CHAPTER 5

CONCLUDING REMARKS AND SUMMARY

SIGNAL TRANSDUCTION IN BIOLOGICAL CELLS

For function and survival, it is crucial for biological organisms to sense and respond to external cues. In nature organisms have developed a wide variety of cell types that are specialized to detect and process external signals. The first step in signal transduction is detection of the external signal. Biological cells may possess a wide variety of different receptor molecules for the detection of external signals. Some pronounced examples are the sensory cells in higher eukaryotes: olfactory cells or taste cells are specialized in sensing specific chemicals, photoreceptor cells in vision are specialized to detect light, whilst mechanosensory cells are specialized to detect mechanical forces in the ear which is essential for hearing.

Many types of receptor molecules appear to be related and belong to the large family of are G-protein coupled receptors, which are part of many signal transduction processes. When this type of receptor molecule is activated by the external signal it is able to activate G-proteins, and subsequently activated G-proteins have the ability to activate other types of target-proteins in the interior of the cell. In many receptor G-protein coupled systems the activated target-protein will produce small signaling molecules via a biochemical reaction. Induced by the external signal this primary cascade of biochemical steps therefore leads to an increase of small signaling molecules in the interior of the cell: the first external signal is transduced into a second internal signal. The second internal signal or second messenger subsequently has the ability to regulate proteins by activation or inhibition. In this way a third signal of signaling molecules may be induced, which also regulates cellular processes. The result of a long evolutionary process may yield a intricate signal transduction process, which is equipped to give an adequate response to a specific external signal.
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Production of small signaling molecules is a central aspect in many signal transduction processes. These molecules regulate directly or indirectly biochemical cell processes, which are important for cell function. The signaling molecules may be membrane bound or dissolved in the cytoplasm. In both cases molecules will move away from their source of production through the process of diffusion and spread into the cell. This means that a signal that is locally generated at one location in the cell after a while will affect other locations in the cell, thereby distantly regulating biochemical cell processes. For understanding signal transduction in general it is therefore important to gain more insight into the spatial and temporal aspects determining signal spreading.

An important research tool in understanding the spatial and temporal aspects of signal spreading is the technique of confocal fluorescence microscopy. This technique makes it possible to follow fluorescent labeled molecules with high spatial and temporal resolution. Additionally to this experimental technique, the more theoretical approach of computational biophysical modeling is important. In these models the diffusion equation describes how signal molecules spatially spread, given the diffusion coefficient and the cell geometry. The diffusion coefficient is a measure for the speed a specific molecule will spread, and the geometry determines the space in which the molecules can move about. Combination of the diffusion equation with the biochemical reactions that govern production and degradation of signaling molecules makes it possible to analyze signaling processes in time and space theoretically.

In this thesis the results are presented of mainly theoretical but also experimental research concerning signal transduction in two different biological cell types, emphasizing the spatial aspects of signal transduction. First phototransduction in the photoreceptor cell of the fruitfly Drosophila melanogaster, and secondly chemotaxis in the single cell amoebae Dictyostelium discoideum. In both systems the localization of signaling molecules play a pivotal role.

**Drosophila Phototransduction**

During evolution the photoreceptor cell of the fruitfly Drosophila melanogaster has been optimized to transduce light into an electrical current. This current gives rise to a change in the cell’s membrane potential, which is subsequently received and processed by the central nervous system. The elementary event during phototransduction is the absorption of a single photon of light, which leads to short-living inward current. The photoreceptor cell is very fast and sensitive, making it able to generate a current with great efficiency within several
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tens of milliseconds. This current, or quantum bump, is typically about 10 pA in size with a duration of about 60 ms duration. Absorption of photons occurs in the so-called rhabdomere, which is a part of the cell with an intricate geometry. It acts as a light guide and is a strongly folded part of the photoreceptor membrane that consists of about 30,000 microvilli that are organised in a tightly packed orderly array. The microvilli are slender, tube-like protrusions from the cell membrane connected to the cell body at one end (the base) and closed at the other end (the tip). A microvillus is typically 60 nm in diameter and 1-2 μm in length. The volume of a single microvillus is about 4.0·10^{-2} m^3 and compared to the volume of the cell body of about 4.0·10^{-15} m^3 is small by a factor of 10^6.

Each microvillus is believed to contain the complete transduction machinery required to produce a single photon response. The first step in phototransduction is absorption of a photon by a rhodopsin molecule. These receptor molecules are abundantly present in the microvillus membrane, and via a photochemical reaction are converted into the metarhodopsin state, which is the active form of the receptor molecule. A single metarhodopsin molecule is able to activate several G-protein molecules, which subsequently activate phospholipase C molecules (PLC). When activated, PLC-molecules will produce small membrane bound diacylglycerol molecules, which are involved in the activation of about 10-15 TRP and TRPL channels in a not yet fully understood way. This whole cascade of biochemical steps ultimately leads to the influx of the positive charged ions, Na^+, Mg^{2+} and Ca^{2+}, and efflux of K^+, giving rise to a net electrical current.

Ca^{2+} is a special ion, as it does not only contribute to the electrical current but it also plays a pivotal role in the biochemical regulation of the single photon response. The calcium influx causes at first a positive feedback on the phototransduction process and subsequently a later negative feedback, that is involved in the termination of the responses. Additionally, the amplitude and duration of a single photon response is strongly dependent on the calcium concentration present in the cell. When the concentration is low at the moment of photon absorption, the responses will be high and long, while at higher concentrations the response becomes smaller and shorter. Experimental measurements show that the basal calcium concentration is about 200 nM, while during prolonged illumination the concentration in the cell may reach micromolar concentrations (μM).
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Mathematical analysis of signal compartmentalization in the photoreceptor cell

A modeling study involving the influx and diffusion of ions in the volume of the microvillus predicts that calcium may reach already millimolar (mM) concentrations after a single photon response. This is a striking result, because most calcium-regulated cell processes in biological cells normally operate in the micromolar concentration range. Most cell types that are exposed to such high millimolar concentrations of calcium for longer periods of time function inadequately, which may lead to severe cell damage and cell death.

From the influx-diffusion equation a simple mathematical relationship can be extracted summarizing this result. The analysis shows that the ion influx $J$ is:

$$J = \frac{I}{V \cdot z \cdot F} \quad (5.1)$$

where $V$ is the microvillus volume, $I$ the current carried by ions with charge $z$ and $F$ is the Faraday constant. If the microvillus would be a closed compartment, the concentration would rise indefinitely. However the microvillus is directly connected to the cell body, and through the process of diffusion the ions will flow from the microvillus into the much larger volume of the cell body. This efflux from the microvillus volume diffusion can be characterized by the following diffusion time constant:

$$\tau_D = \frac{1}{3} \frac{L_m^2}{D} \quad (5.2)$$

where $L_m$ is the microvillus length and $D$ the diffusion coefficient. The concentration in the microvillus during a single photon response is ultimately determined by the balance between influx through the channels and efflux by diffusion. The change in the average concentration $\Delta C_{avg}$ during a (semi-)steady state can simply be expressed as the product of the influx $J$ and diffusion time constant $\tau_D$:

$$\Delta C_{avg} = J \cdot \tau_D \quad (5.3)$$

Because of the small microvillus volume, a 1 pA current carried by calcium ions already gives an influx of $J = 1.2$ mM ms$^{-1}$, and because of the relatively long microvillus length, the diffusion time constant is $\tau_D \approx 3.33$ ms. This yields a rise in average calcium concentration of $\Delta C_{avg} \approx 4$ mM, which is an extremely high value for biological cells.
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The model predicts that during a single photon response the calcium concentration in the microvillus may rise up to a concentration of 10 mM, while experiments done by Oberwinkler show that during prolonged illumination calcium concentrations in the cell body may just reach about 10 μM. Because of the special geometrical properties of the microvillus - e.g. a small volume with a relatively large length and small diameter - the calcium concentration during a single photon response can locally rise to much higher concentrations than the global calcium concentration in the cell body, in spite of the relative high diffusion coefficient of calcium.

The concentration differences locally in the microvillus and globally in the cell body may be a factor of 1000. Therefore, during evolution the proteins that are involved in generating a single photon response are likely to be optimized to operate under high local calcium concentrations. In this way every photon absorption can generate a robust single photon response. Additionally, the compartmentalization makes it possible for other calcium dependent cellular processes to be optimized to operate under lower global calcium concentrations. An important example in the photoreceptor cell is the adaptation process that is regulated by low calcium concentrations and that determines the amplitude and duration of the single photon response causing smaller single photon responses during prolonged illumination conditions. In conclusion, the special geometry of the photoreceptor cell leads to the existence of two subsystems - the microvilli in the rhabdomere and the cell body - which are connected to each other and therefore can regulate each other but are optimized to operate under differential calcium concentrations.

**Dictyostelium chemotaxis**

The amoebae *Dictyostelium discoideum* is a highly motile, bacteria eating single-celled organism, which under specific conditions is able to develop into a multi-cellular organism by means of cell aggregation. For finding bacteria and also during the aggregation process, *Dictyostelium* cells use chemotaxis. During the signal transduction process of chemotaxis an external gradient of a specific chemical substance is converted into directed movement of the cell to the source of this substance.

*Dictyostelium* cells utilize pseudopodia for cell movement. These pseudopodia are temporary protrusions of the cell membrane which are caused by a strong local build up of the actin cytoskeleton. Without any external concentration gradients of chemoattractants, *Dictyostelium* cells are not polarized and exhibit random motion behavior, making pseudopodia in all directions. For *Dictyostelium*
cells to move in the right direction in an external concentration gradient the cells have to form pseudopodia predominantly in the direction of the chemoattractant source. This means that the cell should stimulate the formation of pseudopodia where the chemoattractant concentration is highest, and that it should suppress the formation of pseudopodia at other locations of the cell.

Chemotaxis is an intricate signal transduction process, of which the molecular and biochemical mechanism is not yet fully understood. It is however clear that the membrane-bound receptors FAR for folic acid and cAR for cAMP are essential for the detection of the external concentration gradient of chemoattractant. These membrane receptors are G-protein-coupled-receptors that activate and regulate a number of internal signaling pathways, which are directly involved in the production of signaling molecules that either end up in the membrane or in the cytosol. Additional to receptors and G-proteins, the receptor-dependent production of the following signaling molecules play an important role in chemotaxis: cytosolic cAMP produced by adenyl cyclase, cytosolic cGMP produced by guanylyl cyclase, and membrane bound PIP$_3$ produced by phosphatidylinositol-3-kinase.

Chapter 3

Mathematical analysis of spatial signals in cells with a simple geometry

A chemotactic cell processes the external concentration differences between the front and the back of the cell through spatially differential activation of surface receptors, which ultimately leads to an amplified internal gradient of signaling molecules. This strong internal polarization of the cell subsequently directs the machinery of pseudopodium-formation in the cell. In this process local as well as global signals play an important role. Signals involved in cell polarization must be able to establish the internal gradient in the cell. Additionally, at least part of the signaling molecules should be able to maintain the gradient in the cell that is established during the polarization process. While a photoreceptor cell possesses a permanent internal cell polarization, Dictyostelium cells do not possess an intricate geometry, but rather have a simple spherical or cylindrical shape. Because signaling molecules have the natural tendency to cancel out any gradient in the cell through diffusion, there must apply special biophysical conditions must exist, to provide a signaling gradient in the cell, which causes sufficient internal cell polarization.

A modeling study of the spatial aspects of signals in cells with a simple geometry shows that there are two main factors that determine to what extend
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A signaling molecule is able to maintain an internal gradient for longer periods of time. On the one hand this is the diffusion coefficient and on the other hand the life time of the signaling molecule. From the intricate mathematics of reaction and diffusion it appears that the degree of dispersion of a locally produced signal can be summarized in the following space-constant:

\[ \lambda_D = \sqrt{D \tau} \]  

(5.4)

The space-constant \( \lambda_D \) indicates how far a molecule with diffusion coefficient \( D \) on average will diffuse away from its source of production, before it is degraded after a lifetime \( \tau \).

The diffusion coefficient of signaling molecules is mainly determined by the viscosity of the surroundings and the size of the molecule. Proteins embedded in the strongly viscous membrane, like receptors, possess a diffusion coefficient of about 0.1 \( \mu m^2 s^{-1} \). The diffusion coefficient of small membrane bound signaling molecules like lipids is about 1 \( \mu m^2 s^{-1} \). The diffusion coefficient of proteins in the much less viscous cytosol is about 25 \( \mu m^2 s^{-1} \) and the diffusion coefficient of small signaling molecules in the cytosol is about 250 \( \mu m^2 s^{-1} \). Assuming that all these different signaling molecules possess a relatively short lifetime of 1 s, the space-constants then are: 0.33 \( \mu m \), 1 \( \mu m \), 5 \( \mu m \) en 16 \( \mu m \) respectively. Given the relatively small sized Dictostelium cell, with an average diameter of about 10 \( \mu m \), these values of the space constants strongly suggest that the membrane bound signaling molecules are most suitable for generating local signals which easily maintain spatial information. Conversely, molecules that diffuse in the cytosol are much more suitable to generate global signals that contain information about the average receptor activity.

The external concentration gradient of chemoattractant during chemotaxis only leads to a small difference in the receptor activation between the front and the back of the cell. Subsequently, the cell will in principle produce signaling molecules internally at every location of the cell, and not only at the front of the cell. The space constant analysis shows that slow diffusing molecules are most suitable to reflect the external gradient. However, because Dictostelium cells are able to transduce an external signal with a difference of only a few percent between front and back of the cell into pseudopodia formation exclusively at the front, therefore small space constants appear essential, but not sufficient. Based on a modeling study described in Chapter 3 this observation requires that the ratio between production and degradation of signaling molecules at the front of the cell must be considerably higher than at the back of the cell. Until now several different amplification schemes have been proposed in the literature, under which the diffusion-translocation model described in chapter 3. All models
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utilize signals with short space constants, that are involved in local positive feedback mechanisms that enhance production. Furthermore, the models also hold signals with longer space constants, which are involved in globally acting negative feedback loops, that lower production and enhance degradation at the back of the cell. Combined action of local activation and global inhibition provides the capacity to transduce a small external difference into a strong, internally localized reaction, thereby providing the necessary cell polarization.

Chapter 4

Analysis of spatio-temporal signals in Dictyostelium cells with confocal microscopy

One of the chemical substances to which Dictyostelium cells give a strong chemotactic response is cAMP. During the signal transduction process that is induced upon stimulation with cAMP numerous molecules that are involved in chemotaxis relocalize in the cell, by which non-uniform spatio-temporal concentrations patterns emerge. A pronounced example is formed by the class of proteins that contain a PH-domain. Some of the known PH-domain containing proteins in Dictyostelium are CRAC, PhdA en Akt/PKB, which are all involved in the Dictyostelium aggregation process. All these proteins have the special property that they can be present in the cytosol but can bind to the membrane as well. Membrane association depends on the presence of specific membrane lipids - probably PIP_{3} - to which the pleckstrin homology (PH) domain is able to bind. The production of these lipids depends on the activation of the PI3K pathway by cAMP through the receptor.

The spatial location of PH-domain containing proteins can be visualized by means of confocal fluorescence microscopy. This experimental technique requires the proteins of interest to be labeled with a fluorescent molecule, like for example GFP (Green Fluorescent Protein). With confocal microscopy it is possible to localize GFP labeled PH-domains (PH_{cyt}-GFP) with a spatial resolution of approximately ~200 nm and a temporal resolution of about 1-2 seconds. The spatio-temporal processes induced after cAMP stimulation take place on a time scale of seconds; combined with a cell size of 10 μm diameter, it makes confocal fluorescence microscopy pre-eminently suitable to study cAMP induced spatio-temporal responses in Dictyostelium cells.

Chapter 4.1 describes two different methods, that were developed to analyze confocal fluorescence microscopy measurements in a statistically sound way. Dictyostelium cells appear to create binding sites at the membrane for PH-domain containing proteins after stimulation with cAMP. The proteins that are

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located in the cytosol will translocate and bind to the membrane region. As a consequence, the fluorescence and hence the protein concentration will decrease in the cytosol and increase at the membrane region. The first method that was developed is suitable to analyze the time course of the fluorescence in the cytosol accurately for many cells, and the second method is suitable to analyze and visualize in detail the time course of the fluorescence along the membrane of individual cells with high spatial resolution.

From the literature it is known that Dictyostelium cells respond chemotactically towards cAMP in the physiological concentration range of about 1 nM to 1 μM. After stimulation homogeneously with a saturating dose of 1 μM cAMP, PH-domain containing proteins translocate from the cytosol ubiquitously to the membrane, peaking at 7 s. Adaptation processes will cause binding sites at the membrane to disappear, whereby the PH-domain containing proteins will return to the cytosol and thus disappear from the membrane after 20 s. When the cells are placed in a concentration gradient of cAMP, they respond differently; under these conditions the PH-domain containing proteins translocate selectively and permanently to the front of the cell where the external cAMP concentration is highest, i.e. the cells only partly adapt. These results implied that in the absence of a cAMP gradient, i.e. will homogeneous stimulation, PH-domain containing proteins cannot be permanently bound to the membrane.

A series of experiments described in Chapter 4, Part I and II, where cells were stimulated homogeneously with cAMP, revealed that cells responded differently than previously anticipated. It appeared that cells that were stimulated homogeneously with 1 μM, after the first uniform response may show a second translocation response after about 40 s, which resides as long as cAMP is present. Although the external cAMP concentration after the first uniform response is still homogeneous, the second translocation response is not in the least uniform. It appeared that the PH-domain containing proteins now localized into 2-3 sharply defined areas: patches. The size of a patch covers about 10% of the cell’s plasma membrane; the patch has a diameter of about 8 μm and a lifetime of 1 min. Interestingly, cells that were stimulated with a very low concentration of 1 nM did not exhibit the first uniform translocation response, but directly formed several patches.

A detailed dose-response analysis described in chapter 4.2 revealed that cells are able to form patches in the concentration range of 1 nM to 1 μM cAMP. It appears that the number of patches formed during the response is dose dependent, but the size, intensity and lifetime of the patches is not. This observation lead to the hypothesis that patches are self-organizing ‘signaling units’, which may be triggered and react by an all-or-nothing response. In
search for a possible function of patches in chemotaxis a detailed analysis was performed on the spatio-temporal correlation of patch and pseudopodium formation. In cAMP stimulated cells the formation of patches appear to precede the formation of virtually all pseudopodia. Additionally it appeared that the step size of a pseudopodium formed during cAMP-stimulation is larger than those formed under cAMP-free conditions. Cells that move around in a concentration gradient of cAMP form pseudopodia predominately at the front of the cell and form patches with similar dimensions and lifetimes as patches formed during homogeneous cAMP stimulation. The results suggested that patches initiate and potentiate the formation of pseudopodia. Therefore it is comprehensible that cAMP-induced patches regulate the formation of pseudopodia during chemotaxis as well. This makes it feasible for Dictyostelium cells to effectively transduce, already at very low cAMP concentrations of 1 nM, an external chemoattractant concentration gradient into a directional movement. These observations may clarify to some extent the extreme sensitivity displayed by Dictyostelium cells for shallow chemoattractant concentration gradients.