger than the diffusion length of Ca^{2+}. The spatial setup of the Ca^{2+} release sites combined with the small number of channels per site has far reaching consequences.

Binding as well as unbinding of Ca^{2+} and IP_3 to only a few tens of IP_3 receptors

induces fluctuations in the dynamics of a single cluster. This can result in the random opening of a single channel named blip or the concerted opening of a group of channels termed puff. These are strictly localized events and represent the smallest unit in the Ca^{2+} signaling tool box. When a group of adjacent clusters opens almost at the same time they may set off a Ca^{2+} wave traveling through the cell. That is only possible, if the Ca^{2+} concentration at still closed nearby clusters is sufficiently increased. Then the open probability of the IP_3Rs grows and they may open. This mechanism illustrates what impact the spatial discreteness of release sites has.
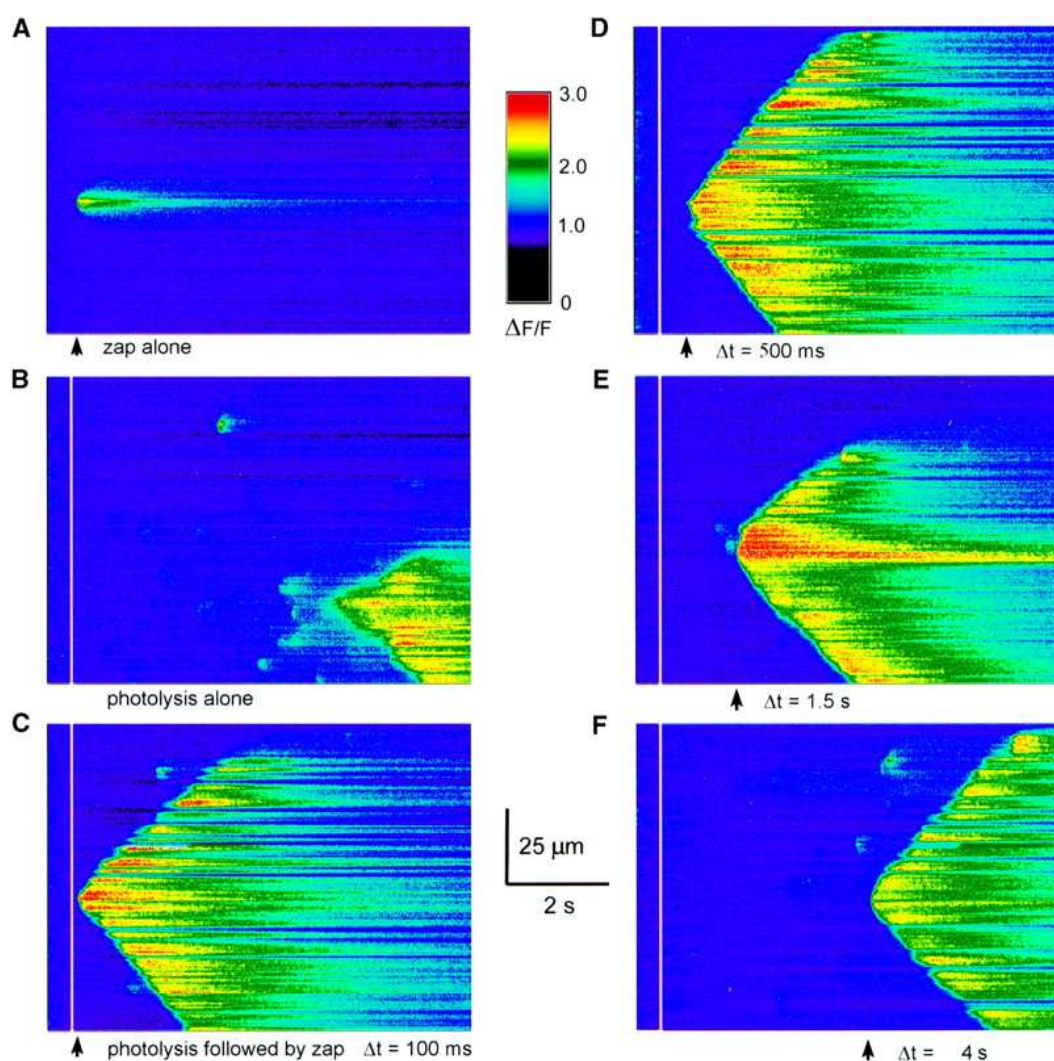

Figure 1.3: Wave initiation in Xenopus oocytes. See text for details. Figure from (Marchant et al. 1999).

Examples for both puffs and waves are shown in figure 1.3. It depicts a typical experiment to study Ca^{2+} dynamics. A cell, in this case a Xenopus oocyte (frog egg), is loaded with a dye that changes its fluorescence intensity upon Ca^{2+} binding. The cell is observed with a scanning confocal microscope and changes in the fluorescence are monitored. The higher the Ca^{2+} concentration, the brighter the signal. The results are plotted as change of the fluorescence ΔF with respect to the base level fluorescence F. They are presented as space time plots. The images of the line that is repeatedly scanned are arranged next to each other. Hence, time runs from left to right and space is drawn vertically.

Puffs appear in panel B and C as tiny turquoise spots. They clearly highlight the small duration and the limited spatial spread. Panels C through F depict the initiation of a Ca^{2+} wave. The wave front corresponds to the two turquoise straight lines that extend diagonally upward and downward. From a physicist's point of view such a wave front indicates a deterministic behavior because the front travels with constant velocity. That is what we deduce from the straightness of the lines. However, deeming intracellular Ca^{2+} waves a deterministic phenomenon does not comply with a more detailed interpretation of figure 1.3.

Parker and collaborators apply the following protocol. They load the cell with caged IP_3, i.e. IP_3 is arrested in a molecular box so that it cannot interact with the environment. Shining a short pulse from an Hg arc lamp on the cell sets IP_3 free. The moment when this photolysis flash is delivered is indicated by the white vertical line. The IP_3 concentration increases and sensitizes the IP_3 receptors. Consequently, channel opening should be more probable after the photolysis than before. Comparing panel A with the other five panels proofs this to be correct. In panel A, only the Ca^{2+} concentration is increased at the time indicated by the black arrow. It is achieved by a short laser pulse causing small holes in the membrane of the ER. They heal over several seconds and no permanent damage to the membrane is done. We see a localized elevation of the Ca^{2+} concentration that ceases after several seconds. In contrast all other panels show nice Ca^{2+} waves. In panels C through F the laser zap is delivered at various times after the photolysis flash. Each time a Ca^{2+} wave starts immediately, even with a time delay of only 100ms. It shows that a cell is capable of supporting a wave at the instant IP_3 is increased. This feature is crucial for the results in panel B. Here, only the IP_3 concentration is increased without additional liberation of Ca^{2+} from the ER. We behold the initiation of a Ca^{2+} wave with some detention to the photolysis flash. Whereas the waves in panels C through F start from the spot of the laser pulse, the initiation in panel B is due to the cooperative action of several puffs. These observations lead to a significant conclusion. The initiation of a Ca^{2+} wave is a stochastic phenomenon (Falcke 2004, Falcke 2003b).

Stochasticity arises from the random occurrence of puffs. The concerted activity of a few puffs connects the stochastic appearance of a single puff with the stochastic nature of wave initiation. Therefore, the beginning of a Ca^{2+} wave may be perceived as a nucleation process. The basic notion of nucleation is the existence of a critical value for the size of the nucleus. If the nucleus is smaller than this critical value it shrinks again. If the size is larger, the nucleus continues to grow. This means in the language of Ca^{2+} waves: If too little puffs take part in the concert, no wave sets off. If the number of cooperating puffs exceeds a threshold, a wave starts. Note that nucleation is by definition a stochastic process because growing and shrinking of the nucleus is driven by fluctuations - in our case, binding and unbinding of Ca^{2+} and IP_3 to the IP_3 receptor.

The central role of puffs in triggering waves demands a deeper understanding of the dynamics at a single cluster. In this thesis, we provide new insights into fundamental properties of puffs. Puffs arise from the opening of IP_3 receptor channels. They are sensitively controlled by the Ca^{2+} concentration at a cluster. We therefore need to know: *What is the Ca^{2+} concentration that IP_3Rs experience at an open cluster?*

Measurements of the Ca^{2+} concentration directly at an open cluster are still missing today. This would require to monitor changes on the length scale of a few tens of nanometers which is far beyond contemporary techniques. The alternative is to model this release event and compare the results with experimental data like signal masses (Marchant and Parker 2001). There is a huge literature about modeling of intracellular Ca^{2+} dynamics. For a recent review see (Falcke 2004). The majority of models assumes a continuous density of IP_3Rs. The gating of the receptor is coupled to an averaged Ca^{2+} concentration. However, IP_3 receptor clusters are not distributed homogeneously. They constitute strongly localized Ca^{2+} sources. This limits the applicability of the before mentioned models and calls for new approaches.

We present a thorough analysis of Ca^{2+} liberation from the ER in chapter 2. The geometry conforms with in vivo conditions, i.e. we consider Ca^{2+} flux through a small hole in a membrane patch. We find that concentrations at an open cluster exceed bulk concentrations by orders of magnitude. This was not investigated before in deterministic studies of intracellular Ca^{2+} dynamics based on the IP_3 receptor. Hence, we have to ask: *What consequence arise from these extremely elevated Ca^{2+} concentrations at an open cluster in deterministic models?*

We answer this question in chapter 3. We introduce a new approach to the dynamics of strongly localized reactions to which Ca^{2+} release through an IP_3R cluster belongs. The method proofs to be very efficient and allows for analytic results. It is based on rather general assumptions only and may therefore be used

in a variety of problems. We show that linear stability analysis is reduced to one algebraic equation. Applying it to the Ca^{2+} dynamics with the new realistic parameter values of chapter 2 shows that the oscillations that have been found in earlier works (De Young and Keizer 1992, Li and Rinzel 1994) disappear. A deterministic description of an IP_3R cluster respecting the high Ca^{2+} concentrations at an open cluster does not show the experimentally observed Ca^{2+} concentrations. This agrees with recent stochastic simulations in which oscillations occur for a non-oscillatory or non-excitable deterministic limit. (Falcke 2003b). Chapter 3 eventually consolidates the necessity for a stochastic treatment of intracellular Ca^{2+} dynamics.

The stochasticity is directly reflected in the distribution of puff periods. There is only a certain probability that a cluster fires a puff in the time interval $[t, t + dt]$ after the preceding puff. *How can we calculate this distribution and its properties?*

Different stochastic methods have been already applied to investigate Ca^{2+} dynamics. Stochastic simulations (Falcke 2003b, Falcke et al. 2000b, Bär et al. 2000) and studies of the stochastic version of the Li Rinzel model (Meinhold and Schimansky-Geier 2002, Shuai and Jung 2002a, Shuai and Jung 2002b) rank among them. The last group of authors have provided valuable results for single IP_3R clusters. However, our results in chapter 2 ask for more. The high values of the Ca^{2+} concentration at an open cluster have to be respected. We comply with this by using the appropriate parameter values in a stochastic version of the Ca^{2+} model of chapter 3. Moreover, we extend the Li Rinzel model in adding the Ca^{2+} activating processes. The fluctuations induced by the binding and unbinding to the activating Ca^{2+} site must not be neglected when calculating puff frequencies. Noise is what initiates a puff. We derive a master equation and two corresponding Fokker-Planck equations for a cluster of N IP_3Rs. We apply these equations to estimate the stochastic part of the puff frequencies. This is feasible by mapping the initiation of a puff to an escape problem. The mean first passage time corresponds to the stochastic fraction of the puff period. We find that no channel opens during the whole period that determines the stochastic fraction. Hence, there is more to puff initiation than has been thought by now.

Remembering the importance of puffs for Ca^{2+} waves brings us back to the motivation of chapter 2. We here close the circle and now start the journey through the world of intracellular Ca^{2+} dynamics. A journey at whose end some of the open questions in the field are finally settled.