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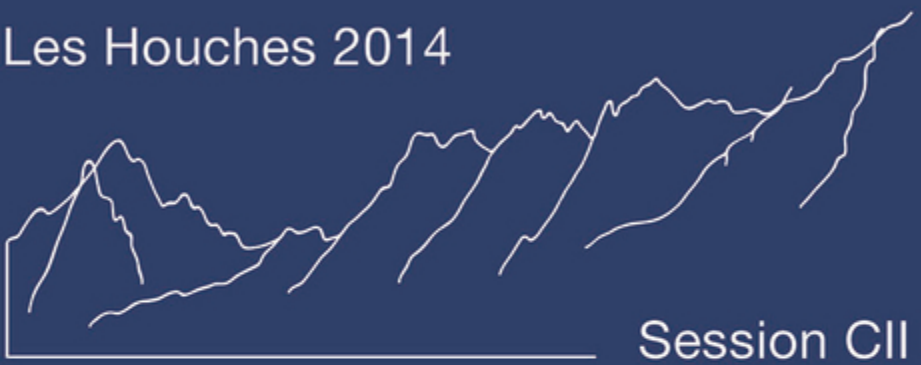

Session CII

From Molecules to Living Organisms: An Interplay Between Biology and Physics

Eva Pebay-Peyroula
Hugues Nury
François Parcy
Rob W. H. Ruigrok
Christine Ziegler,
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Editors

From Molecules to Living Organisms: An Interplay Between Biology and Physics

École de Physique des Houches

Session CII, 7 July–1 August 2014

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Edited by

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CII:	2014:	From molecules to living organisms: An interplay between biology and physics

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Sessions IX and X: Herman, Wiley
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Sessions XII–XXV: Gordon and Breach
Sessions XXVI–LXVIII: North Holland
Session LXIX–LXXVIII: EDP Sciences, Springer
Session LXXIX–LXXXVIII: Elsevier
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Preface

The 4-week school held in July 2014 addressed current approaches and concepts for understanding the relation between the regulation of gene expression, synthesis and structural assembly of proteins, the forces that dictate the structural, dynamic and functional properties of protein complexes and the properties of cells and their interactions in the formation of tissues and organisms. Students and early stage researchers from different fields of biology, physics and chemistry attended these multidisciplinary lectures. Several practical courses and seminars were also organized.

From molecules to cells and organisms

With the generalized application of genomics and proteomics the molecular level has become an important aspect of biology. At the core of molecular biology is the structure–function hypothesis. In recent years, new imaging approaches allowing the visualization of single molecular complexes within cells have paved the way for further understanding of biological processes at the molecular level. Integrating the detailed molecular description of individual proteins or complexes into the cellular environment and understanding the structures, architectures and interactions that guide fundamental cellular pathways and cellular responses to external stimuli are major challenges for the near future. At higher levels of organization, organisms that adopt precise shapes are built up from cells. Although several signaling pathways that guide development have been identified, many aspects cannot be explained just by the chemical nature of the molecules involved. Indeed, in several examples, physical forces have been shown to be responsible for the shapes of cells or cellular compartments and cellular the assemblies forming organisms. The generation of these forces is often related to structural assemblies of macromolecules and their dynamical rearrangement.

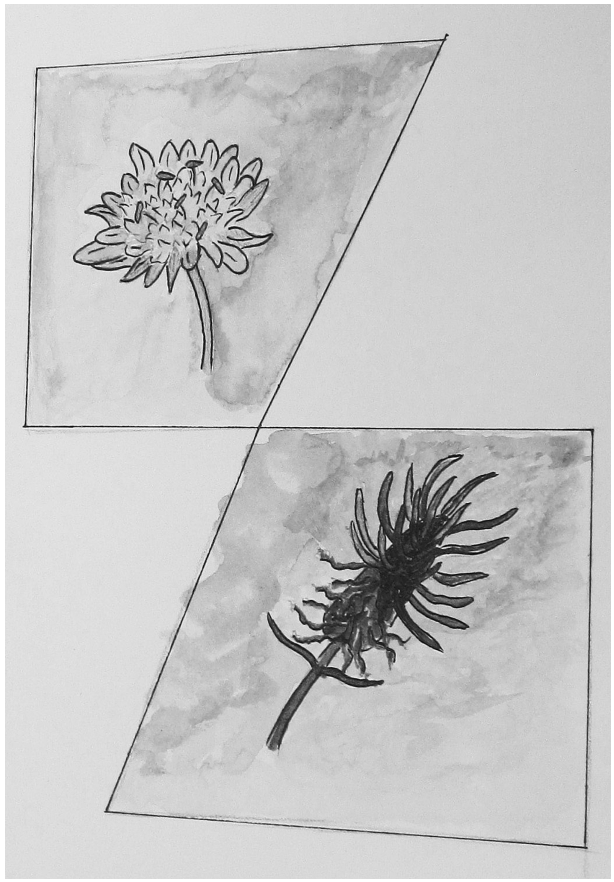
The context of this school is the emerging field of integrated biology (biomolecule↔cell↔organism) in the light of recent advances in cellular biophysics and modeling approaches. In particular, bridging data from different types of approaches and the provision of information on various scales (space and time) are far from well established. The aim of the school was to teach these new topics. The audience comprised structural biologists looking towards cell and organismal biology, biologists interested in the molecular view of biological pathways and physicists interested in biological processes for both the biology and the physics underlying the biology. Understanding the principles behind each method, and also their limitations and the complementarity between methods, was an important aim of the lectures. A large number of the interdisciplinary lectures were on subjects at the frontier between biology and physics.

A new way of thinking and teaching biology: a combination of interdisciplinarity and cutting-edge methods

Important new developments are expected in the coming years that may well introduce paradigm shifts in biological science. This school aimed to prepare participants to become major actors in these breakthroughs. It looked at opening a new way of teaching (and thinking) biology, bridging physics and biology beyond current biophysics. This book contains the proceedings of the main lectures given at the school. After an introduction to cell biology in Chapter 1 (Franz Bruckert), the power of integrated approaches from molecules to cells and organisms, including imaging, biophysics and structural biology, is illustrated through two examples. In Chapter 2 Hans-Georg Kräusslich and colleagues, demonstrate how the interactions between HIV and host cells can be deciphered, while in Chapter 3 François Parcy and his team highlight how floral development can be understood from the gene to the flower. Concepts in physics such as thermodynamics, important for understanding the behavior of biological macromolecules in solution, are recalled by Giuseppe Zaccai in Chapter 4, and in Chapter 6 Albert Guskov and Dirk Jan Slotboom show how some aspects of these behaviors can be experimentally characterized. Emerging and novel approaches such as in-cell NMR are described by Enrico Luchinat in Chapter 5. The next part of the book is dedicated to plant development, from innovative biological approaches, described by George Coupland in Chapter 7, to experimental evidence of the role of forces in plant development by Olivier Hamant in Chapter 8, and mathematical modeling based on this experimental knowledge by Christophe Godin and colleagues in Chapter 9. Forces also drive the shapes of membranes and their remodeling, as described in Chapter 10 by Michael Kozlov, Winfried Weissenhorn and Patricia Bassereau. These authors nicely illustrate the complementarity between experiments exploring physical parameters of proteins embedded in membranes, theoretical modeling based on physical principles and applications to a biological question, namely the budding of viruses out of host-cell membranes. The most predominant molecules in cells are proteins, and their shapes but also their conformational changes are responsible for their functional properties, as analyzed in Chapter 11 by Yves Gaudin. Membrane proteins are naturally embedded in a lipid bilayer with mesoscopic properties. Their handling necessitates special treatments, as shown by Christine Ziegler in Chapter 12. François Dehez, in Chapter 13, illustrates how such studies have benefited from an ensemble of tools based on molecular simulations in the light of experimental work. Chapters are grouped into six parts as indicated in contents list to facilitate structured reading. Altogether, the chapters show how the examination of a biological system from different viewpoints in a multidisciplinary fashion often brings new ideas to controversial arguments. Please note, that the online version of this book provides color figures that will be helpful to the reader.

Science and art

Observation was a key element in the development of biology, and more specifically botany. By drawing what they observed in the field, botanists could deduce important features in plants and define the various classifications. Nowadays, structural biologists spend a substantial amount of their time examining three-dimensional protein



structures on a computer screen in order to relate structural features to function by comparison with structures that are already known. Serge Aubert guided the participants through the alpine garden of the Col des Montets, illustrating the recent findings on molecular-level adaptation of plants to harsh mountain conditions that he presented during the conference. Anja Kieboom organized a few afternoon sessions during which she demonstrated some basic techniques for drawing flowers. These practical sessions were very successful and contributed to social team building. Graphic printouts of protein structures as well as plant drawings are not only informative, they are also intended to be attractive in themselves. Art in science contributes to the message that scientists aim to deliver.

While we were finishing this book, our colleague Serge Aubert died. Serge was a very talented and passionate scientist and he liked to share this passion with students. We would like to dedicate this book to him.

Acknowledgments

This Les Houches summer school was made possible by substantial financial support from:

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This financial support made a large contribution to the summer school, in particular to the funding of several students, and permitted a broad international participation.

The organizers wish to express their gratitude to the scientific committee. In particular, discussions with Yves Gaudin, Giuseppe Zaccai, Lucia Banci and Gideon Schreiber helped to refine the program and find appropriate speakers. The organizers are very grateful to the speakers who dedicated their time to the school and gave excellent and highly appreciated courses. The participants also deserve thanks: by their questions during the lectures, coffee breaks and at other times and discussion sessions they organized in the evening they were the major contributors to the excellent ambience during the month, despite some very unpleasant weather. Finally, the organizers would also like to thank the administrative staff of the "Les Houches" physics school, Murielle Gardette, Isabelle Lelièvre and Flora Gheno, for their support before, during and after the session, as well as the restaurant staff; technical assistance from Jeff Aubrun also contributed to the success of the school.

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Part 1

Concepts in cell biology and examples of multiscale studies in biology

1
Introduction to cell biology

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Abstract

Living cells are complex: they are made of a myriad of different molecules and their structure results from the dynamics of the interactions between these molecules. In this short introductory chapter some levels of cellular organization are first briefly described: membranes, cytoskeletons, adhesion structures and signaling pathways. Then, some mechanisms that specifically localize proteins in the cell are reviewed: signal and targeting sequences, vesicular transport. A third section deals with protein activation, emphasizing how energy is consumed to drive cycles of assembly and disassembly of protein complexes. A key problem is how the different parts and processes of the cell are coordinated. Some general mechanisms can help with that: changes in the transmembrane potential that spread rapidly along large distances and the bistable behavior of biochemical reactions combining non-linear activation and positive feedback. A remarkable example is the well-ordered pattern of gene expression that appears during cell differentiation.

Keywords

Cell structure, cytoskeletons, cell adhesion, cell differentiation, membrane proteins, protein targeting, protein activation, vesicular transport, synchronization of cell activity, control of gene expression

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Chapter Contents

1.1 Levels of organization in cells

The organization of living cells is usually described at two complementary levels, structural and functional. There are multiple levels of cellular structure: molecular complexes made of proteins and small molecules (ribosomes, proteasomes, etc.), larger polymeric protein structures (cytoskeletons, cilia) and membrane-based structures (vesicles, organelles, nucleus, plasma membrane). Membranes delineate cell compartments in which different reactions take place. Cytoskeletal filaments are often used for directed movements within the cell. These different structural elements collaborate to carry out the many functions of the cell: energy production, macromolecule synthesis and degradation, intracellular transport, uptake and secretion of molecules, cell movement, cell replication and division. A single structural element may therefore fulfill several functions—for example, both lipid synthesis and the first steps of protein secretion take place in the endoplasmic reticulum.

1.1.1 Membrane structure and cell compartments

Biological membranes are composed of proteins inserted into a lipid bilayer. The bilayer is only 5 nm thick, but vesicle diameters range from 50 nm to 5 μm. The protein content of biological membranes varies between 25 and 75%. It should be noted that although 70% of all proteins interact with membranes they constitute only 20% of total proteins by weight—this shows that membrane proteins are less abundant than other proteins but that they perform important roles. The main classes of lipids are phospholipids, sphingolipids, glycolipids and cholesterol. The composition of membrane lipids is highly complex, for several reasons:

- the diversity of alcohol groups found in the hydrophilic head;
- the diversity of fatty acids in the hydrophobic part;
- the lipid composition of biological membranes is asymmetric, meaning that the two layers do not have the same composition;
- the two-dimensional (2D) composition of membranes is also not uniform, with lipid rafts having a different composition from the surrounding bilayer.

One of the main reasons for this diversity is to prevent the solidification of lipid membranes. Pure lipids freeze at a defined melting temperature. A lipid mixture does not have a clear phase transition, which is beneficial because it allows conformational changes of the proteins embedded in the bilayer. Proteins such as ion channels indeed change their shape when they catalyze chemical reactions or transport. The fluctuations of lipid atoms in the layers create holes that can accommodate these changes. In other words, below the melting temperature the lipid structure is rigid and protein activity is hindered. These fluctuations also explain why water molecules diffuse rather easily through lipid bilayers.

Two types of proteins are associated with membranes: integral and peripheral.

Integral membrane proteins are embedded in the lipid bilayer. They necessarily span the entire hydrophobic interior of the bilayer and protrude from both sides of the membrane. Many of them contain one or several transmembrane alpha-helices containing mostly hydrophobic amino acids. This structure is stabilized by internal H-bonds

and exposes the hydrophobic side chains to the hydrophobic interior of the membrane. Twenty-one amino acids correspond to six helical turns, or about 4 nm, which is the thickness of the hydrophobic part of the bilayer. The presence of these hydrophobic alpha-helices is easily predicted by sequence analysis software, such as TM Pred (http://www.ch.embnet.org/software/TMPRED_form.html). Some transmembrane proteins, for instance some transmembrane carriers, adopt another structure; they have a cylindrical shape formed from adjacent beta-sheets (beta-barrel). This structure also exhibits hydrophobic amino acids on the external side of the beta-barrel that faces the interior of the membrane.

Other important examples of transmembrane proteins involving beta-sheets are pore-forming toxins. These molecules are secreted by cells in soluble form in an alpha-helical conformation. Upon binding to the membrane, the proteins self-associate and experience a cooperative conformational change that results in the formation of intermolecular beta-sheets that form a transmembrane pore. The formation of this pore induces the release of ions and small molecules, which kills the cell. These pore-forming toxins are secreted by bacterial pathogens and by certain cells of the immune system that kill other cells (cytotoxic cells).

Peripheral membrane proteins are attached to one side of the lipid bilayer only. Different mechanisms of interaction exist. Positively charged protein domains can interact with negatively charged phospholipids. Proteins can also be post-translationally modified by lipids, which themselves are inserted in a membrane layer. Finally, some proteins interact with membrane proteins, and this interaction anchors them to the membrane. Experimentally, it is often possible to separate peripheral membrane proteins from the membranes themselves without destroying the membrane, for instance by varying the salt concentration or changing the pH (electrostatic interactions and H-bonds). Integral membrane proteins, however, cannot be separated from the membrane without destroying the membrane, because the separation would expose hydrophobic parts of the molecules to water.

In organelles, membranes delineate several compartments, the interior and exterior of the organelle, and each side of the membrane. For interacting proteins, these are regions that have different biochemical compositions.

1.1.2 The cytoskeleton

Animal cells possess two or three cytoskeletal networks made of polymerized proteins: the actin cytoskeleton (microfilaments), the tubulin cytoskeleton (microtubules) and intermediate filaments. The cycle of polymerization and de-polymerization of microfilaments or microtubules is coupled to hydrolysis of ATP or GTP, respectively. Intermediate filaments, for their part, are controlled by protein phosphorylation and de-phosphorylation. Microfilament and microtubule polymers have a "plus end", where the association and dissociation of monomers is fast, and a "minus end", where it is slow. The complexity arises from the fact that, upon integration in the filament, actin or tubulin hydrolyzes ATP or GTP, respectively. The linear composition of the filament is therefore not uniform. Furthermore, about 100 proteins control the growth and dissociation rates or are associated with microfilaments and microtubules:

actin-binding proteins, microtubule-associated proteins, bundling proteins, severing proteins, molecular motors and so on. Some of these influence the nucleation of new polymers, the nucleotide state of monomers within the filament.

Microfilaments are oriented polymers of actin, an ATP-binding and ATP-hydrolyzing protein. They constitute the cortex of the membrane, which allows the plasma membrane to deform. Stress fibers are actin microfilaments linking adhesion focal points. Podosomes are structures involved in cell motility. Actin microfilaments are reversibly linked to the plasma membrane by specific proteins (e.g., talin, catenin, ezrin). Mechanical forces are exerted at the plasma membrane by the polymerization of actin and between actin microfilaments by molecular motors.

Microtubules are oriented polymers of tubulin, a protein that binds and hydrolyzes GTP. They are organized radially around the centrosome, also called the microtubule organizing center (MTOC), which is usually located near the nucleus. The minus ends of microtubules are locked at the centrosome. Tubulin is incorporated in protofilaments as $T\alpha_{GTP}T\beta_{GTP}$, then the tubulin beta subunit rapidly hydrolyzes GTP into GDP. A ring of $T\alpha_{GTP}T\beta_{GTP}$, called a GTP cap, is thus present at the extremity of growing microtubules and can be revealed by proteins that specifically bind the GTP form of tubulin, such as EB1 (Mimori-Kiyosue et al. 2000). Since the $T\alpha_{GTP}T\beta_{GDP}$ protofilaments are unstable, the plus end of the microtubule depolymerizes about 100 times faster when it contains GDP tubulin than when it contains GTP tubulin. A GTP cap therefore favors growth and when it is lost rapid depolymerization occurs. Individual microtubules therefore alternate between a period of slow growth and a period of rapid disassembly, a phenomenon called dynamic instability. Microtubules are rigid, and their polymerization exerts mechanical forces that allow the centrosome to reach a position along the cell cortex determined by the microtubule depolymerization activity. They allow centripetal or centrifugal transport of organelles within the cell. During cell division, the centrosome replicates and organizes the separation of chromosomes by forming the mitotic spindle.

1.1.3 Cell adhesion

Tissues consist of differentiated cells and of extracellular matrix (ECM) bathed in interstitial fluid. Cells are attached to other cells (via homotypic or heterotypic cell–cell interactions) or to the ECM by means of specific adhesion receptors.

The ECM is made of macromolecules (proteins and polysaccharides) synthesized and secreted by cells. It provides a specific mechanical and chemical environment for the cells. Cell adhesion molecules are proteins expressed at the surface of cells that mediate cell–cell binding (e.g., cadherins) or binding to the ECM (e.g., integrins). They trigger intracellular signals and therefore act as receptors. These adhesion receptors interact with the cytoskeleton. Cadherins and integrins are indeed able to influence the growth of microfilaments and microtubules.

The interstitial fluid is a solution that surrounds cells and the ECM. Its composition depends on exchanges between the cells, permeability barriers and the blood, and is similar to that of blood plasma. The interstitial fluid also contains growth factors and

Table 1.1 The main receptor families involved in signaling in eukaryotic cells

RECEPTOR FAMILY	MAIN FUNCTION OF THE RECEPTORS	MAIN CELLS	APPROXIMATE NUMBER OF RECEPTORS
G-protein-coupled receptors	Differential detection, fine tuning, adaptation	Sensory systems (vision, hearing, taste, etc.)	907
Ion channel receptors	Fast responses, "digital" communication, frequency-dependent responses	Neuron and muscle action potentials and synaptic communication	400
Receptors with tyrosine kinase activity	Threshold detection, coincidence detection, multiple inputs, cell fate decisions	All cells	58
Receptors with associated enzyme activity (including kinases)	Threshold detection, coincidence detection, multiple inputs, cell fate decisions	All cells	115
Intracellular receptors	Response to hydrophobic molecules	All cells	48

hormones that influence cell behavior by the means of specific receptors present at the plasma membrane. (These receptors are described in Section 1.1.4 and Table 1.1.) Note that many growth factors strongly and specifically interact with the ECM, which therefore acts as a reservoir of bioactive molecules.

Connective tissue (mesenchyme) contains fibroblasts and is rich in fibrous ECM. Muscle tissues contain muscle cells and have strong contractile activity. Nervous tissues are made of neurons and glial cells and conduct action potentials. Epithelial tissues consist of monolayers of cells that provide a selective barrier; for instance, endothelial cells line the inner surface of blood vessels and separate them from the blood. Similarly, a complex epithelium delimits the border of the intestine (see Section 1.1.5 and Fig. 1.1). Organs are complex structures formed by several tissues carrying out specific functions. Different tissues are separated by specific basement membranes, also called basal lamina; these in fact are not membranes as already defined, but a very thin layer of ECM.

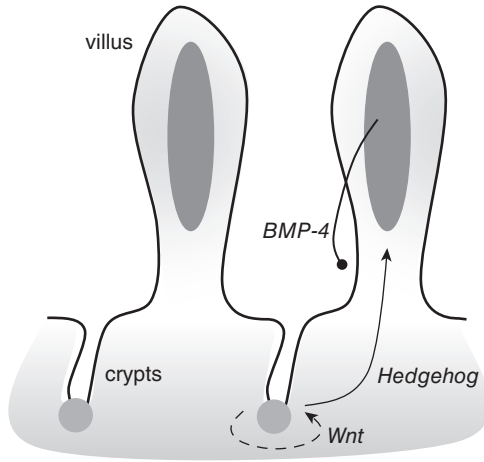

Fig. 1.1 Self-organization of the crypt–villus axis. The epithelium (black line) consists of a layer of cells covering the villi and the crypts. Stem cells at the bottom of the crypts (gray balls) are exposed to Wnt, locally secreted by Paneth cells and underlying mesenchymal cells. Wnt stimulates cell proliferation while preventing cell differentiation. The differentiation of stem cells into Paneth cells provides a positive feedback mechanism for their own proliferation. Mesenchymal cells (dark gray ovals) in the villi secrete BMP-4, that diffuses and stimulates the differentiation of epithelial cells in the villi. As a result, cell proliferation in the villi stops. Crypt cells secrete Hedgehog, a molecule that diffuses toward the mesenchymal cells of the villi and induces secretion of BMP-4.

1.1.4 Cell–cell communication

Cells are highly sensitive to their extracellular environment. Molecules dissolved in the interstitial fluid, in the blood, or eventually in air, bind to cell surface receptors where they elicit specific responses or enter the cell through channels or transporters. Hydrophobic molecules can even enter cells directly because they cross membranes spontaneously. All cells are highly sensitive to temperature and oxygen concentration. In tissues cells are less than 200 μm from a capillary to ensure a sufficient supply of oxygen. Conditions of chronic hypoxia cause the activation of hypoxia-inducible factors (HIF) that result in the secretion of vascular endothelial growth factor (VEGF), a growth factor that attracts endothelial cells and stimulates the growth of new capillaries (angiogenesis). The geometry of the ECM adhesion zone (because of the adhesion receptors such as integrins) influences the organization of the cellular cytoskeleton, and therefore the positioning of the nucleus and organelles (Théry et al. 2006). In addition, cells respond to mechanical forces, for instance transmural pressure and the flow shear stress in blood vessels and nephrons. They are also sensitive to the stiffness of the ECM. Discher's group (Discher et al. 2005) were the first to demonstrate that cell differentiation is influenced by this parameter.

Cells also sense and respond to neighboring cells. Gap junctions are cellular structures that allow the direct exchange of small molecules and ions between adjacent cells. Cells secrete diffusible molecules either in the interstitial fluid or in the blood; these bind to receptors on the surface of another cell or enter other cells through channels or transporters. Many cells express specific molecules that can be recognized by other cells as ligands to their own receptors. Well-known examples are the Fas/FasL pair that control apoptosis, the notch receptors and Delta-like and Jagged ligands, implicated in organ development. As with cell–ECM adhesion, cell–cell adhesion also induces cytoskeletal remodeling. Cells are therefore sensitive to mechanical forces (stress) exerted within living tissues (Desprat et al. 2008, Martin et al. 2010).

Cell responses can be classified as early or delayed depending on the involvement of *de novo* protein expression. Early responses involve none or limited new protein expression and include:

- cytoskeletal reorganization and changes in cell morphology,
- reorganization of cell–cell or cell–ECM contacts,
- metabolic changes (a shift in energy sources),
- cell migration (directional motility or chemotaxis),
- secretion of specific molecules (exocytosis)
- uptake of specific molecules (endocytosis).

These changes are induced by protein phosphorylation or other post-translational modifications, degradation signals or changes in the concentration of ions and second messengers. (Second messengers are small molecules produced in the cell in response to an external stimulus, e.g., cAMP.) These modifications can appear rapidly, in less than a second.

Delayed responses involve major changes in protein expression. These include cell division, the arrest of cell proliferation, cell differentiation and apoptosis. In addition to the mechanisms found in early responses, these changes also involve the activation of transcription factors and *de novo* protein synthesis. They are usually rather slow (20 min to 1 hour). Cell division is a complex mechanism consisting, first, of the replication of all cell components, including the genomic DNA. A set of several specific kinases (cyclin-dependent kinases) control the different stages of cell division. Cell differentiation involves the activation of "master genes" that control many other genes. Finally, during apoptosis, caspase genes are expressed. Caspases degrade specific intracellular cell components.

Proteins involved in cell signaling activity are usually grouped together in "signaling pathways". Many cell signaling proteins exist, because there are an enormous number of cell surface receptors. Table 1.1 gives an overview of the main categories of receptors, their main tasks and cell types and how many of then there are in the human genome.

1.1.5 Cell culture

Most biological experiments are performed with isolated cells or with wild-type or genetically modified organisms.

Primary cell cultures consist of cells that have been extracted directly from tissues, generally using trypsin and ethylenediaminetetraacetic acid (EDTA) to dissociate them. They divide a limited number of times *in vitro*. Note that some differentiated cells do not divide (neurons, myofibers). The tissues are often obtained from genetically modified animals; this allows the protein of interest to be labeled with green fluorescent protein (GFP), the knocking-down some gene of interest or the expression of mutations.

Secondary cell cultures consist of cancer cells extracted from tumors or "immortalized" cells modified by the expression of an oncogene (a gene whose permanent activity induces tumor growth and proliferation). These days many cell types are available as secondary cultures; their properties can nevertheless be different from those of primary cell cultures.

Stem cells are increasingly being used to obtain differentiated cell types. More precisely, in adult organisms, different classes of stem cells (in plants, meristem cells) exist that allow the renewal of different types of tissues. Pluripotent stem cells are able to divide and differentiate into any cell type. Multipotent progenitor cells are able to divide and differentiate into a limited number of cell types once an irreversible signal has been received (commitment). Note that, quite often, cells obtained from a primary cell culture will have originated from the stem cells present in the starting culture. A feature common to all stem cells, which differentiates them from ordinary cells, is that they divide asymmetrically. This means that, after division, one cell remains a stem cell whereas the other undergoes a series of symmetrical divisions as a multipotent progenitor cell. Stem cells reside in a "niche", with a very specific molecular and cellular environment that defines a polarity axis orienting cell division in such a way that two different cells are produced. One is a progenitor cell that will differentiate and proliferate, the other remains in the niche as a stem cell.

Intestinal crypts are a good example of an epithelial stem cell niche. The intestine contains four main epithelial cell types: enterocytes, or absorptive cells, goblet and Paneth cells that secrete mucus and anti-microbial molecules, respectively, in the intestinal lumen, and enteroendocrine cells that secrete hormones into the blood and neurotransmitters. Enterocytes, goblet cells and enteroendocrine cells are found in protruding structures called villi, consisting of an epithelium covering mesenchymal cells. Intestinal crypts are small recesses located between the villi where new cells are produced. These crypts are surrounded by connective tissue, itself supported by layers of smooth muscle cells whose waves of contraction ensure the movement of food boluses within the lumen of the intestine. Paneth cells are specifically located at the bottom of the intestinal crypts. Intestinal stem cells also reside in the crypts, in contact with Paneth cells, and divide asymmetrically to produce transit amplifying cells that divide rapidly (a 10-h cell cycle). Most of these transit amplifying cells move out the crypt and differentiate into enterocytes, goblet cells and enteroendocrine cells, while a few of them move down the crypt and differentiate as Paneth cells. Cell proliferation and differentiation are controlled by two signaling pathways, Wnt and BMP-4, respectively. Wnt is secreted by Paneth cells and underlying mesenchymal cells, and acts on stem cells and transit amplifying cells to stimulate their division. This provides a positive feedback mechanism that maintains cell proliferation. BMP-4 is secreted

by mesenchymal cells in the villi and blocks the proliferation of epithelial cells while stimulating their differentiation. Intestinal epithelial cells indeed express cell surface receptors for both Wnt and BMP-4 and are thus sensitive to the relative concentration of these molecules. BMP-4 is called a morphogen because it controls the location of differentiated cell types in a tissue. Crypt cells also secrete Hedgehog, a signaling molecule that activates the secretion of BMP-4 by mesenchymal cells in the villi. The self-organization of crypts and villi results from the interplay between these three signaling pathways and these different cell types (Fig. 1.1). The concept of "stem cell niche" in this case is therefore relatively complex.

Eukaryotic cells are often grown on solid surfaces covered by ECM macromolecules. Surface stiffness is quite important for cell growth and differentiation (Discher et al. 2005). ECM molecules are often provided exogenously but they can also be secreted by the cells themselves. The exact nature of the ECM molecules is therefore often not known with precision. Cells require a specific medium (e.g., Dulbecco's modified Eagle's medium, DMEM) for growth, often supplemented with growth factors, vitamins and antibiotics. Initial ECM molecules and growth factors are often supplied by a certain proportion of fetal calf serum (FCS), the exact composition of which is usually not defined and probably varies from batch to batch. Later cells can secrete their own growth factors and their own ECM. Eukaryotic cells are usually grown at 37°C, in the presence of saturating H_2O, air and 5% CO_2 to maintain the pH. Chemical buffers can also be used to stabilize the pH. When desired, cells are dissociated from the ECM and other cells using trypsin and EDTA, a Ca^{2+}-chelating molecule. When the cell density reaches saturation (meaning that cells enter into contact), they should be dissociated, diluted and seeded onto a new plastic surface at a lower concentration. The current trend is to switch from 2D cultures to 3D, and possibly stem cells, using micro- and nanotechnology to engineer their specific environment. Note that the same cell type can switch from 2D to 3D culture simply by changing the nature of the ECM molecules provided. On fibronectin-coated surfaces, MDCK cells for instance form a monolayer. On Matrigel®, a complex set of macromolecules secreted by tumor cells, the same cell type forms spherical or cylindrical structures called cysts that resemble glands (acini) or vessels (Kleinman and Martin 2005).

Two *in vitro* cell culture systems have been described for intestinal cells that allow the growth and differentiation of stem cells. Intestinal *tissue fragments* can be grown on a collagen gel (Ootani et al. 2009). This gives rise to a 3D culture consisting of a hollow spherical epithelium growing on a layer of mesenchymal cells embedded in ECM. The spherical epithelium contains all cell types, and the culture does not need externally added growth factors because they are supplied by the mesenchymal cells. Alternatively, *intestinal crypts* can be grown on a laminin-rich gel (Matrigel®) (Sato et al. 2009). In this 3D culture, cysts form that look like hollow spheres with extending crypt-like protrusions. The lumen of the cysts therefore resembles an intestinal lumen while the newly formed crypts are similar to the initial ones and can even be grafted into a host tissue. Three growth factors are necessary for this: EGF (a broad-spectrum growth factor), Noggin (to block BMP-4 signaling) and R-spondin (to enhance Wnt activity). These cell cultures recapitulate the differentiation pattern of epithelial cells in the intestine and are thus very useful for studying their physiology and pathologies.

1.1.6 Purification of intracellular compartments

It is difficult to purify intracellular compartments. First, one needs to prepare a cell suspension. Since most cells are adherent, this implies using trypsin, a protease, and EDTA to cleave adhesion proteins and the base structure. Then, the plasma membrane needs to be gently disrupted, to free internal components. Mechanical or chemical techniques can be employed (Goldberg 2008). Mechanical disruption often relies on the shear stress created by passing the cell suspension through a narrow space between two surfaces (e.g., a Dounce homogenizer or Balch homogenizer, high-pressure devices, etc.). Chemical techniques use mild detergent to solubilize the plasma membrane or osmotic shock. Once the plasma membrane has been disrupted, intact cells and nuclei are separated from the cytoplasm by low-speed centrifugation. The resulting suspension is called a "post-nuclear supernatant". It can be further fractionated by a combination of high-speed centrifugation techniques: velocity centrifugation that separates organelles, vesicles and particles according to their sedimentation coefficient S and equilibrium centrifugation that separates them according to their buoyant density ρ. The sedimentation coefficient S depends on the size and the shape of the object as well as the difference between the density of the particle and that of the surrounding fluid. Note that the buoyant density of an organelle may vary because of osmotic effects.

All these purification methods are rather time-consuming and cumbersome. Furthermore, it is difficult to work with fewer than 10^8 cells. It is anticipated that microfluidic techniques will allow more rapid preparation of intracellular compartments with less starting material.

1.2 Protein localization within cells

At a given time, there are at least 10^4 different proteins expressed in a given cell. Since protein interaction only occurs over short distances (less than a few nanometers), the proper localization of a protein within the cell is essential. Many proteins are therefore bound to specific membrane surfaces or at specific places along cytoskeletal filaments. In this section we review the basic features that define the localization of a given protein as well as some common mechanisms used in eukaryotic cells for protein localization, focusing on the main cellular organelles.

1.2.1 Cell targeting signals

Targeting or sorting signals are stretches of amino acids encoded in the primary sequence that define the journey of a given protein in the cell and its final localization. A single protein may contain several targeting and sorting signals. Specific to a given compartment and well conserved in eukaryotes, they allow reversible interaction with the proteins that organize intracellular protein transport. They are generic and allow targeting of a protein of interest, for instance a fluorescent protein. In the absence of any signal, a protein is targeted to a default localization: the cytosol

Table 1.2 The main targeting signals in eukaryotic cells

TARGET COMPARTMENT	TYPICAL SEQUENCE	TRANSPORT MECHANISM
Import into the ER (signal sequence)	+MMSFVSLLLVGILF WATEAEQLTKCEVFN	Translocation through the membrane
Import into mitochondria (signal sequence)	+MLELRNSIRFFKPATRTLCSSRYLL	Translocation through the membrane(s)
Import into the nucleus	PPKKKRKV	Gated transport
Retention in the ER	KDEL-	Vesicular transport
Sorting to endosomes or lysosomes	YxxP, ExxLL	Vesicular transport
Plasma membrane	+GSSKSKPK, CxxL-, CCxx- and acylation (modification by a lipid group)	Direct binding of a peripheral membrane protein
Plasma membrane	Glycosylation at NxS/T sequence of membrane proteins and secreted proteins	Vesicular transport

ER, endoplasmic reticulum.

for a soluble protein and the plasma membrane for an integral membrane protein. Table 1.2 summarizes the main targeting signals used to address proteins in eukaryotic animal cells. Targeting signals are either constitutive (always active) or may be activated by phosphorylation and/or conformational changes. Their efficiency will depend on the accessibility of such sequences to the proteins of the targeting and sorting machinery.

A signal sequence consists of about 20 amino acids at the N-terminal end of the primary sequence of a protein. It allows insertion of the protein in the membrane of an organelle (e.g., endoplasmic reticulum, mitochondrion) or translocation of the protein through one or several organellar membranes. Once the protein has been imported into the lumen of the organelle, the signal sequence is often cleaved by a specific protease and degraded.

Retention signals maintain some proteins in a given compartment, usually by interacting with specific membrane receptors. The steady-state localization of these receptors results from the balance of anterograde and retrograde traffic.

1.2.2 Vesicular transport mechanisms

Vesicular transport is a complex intracellular mechanism that carries membrane proteins or soluble proteins contained within the lumen of an organelle to other locations in the cell. The basic mechanisms of vesicular transport are reviewed in Fig. 1.2. First, the proteins to be transported (the cargo) are sorted in budding vesicles, with the help of cargo receptors in the case of soluble proteins or by the direct interaction of transmembrane cargo proteins with coat proteins (1 and 2 in Fig. 1.2). Coat proteins (clathrin, coatomer) assemble around the nascent vesicle in large multiprotein structures that deform the membrane. Activation of a small G protein such as ARF is necessary for the assembly of the protein complex. Specific proteins such as dynamin pinch off the vesicle from the donor membranes (3). Dissociation of the coat is coupled to nucleotide hydrolysis by ARF (4). The vesicle is then carried along microtubules by specific molecular motors, possibly over several tens of micrometers. The vesicle then interacts with target membranes, involving another set of small G proteins called Rab, which ensures the specific docking of the vesicle with the proper target compartment (5 and 6). Membrane fusion between the incoming vesicle and the target membrane is performed by the action of SNARE proteins (7). These proteins are present on transport vesicles (v-SNAREs) and on target membranes (t-SNARE). The specificity of membrane fusion in ensured by specific v–t SNARE complexes that form a coiled-coil structure consisting of four alpha-helices. v-SNAREs and t-SNAREs are also called R- and Q-SNAREs, referring to the identity of the amino acid at the center of this coiled-coil structure. The positioning of v- and t-SNAREs in the vesicle and in the target membrane is strict: exchanging protein positions abolishes the membrane fusion activity. After membrane fusion, SNARE disassembly is catalyzed

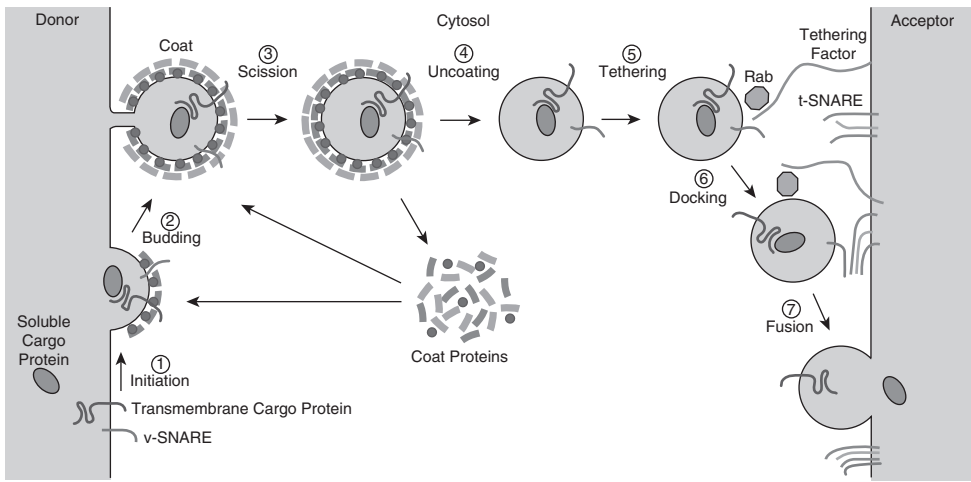

Fig. 1.2 Basic mechanisms of vesicular transport (Bonifacino and Glick 2004 *Cell* **116**, 153–166). See text for details.

by NSF and SNAP proteins. The cycle of SNARE complex assembly and assembly is driven by ATP hydrolysis.

1.3 Protein activation in cells

Protein activity is tightly regulated, either by post-translational modifications (Table 1.3) or by interaction with other proteins, resulting in conformational changes. This regulation provides another level of complexity, because for two proteins to interact they must not only be at the same place at the same time (control of protein expression and localization signals) but must also both be active at the same place at the same time! Protein activity is difficult to monitor *in situ*: mass spectrometry techniques allow monitoring of post-translational modifications and fluorescent reporter techniques have been developed, but they only monitor a single protein species at a time.

1.3.1 Phosphorylation as an example of a protein activation mechanism

Phosphorylation of the amino acids serine, threonine, tyrosine or, less frequently, histidine is a widespread mechanism for regulating protein activity. This biochemical reaction is catalyzed by specific kinases that bind both ATP and the protein (polypeptide) to be modified. Kinases catalyze the transfer of the γ-phosphate of ATP to the hydroxyl group of the amino acid. The phosphorylated protein is then released. Conversely, phosphatases catalyze the release of the phosphate group from the amino acids. Kinases are specific and recognize "consensus sequences". The accessibility of these consensus sequences on a potential target protein may be regulated by conformational changes. Kinase activity is often regulated, and kinases themselves are often regulated by phosphorylation. The main targets of regulation by kinase activity are the substrate-binding site and the catalytic site. The binding of the substrate can be controlled by the position of a "regulation loop". The catalytic transfer of the γ-phosphate of ATP may also depend on the position of some critical amino acids. Kinases are therefore regulated by many different molecules: small molecules such as cAMP in protein kinase A (PKA; cAMP-dependent kinase) or other proteins such as cyclin in cyclin-dependent kinase (CDK). Kinase domains are also present in proteins and are often regulated by conformational changes (e.g., receptors with tyrosine kinase activity). A total of 518 kinases and 73 phosphatases have been described in the human genome.

1.3.2 Formation of protein complexes

Cellular mechanisms often involve the formation of protein complexes. A remarkable feature is that several components can reversibly associate, have an effect and then dissociate. This cycle of complex formation and dissociation is linked to energy consumption. For instance, a small G protein containing a non-covalently bound GTP molecule can be incorporated during the formation of the complex. Completion of

Table 1.3 The main post-translational modifications that affect protein activity

POST-TRANSLATIONAL MODIFICATION	BIOCHEMICAL MODIFICATION	EFFECT
Proteolytic cleavage	Polypeptide bond cleavage	Introduces two new N-terminus and C-terminus ends. Possibly releases one of the polypeptides
Co-factor addition	Covalent or non-covalent bonding of a small molecule	Expands the span of catalytic activity of proteins
Lipid addition	Covalent binding of one or several fatty acids to specific amino acids	Addition of hydrophobic fatty acids anchors the protein or a portion of the protein into the membrane where the modification takes place
Acetylation	Covalent addition of an acetyl group, either at the N-terminus or at lysine residues	Eliminates the positive charge and enhances the hydrophobic character of the amino acid; in histones, this regulates DNA accessibility
Phosphorylation	Covalent addition of a phosphate group to specific amino acids (serine, threonine, tyrosine, histidine)	Change in H-bonding capacity and in the charge of the amino acid. Modifies protein conformation and interaction with partners
Ubiquitination	Covalent linkage of ubiquitin (a small protein) to specific lysine residue	Target the protein for degradation. Modifies protein conformation and interaction with partners
SUMOylation	Covalent linkage of the SUMO protein (small ubiquitin-related modifier) to a lysine in the consensus motif Ψ-K-x-D/E	Modifies protein conformation and interaction with partners—protein localization may change

complex formation triggers GTP hydrolysis by the small G protein and the release of phosphate. The energy released is used to disassemble the complex. This mechanism is at work in vesicular budding and docking (Arf and Rab proteins), delivery of tRNA to the ribosome and translocation of mRNA (EF-Tu, EF-G).

Another common mechanism involves the disassembly of protein complexes by AAA ATPases (AAA stands for ATPases associated with diverse cellular activities). These proteins often assemble in hexamers that form a toroidal structure. Good examples of this protein family are NSF, which disassembles SNARE complexes in collaboration with SNAP proteins, chaperones and proteasome AAA ATPases that unfold or refold proteins.

The provision of energy for the formation of protein complexes may also enhance the specificity of molecular recognition, in a manner similar to proofreading mechanisms (Hopfield 1974, Ninio 1975). Another way to achieve a high level of specificity is the presence of "scaffold proteins" that bind the components of a complex but do not play an active role in the process. They restrict the number of interacting molecules and avoid losing time in non-productive dissociation–reassociation steps. Scaffold proteins were initially described in cell signaling pathways, where they play an essential role in the response kinetics (Harris and Lim 2001).

Once the core proteins involved in cellular processes have been identified, and the basic biochemical mechanisms deciphered, it is interesting to reconstitute the process or part of the process in a simplified system, with purified components (lipids, proteins, small molecules). Nevertheless, the kinetics of the reconstituted process is often much reduced from that of intracellular processes. This can be due to a lower concentration of active proteins and difficulties in reconstituting the proper orientation of molecules in membrane structures.

1.3.3 Synchronization of cell activity

Time is an essential factor in self-organization. Although at the level of individual molecules, movement is governed by diffusion, things can be different at the level of cells, and information can propagate much faster than individual molecules. This is similar to the case of a metallic conductor. The velocity of individual charges is much slower (100 μm/s) than the propagation of the electrical field across the wire (the speed of light). In the following, we will give two examples showing how information can spread rapidly throughout a cell.

The first example corresponds to the well-known case of contraction of skeletal muscle. Muscle fibers (myofibers) are centimeter-long cells created by the fusion of smaller myoblasts. They contain myofibrils, an intracellular contractile structure made of repeated units called sarcomeres. The active part of the sarcomere consists of myosin bundles that move along actin microfilaments when their ATPase activity is unleashed in the presence of intracellular Ca^{2+}. The movement of myosin molecular motors along actin filaments reduces the length of the sarcomere, exerting force at the ends of the myofibrils. Ca^{2+} is mainly contained in intracellular compartments, called sarcoplasmic reticulum, that extend throughout the cell. How can a centimeter-long cell control the release and uptake of Ca^{2+} in a synchronized manner? This is the result of three fast biochemical mechanisms: (1) propagation of an action potential along the plasma membrane, (2) local release of Ca^{2+} at the T-tubules and (3) Ca^{2+}-induced release of Ca^{2+} at the surface of the sarcoplasmic reticulum.

Action potentials originate from a motor plate, a specialized synapse between a motor neuron and a muscle, and propagate along the myofiber plasma membrane, at a

very high speed (around 1 m/s). This electrical signal is due to the fast oscillation of the transmembrane potential induced by the rapid transient opening of Na^+ ion channels, followed by the transient opening of K^+ ion channels. In this process, adjacent ion channels interact through the variation in transmembrane potential they generate, which propagates rapidly and passively along membrane structures.

The plasma membrane of myofibers is invaginated and forms T-tubules that come into close contact with the sarcoplasmic reticulum inside the myofiber (Fig. 1.3). Voltage-sensitive Ca^{2+} channels open upon membrane depolarization induced by the action potential. The initial rise in Ca^{2+} in the cytoplasm activates specific calcium channels in the sarcoplasmic reticulum (RyR) that release more Ca^{2+} from the calcium stores (calcium-induced calcium release). These calcium channels therefore open each other, and very rapidly the entire sarcoplasmic reticulum releases Ca^{2+}. The activity of these channels is transient since they rapidly inactivate, and the cytoplasmic calcium is pumped back into the sarcoplasmic reticulum by a specific ATPase (SERCA).

Using this example we can recall that propagation can be speeded up by two simple mechanisms: (1) the activation of one protein activates others nearby (amplification or positive feedback) and (2) some proteins spontaneously deactivate, preventing the full simultaneous activity of all molecules (delayed negative feedback). Instead, a traveling wave propagates rapidly.

A second example comes from signal transduction pathways. The mitogen-activated protein (MAP) kinase (MAPK) cascade consists of a set of three kinases that regulate each other and relay signals from the plasma membrane to the nucleus: kinase 1 phosphorylates and activates kinase 2, kinase 2 phosphorylates and activates kinase 3. At one end, kinase 1 is activated by receptor tyrosine kinase (RTK) receptors, and at the other kinase 3 activates transcription factors that determine cell differentiation, among other targets. *Xenopus* oocyte development involves such a cascade that mediates the response of oocytes to progesterone. *Xenopus* oocytes are millimeter-sized cells: Ferrell (1998) demonstrated that for an intermediate level of progesterone, at the level of a single cell, either all kinase molecules were active or none were. This remarkable result shows that these big cells somehow synchronize the activation state of the MAPK cascade molecules. This bistable behavior is explained by two features of the *Xenopus* MAPK pathway: a non-linear response and a positive feedback loop that provides an amplification mechanism. The non-linear response, also called ultrasensitivity, results from the successive activation of the three kinases in the presence of constant antagonist phosphatase activity (Huang and Ferrell 1996). At a low signal concentration phosphatase activity overcomes kinase activity, whereas above a certain threshold the opposite occurs. The positive feedback loop is provided by a specific serine–threonine protein kinase (Mos), which activates MAPK 2 (Matten et al. 1996). As a consequence, activation of the full cascade requires a minimum activation signal (threshold) which can even be transient (hysteresis effect). This is explained by a simplified biochemical model which is presented in Fig. 1.4. Cooperative effects in protein interactions therefore provide non-linear responses that help cells take decisions. In this manner, all proteins in the same cell may have the same activation state at the same time.

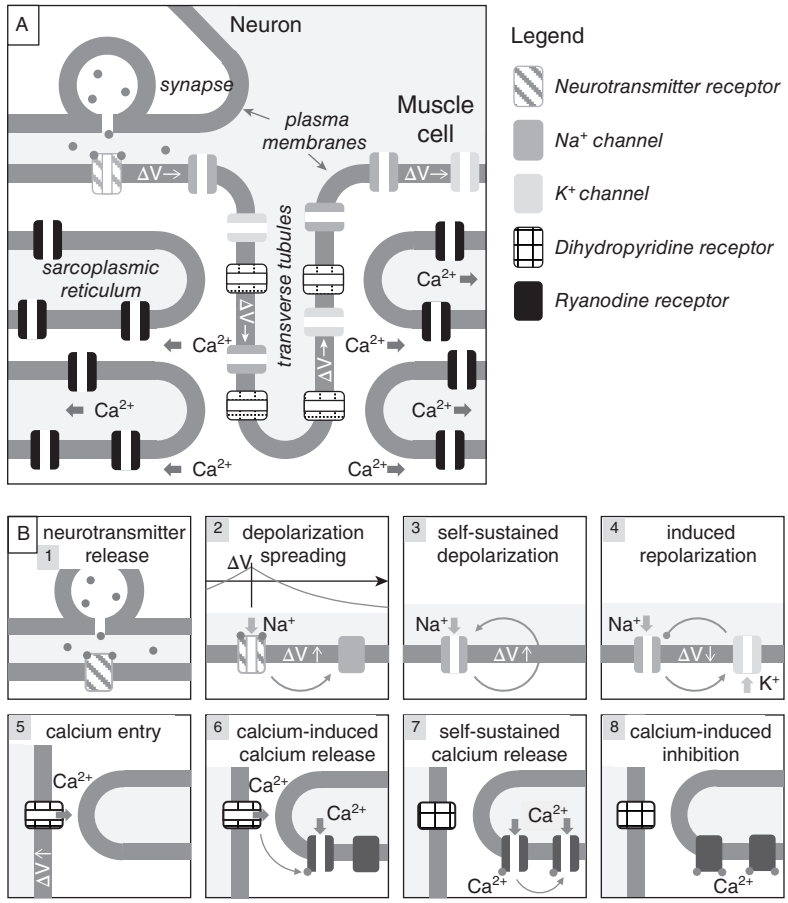

Fig. 1.3 Mechanisms of muscle cell activation. A. Structure of muscle cells: a motor neuron forms a synapse at the surface of the plasma membrane. The muscle plasma membrane is folded in T tubule. A specialized compartment, the sarcoplasmic reticulum, contains Ca^{2+} ions necessary for myosin-actin interaction and muscle contraction. B. The molecular mechanisms of the action potential and the wave of Ca^{2+} release illustrate the role of **positive feedback** in the fast propagation of activation signals. The synchronous Ca^{2+} release from sarcoplasmic reticulum ensures proper muscle cell contraction. The motor neuron synapse induces an action potential that propagates along the plasma membrane and the T tubules, inducing Ca^{2+} release from the sarcoplasmic reticulum by Ca^{2+}-induced Ca^{2+} calcium release mechanisms. The initial release of neurotransmitter at the synapse (1) induces a depolarization of the plasma membrane (2) that opens nearby Na^+ channels (grey). Na^+ entry further depolarizes the membrane that allows further signal spreading (3, **positive feedback**). After a delay, membrane depolarization induces K^+-channel (light grey) opening, that brings back the transmembrane potential to resting value (4). In T tubules, the plasma membrane depolarization due to the incoming action potential opens dihydropyridine receptors (5, square filling) that mediate Ca^{2+} entry. Ca^{2+} entry induces the activation of ryanodine receptors (6, dark grey). Ryanodine receptors are Ca^{2+} channels activated by cytosolic Ca^{2+}. The self activation of ryanodine receptors spreads rapidly in the sarcoplasmic reticulum (7, **positive feedback**). After a delay, ryanodine receptors get inactivated (8).