Senthilkumar Rajagopal
Murugavel Ponnusamy

Calcium Signaling: From Physiology to Diseases

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Foreword by Sabu Thomas

It is a distinct honor to have been invited by my colleagues in the calcium channel regulation arena to write the foreword to this very articulate and scientifically state-of-the-art book entitled *Calcium Signaling: From Physiology to Diseases*. The book covers all the areas required to create a robust category and perform a read-across. I am certain that the readers including faculties, researchers, and students will find this book extremely informative, interesting, and inspiring. Hence, I hope that you will find this book as possessing sufficient disclosure and adequate utility. This book aims to simplify the revolution and to fortify the researcher with the information needed to use calcium channel antagonists with complete confidence and the best compound that can be applied for therapy of the individual. The book explores in many ways and makes good sense of the further investigation of channelopathies, and this valuable text opens the doors for the progression that occurs when one discovers a fact, becomes interested, and then begins investigation and discovery of the natural process.

I have 30 years of experience in polymer science and technology, and I contributed greatly to the research and development of nanoscience and nanotechnology. I have received my PhD from IIT–Kharagpur, and then joined as a senior visiting researcher in Katholieke Universiteit, Leuven, Belgium, and Laval University, Quebec, Canada. Subsequently, I served as associate professor and professor at Mahatma Gandhi University, Kottayam, Kerala, India. I am one of the pioneers of the field of polymer science and technology and have published over 750 peer-reviewed research papers, reviews, and book chapters. I have co-edited nearly 60 books, and I am the inventor of 5 patents. I have supervised 79 PhD students, and my h-index is 78 with nearly 28,675 citations. I have delivered over 300 plenary/inaugural and invited lectures in national/international meetings in over 30 countries. I am presently the Director of the International and Inter University Centre for Nanoscience and Nanotechnology and a full professor of polymer science and engineering at the School of Chemical Sciences of Mahatma Gandhi University, Kottayam, Kerala, India. I am an outstanding leader with sustained international acclaims for my work in polymer science and engineering, polymer nanocomposites, elastomers, polymer blends, interpenetrating polymer networks, polymer membranes, green composites and nanocomposites, nanomedicine, and green nanotechnology. My groundbreaking inventions in polymer nanocomposites, polymer

blends, and green nanotechnological and nano-biomedical sciences have made transformative differences in the development of new materials for automotive, space, housing, and biomedical fields.

As a consequence, a large number of books, thick and thin, have been and will continue to be published on various aspects of dysregulation of calcium channels. This monograph is intended to give an overview about the current knowledge of Ca^{2+} signaling in essential physiological processes and aspects of pharmacological targeting of Ca^{2+} channels and other Ca^{2+} handling machineries to attenuate or prevent the progression of certain common disorders associated with Ca^{2+} dysregulation, based on the impressive growth of knowledge in all aspects (cellular, organic, hormonal, structural) of calcium channel proteins that interfere with the physiology of the calcium ion. The introductory chapter describes the influence of Ca^{2+} level and its associated signaling pathways on developmental process and physiological functions. This chapter also gives an overview about the Ca^{2+} homeostasis mechanism and impact of abnormal Ca^{2+} level.

The book analyzes trends in the processing of natural products by using nanotechnology and their implications in calcium-related disorders. It covers some of the most interesting aspects of research in calcium signaling disorders and provides a trustworthy source of current information in this area of research. The elaborated description in chapters will enhance the understanding of calcium ion deficiencies, which will help the readers to gain an in-depth and latest development in the field of nanotechnology in channelopathies. The fifth chapter presents the various calcium channelopathies identified to date and discusses the current knowledge of calcium-regulating diseases. The sixth chapter has discussed the nanotechnology strategies that could help to overcome challenges in treating channelopathies and ease the translation of natural products from bench to clinical application. The better understanding of regulation of oxidative stress can be utilized for devising strategies for the development of novel therapeutic preparations for clinical interventions in oxidative stress and pathogenesis-induced calcium deficiency disorders.

With best wishes,

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Foreword by Noah Weisleder

The field of calcium signaling research is vast and evolving rapidly in both basic research and clinical therapeutics. When I was asked to write a foreword for this book, my immediate thought was that there are many monographs and comprehensive textbooks focusing on Ca^{2+} signaling that cover a wide variety of topics at various levels of detail, so why the need for another book in this increasingly crowded field? This book is distinguished from the currently available literature in that it provides a concise summary of the basic mechanisms of cellular Ca^{2+} signaling and how these mechanisms are linked with the emergence of common, devastating pathological disorders. The authors build on current knowledge to detail the link between Ca^{2+} deregulation and the molecular pathology of diseases. They explain these complex concepts with straightforward language that allows greater accessibility to a wide audience. The succinct text will assist the novice in understanding Ca^{2+} signaling research, while the up-to-date information on the current state of Ca^{2+} signaling and pathophysiology will be of interest to experts in the field.

I have been interested in Ca^{2+} signaling research at all stages of my career. I received my BS in biotechnology and molecular biology from Worcester Polytechnic Institute and a PhD in cell biology from Baylor College of Medicine. This led to my postdoctoral studies at Robert Wood Johnson Medical School where I went on to join the faculty as assistant professor in the Department of Physiology and Biophysics. Currently, I am an associate professor and director of graduate studies in the Department of Physiology and Cell Biology at Ohio State University, as well as an investigator in the Davis Heart and Lung Research Institute. I have published numerous peer-reviewed publications or book chapters in the fields of muscle physiology, cardiovascular disease, cytoskeletal dynamics, membrane repair, and cellular Ca^{2+} homeostasis in normal physiology and disease states. I have chaired sessions at national and international meetings on Ca^{2+} signaling and muscle physiology. Additionally, I am an inventor of numerous US and international patents. These inventions became the basis for the formation of TRIM-edicine, a biotechnology company developing protein therapeutic targeting regenerative medicine applications, where I was a founder and served as chief scientific officer. Thanks to these accomplishments, I received a fellowship from the American Heart Association, a Pathway to Independence Award from the National Institutes of Health, and the Kauffman Foundation Outstanding Postdoctoral Entrepreneur Award.

As a researcher and teacher, I particularly appreciate the accessibility and simplicity of the contents of this book which covers many aspects of Ca^{2+} signaling including its role in physiological processes, dysregulation of Ca^{2+} gradients, and Ca^{2+} handling molecule contribution to the pathogenesis of several disorders like neurodegenerative diseases, muscle disorders, and chronic pain. Furthermore, they discuss the promise of targeting Ca^{2+} transporting receptors and select proteins for treating neurological, muscle, and other disorders. Finally, they address the benefits of natural products in treating Ca^{2+} disorders and how nanotechnology can help improve the therapeutic effects of naturally available Ca^{2+} channel modulators. The authors present their multidisciplinary approaches in a single, readily accessible book to provide a reliable reference for students and investigators interested in Ca^{2+} signaling research.

Each chapter of this book contains insight that will be useful to scientists at all levels. The introductory chapter describes the influence of intracellular Ca^{2+} levels and how changes in this critical variable affect signaling pathways that influence developmental processes and physiological functions. This chapter also gives an overview of Ca^{2+} homeostasis regulatory mechanisms and the impact of abnormal Ca^{2+} levels. Chapter 2 focuses on regulation of Ca^{2+} in muscle physiology by summarizing the involvement of Ca^{2+} ion channels in muscle physiology and pathophysiology. The third chapter provides a broad overview of Ca^{2+}-permeable ion channel contributions to nociception pathways. Chapter 4 expands on the role of Ca^{2+} signaling in the nervous system by examining the contribution of altered Ca^{2+} regulation in the progression of neurological disorders. This focus on pathophysiology continues in Chap. 5 to summarize the physiological function of voltage-dependent Ca^{2+} channels and how various channelopathies develop due to changes in Ca^{2+} signaling through these channels. The final chapter expands on the channelopathy theme by detailing nanotechnology strategies that could help to overcome challenges in treating channelopathies and ease the translation of natural products from bench to clinical applications.

This book will provide a useful reference for those interested in the role of Ca^{2+} signaling in physiology and pathophysiology, as well as for those who are interested in targeting Ca^{2+} signaling as a therapeutic approach for various disease states. I congratulate the authors on producing a straightforward text that can be useful to researchers with different levels of expertise. I hope that this work will help to expand interest in the essential field of Ca^{2+} signaling research.

With best wishes,

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Preface

The evolution process of living organisms offered a well-established communication between millions of cells in higher organisms like humans. Calcium (Ca^{2+}) is one such element involved in the cellular communication system. This versatile bio-element contributes to nearly all the aspects of developmental and physiological processes of all living organisms. In fact, there is no genesis or movement of organisms without Ca^{2+} due to its fundamental role in embryogenesis, skeleton formation, and muscle function. Unlike other ions, Ca^{2+} has the capability to translate the extracellular signal into a different type of response which depends on a very tight spatial and temporal control of intracellular Ca^{2+} level. Thus, Ca^{2+} homeostasis is an integral part of normal physiological functions of any eukaryotic organism including humans. In the last several decades, a dramatic progress has been made to understand the complex process of Ca^{2+} signaling and homeostasis. However, the molecular events that initiate/regulate the Ca^{2+} influx and efflux remain in need of further study for better understanding of the activity of Ca^{2+} in a biological system. This monograph is intended to give an overview about the current knowledge of Ca^{2+} signaling in essential physiological processes and aspects of pharmacological targeting of Ca^{2+} channels and other Ca^{2+} handling machineries to attenuate or prevent the progression of certain common disorders associated with Ca^{2+} dysregulation.

As the Ca^{2+} signaling is a diverse field, it is difficult for us to cover all the areas, and more specifically, we felt difficulties in determining the focus and target readers. We have mainly focused on the functional role of Ca^{2+} in muscle and neural physiology and providing current knowledge on the link between Ca^{2+} dysregulation and the development of pathological conditions in muscle disorders, neurodegenerative diseases, and pain. Although we have not covered all the molecules and signaling pathways involved in the regulation and activation of Ca^{2+} signaling, we have summarized the characteristic features and functions of major molecules and receptors involved in the transport, activation, and regulation of Ca^{2+}. In recent years, the advancement of molecular studies has not only unveiled the structural basis of Ca^{2+} channels but also expanded the list of human diseases associated with defects in Ca^{2+} channels and other Ca^{2+} handling machineries. Taking this into account, we have illustrated in the sixth chapter about the naturally available Ca^{2+} channel activators and inhibitors and their pharmacological benefits in treating various Ca^{2+} dysregulation-associated disorders. The most important part of this monograph covers the advantage of integration of nanotechnology to the preparation of

natural product-based therapeutics. We have provided a crisp up-to-date account on the different methods of nanocarrier preparation. This chapter also describes the efficiency of the nanocarrier delivery system, in the aspect of targeted delivery, improved bioavailability, and therapeutic efficacy, for the administration of phytoactive constituents with Ca^{2+} channel-modulating capability.

The central theme of this monograph is giving the fundamental mechanisms of Ca^{2+} influx and efflux from cells, the pathological consequences of defect and/or disturbance of Ca^{2+} handling machineries and Ca^{2+}-associated signaling molecules, and the efficacy of nanotechnology system in delivering natural product-based Ca^{2+} channel modulators. We have made an effort to unify all the content of scattered research literature in this area of research and tried to provide in-depth contents about the topics we have chosen. Overall, this monograph is not just a collection of papers, but it is an essence of the diverse Ca^{2+} signaling field and demonstrates that Ca^{2+} channels and other Ca^{2+} homeostatic machineries can be a therapeutic target for multiple disorders. In all chapters, we have provided the basic information relevant to the topics, and at the same time, we have described the perspective knowledge about Ca^{2+} homeostasis. Thus, we believe that this monograph could be an informative resource, in the form of a condensed handbook, for research students as well as advanced researchers. As nanotechnology has broken many barriers in natural product-based medicine to bring them into clinical settings, we believe that the contemporary contents of this monograph may present some useful information and new ideas to all categories of readers, in particular, to beginners. If this is indeed achieved, then our efforts will have succeeded and we would be happy.

Kurnool, India — Senthilkumar Rajagopal
Qingdao, China — Murugavel Ponnusamy

Acknowledgement

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Contents

About the Authors

Senthilkumar Rajagopal is presently a recipient of Ramalingaswami Re-entry Fellowship (Department of Biotechnology, Ministry of Science and Technology, Government of India) and coordinator at the Department of Biochemistry in Rayalaseema University, Kurnool, AP, India. He has handled the project entitled "G-Protein-Coupled Receptor-Mediated Intracellular Mechanisms by Alcohol-Induced Digestive Disorders in the Gastrointestinal Tract" while working as DBT-Ramalingaswami re-entry fellow (Scientist D) from 2013 to 2015 at the Department of Zoology, Nizam College, Osmania University, Hyderabad, India, and later he moved to the Department of Biochemistry, Rayalaseema University, Kurnool, AP, as a Ramalingaswami re-entry fellow. Previously, he had worked as postdoctoral research fellow at the University of Virginia, Dana-Farber Cancer Institute, Harvard University, and Virginia Commonwealth University. His research interests are majorly focused on deciphering the role of various signaling molecules in the patho-physiology of organisms and their clinical implications. His doctorate research involved studying the role of glycine in ethanol-induced hepatotoxicity. This work was carried out at the Department of Biochemistry and Biotechnology, Annamalai University, Tamil Nadu, India. Till now, he had published more than 47 papers in various international peer-reviewed journals. He has been editor of two book series: Digestive Diseases – Research and Clinical Developments and Hepatology Research and Clinical Developments. Furthermore, he has contributed chapters to more than 14 books. He is an active member of various international scientific organizations and societies and serves as an associate editor; a review, technical, and lead guest editor; and an editorial board member on various prestigious international journals. His contribution toward neuroscience field has been applauded in the form of a Gourmand World Cookbook Award which was presented as a special award by the Society of Neuroscience. He is an active life member of various societies including the Indian Society of Cell Biology (ISCB), Society of Biological Chemists (SBC) India, Indian Society for Atherosclerosis Research (ISAR), Indian Science Congress Association (ISCA), Society for Free Radical Research (SFRR) India, Indian Society of Cell Biology (ISCB), Indian Biophysical Society (IBS), and Science Advisory Board of Bioinformatics, LLC (USA), and a member of the Royal Society of Chemistry (RSC), UK.

Murugavel Ponnusamy is working as postdoctoral research associate at the Center for Developmental Cardiology, Institute for Translational Medicine, Qingdao University, China. His research interest includes molecular pharmacology, cellular signaling, tissue repair and regeneration, and experimental therapeutics. Previously, he had worked as postdoctoral fellow at the Division of Renal Diseases, Department of Medicine, Rhode Island Hospital, Brown University-Alpert Medical School, USA, under the supervision of Dr. Shougang Zhuang. His PhD research was focused on elucidating the effect of diallyl tetrasulfide on cadmium-induced toxicity (an in vivo and in vitro study), which was carried at Annamalai University, India. In a short time, he has been able to publish more than 28 papers in various national and international peer-reviewed journals and contributed four chapters to different books. He is an active member of various societies including the Society of Biological Scientists of India (SOBSI), Indian Society of Cell Biology (ISCB), and Society of Biological Chemists (SBC) India.

Calcium Ion in Biological Systems

1

Abstract

Calcium is a fundamental element in all biological system. It plays indispensable role in almost all biochemical signalling and physiological functions. Ca^{2+} signalling is a tightly regulated process. The concentration and duration of intracellular Ca^{2+} signalling are the crucial determinants of which signalling should be 'turn on' and/or 'turn off'. Cells are well equipped with molecular machineries to precisely regulate the spatiotemporal dynamics of Ca^{2+} signals. Any disturbance/defect in Ca^{2+} signalling cascade can affect Ca^{2+} homeostasis and eventually lead to the development of many pathological abnormalities. This introductory chapter describes the influence of Ca^{2+} level and its associated signalling pathways on developmental process and physiological functions. This chapter also gives an overview about Ca^{2+} homeostasis mechanism and impact of abnormal Ca^{2+} level.

Keywords

Bio-elements • Calcium phosphate • Ca^{2+} signalling • Embryogenesis • Proliferation and death • Homeostasis

1.1 Background

The elements, in the form of free ions or combined with other elements, are fundamental building block of all living organisms. There are at least 19 elements that have an essential role in all biological system, which are called as bio-elements. The human and animal body is mainly composed of nine elements (H, C, N, O, Ca, P, K, S, Na, Cl), which constitute more than 99% of the total body mass. They have indispensable role in every step of cell growth, proliferation as well as differentiation by contributing to the activity of enzyme system (as a cofactor and catalyst), oxidation-reduction reactions of energy metabolism, stabilization of structure of biological

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molecules, buffering of biological fluids and most importantly in hormone action. For example, Fe is required for oxygen transportation by red blood cells (RBC). The balance of Na, K and Cl is important for buffering of biological fluids. Iodine (I) is vital for the thyroid hormone function. The sulphur bridge is responsible for the maintenance of protein structure and nativity (Huskisson et al. 2007).

Beside the big four elements (C,H, O, N) of biological system, calcium (Ca^{2+}) is the fifth most abundant element by weight (1.2–1.5 kg) in the human body. Interestingly, Ca^{2+} is the fifth most abundant element by mass in the earth's crust and dissolved ions of seawater. This highlights the connection between the nature and evolution of living organisms. Ca^{2+} is one of the imperative elements for the growth and development of all living organisms including plants. This ubiquitous divalent cation is highly required in vertebrates, from the developmental stage to adult stage, mainly for the formation of hard tissues such as the bone, teeth as well as protective exoskeleton in some species (e.g. armadillo, molluscs, turtles, etc.). Calcium phosphate is the predominant form of calcium naturally available from milk. The calcium lactate from milk products and other supplemental forms (tricalcium phosphate, calcium carbonate or calcium citrate) from fortified food products are other common types of naturally available calcium (Straub 2007). The phosphate form of calcium, hydroxyapatite [Ca_{10} (PO_4)$_6$ (OH) $_2$], is the major form (>98%) of Ca^{2+} in the human body and that is mainly found in the hard tissues such as bone.

Ca^{2+} is an integral part of many essential physiological processes in the biological systems, including contraction and relaxation of muscles, neurotransmission and synaptic plasticity, secretion of hormone and its action, blood coagulation, cell proliferation, differentiation, motility and survival (Sharma et al. 2016). The saga of calcium signalling began from the late eighteenth century. Based on available reports, the very first experiment with tap water (rich in calcium) and suspension medium containing Ca^{2+} salts maintained the contractility of the isolated heart from rats. This landmark observation by Sydney Ringer in the 1880s revealed the involvement of Ca^{2+} in cardiac tissue contraction (Carafoli 2003). However, a wide interest to study the impact of Ca^{2+} on cell function only boomed in late 1940s, following the notable findings of Heilbrunn and Bailey. Heilbrunn (1956) observed that isolated frog muscle fibres could contract when Ca^{2+} is applied. In one-step advancement, Bailey (2009) found that Ca^{2+} stimulates myosin ATPase activity, and liberation of Ca^{2+} from the neighbourhood of myofilaments promotes muscle contraction (Carafoli 2003). Then, the field of Ca^{2+} signalling gained much attention, and rapid progressions were made in the next two decades (1950–1970). Several researchers found that Ca^{2+} integrates the electrical excitation of muscles and nerves, which provided the mechanism of neuromuscular functions during the heart contractility. The machineries of Ca^{2+} signalling such as calcium-binding proteins and receptors were identified, and the activity of Ca^{2+} in mitochondria and endoplasmic reticulum [ER] was demonstrated (Carafoli 2003). However, the earlier studies were much around the influence of Ca^{2+} signalling in muscle contraction mechanisms.

In the last two decades, the technical advancement has made a tremendous progress in the field of Ca^{2+} signalling, and the unavoidable role of Ca^{2+} signalling in the

entire lifespan of all living organisms has been recognized, beginning from the fertilization stage to the eventual death at the end of their life cycle. It acts as both messenger and cofactor to coordinate many intracellular signalling pathways. It is quite interesting that Ca^{2+} can trigger various specific cellular responses by virtue of differences in amplitude, frequency and duration of intracellular Ca^{2+} concentration. Thus, the spatial and kinetic properties of Ca^{2+} signalling discretely regulate a variety of signalling in the intracellular environment. For example, the endothelin-1-mediated Ca^{2+} signalling promotes contraction, while the growth factor such as platelet-derived growth factor-mediated Ca^{2+} signalling primarily induces cell proliferation and growth in vascular endothelial cells. Thus, the changes in intracellular Ca^{2+} level orchestrate the cells to secrete, divide or die.

1.1.1 Calcium in Fertilization, Embryogenesis and Morphogenesis

Calcium dynamics has a great variety of function in the developmental processes. Many intracellular signalling pathways and Ca^{2+}-sensitive factors coordinate with Ca^{2+} waves during embryogenesis. The intracellular Ca^{2+} level is the vital factor of sperm and oocyte activation process, which is the integral part of fertilization in the mammalian system. The fertilizing sperm-mediated oscillation of intracellular Ca^{2+} level is crucial for the egg activation and initiation of embryogenesis (Shirakawa et al. 2016). Recent molecular studies found that the mammalian sperm harbour a specific phospholipase C (PLC zeta) to promote the oscillation of intracellular Ca^{2+} in activated eggs. The point mutations in PLC zeta contribute to male infertility (Nomikos et al. 2017; Swann and Lai 2016). The fluctuation of Ca^{2+} level (in the form of pulses, waves and steady gradients) is crucial for the establishment of early embryonic processes including cell differentiation, blastulation, gastrulation, body axis determination and emergence of organ systems (Slusarski and Pelegri 2007; Webb and Miller 2006). Ca^{2+} is an important regulator of the cell polarization and movement, a key event for the axial extension as well as right-left patterning of organs across the axis. Any disturbances in Ca^{2+} oscillations and/or Ca^{2+}-dependent responders cause developmental abnormalities in vital organs such as the heart, lungs and brain (Brennan et al. 2013; Langenbacher and Chen 2008; Slusarski and Pelegri 2007). The low intracellular Ca^{2+} level leads to abnormal anterior cardiac looping, lack of right ventricle and enlarged left ventricle in the heart (Porter et al. 2003). In the case of the lungs, a tightly regulated level of Ca^{2+} is required for the airway branching morphogenesis during embryo development (Brennan et al. 2013). During the brain development, a specific connection between neural cells is established by Ca^{2+}-dependent signalling. Although a dramatic change occurs in the molecular composition and function of an emerging synapse, the spatiotemporal specificity of calcium signalling is maintained at every step of synapse formation. Thus, the local calcium dynamics helps for the development of accurate synapse connection, and any impairment in Ca^{2+}-dependent network of neural cell maturation leads to neural developmental disorders (Lohmann 2009).

1.1.2 Action of Calcium in Physiological Functions

Many basic physiological processes in the biological system are coupled to the level of Ca^{2+} in the cytoplasmic compartment of the cell. The contraction and relaxation of muscle tissue are fundamental processes for the functioning of the heart as well as physical movement and activity of all living animals. During contraction, the release of Ca^{2+} from calcium store of muscle cells increases cytoplasmic Ca^{2+} concentration, which promotes troponin C action for the initiation of contraction. While the muscle comes to a resting state (relaxation), SR sequesters cytosolic Ca^{2+} to bring down to normal cytosolic level (Eisner 2014). The blood coagulation process (thrombosis) is the immediate response of the body to the vascular injury. The elevation of intracellular free Ca^{2+} level is the key for the platelet activation, which is the initial event in the thrombosis. The raise of Ca^{2+} level in this cell type is achieved by release from intracellular storage sites such as dense tubular system and influx of extracellular Ca^{2+} through plasma membrane channel system (Varga-Szabo et al. 2009). In neural cells, the changes in intracellular Ca^{2+} regulate the neuritis growth, remodelling, neuronal excitability and synaptic connection (Fig. 1.1).

The transient raise of cytosolic Ca^{2+} level serves as a signal for the communication between neural cells and synaptic conductance. A spatial localized calcium signalling, in particular, at presynaptic active zones selectively triggers this action (Augustine et al. 2003). The alterations in intracellular Ca^{2+} homeostasis contribute to many neurodegenerative disorders such as Huntington's and Alzheimer's disease (Magi et al. 2016). The intracellular Ca^{2+} concentration also has a considerable role in haematopoiesis. Ca^{2+} acts as versatile ion for the proliferation and differentiation of haematopoietic cells, especially myeloid lineage cells (Paredes-Gamero et al. 2008). Thus, calcium ion virtually regulates all cellular process. Its concentration in intracellular compartments such as cytoplasm, mitochondria and ER is the deciding factor of the fate of various cellular events including survival, proliferation and differentiation.

1.1.3 Calcium Homeostasis

The maintenance of Ca^{2+} level in circulation as well as in intracellular compartments is crucial for the normal physiological functions of the cell and organs. In general, the Ca^{2+} homeostasis refers to the balancing of normal circulatory calcium level, which is mainly determined by dietary intake, intestinal absorption, bone remodelling and renal excretion. The Ca^{2+} homeostatic process largely relies on the integrated system of hormones and Ca^{2+} transporters of the gut, kidney and bone. A network of ion channels, calcium-sensing molecules/receptors and Ca^{2+}-dependent intracellular proteins is involved in this process. Any disturbance to this system can affect Ca^{2+} balance and eventually leads to the development of many pathological conditions as mentioned in previous sections.

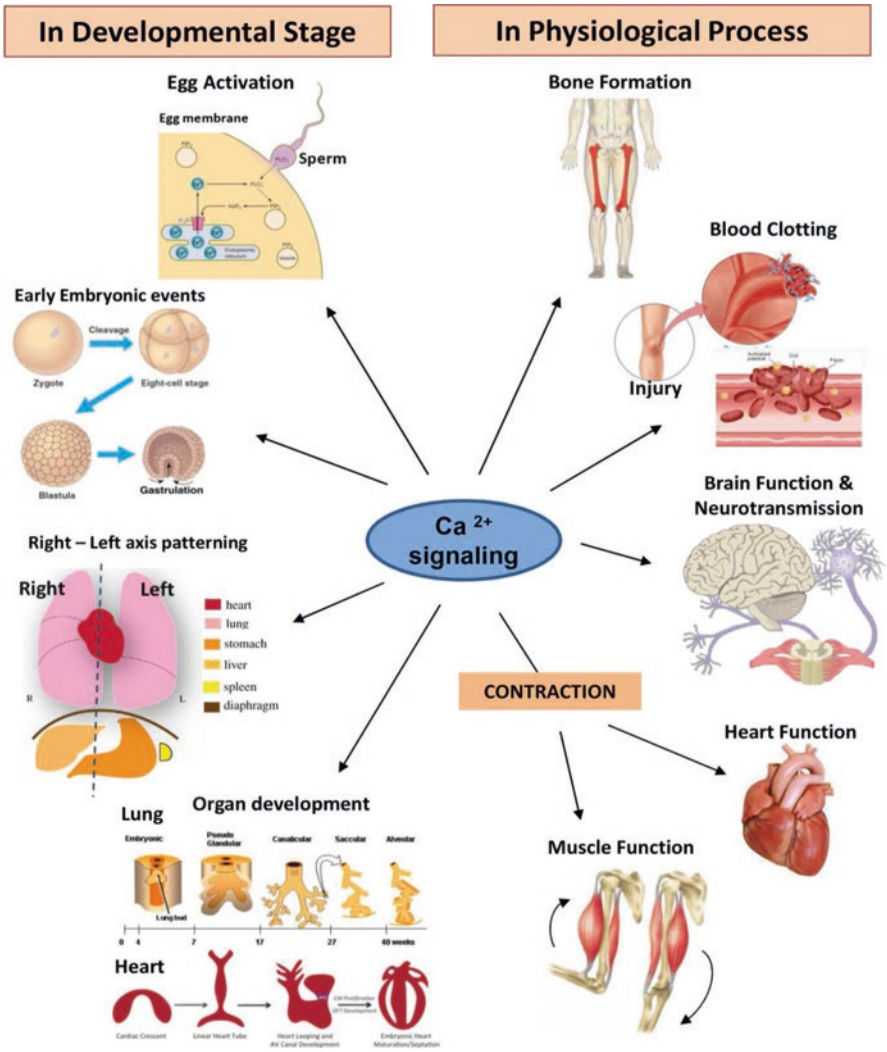

Fig. 1.1 Involvement of Ca^{2+} in important developmental processes and physiological functions

1.1.3.1 Factors Associated with Calcium Homeostasis

The supply of Ca^{2+} in mammalians and other vertebrates is primarily from their food source. However, the required level varies depending on stages of life. In human population, the growing child (9–18 years old) and pregnant woman need comparatively more calcium (about 1.3 g of Ca^{2+} per day) than other age groups. Milk and milk products are the richest source of Ca^{2+} that provides about 30–40% of daily Ca^{2+} recommended intake. The seafood (10–50%), vegetables, grains and fruits also supply a significant amount of daily Ca^{2+} requirement. The fortification of foods and beverages with calcium as well as intake of calcium supplements (chewable calcium

carbonate or calcium citrate) can compensate the daily required level of Ca^{2+}. Although an adequate amount of Ca^{2+} is taken in the food, the balance in intestinal absorption and renal excretion process is the basis for the body Ca^{2+} balance (Peacock 2010). The combined average Ca^{2+} absorption rate is about 30–40%. However, the supplements like calcium citrate have higher absorption rate (60–70%). The components of milk such as lactose, casein and vitamin D can promote calcium absorption. In contrast, the fat-rich foods and some leafy vegetables rich in phytic and oxalic acid (spinach, fibre-rich foods, etc.) inhibit/delay calcium absorption (Straub 2007).

The blood contains only Ca^{2+} in a range between 2.1 and 2.5 mM (8.5–10.5 mg/dL) in which about 50% of them are bound to albumin and other macromolecules. The circulatory ionized Ca^{2+} level is tightly maintained in a narrow range of 4.4–5.4 mg/dL, which is an active form of calcium in biological system, and its alteration can cause a wide range of metabolic and physiological disturbances (Peacock 2010). The circulatory level of Ca^{2+} is monitored and maintained by the coordinated system of vitamin D derivative (1,25-dihydroxycholecalciferol; calcitriol; 1,25-$(OH)_2$-D_3) and its receptor, parathyroid hormone (PTH) and its receptor and calcitonin. The negative feedback loop system is involved in Ca^{2+} homeostasis process (Peacock 2010). Ca^{2+} absorption is functionally controlled by 1,25-$(OH)_2$-D_3, which is a metabolite of vitamin D produced by the kidney. Thus, sufficient amount of dietary vitamin D is also important for the Ca^{2+} homeostasis. A slight change in the level of circulatory Ca^{2+} is sensed by Ca^{2+}-sensing receptor (CasR) in the parathyroid gland and that promotes the secretion of PTH. The PTH acts in three ways to balance the blood Ca^{2+} level. The surge of PTH stimulates the synthesis of 1,25-$(OH)_2$-D_3 in the kidney in order to stimulate the intestinal epithelial transport system of Ca^{2+} absorption.

Calcium is absorbed in two ways: passive diffusion and active transport. The passive diffusion (paracellular) is the predominant mode of calcium entry, when the dietary calcium is high, while calcium active transports (transcellular) are the predominant mechanism of Ca^{2+} entry during the normal and low intake of calcium. Normally, the soluble ionic Ca^{2+} is efficiently absorbed by passive diffusion throughout all segments of the intestine. 1,25-$(OH)_2$-D_3 promotes paracellular calcium transport by increasing permeability through suppression of many tight junction proteins including claudin 2, claudin 12, cadherin 17 and aquaporin 8 (Pua et al. 2016). The active transport largely occurs in the duodenum and jejunum of small intestine and that is mainly by Ca^{2+} ATPase system and a lesser extent by Na^+/Ca^{2+} exchanger (NCX1). In this process, Ca^{2+} entry is initiated by transient receptor potential vanilloid type 6 (TRPV6), a Ca^{2+}-selective channel, on brush border side of the intestinal epithelia. In intracellular location, the binding of Ca^{2+} with a calcium-binding protein (calbindin D9K) mediates Ca^{2+} excluding out from the cell to the blood vessel via plasma membrane ATPase 1b (PMCA1b), in an energy-dependent manner. Notably, 1,25-$(OH)_2$-D_3 can regulate the expression and activation of both calbindin D9K and PMCA1b in intestinal cells (Peacock 2010; Pua et al. 2016).

In the kidney, PTH prevents the loss of Ca^{2+} in the urine by a mechanism similar to the active transport process in intestinal cells. The trans-epithelial resorption process also proceeds in three major steps. First, Ca^{2+} is transported to intracellular space by TRPV5 in the epical membrane, where it binds with calcium buffering proteins (calbindin D9K and calbindin D28K) and passively diffused towards basolateral membrane by calcium gradients. The basolateral membrane bound to PMCA1b and NCX1 transports it into the blood (Pua et al. 2016). However, PTH and 1,25-$(OH)_2$-D_3 can block this active transport process in kidney cells by blocking the expression of TRPV5, calbindin D28K, PMCA1b and NCX1 (Jeon 2008; Pua et al. 2016). Apart from the intestine and renal tissue, the bone is an important contributor in maintaining the blood calcium level. The bone is constantly under remodelling by osteoblasts (a cell type that forms bone) and osteoclasts (cell type that breaks bone tissue and releases calcium). The activation of osteoclast is mediated by receptor activator of NF-κB ligand (RANKL). PTH and vitamin D3 promote bone formation and balance circulatory calcium by upregulating expression of this molecule in osteoclast. In addition, 1,25 (OH) 2-D and PTH suppress osteoprotegerin (OPG), a competitive inhibitor of RANKL activity. Calcitonin has opposite role to PTH and vitamin D. Calcitonin is secreted by parafollicular cells of the thyroid gland when the ionic form of Ca^{2+} increased in the circulation. The gastrin is a gastrointestinal peptide that can stimulate calcitonin secretion, particularly after food intake. Calcitonin regulates blood Ca^{2+} by mainly acting on osteoclasts and inhibiting bone resorption as well as promoting osteoblast activity (Pua et al. 2016). Thus, the PTH, 1,25 (OH) 2-D and calcitonin play a central role in the maintenance of calcium homeostasis.

1.1.3.2 Intracellular Calcium Homeostasis

All types of cells contain a sustained amount of Ca^{2+} due to its vital role in many fundamental cellular processes. The intracellular concentration of free Ca^{2+} level in all cells is at least 20,000-fold lower than its level in the extracellular environment. The total intracellular Ca^{2+} level (including protein bound and buffered) is usually in μM range, while the free Ca^{2+} ion level is maintained in nM range. For example, erythrocyte has only about 30–60 nM free Ca^{2+} ion, but the total of intracellular calcium is about 5.7 μM (Bogdanova et al. 2013). However, this level varies depending on the cell type and its functions. For example, the human osteoblast contains a large internal Ca^{2+} stores, and they have a cytosolic concentration about 245 nM (Zerwekh et al. 1990). The skeletal muscle cells maintain an intracellular concentration ~ 130 nM under resting state, and it elicits to μM level during excitation (Benders et al. 1997, Fig. 1.2).

The vascular smooth muscle cells and endothelial cells consist of <100 nM Ca^{2+} ions under resting state, and it increases up to 1 μM during active state (Moccia et al. 2012). Similarly, the cytosolic concentration of free Ca^{2+} ion in resting neurons is about 200 nM, but this level increases more than 100 μM upon electrical stimulation (Mark et al. 2017). However, the intracellular compartments such as endoplasmic reticulum (ER), mitochondria and lysosomes maintain different concentration of Ca^{2+} under resting as well as active state of cells. Emerging evidences reveal that

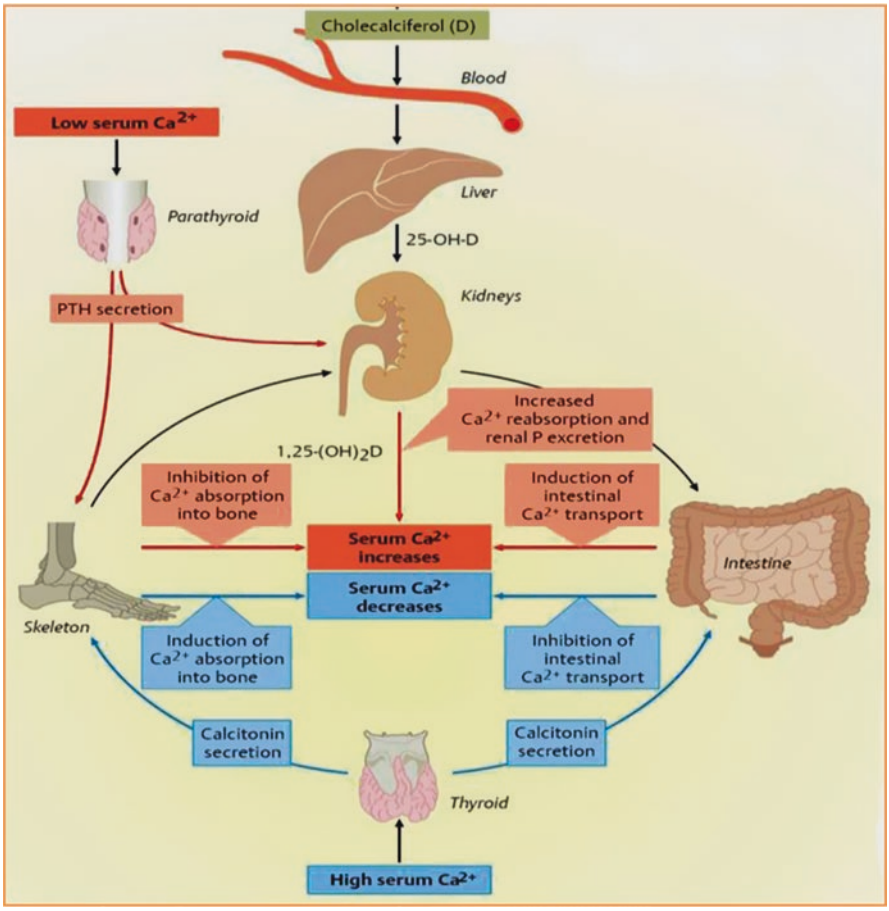

Fig. 1.2 Overview of calcium homeostasis in circulation. 25-OH-D, 25, hydroxycholecalciferol; 1,25 $(OH)_2D$, 1,25 dihydroxycholecalciferol; PTH, parathyroid hormone

they serve as a cellular and intracellular Ca^{2+} stores (La Rovere et al. 2016). Among them, ER is the primary intracellular Ca^{2+} pool that consists of Ca^{2+} level in a range between 250 μM and 600 μM, which is more than 10,000 times higher than cytosolic level (Demaurex and Frieden 2003; Raffaello et al. 2016, Fig. 1.3).

The free ionic Ca^{2+} level is highly controlled in biological tissues because a small variation in its level can cause disturbance in normal cellular and tissue functions. Normally, the cytosolic free Ca^{2+} level reaches up to 10 mM under activated/excited condition, and it drops down to normal level, when cells reach resting state (Berridge et al. 2003; Usachev et al. 1995). The intracellular organelles such as ER, mitochondria and lysosome are central players in Ca^{2+} cellular homeostasis. The coordination and interaction of these organelles by a tight network of Ca^{2+} sensors, receptors and Ca^{2+}-binding proteins are crucial in regulating Ca^{2+} dynamics (La Rovere et al. 2016). In resting cells and non-excitable cells, the cytosolic Ca^{2+} is increased, to

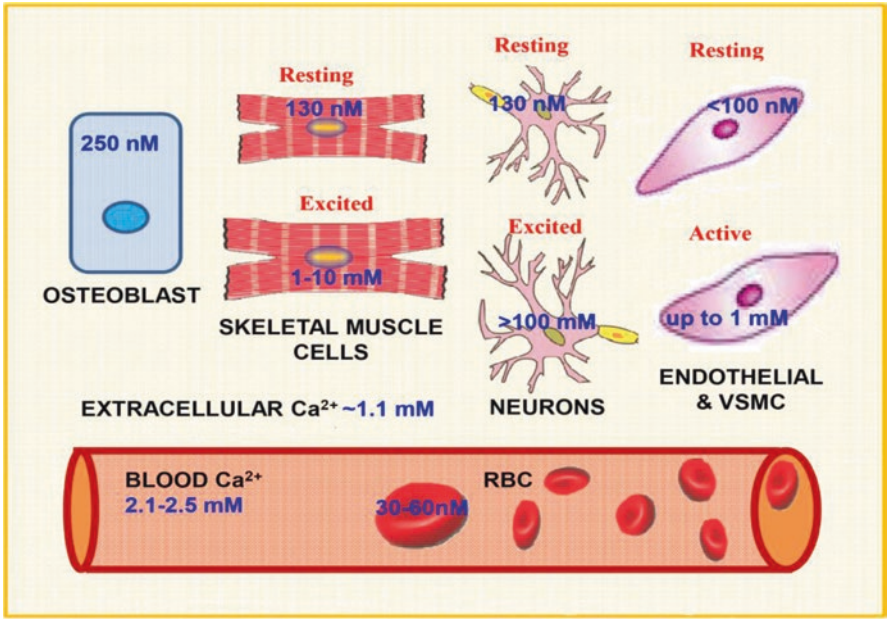

Fig. 1.3 Ca^{2+} concentration in different types of cells and circulation

perform specific cell function, by rapid release of Ca^{2+} from intracellular calcium stores (ICS) such as ER and Golgi apparatus, through activation of inositol-1,4,5-trisphosphate (IP3) receptor as well as ryanodine receptor (RyR) (Raffaello et al. 2016). However, the influx of Ca^{2+} from extracellular space by turning on different types of calcium channels depends on the stimuli and cell type, when there is a depletion of intracellular Ca store or cells under persistent excitation state. Among different types of Ca^{2+}-permeable channels that coexist in the plasma membrane, voltage-dependent or voltage-gated calcium channels (VDCC/VGCC) and store-operated Ca^{2+} channels (SOC) are predominant functional units in all types of cells. In excitable cells like muscles and nerves, VDCC dominate the influx of Ca^{2+} due to constant alterations in membrane polarization and Ca^{2+} gradients inside the cells, while receptor-operated channels (ROCs) are rapidly activated in excitable cells by binding of external ligands (e.g. neurotransmitters and hormone agonists) (Parekh and Putney 2005). In certain cell types, the second messenger-operated Ca^{2+} channels (SMOC) such as transient receptor potential channels (TRP channels) are activated mainly by cyclic nucleotides (cAMP and cGMP), IP_3 and lipid-derived messengers (diacylglycerol and arachidonic acid). In some conditions, Na^{+}-Ca^{2+} exchanger (NCX) works in a reverse way to increase Ca^{2+} entry into cells (Parekh and Putney 2005). The Ca^{2+} influx generates a net inward current and triggers the biochemical signals for a variety of cellular events such as secretion of neurotransmitter, contraction and hormone secretion (Fig. 1.4).

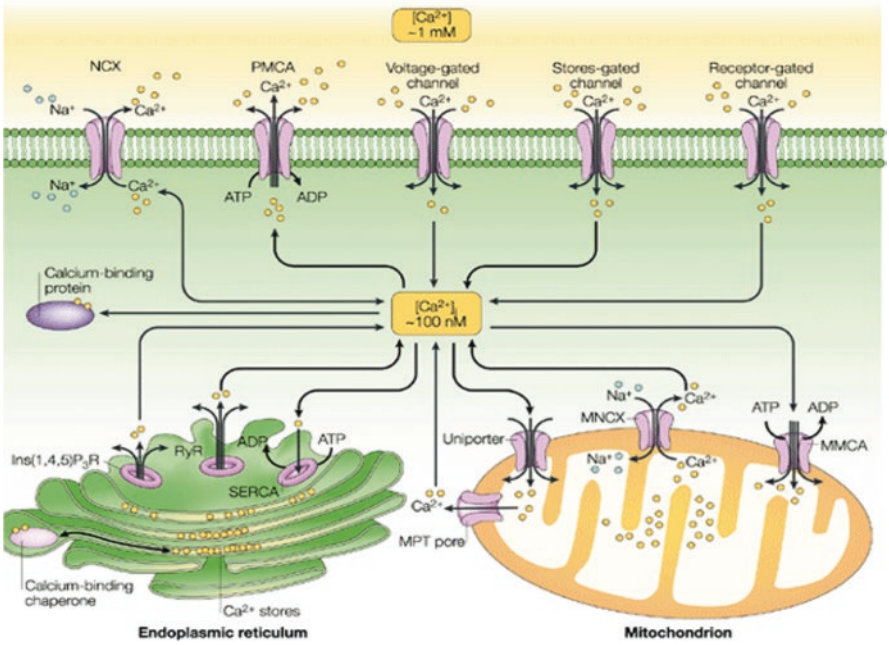

Fig. 1.4 General mechanism of intracellular Ca^{2+} homeostasis. Under normal condition, the intracellular Ca^{2+} concentration is tightly regulated within narrow limits. The Ca^{2+} calcium influx from extracellular space is carried out through various channels (voltage-, ligand-/receptor- or Ca^{2+}concentration-gated channels). NCX is the predominant Ca^{2+} efflux pathway during normal conditions. However, it can also contribute to Ca^{2+} influx (reverse mode exchange) especially during strong depolarization and increased intracellular sodium. The intracellular Ca^{2+} concentration primarily increases through release from endoplasmic reticulum stores, through the ryanodine (RyR) and inositol-1,4,5-trisphosphate receptors (Ins (1,4,5)P_3R). The cytoplasmic increase of Ca^{2+} is counterbalanced by the plasma membrane calcium pump (PMCA), NCX and sarco-endoplasmic reticulum Ca^{2+}ATPase (SERCA), which restore normal calcium levels. Increased intracellular Ca^{2+} concentration drives calcium overload at mitochondria, through activation of PMCA, and relaxed specificity channels (mitochondrial uniporters). This triggers secondary release of calcium from mitochondrial stores, through the mitochondrial NCX (MNCX) and mitochondrial pores opened during mitochondrial permeability transition (MPT). The cytoplasmic and ER Ca^{2+}-binding proteins sequester Ca^{2+} ions and provide additional Ca^{2+} buffering capacity. MMCA – mitochondrial membrane Ca^{2+}ATPase (Adapted from Syntichaki and Tavernarakis 2003)

Apart from them, SOC is a ubiquitous mechanism of Ca^{2+} entry that mainly operates to refill the intracellular Ca^{2+} stores in non-excitable cells. The depletion of Ca^{2+}in ER stimulates SOC activity by interaction of stromal interaction molecule-1 (STIM1), a Ca^{2+}-sensing protein on ER and channel-forming Orai protein on plasma membrane. This process is known as capacitative or store-operated Ca^{2+} entry (SOCE), which is regulated by filling state of intracellular sensitive Ca^{2+} compartments. This mode of Ca^{2+} entry contributes to sustained elevation of cytosolic Ca^{2+} as well as supports the maintenance of amplitude of Ca^{2+} oscillation (Raffaello et al.

2016; Smyth et al. 2010). In addition to ER, the Ca^{2+} homeostasis is couple to mitochondrial Ca^{2+} signalling. The mitochondrial Ca^{2+} uptake system has low affinity for Ca^{2+}, and it is active only at high Ca^{2+} environment. Mitochondria act as a local Ca^{2+} buffering system by its high capacity to taking up Ca^{2+} from micro domains of local environment, particularly at the sites close to the mouth of cytosolic Ca^{2+} pumps (free Ca^{2+} level about 10–20 μM) of plasma membrane and ER. The mitochondrial calcium uniporter (MCU) and mitochondrial specific RyR are involved in the Ca^{2+} uptake process that works in a distinct way by their capacity to sense the local Ca^{2+} microenvironment. In this way, mitochondria prevent the Ca^{2+}-dependent inactivation of the channels. Notably, the mitochondrial Ca^{2+} uptake is coupled to the energy metabolism (Garcia-Sancho 2014). The Ca^{2+} influx is transient in non-excitable cells, and this can be inhibited within fractions of seconds. Once the target cellular activity is accomplished, the cytosolic Ca^{2+} concentration reverts to resting level by P-type Ca^{2+} ATPase system of plasma membrane as well as intracellular stores. The cytosolic Ca^{2+} is rapidly pumped to extracellular space by plasma membrane Ca^{2+} ATPase (PMCA) and Na^{+}/Ca^{2+}-K^{+} exchangers (NCX) or to intracellular storage organelles predominately to ER/SR by ER Ca^{2+} ATPase (SERCA) system. The PMCA has high affinity to Ca^{2+} but low capacity for transport, while NCX has low affinity to calcium but possesses high capacity for transport of Ca^{2+}. The NCX pump is abundantly expressed in highly excitable tissues like the heart and brain (Berridge et al. 2003; Brini and Carafoli 2011; Raffaello et al. 2016). In addition to this, the ligand-dependent inactivation system partly contributes to the clearance of Ca^{2+} from cytosol. For a short time, the cells can survive in this raised level of Ca^{2+}. However, a persistent rise in Ca^{2+} level causes ER stress, alterations in outer membrane integrity and mitochondrial dysfunction and eventually leads to cell death. The pathological damage to cell provokes cytosolic free Ca^{2+} level up to 100 μM due to disturbance and/or imbalance in the Ca^{2+} cellular homeostasis.

1.1.4 Impact of Calcium Deficiency and Overload

The bone is the storage site for excessive Ca^{2+}. If dietary intake is insufficient, bone acts as a resource to maintain the constant level of Ca^{2+} in the circulation. The mild and transient calcium deficiency is asymptomatic. Nevertheless, a prolonged inadequate intake or malabsorption of calcium leads to many physiological abnormalities and detrimental pathological consequences in human as well as in animals. The persistent calcium deficiency/absorption disorders cause bone deformities (osteoporosis), muscle spasms, tetany and heart failure. This is mainly due to the deficiency of factors required for the Ca^{2+} absorption such as parathyroid hormone and vitamin D (Moe 2008). In pregnant women, calcium deficiency is responsible for the causes of pre-eclampsia, which is one of the major causes of maternal and foetal morbidity and mortality. The calcium supplements can prevent this problem in pregnant women (Hofmeyr et al. 2014). On the other hand, the excessive intake of calcium or hypercalcemia also leads to the development of many disorders and pathological conditions. The most common cause of hypercalcemia is hyperparathyroidism,

malignancy, chronic kidney diseases and regular usage of certain medications (thiazide diuretics, antioestrogens, etc.). There are two types of pathological calcification occurring in the human system. One is dystrophic calcification, which is due to abnormal deposition of calcium in the dead or damaged soft tissues. Another type is metastatic calcification resulting from the disturbance of whole body calcium metabolism (Irnell 1969; Moe 2008; Proudfoot et al. 2001). The major pathological calcification is the formation of stones (calculi) in the kidney and gall bladder as well as vascular calcification. The dysregulation of intracellular calcium homeostasis as well as transformation of cell phenotype during degenerative tissue damage is mainly responsible for the excessive accumulation of calcium in cells and dystrophic calcification, despite circulatory calcium level being in normal range (Kalantari et al. 2007; Leopold 2012). The severe and persistent hypercalcemia causes brain dysfunction, abnormal heart rhythm and heart failure (Moe 2008). Regardless of origin, the overload of intracellular Ca^{2+} in RBC is linked to hereditary haemolytic anaemia. The dysregulation of Ca^{2+} transport machineries causes accumulation of Ca^{2+} in RBC and leads to sickle cell diseases, thalassemia and other forms of anaemia (Bogdanova et al. 2013). Similarly, the disturbance of Ca^{2+} homeostasis in muscle cells causes various skeletal muscle diseases, which is mainly due to dysregulation of a skeletal muscle cell Ca^{2+} release channel called as ryanodine receptor type 1 (RyR1) (Hernandez-Ochoa et al. 2015).

1.1.5 Summary

The molecular physiology of calcium signalling is a complex process, and it remains largely unknown, but it is quite interesting in the aspect that it activates particular signalling cascade at micro domains in the multifaceted intracellular environment. The aim of this book is to present a precise summary of current knowledge on all aspects of calcium signalling in normal physiology, health and disease. In this book, we describe the updates on molecular and regulatory machineries associated with calcium signalling and how they are linked to the pathophysiology of different disorders. The most important part of this book is it covers the advantages of the use of natural products as an agonist/antagonist for calcium signalling-associated disorders and perspectives of using nanotechnology to process natural products in order to utilize them as therapeutics.

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Regulation of Calcium in Muscle Physiology

2

Abstract

The physiological and biochemical roles of cells are controlled by calcium ions. Calcium acts as a second messenger in signal transduction pathways, in neurotransmitter release regulation, in fertilization and in all muscle cell type contraction. Calcium can act as a cofactor for many enzymes. The bone formation and the potential difference across excitable cell membranes are regulated by extracellular calcium. An abnormal change in the intracellular Ca^{2+} concentration results in defective muscle contraction and/or relaxation, and any alteration in structural, metabolic or contractile proteins causes muscle diseases. Plasma membrane elements involved in membrane potentiation are induced by voltage and signal transduction. This potentiation leads to excitation of the cells followed by muscle contraction and relaxation. This cycle is called as excitation-contraction-relaxation cycle. Ca^{2+} flux modified by mutations of membrane proteins causes severe pathophysiological effects in muscle. In this chapter, we reveal the different types of muscle functions and involvement of calcium channel in muscle physiology and disorders.

Keywords

Calcium channels • Ca^{2+} homeostasis • Cardiac muscle • Excitation-contraction • Skeletal muscle • Smooth muscle

2.1 Introduction

The muscles help us to move and maintain the tissue grade of organization. It can be classified depending upon their work and morphology. Muscles provide strength to the body and encapsulate the skeleton – framework of higher organisms. Muscles are made of cells rich in fibres which can provide flexibility and ability to contract and relax. A good example of muscle is the heart which pumps blood to all over the

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body. Muscles are the major tissue, which has granted us locomotion, something that plants can't do. Lack of muscle function might lead to several disorders like paralysis, weakness, muscular dystrophy, etc. The following muscle disorders occurs in our body: injury or overuse, such as strains, muscle cramps and genetic disorder such as muscular dystrophy, inflammation and diseases of nerves that affect muscles; sometimes the cause is not known (Amici et al. 2017; Ozono 2017). The flexibility of muscles comes from the polymerization and self-assembly of cytoskeleton protein in cytoplasm like actin and myosin. The cytoskeletal protein and muscles fibres need small cations like calcium to mediate this process of contraction and relaxation; hence, inorganic chemistry plays a predominant role in the muscle physiology.

An intracellular Ca^{2+} is maintained by a set of membrane proteins present on endoplasmic reticulum (ER) membrane (called as sarcoplasmic reticulum [SR] in muscles) and plasma membrane and also by some cytosolic kinases like PKC. The lack of Ca^{2+} homeostasis which can be due to perturbation or functional loss of ion regulatory proteins might lead to the severe muscle disorders. Taken together, the alpha-1 DHPR, rynodine receptor type 1 and SERCA1 mutations cause malignant hyperthermia, central core disease, and hypokalaemic periodic paralysis or Brody disease. However, dysregulation of key Ca^{2+} regulatory muscle proteins cause extreme distraction in excitation-contraction coupling in few animal model studies (Goody et al. 2017).

2.2 Types of Muscles

- Smooth muscle performs mostly involuntary functions. Smooth muscle cells are uni-nucleated spindle-shaped morphology and is abundantly present on the walls of organs of respiratory and digestive systems and also present in bladder and blood vessels (Windmaier et al. 2004).
- Skeletal muscle largely performs voluntary actions and is anchored to the bones by tendons. It facilitates locomotion with the help of bones as rigid framework. The skeletal muscles consist of multinucleated cells with striated morphology due to strips (Chal et al. 2015).
- Cardiac muscles are exclusively found in the mitochondria and are rich in mitochondria. It has striated morphology but performs involuntary actions (Olaf et al. 2009, Table 2.1).

2.3 Symptoms of Calcium Deficiency

The calcium deficiency listed below:

- Muscle cramps
- Muscle pain
- Muscle twitching
- Muscle spasms

Table 2.1 Properties of different muscles

Properties	Type of muscles		
	Skeletal	Cardiac	Smooth
Control	Voluntary	Involuntary	Involuntary
Striations	Present	Present	Absent
Gap junctions	Absent	Present	Present
Nucleus	Many	Single	Single
T-tubules	Present	Present	Absent
Troponin	Present	Present	Absent
Ca^{2+} source	Release	Influx and release	Influx and release

A muscle cramp is one of the initial symptoms of calcium deficiency. This sign of deficiency of calcium occurs as the initial alarm towards the decrease of calcium in the body. While moving and walking around, if you feel muscles ache, especially those of the thighs, arms and underarms, this may be a sign of calcium deficiency. Calcium plays a pivotal function in both neurotransmission and muscle contraction. Therefore, deficiency of calcium can bring on seizures in healthy people (Katzberg et al. 2010; Sinzinger and O'Grady 2004).

2.4 Action of Calcium in Smooth Muscle Physiology

SR acts as the intracellular stores of Ca^{2+} inside the cells with limited role of mitochondria under few pathological conditions where it is found to have reversible accumulation of large amounts of calcium. The intracellular amount of Ca^{2+} changes in stage-specific manner, and the calcium concentration ranges from 80 to 200 nM, at resting stage, and is lower in phasic than in tonic smooth muscles (Somlyo and Himpens 1989). During development process of an embryo, it needs to create a large amount of smooth muscles for developing gastrointestinal system, and any lack of smooth muscles at this stage leads to fatal condition called as "smooth muscle condition". An auto-immune disorder such as hepatitis, cirrhosis or lupus has been identified by anti-smooth muscle antibodies (ASMA).

To name some of the diseases due to loss of flexibility/plasticity of smooth muscle, a good example is achalasia, where the lower oesophagus and the lower oesophageal sphincter are unable to contract or relax properly. Lack of peristaltic wave doesn't allow lower sphincter to relax, and consequently, the food bolus can't pass through the oesophagus. The molecular mechanism behind such defects is largely unknown, but there is speculation that it could be due to the loss of nerve fibres from smooth muscles (Arif et al. 2006; Roland et al. 2016, Fig. 2.1).

Rajagopal et al. (2013) shown that G protein-coupled receptor, TGR5 expression in gastric smooth muscle and its function in smooth muscle relaxation. They have studied that the TGR5 receptor coupled with $G_{\alpha}s$ subunit causes gastric muscle relaxation via inhibition of RhoA/Rho kinase pathway that leads to stimulation of

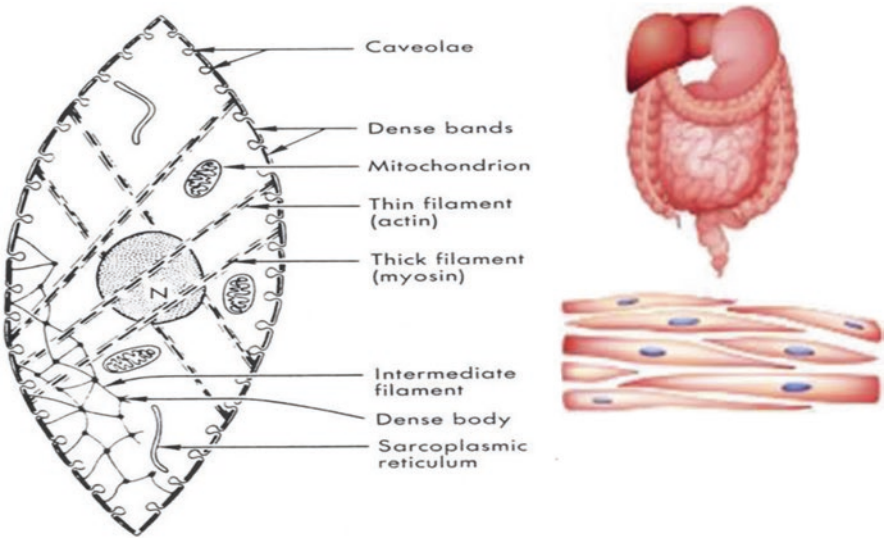

Fig. 2.1 Intracellular structure of smooth muscle

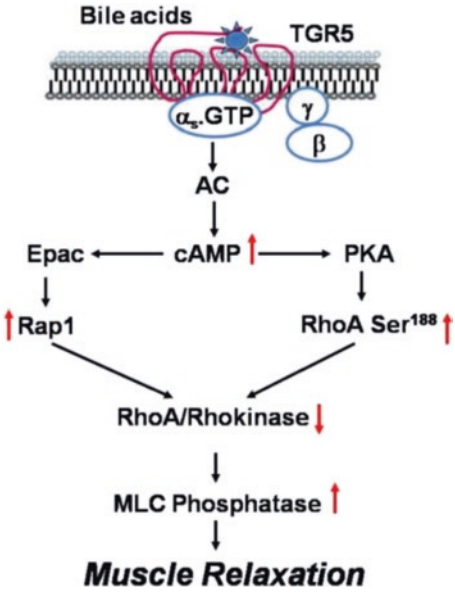

Fig. 2.2 Mechanism of TGR5-mediated muscle relaxation

myosin light-chain phosphatase (MLCP) activity and MLC20 dephosphorylation. Suppression of Rho kinase activity was mediated by both PKA-dependent phosphorylation of RhoA at Ser^{188} and PKA-independent mechanism involving stimulation of Rap1 via Epac [Fig. 2.2]. An activation of TGR5 receptor on smooth muscle showed a novel mechanism for the regulation of gut motility by bile acids through

stimulation of Ca^{2+} in both ways such as calcium influx and intracellular release of Ca^{2+} from SR (Rajagopal et al. 2013).

TGR5 receptors are GPCRs expressed in gastric smooth muscle, which involves G protein and cAMP downstream of activation. TGR5 activation inhibits contractions mediated by RhoA/Rho kinases. The mechanism of RhoA/Rho kinase inhibition is mediated by phosphorylation at Ser^{188} in PKA-dependent and PKA-independent manner and activation of Rap1. In turn, RhoA phosphorylation and Rap1 activation inhibit Rho kinase and stimulated MLCP activity which leads to muscle contraction.

2.5 Function of Calcium in Skeletal Muscle Physiology

Skeletal muscle coordinates with the skeleton made up of bones/cartilages, and these muscles are under the "voluntary" control of the somatic nervous system. Skeletal muscle is comprised of the multiple bundles of muscle fibres, and they are packed with the help of collagen-based connective tissue called fasciae, which also stabilizes muscles and few other tissues. Muscle fibres are syncytial and cylindrical in structure. The striated appearance of muscle fibres is due to the sarcomeres which are composed of actin and myosin filaments. Several sarcomeres are assembled to form myofibrils which in turn form skeletal muscles. Sarcomeres mediate the contraction relaxation process with the help of small cations like Ca^{2+} (Birbrair et al. 2013, Fig. 2.3).

The mutations in ion channels or related proteins lead to severe muscle/neurological disorders, and their studies are today called as channelopathies. RyR in skeletal muscle seems to modulate Ca^{2+} homeostasis as its mutation causes disturbances in Ca^{2+} levels. Malignant hyperthermia episodes occur due to the structural perturbation of Ca^{2+} release channel and mutations in RyR isoform that increase Ca^{2+} release in central core disease. Brody disease, a disorder of impaired muscle relaxation, correlates with abnormalities in SERCA1 (Groom et al. 2011). As discussed in the previous section, Ca^{2+} not only sets electrochemical gradient across the cell, but it also acts as the secondary messenger in signal transduction pathways. The fluctuations in the cytosolic Ca^{2+} concentration, which act as a second messenger system, cause sarcomeric contraction and relaxation which are regulated by complex multicomponent mechanisms. Several Ca^{2+} regulatory proteins/enzymes and their working molecular/structural mechanism are identified. The changes in membrane polarity and interact via its II-III loop domain with the junction of SR RyR, in transverse tubular DHPR, which in turn releases Ca^{2+} ions that trigger contraction via binding to the troponin/tropomyosin system (Al-Qusairi and Laporte 2011). Ca^{2+} homeostasis is re-established by SR Ca^{2+} ATPases of the longitudinal tubules by pumping Ca^{2+} ions back into the SR lumen and is stored with the help of Ca^{2+} binding proteins (Beard et al. 2004; Kwan et al. 1994). The direct protein-protein interactions are very important for signal transduction and that the RyR Ca^{2+} release channel is balanced by a variety of factors that are studied by a variety of techniques such as electron microscopical studies, domain binding and chemical cross-linking

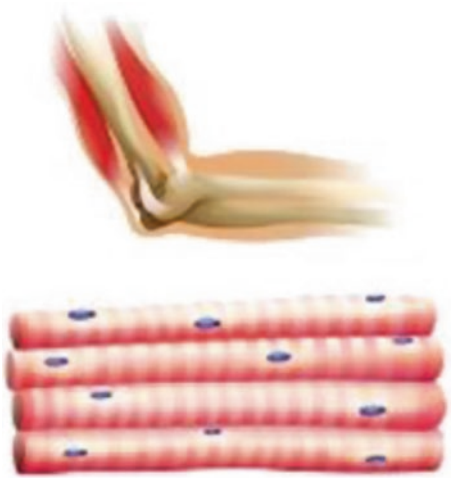

Fig. 2.3 Structure of skeletal muscle

experiments. By using these techniques and other techniques, the researchers have identified and biochemically characterized novel triad proteins, which increases the number of modulating components (Murray and Ohlendieck 1997).

The main important function of cells such as contraction-relaxation, secretion and gene regulation are controlled by voltage-dependent calcium channels (VDCC), which convert electrical signals of excitable cells into other cellular functions. These channels have more peculiar voltage-sensing domains that detect changes in membrane potentials and control channel gating. Exceptionally, skeletal muscle does not have the flow of Calcium ions and these ions regulating channel functions as second messengers in all the excitable cells. The studies have shown that the skeletal muscle contraction is not controlled by calcium currents. Ca_V 1.1 is essentially not functioning as channel but activates calcium release from intracellular stores. Recently, the researchers discovered the splice variants of Ca_V 1.1 with normal channel functions. This dicovery provided us the molecular mechanisms regulating the channel gating and led to the understanding that in skeletal muscle calcium currents. This channels allow proper regulation of fibre-type specification and prevent mitochondrial damage (Flucher and Tuluc 2016, 2017; Jeftinija et al. 2007). As discussed in previous part, skeletal muscles are in the direct control of autonomous nervous system and mostly under voluntary control. Muscle fibres contact with electrically excitable cells – motor nerve at surface and transverse tubular system of muscle fibre. The motor neuron mediated action potential spreads across the muscle fibre. Membrane depolarization due to action potential alters conformation of sensor protein receptor protein (DHP) which in turn activates RyR leading to the release of Ca^{2+} from the SR which interacts with troponin and activates the contraction process (Jurkat-Rott and Lehmann-Horn 2005).

2.5.1 Hereditary Muscle Diseases

Any perturbations in the metabolic or structural proteins might lead to the muscle diseases. Excitation-contraction cycle of sarcomeres in the muscle fibre is a complex process, and mutations in any proteins, which are part of this complex process, e.g. voltage-gated ion channels, lead to pathophysiological diseases (Marston 2011).

2.5.2 Malignant Hyperthermia

Malignant hyperthermia (MH) is an autosomally inherited dominant disorder characterized by uncontrolled hypermetabolism on exposure to volatile anaesthetics or muscle relaxants (Groom et al. 2011; Kim et al. 2011). Halothane and depolarizing muscle relaxant succinylcholine (containing the preservative 4-chloro-m-cresol) are triggering the MH. Their mechanism of action involves the increased release of Ca^{2+} ions from the SR lumen into the cytosol, thus increasing cytosolic levels of Ca^{2+} which leads to sustained muscle contracture (Naguib 2007; Sudo et al. 2008). MH is a symptomatic disease which includes muscle rigidity, hypoxia, hyperkalaemia and hyperthermia associated with acidosis. MH is treated by Ca^{2+} release inhibitors dantrolene; if not treated, it might lead to fatalities. In combination with dantrolene, anaesthesia is avoided as precaution to MH. In spite of being a metabolomic disorder, MH patients have been found to have pathophysiological discomforts in other tissues such as heart, kidney, muscles and lungs (Fruen et al. 1997; Krause et al. 2004).

Mutational hotspots on RyR1 gene in human chromosome were recognized for MH using genetic linkage analysis. Initial studies showed that mutations in RyR1 isoform of SR Ca^{2+} release channel might lead to MH (Chaube et al. 2014; Lanner et al. 2010), which is because mutated RyR1 exhibiting increased ion channel opening time and leading to transient accumulation of cytosolic Ca^{2+} during excitation. Since Ca^{2+} is ubiquitous secondary messenger, it might trigger other processes like glycogenolysis and ultimately perturbed Ca^{2+} homeostasis with muscle cell damage as consequence (Bellinger et al. 2008; Gehlert et al. 2015). Following these, several other MH-related mutations have been found in chromosome 19q13.1, encoding for the Ca^{2+} release channel. There are several mutational hotspots in RyR1 domain, which include 12 such sites at centre receptor domain (Region-2) and 9 at extreme N-terminal region (Region-1) and 1 at extreme C-terminal region. Mutations in RyR only contribute in the 50% of the MH cases. There are several additional mutation sites which contribute to MH, and these mutations occur in the alpha-1 and the alpha-2/delta subunits of the DHPR (Bellinger et al. 2008; Lanner et al. 2010; Nilius and Owsianik 2011). Interestingly, these mutations are linked to SR RyR as the alpha-1 subunit directly interacts with RyR during excitation-contraction activities in muscles. So, DHPR mutants might delay RyR closure leading to increase in intracellular levels of Ca^{2+} (Lanner et al. 2010). Mutations in Ca^{2+} homeostatic proteins other than RyR1 and DHPR might also contribute to the MH, but currently they

need investigation. One can only conclude that MH is a channelopathy related to contraction-relaxation coupling in muscle fibre (Ullrich et al. 2011).

MH is also reported other than humans, like pigs, and it is called porcine stress syndrome (PSS) (Oliveira Band et al. 2005). It is characterized by tachycardia, fever and muscle rigidity and might be due to physical/emotional stress like transportation, fear and overheating. Molecular studies show that pigs suffering from MH bear point mutation (homozygous) in the RyR1 gene (Droval et al. 2012). These RyR1 mutants need to be homozygous to cause any PSS-like symptom but in pigs heterozygous for this mutation; external substances or environmental factors might trigger PSS-like symptoms. (Rosenberg et al. 2015).These findings emphasize on the suitability of pig as a model system to study MH/PSS. Other than pig, studies have been done on rabbit, and there are evidences which suggest that halothane might trigger abnormal Ca^{2+} homeostasis leading to MH; mechanistically, it could be halothane oligomerisation of mutated RyR. Other than RyR, halothane is also found to induce aggregation of other proteins like Ca^{2+} binding proteins CSQ and DHPR-1(Nelson 1990; Rosenberg et al. 2015). Another possible mechanism includes Ca^{2+} release modulation by direct interaction between halothane and voltage sensor RyR, which halothane-induced RyR oligomerisation is isoform specific and it doesn't induce any aggregation of RyR-2 isoform found in cardiac muscles (Capes et al. 2011; Perez et al. 2003). So MH can be triggered by external substances like halothane in isoform-specific manner (Hernández-Muñoz et al. 2000; Stutzmann and Mattson 2011).

For patients without a family history of MH, in vitro contracture test is done to know if the ion channel modulatory drugs are safe for them. This test is done on the biopsy samples and relies on the abnormal behaviour to drugs like halothane, caffeine, etc. (Sudo et al. 2008). This test has a problem of being false positive even for the diseases closely related to MH-like central core disease (Jungbluth 2007), King-Denborough syndrome (Dowling et al. 2011) and Evans myopathy. The underlying molecular mechanism is different for these pathophysiological conditions, but MH-like symptoms are also seen in Duchenne muscular dystrophy (Tayeb 2010), myotonia fluctuans (Hahn and Salajegheh 2016), myotonia congenita (Novak et al. 2015) and other myopathies (Cannon 2015).

2.5.3 Central Core Disease

Central core disease (CCD) is closely related to MH and shares some symptoms as well; hence, it would be good to differentiate both diseases at molecular level. CCD is also a channel-related disorder caused by the mutations related to Ca^{2+} release proteins in skeletal muscles, e.g. mutations in RyR (Avilaa and Dirksena 2001; Kraeva et al. 2013). This disease is characterized by hypotonia, delayed motor development and muscle weakness, and it is a nonprogressive disorder, but muscle weakness can be improved with exercise with age onset. Hypotonia is early symptom of CCD in child. Muscles suffering from CCD have distorted myofibrils which less mitochondria (Keeton 1976). Due to loss of mitochondria, there is a lack of

oxidative enzymes muscle cells (Gehlert et al. 2015) and structural disintegration of the sarcomere which is employed in the diagnosis of CCD. Increased cytosolic Ca^{2+} due to mutant RyR might be the cause of mitochondrial damage, which in turn leads to reduced metabolic activity and disorganization of sarcomere. The high concentration of cytosolic Ca^{2+} is removed by SERCA-type Ca^{2+} pumps and the Na^{+}/Ca^{2+} exchanger. The intracellular Ca^{2+} level is elevated by the SERCA-type Ca^{2+} pumps and the work load for surface Na^{+}/Ca^{2+} exchanger is more, if the RyR is defective. Hence, mitochondria might participate in Ca^{2+} removal from cytoplasm to prevent Ca^{2+} induced necrosis and in this process mitochondria is damaged due to increased Ca^{2+} levels (Bers 2002; Borowiec et al. 2014; Ferdek et al. 2017). Damaged mitochondria also reduce ATP production; hence, it might be the reason for muscle weakness in the CCD (MacLennan and Zvaritch 2011; Paolini et al. 2015).

2.5.4 Hypokalaemic Periodic Paralysis

Hypokalaemic periodic paralysis (HypoPP) is a progressive autosomal dominant genetic disorder characterized by muscle weakness and increased K^{+} levels after carbohydrate rich diet or rigorous physical activity (Finsterer 2008; Kim and Kim 2007; Kim et al. 2010). It mostly affects proximal lower extremities and upper parts of the body like respiratory system, but cardiac muscles are unaffected. It can be treated with oral or intravenous K^{+} repletion and administration of K^{+} supplements, and carbonic anhydrase inhibitors are used as prophylaxis (Tricarico et al. 2004). It can be controlled by avoiding triggering agents.

Alpha-1 subunit of the DHPR mutation causes HypoPP, which senses voltage in skeletal muscles (Piétri-Rouxel et al. 2010; Samsó 2015). The mutations relevant to HypoPP appear in highly conserved S4 regions of the repeats II and IV of the Ca^{2+} channel which might alter its interactions with the RyR and thus interfere with excitation-contraction coupling (Dayala et al. 2013). Primary culture of myotubes from HypoPP exhibits reduction in dihydropyridine [DHP]-sensitive Ca^{2+} currents (Morrill et al. 1998; Yawo and Akiko 1993) and slower activation of the DHPR Ca^{2+} channel (Gehlert et al. 2015). Increased cytosolic Ca^{2+} levels might perturb functioning of sarcolemmal and t-tubular Na^{+}-channels leading to reduced K^{+} fluxes and hypokalaemia (McCallum et al. 2015).

2.5.5 Brody Disease

The painless muscle cramps and impairment of muscle relaxation is the main properties of Brody disease. SR vesicles isolated from patients showed reduced Ca^{2+} ATPase activity and Ca^{2+} uptake. (Desmond et al. 2015; Lax et al. 2002). In contrast to reduced Ca^{2+} ATPase activity up to 50%, there is no significant change in the number of Ca^{2+} pumps (García-Martín and Gutiérrez-Merino 1996). The symptomatic muscle stiffness and cramps can be explained by slow restoration of normal

cytosolic Ca^{2+} concentration and muscle relaxation. It can be treated by the administration of drugs which can reduce cytosolic Ca^{2+} levels like dantrolene. Dantrolene blocks RyR hence inhibits Ca^{2+} release from ER lumen (Stutzmann and Mattson 2011). Karpati et al. (1986) reported that fast-twitch muscle fibres are the major muscles affected and which could be due to the SERCA1 dysfunction. Later, it was confirmed by Odermatt et al. (1996) who have found mutations in ATP2A1 gene in patients of Brody disease. Zhang et al. (1995) found that few patients of Broody disease doesn't have perturbed SERCA1; hence, Brody disease might be genetically heterogeneous disease and might include regulators of SERCA pump units (Periasamy and Kalyanasundaram 2007).

2.6 Cardiac Muscle Physiology

Similar to skeletal muscles, major physiological functions of cardiac muscles are also regulated by calcium. The stimuli for contraction of cardiac muscles starts at the sinoatrial node (SA node) consisting of cells called node cells. Node cells begin depolarization by influx of Na^{+} ions, and once it reaches −40 mV threshold, fastening Ca^{2+} channels open and lead to the influx of Ca^{2+} in node cells. Due to this calcium, SA node fires action potential and stimulates the other cardiac muscles to beat (Germani et al. 2007; Göktepe et al. 2010; Openstax 2013, Fig. 2.4).

2.6.1 Regulation of Calcium Channels in Cardiac Muscle Function

Cardiac muscle contraction and initiation of its excitation is regulated by Ca^{2+} ions. The intracellular cyclic nucleotide levels can activate slow channels which depend on metabolic energy and are selectively blocked acidosis. Slow channels have a special property which allows it to be modulated by several intrinsic and extrinsic factors. Slow cardiac Ca^{2+} channels are also regulated by cAMP; hence, agents affecting cAMP levels might affect cardiac physiology. During excitation, cAMP elevation increases probability of a slow channel opening and the mean open time of the channel, thus potentiating Ca^{2+} influx and contraction. cGMP acts in reverse of cAMP on slow Ca^{2+} channels. Other than cAMP and cGMP, several other associated regulatory proteins modulate the slow Ca^{2+} channels. GPCRs might directly regulate these channels (Anne-Marie et al. 1994; Katz 1996; Sperelakis 1990; Wang et al. 2004).

2.6.2 Cardiac Muscle's Disease

RyR has three multiple isoforms: RyR1, RyR2 and RyR3. RyR2 is mainly expressed in cardiac muscle (myocardium). RyR arbitrates the release of calcium ions from the SR and endoplasmic reticulum (ER) which is an essential step in muscle

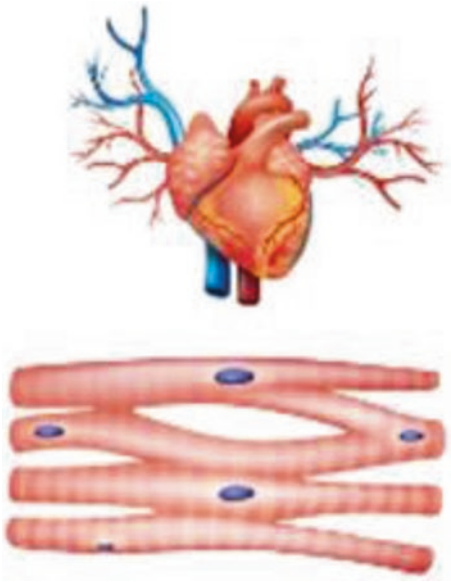

Fig. 2.4 Structure of cardiac muscle

contraction. In cardiac muscle, calcium-induced calcium release is a primary mechanism of activation, which causes calcium outflow from the SR. The cardiac-specific isoform RyR2 forms quaternary complex with luminal calsequestrin, junction and triadin. Calsequestrin has multiple low-affinity Ca^{2+} binding sites to facilitate their easy release. RyR2 mutations play a role in stress-induced polymorphic ventricular tachycardia and arrhythmogenic right ventricular dysplasia (ARVD) (Fabiato 1983; Santulli and Andrew 2015; Zucchi and Ronca-Testoni 1997).

2.6.2.1 Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

The mutations in calcium channels or the associated proteins in SR of cardiac muscles cause a muscle disorder known as catecholaminergic polymorphic ventricular tachycardia (CPVT). The natural release of catecholamine from nerve endings on the cardiac muscle and from the adrenal glands into the circulation leads to reduced electrical stability of cardiomyocytes that may cause the heart to enter a life-threatening state of ventricular arrhythmia. This rhythm disturbance prevents the heart from pumping blood appropriately. The beta blockers and verapamil may use to treat CPVT, and flecainide inhibits the release of the cardiac ryanodine receptor-mediated Ca^{2+} and is to medicate the underlying molecular cause of CPVT in humans (Priori and Chen 2011; Priori and Lui 2008; Sumitomo et al. 2003; Watanabe et al. 2009).

2.6.2.2 Arrhythmogenic Right Ventricular Dysplasia (ARVD)

Ventricular arrhythmias occur due to abnormal function and structure of the right ventricle (RV), and it is an inherited cardiomyopathy. ARVD remains a major cause of ventricular arrhythmias in children and young adults. It is predominant in males

and familial distribution. Beta blockers such as sotalol and a class III antiarrhythmic agent are used for ARVD (Corrado and Fontaine 2000; Jain 2010; Sen-Chowdhry et al. 2007).

2.7 Summary

Calcium consumption can influence the function of all types of muscles. This chapter discusses the present knowledge of the molecular interaction between cellular and molecular aspects of calcium and its influence on muscle's physiology. In addition, this chapter adds to the growing body of evidence supporting a key role for calcium ion as a regulator of muscle physiology and muscle's diseases. We should learn, in the coming years, in more detail about the molecular mechanisms of calcium ion and intracellular cascades of muscle physiology.

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Regulatory Action of Calcium in Pain Pathway

3

Abstract

The expression and functions of variety of voltage- and ligand-gated ion channels, including sodium, calcium and TRP channels, are altered during pain processing. These receptors involve pain sensitization and processing in response to painful stimuli. The increase of $[Ca^{2+}]_i$ contributes to the development of chronic pain by increasing neural activity, which is relayed to the central nervous system and leads to increased pain perception. The inflammation mediators such as bradykinin, serotonin, substance P and prostaglandin E2 stimulates pain pathway by increasing Ca^{2+} influx through Ca_v activation. These channels regulate the membrane potential and excitability of neural cells in addition to their role in cell-signalling pathways. In neurons, they involve activation of calcium-dependent enzymes, release of neurotransmitters, changes in plasticity and gene transcription. This chapter provides a broad overview of the contribution of different calcium-permeable ion channels in the pain pathway.

Keywords

Central nervous system • Ion channels • Pain • Store-operated calcium channels • Voltage-gated calcium channels

3.1 Introduction

Most of the ion channels have the capability to transport Ca^{2+} across the membrane. However, these channels perform unique role in normal cellular events and physiological functions of tissues. Ca^{2+} and voltage-dependent calcium channels (VDCCs) are one of the major factors involved in the regulation of pain processes. Many pain stimuli accelerate pain processes by increasing expression and activity of calcium channels and influx of calcium ion ($[Ca^{2+}]_i$). In neurons, calcium channels act as integrators of G protein-mediated signalling. The phosphorylation of Ca_v

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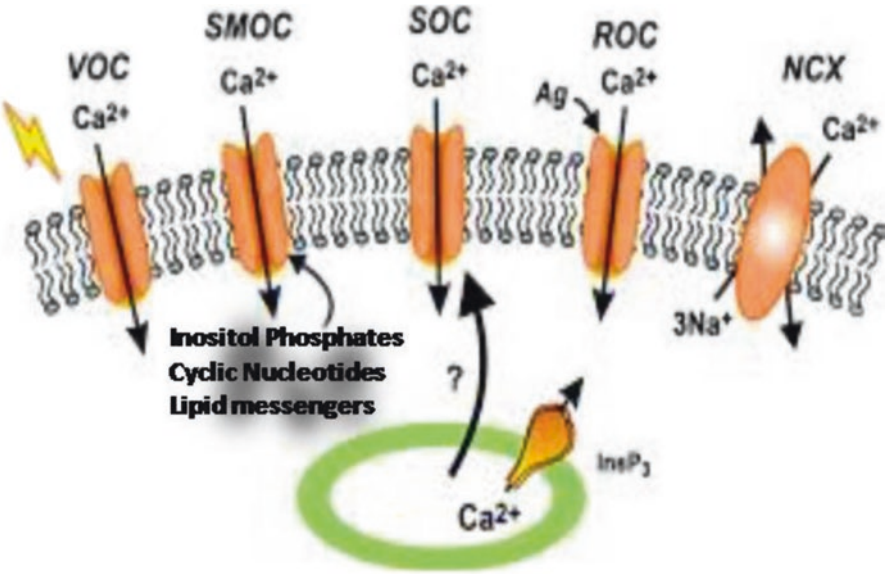

Fig. 3.1 Modes of regulated calcium entry across the plasma membrane

channels modulates its activity. The protein kinases such as PKA, PKC and tyrosine kinases phosphorylate Ca_v channels, and these post-translational modifications either increase or decrease their activities. Under pathological conditions, the alterations in Ca_v channel expression level and/or biophysical properties cause malfunctions of VDCC-mediated activities and result in disease states. Numerous studies indicate that there is a casual relationship between alterations in VDCC properties and the development of chronic pain, including nerve injury-induced pain or neuropathic pain. In preclinical and clinical research, several VDCC blockers or modulators have been identified as effective therapeutic agents for the management of chronic pain, and some of them of are approved by US Food and Drug Administration for clinical use. However, their efficacy and long-term use in pain relief is limited in some patients due to their side-effect profiles. However, these setbacks can be improved by developing more specific blockers or modulators of subtypes of VDCC (Danielle and Luo 2009; Park and Luo 2010; Rajagopal et al. 2015).

The differential sensitivity of subtypes of calcium channel is mainly due to the fact that α1 subunits are encoded by multiple genes and variants are derived by alternative splicing, and this subunit co-assembles with a variety of ancillary subunits of calcium channels. This variation in their structure is responsible for the highly specialized role in the neuronal subtypes, particularly at subcellular loci. As the calcium channels are critically involved in the brain function, the changes in their expression or functions give rise to a variety of neurological disorders, including pain, epilepsy, migraine and ataxia (Park and Luo 2010; Simms and Zamponi 2014, Fig. 3.1).

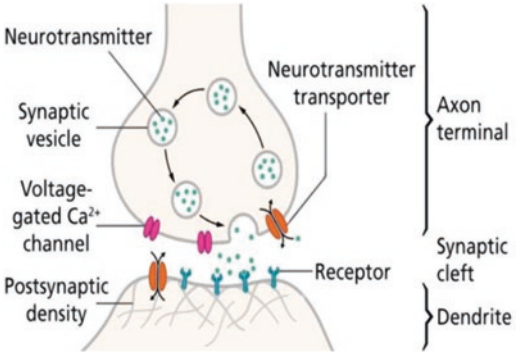

Fig. 3.2 Schematic representations of the major elements in a prototypical synapse

Ca_v 2.2 channels have crucial role in pain transduction. The PKC-dependent phosphorylation of Ca_v 2.2 channels at Ser-1757 in response to phorbol-12-myristate, 13-acetate (PMA)-induced Ca_v current could have a significant role in neural cells. A splice variant of calcium channel α subunit (α1 2.2) found in superior cervical ganglion possesses Ser-1757 in exon 37b. In dorsal root ganglia, Ser-1757 is replaced by Ala in the exon 37a of α1 2.2 subunit. This splice variant is more specific to dorsal root ganglia and preferentially presents in neurons that contain nociceptive markers, vanilloid receptor 1 and voltage-gated sodium (Na_v 1.8) channels. The cytoplasmic domain of α1 subunits is essential for recognition and activation of second messengers and enzymes that modulates the activity and functions. Despite there is a variation in their structure, all these three classes of Ca_v contribute to the pathophysiology of nociception. The differential localization of two α1 2.2 subunit splice variants and the modulation of one of them by PKC phosphorylation are closely associated with the regulation of the signal transduction for pain. The regulation of active sites in α1 subunit is more complex. The availability or phosphorylation of one site may determine the availability or activity of another. The activation could be a well-organized process, which might be carried out by sequential phosphorylation events (Rajagopal et al. 2009).

The volatile anaesthetics (VAs) impose anaesthetic state by blocking of synaptic transmission by suppressing excitation or enhancing inhibition or the combination of these two modes (Richards 1998). The intracellular Ca^{2+} level has a vital role in synaptic transmission by promoting fusion of neurotransmitter-loaded vesicle with membrane and its release (Fig. 3.2) (Catterall 2010; Dolphin 2009). The influx and efflux of Ca^{2+} from cell is controlled by VDCC whose activity is partly mediated by PKC isozymes (Rajagopal et al. 2008, 2009). VAs alters the activity of both Ca_v and PKC. Isoflurane is the most commonly used anaesthesia that induces anaesthetic state by presynaptic depression of neurotransmitter release (Larsen et al. 1994; Liachenko et al. 1999; Mantz et al. 1994). Isoflurane and other volatile anaesthetics can inhibit Ca_v channels in hippocampal neurons (Study 1994) as well as in *Xenopus* oocytes expressing those Ca_v channels (Kamatchi et al. 1999).

Synapses allow nerve cells to communication with one another through axons and dendrites converting electrical impulses into chemical signals.

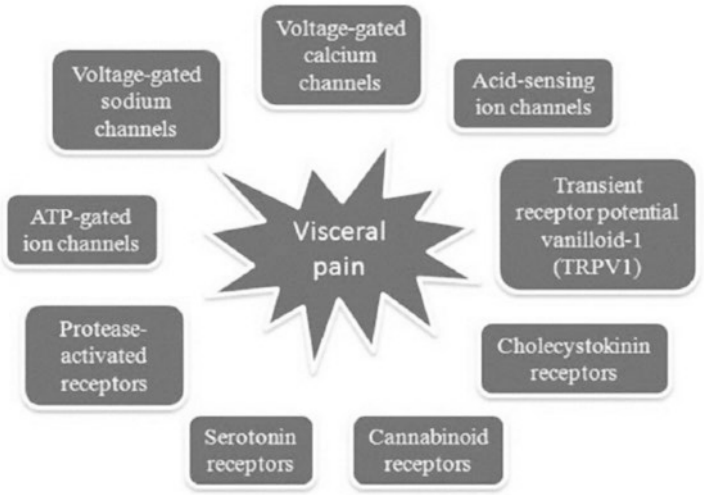

Fig. 3.3 Main channels and receptors involved in pain

3.2 Role of Ion Channels in Pain

Many types of voltage- and ligand-gated ion channels involve the generation and propagation of neuronal action potentials. Most of these channels are ubiquitously expressed in different types of neurons (Bourinet et al. 2014; Vacher et al. 2008). Certain ion channels, however, appear to be localized to neurons conveying nociceptive information. Such channels are potential therapeutic targets; selective blockade could produce analgesia (Di Marzo et al. 2002; Djouhri and Lawson 2004). Many numbers of ion channels and receptors also contribute pain signalling pathways (Fig. 3.3).

3.3 Regulation of Store-Operated Calcium Channels (SOCCs) in Pain

The store-operated calcium channels (SOCCs) are more selective gateway of Ca^{2+} influx found in the plasma membrane. The mechanisms of activation of SOCC and the role of this process in cellular function during normal physiological and disease states are not yet well defined. However, SOCCs and its components play a fundamental role in influx of Ca^{2+} and Ca^{2+}-dependent processes in non-excitable cells. The abnormal activity of this channel is implicated in a wide range of disorders including allergies, multiple sclerosis, cancer and inflammatory bowel disease (IBD). Apart from this, the expression of SOCCs in the central nervous system (CNS) contributes to normal neuronal functions as well as pathological conditions, including chronic pain (Prakriya and Lewis 2015).

3.3.1 The Possible Mechanisms of SOCCs in the Pain Modulation

The alteration in store-operated calcium entry (SOCE) is associated with the development of CNS disorders. However, its role in the pain process is not well recognized. In a condition such as spared nerve injury (SNI), the activity of SOCCs is highly increased in DRG neurons (Gemes et al. 2011). Substance P is an endogenous neurotransmitter that signals through the neurokinin 1(NK1) receptor, a $G_{\alpha q/11}$-protein-coupled receptor, to produce nociceptor sensitization and pain. Substance P induced a transient calcium response in the absence of Ca^{2+}; however, addition of 2 mM Ca^{2+} caused robust calcium influx (Xia et al. 2014).

The inflammatory mediators, such as TNF-α, IL-1β and PGE-2, contribute to the generation of pain and peripheral pain sensitization (Parada et al. 2003; Woolf et al. 1997). The sensitization of CNS is responsible for the persistent pain. The activation of mitogen-activated protein kinases (MAPKs) and calcium-/calmodulin-dependent protein kinase II (CaMKII) pathways contribute to the central sensitization (Ji et al. 2009). SOCCs and their respective components have also been well studied in immune and other non-excitable cells. Recent studies reveal that SOCE has a crucial role in CNS disorders (Ji et al. 2009; Xia et al. 2014); the study of SOCCs in chronic pain conditions remains a new and emerging field. Inhibition of SOCE can attenuate or completely abolish pain hypersensitivity, suggesting that SOCE plays a role in chronic pain. While much remains elusive regarding the role of SOCCs in the nervous system, current evidence encourages the possibility that SOCCs may be used as potential drug targets for neurological diseases including pain. The identification of the individual SOCC components provides new and more specific tools to allow us to explore the different key players in chronic pain conditions.

3.4 Involvement of Voltage-Dependent Calcium Channels in Pain Regulation

VDCCs are differentially distributed at the neuronal level. P/Q-type VDCCs are predominantly found at presynaptic locations (Christianson et al. 2009, 2010), while N-type are found both in pre- and postsynaptic neurons (Choi et al. 2007), and T- and L-type channels mainly exist on the proximal dendrites and soma of neurons (Manuel et al. 2014). Synaptic release of neurotransmitters depends on the influx of calcium ions through VDCC. P/Q-type VDCCs are the most prevalent exocytotic calcium channel expressed in the CNS that control the release of excitatory amino acids, monoamines and peptide neurotransmitters (Simms and Zamponi 2014). In experimental studies, the excitatory glutaminergic neurotransmission is not completely prevented by blockade of N-type channels, which reveals that several types of VDCCs are required for the release of neurotransmitters (Kiyonaka et al. 2007; Urbano et al. 2002). Thus, the antagonism of one type of calcium channel may therefore not significantly affect sensory signalling, especially if the presynaptic neuron is strongly depolarized. L-type channels do not appear to have a very

significant role in neurotransmitter release in the CNS. Nifedipine is a calcium channel blocker that inhibits the release of substance P from dorsal root ganglion cells when they are depolarized with KCl but not by electrical stimulation (Holz et al. 1988). L- and N-type channels may have a more important role regulating neuronal membrane properties and synaptic integration. Calcium-channel conductance is also subject to the modulatory effects of various neurotransmitters and peptides, which can further affect a neuron's response characteristics at any given voltage (Myongkeun et al. 2012; Tritsch and Sabatini 2012).

It is unsurprising that VDCCs may be targets for compounds with potential analgesic properties. Gabapentin is a commonly used drug for the treatment of neuropathic pain and is also used as a prophylactic treatment for migraine. This calcium-channel modulator inhibits high-threshold VDCCs in dorsal root ganglion neurons (Sutton et al. 2002), and it could influence excitatory and inhibitory neurotransmitter release in the spinal dorsal horn (Fink et al. 2000). The N-type channel blocker ziconotide has also shown promise in clinical trials as a treatment for postoperative and cancer pain (McGivern 2007).

3.4.1 L-Type VDCCs' Regulation in Pain Pathway

L-type VDCCs' blockers have demonstrated analgesic properties against nociceptive stimuli (Kato et al. 2002; Zamponi et al. 2009). Behavioural studies on mice, in which the gene encoding the $Ca_V1.3$ (α_{1D}) subunit has been ablated, do not appear to support a role for L-type channels in acute thermal nociceptive signalling (Yamasaki et al. 2012). Visceral pain transmission may rely in part on L-type channels. Several studies show an analgesic effect for selective antagonists (such as verapamil and diltiazem) (Striggow and Bohnensack 1993; Yousef et al. 2005); however, this has not been universally demonstrated (Striggow and Bohnensack 1993). L-type channels have also been implicated in inflammatory pain signalling, at least in models that use chemical irritation of peripheral nerves and joints as a nociceptive stimulus. In these models, two behavioural phases are noted, an early and a late phase, the latter of which correlates with the onset of central sensitization. Although L-type VDCCs have a doubtful role in the early phase, they do have a modest effect reducing behavioural responses associated with the late inflammatory phase (Danelli et al. 2017; Hunskaar and Hole 1987).

3.4.2 Action of N-Type VDCCs in Pain Pathway

Blockers of N-type channels have demonstrated analgesic properties against acute mechanical and thermal nociceptive stimulation in several animal studies (Patel and Dickenson 2016; Snutch 2005), although again these results have not been replicated in all experiments. Preliminary clinical data suggest that N-type channels have important role in human pain pathways. The selective blocking agent ziconotide has analgesic properties when it is intrathecally administered (McGivern 2007);

however, side effects in the initial titration period remain a serious problem. N-type channels also have an important function in neuropathic and chronic inflammatory pain. Upregulation of the Ca_v 2.2 subunit is correlated with pain behaviour following neural or chemical injury (Basbaum et al. 2009; Costigan et al. 2009). The delayed response to inflammatory agents is attenuated by treatment with N-type blockers, and they are still effective at reducing nociceptive behaviour, even if central sensitization has become established. The advent of genetically modified mice lacking functional N-type channels confirms their importance in the development of chronic pain states (Adams and Berecki 2013; Campbell and Meyer 2006; Costigan et al. 2009). Studies on the behavioural responses elicited by acute noxious thermal and mechanical stimulation, however, have yielded conflicting results (Dubin and Patapoutian 2010; Smith-Edwards et al. 2016). In general, these mice do not manifest significantly altered behavioural responses, which questions their role in the transmission of acute pain (Campbell and Meyer 2006; Price et al. 2014).

In experimental animals, the formaldehyde solution (formalin) is commonly used to induce acute pain. It cross-links protein amine groups and generally causes cytotoxicity (Rajagopal et al. 2010). However, the mechanism of pain induction by formalin and its long-term effects on neuronal cells remain largely unknown. By utilizing *Xenopus* oocyte expression system, it is possible to more specifically identify the interaction between Ca_v channels and some of the constituents of the intracellular signalling pathway such as G proteins, PLC and PKC. A study by Rajagopal et al. (2010) found that increased activity of PKC is involved in formalin-induced increase in Ca_v currents. This finding correlates with observations of in vivo pain investigations in which Ca_v currents and PKC activation is increased. Thus, oocyte exposure to formalin could serve as an in vitro model for some cellular mechanisms of pain.

3.4.3 Regulatory Action of P/Q-Type VDCCs in Pain Pathway

As the P/Q-type channels are involved in both excitatory and inhibitory synaptic neurotransmission (Lei and McBain 2003; Voglis and Tavernarakis 2006), it is not surprising that P/Q-type channel blockers are reported to have inhibitory, facilitatory or even no effects on the responses of spinal neurons to nociceptive stimulation (Lee and Jones 2002; Nimmrich and Gross 2012; Park and Luo 2010). It is probably simplistic to think that P/Q-type channels are only involved in excitatory mechanisms of pain transmission. Behavioural studies using natural mutant "leaner" mice confirm that mutations of P/Q-type channels may modulate noxious sensory information in complex ways. Although analgesic behaviour is demonstrated following mechanical testing, hyperalgesic responses are observed after noxious thermal stimulation (Baliki et al. 2005). It is also becoming apparent that P/Q-type channels have important actions in GABAergic inhibitory circuits. When mutant familial hemiplegic migraine type 1 (FHM1) P/Q-type channels are expressed in inhibitory interneuron, it has been reported that they are less able to sustain GABAergic synaptic currents (Xie and Manis 2014). Application of ω-agatoxin GIVa to the brainstem leads to an increase in spontaneous firing of medullary dorsal horn neurons

(while also inhibiting the responses to noxious stimulation of the dura mater), possibly because of an action on GABAergic interneuron. A similar disinhibitory action may also be observed following microinjection of P/Q-type blockers into the periaqueductal grey (PAG) (Knight et al. 2002). P/Q-type channels may therefore have a role in both inhibitory and excitatory neurotransmission, influencing the gating of sensory information at multiple levels in the nervous system. P/Q-type channels also contribute to the perception of inflammatory pain. Primary and secondary hyperalgesia resulting from chronic inflammation is prevented by pretreatment with P/Q-type blockers (Bourinet et al. 2014; Mager et al. 2001; Stein et al. 2009), which suggests that P/Q-type channels may have an important role in the development of central sensitization.

3.4.4 Modulatory Effect of R-Type VDCCs in Pain Pathway

Until recently, the studies about the role of R-type VDCCs was limited by a lack of specific blocking agents. Attempts have been made to circumvent this limitation by generating $Ca_V2.3$ (α_{1E}) knockout mice. Both homozygous Ca_V 2.3-null and heterozygous mice exhibit normal responses to acute noxious thermal, mechanical and chemical stimuli, homozygous mutant mice demonstrate reduced behavioural responses to somatic inflammatory pain. Heterozygotes but not $Ca_V2.3$-null mice also appear to have impaired responses following nociceptive stimulation of the viscera (Cregg et al. 2010; Dubin and Patapoutian 2010). Recently, it has been suggested that the peptide SNX-482 may act as a relatively selective R-type blocker (Matthews et al. 2007). Intrathecal administration of SNX-482 appears to have complex actions on nociceptive behaviour. In the formalin test, the late-phase response is attenuated in a dose-dependent manner, but the early phase is either unaffected or even potentiated (Matthews et al. 2007). At present, it appears that R-type channels have a critical role in the development of somatic inflammatory pain and possibly also visceral pain, but it is not clear what—if any—role they play in the transmission of acute nociceptive information.

3.4.5 Role of T-Type VDCCs in Pain

The inhibition of T-type channels with ethosuximide reduced spinal dorsal horn neuronal firing in response to electrical, mechanical and thermal stimulation in a dose-dependent manner in a model of neuropathic pain (Lee 2013). Such hyperalgesic behaviour may be mediated in part by a synergistic interaction between T-type VDCCs and neurokinin 1 receptor activation in lamina I neurons (Drdla and Sandkühler 2008). In addition to mechanically induce neuropathic pain (Cortright et al. 2007), T-type blockers also appear to be effective in combating nociceptive behaviour resulting from chemotherapy-induced neuropathy (Park 2014). Further evidence supporting a role for these channels in nociceptive transmission is provided by the antisense targeting of $Ca_V3.2$ mRNAs. This results in a significant

reduction of T-type channel currents with a concomitant antinociceptive effect in models of both acute and chronic somatic pain (Todorovic and Jevtovic-Todorovic 2011). T-type currents also have a central function modulating thalamic neuronal firing. The transition from tonic to burst mode may have important sensory-gating properties, regulating the flow of visceral nociceptive information (Theile and Cummins 2011).

3.5 Summary

Given the central contribution that VDCCs make towards action potential generation and synaptic neurotransmission, it is hardly surprising that VDCCs have a critical role in pain signalling. Blockade of individual channels may not necessarily prevent transmission of nociceptive information, but certain channels appear to serve a more integral part in pain transmission than others. All VDCCs appear to have complex actions, and in some cases, this may involve modulating activity in GABAergic interneurons as well as descending inhibitory circuits. Whether this can be translated into further successful therapeutic interventions remains to be seen.

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Calcium Signalling in Neurological Disorders

4

Abstract

Neurotransmitters are broadly classified as excitatory neurotransmitters and inhibitory neurotransmitters based on their function. Ca^{2+} has a fundamental role in the neuronal physiology and brain function by governing the synthesis and secretion of neurotransmitters. The intracellular Ca^{2+} concentration is not only important for the release of neurotransmitters but also essential for the regulation of their action potential in postsynaptic membranes. Ca^{2+} signalling has been implicated in almost all neural activities including neural cell membrane excitability, synaptic transmission, synaptogenesis and dendrite development and most importantly in learning, memory processing and storage. Molecular studies found that the Ca^{2+}-dependent astrocyte hyperactivity is associated with the development of many neurodegenerative disorders including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), fragile X syndrome (FXS) and Parkinson's disease (PD). In addition, persistent Ca^{2+} waves in astrocytes in acute conditions such as stroke, traumatic brain injury and epilepsy cause neurological problems by increasing gliotransmitter (glutamate and ATP)-induced neural cell death. Although the secretion of neurotransmitters is normal, the impairment of Ca^{2+} activity-dependent responders is also associated with the development and progression of several neurodegenerative diseases. In this chapter, we focus on regulatory function of calcium in neurological disorders.

Keywords

Acetylcholine • Biogenic amines • Epilepsy • Fragile X syndrome • Neurotransmitters

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4.1 Introduction

The central nervous system (CNS) is mainly composed of neurons, astrocytes, oligodendrocytes and microglia. Among them, astrocytes are the largest population of cells in the brain that compromise nearly 50% volume of the brain cell population. In fact, astrocytes tile the entire CNS and mediate many central events of neurons such as synapse formation and removal, neurovascular coupling and neurotransmitter clearance as well as ion homeostasis (Simard and Nedergaard 2004; Zundorf and Reiser 2011). The bidirectional communication between neuron and astrocytes is an integral part of information processing in the brain. The neurons use two different codes such as electrical signal (action potential) and chemical signal (neurotransmitters) to communicate the message to neurons as well as nonneural cells. Neurotransmitters are broadly classified as excitatory neurotransmitters and inhibitory neurotransmitters based on their function and grouped as biogenic amines, neuropeptides, acetylcholine, nucleotides (ATP, AMP) and amino acids based on their chemical nature. Among them, the monoamines such as acetylcholine, dopamine, adrenaline, serotonin, glutamate, γ-aminobutyric acid (GABA), ATP, etc. are central players in the synaptic dynamics under normal physiological processes. However, several neurotransmitters, particularly neuropeptides, respond only in certain physiological conditions. For example, neurostatin is involved in the regulation of blood pressure, food intake and drinking behaviour. Vasoactive intestinal peptide (VIP) is involved in relaxation of smooth muscles especially the stomach, intestine and vascular system of the heart and lungs, while neuropeptide Y acts as a strong vasoconstrictor (Eguiagaray et al. 2004).

Ca^{2+} has a fundamental role in the neuronal physiology and brain function by governing the synthesis and secretion of neurotransmitters. The intracellular Ca^{2+} concentration is not only important for the release of neurotransmitters but also essential for the regulation of their action potential in postsynaptic membranes. Ca^{2+} signalling has been implicated in almost all neural activities including neural cell membrane excitability, synaptic transmission, synaptogenesis and dendrite development and most importantly in learning, memory processing and storage (Kawamoto et al. 2012). In neural cells, Ca^{2+} displays multiple actions by maintaining different gradients depending upon the circumstances. The spatiotemporal pattern of intracellular Ca^{2+} and its positive effects on synaptic transmission rely on intracellular processes such Ca^{2+} buffering, intracellular sequestration and extrusion out of cells. Even small disturbances in Ca^{2+} homeostasis cause profound alterations in neural signalling, and it has been linked to many neurodegenerative diseases (Kawamoto et al. 2012). For example, a tightly regulated intracellular Ca^{2+} increase in astrocyte is essential for its communication with neighbouring astrocytes as well as neurons. The Ca^{2+}-dependent excitability and release of glutamate d-serine and ATP from astrocytes can regulate excitatory and inhibitory synapses, and thereby it controls neuronal synaptic circuits (Harada et al. 2015). The astrocytic hyperexcitability due to disturbance of Ca^{2+} homeostasis leads to excitotoxicity, a pathological process causing damage and destruction to neural cells, by relentless release of glutamate. Molecular studies found that the Ca^{2+}-dependent astrocyte hyperactivity is

associated with the development of many neurodegenerative disorders including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), fragile X syndrome (FXS) and Parkinson's disease (PD). In addition, persistent Ca^{2+} waves in astrocytes in acute conditions such as stroke, traumatic brain injury and epilepsy cause neurological problems by increasing gliotransmitter (glutamate and ATP)-induced neural cell death (Atherton et al. 2014; Harada et al. 2015). Although the secretion of neurotransmitters is normal, the impairment of Ca^{2+} activity-dependent responders is also associated with the development and progression of several neurodegenerative diseases. Acetylcholine (Ach) is an excitatory neurotransmitter, which implements its action through muscarinic acetylcholine receptor (mAchR)- and nicotine acetylcholine receptor (nAchR)-mediated increment of intracellular Ca^{2+} level. The impairment/dysregulation of these cholinergic receptors contributes to the progression and cognitive decline in AD and PD (Guo-PingLiu et al. 2012). Similarly, the over-activation of NMDA receptors by amyloid plaques in AD provokes neural loss by increasing Ca^{2+} accumulation (Guo-PingLiu et al. 2012). Thus, Ca^{2+} signalling is an integral part of CNS function, and dysregulation of Ca^{2+} concentration directly or indirectly contributes to the development of many neurological disorders (Guo-PingLiu et al. 2012; Lombardo and Maskos 2015; Quik et al. 2012).

4.2 Ca^{2+} Signalling in Neurotransmitter Release

The neuron communication is sparked by flooding of Ca^{2+} into cells in response to action potential at the first neuron (presynaptic) and release of neurotransmitters to the synaptic cleft. This, in turn, activates receptors on the postsynaptic neuron and consequently the action potential transfer to the next synapse. The neurotransmitter release occurs through three different mechanisms: synchronous, asynchronous and spontaneous. All three modes of release share a common key fusion processes, but they differ in the Ca^{2+} source (extracellular or intracellular stores) and identity of Ca^{2+} sensors. So, the Ca^{2+} concentration and sensitivity of Ca^{2+} sensors play a crucial role in switch on different modes of neurotransmitter release (Kaeser and Regehr 2014). The synchronous mode of release is the predominant and rapid event, which is stimulated by action potential of presynaptic bouton and that is highly dependent on the influx of Ca^{2+}. The synchronous release is initiated by activation of relatively low-affinity Ca^{2+} sensors (synaptotagmins (Syt)), which requires a high concentration of Ca^{2+}. This increase is only a very short time (a millisecond or less), and that is spatially constrained to the vicinity of open Ca^{2+} channels. A very sharp and precise synchrony of neurotransmitter release is achieved by quick activation and deactivation of voltage-gated Ca^{2+} channels. In synchronous release, $Ca_v2.1$ (P-/Q-type Ca^{2+} channel) and $Ca_v2.2$ (N-type Ca^{2+} channel) channels are mainly responsible for Ca^{2+} entry and initiation of fast synaptic transmission. There are two key events in the synchronous mode of release: the formation and rapid fusion of intrinsic releasing apparatus in response to the elevation of intracellular $Ca^{2+}$and placement of docked vesicles in very close (<100 nm) distance from VDCC. In

synchronous release, Ca^{2+}-triggered assembly and exocytosis of synaptic vesicles are orchestrated by soluble NSF attachment protein receptor (SNARE) and its associated proteins. The Ca^{2+} sensors such as Syt1, Syt2 or Syt9 and its cofactor complexion bind with phospholipids of synaptic vesicles and drive them to fuse with plasma membrane during which vesicle SNARE proteins (synaptobrevin/VAMP) form complex with plasma membrane SNARE-associated proteins (syntaxin1 and SNAP25). The $Ca_v2.1$ and $Ca_v2.2$ have interaction sites for SNARE proteins, and thus they can initiate and regulate synaptic transmission. At final step, Ca^{2+} triggers the exocytosis of synaptic vesicles by opening of Ca^{2+} channels (Kaeser and Regehr 2014; Sudhof 2012).

In some type of neurons such as hippocampal cholecystokinin (CCK) containing neurons, dorsal horn neurons, parallel fibre neurons and synapses from deep cerebellar nuclei (DCN) to the inferior olive (IO), the release of neurotransmitters can persist long at least few hundred milliseconds after ceasing of first action potential. This is known as asynchronous mode of release. This mode of Ca^{2+} action provides a high synaptic activity and sustained release of neurotransmitters. The asynchronous release is carried out by smaller amplitude and very slow variation of Ca^{2+} increase, which accelerates the recruitment of neurotransmitter-loaded vesicles to plasma membrane and promotes exocytosis. In fact, asynchronous release effectively activates postsynaptic Ca^{2+} channels and NMDA receptors and evokes neuropeptide release from dendrites by generating strong Ca^{2+} signals (Wen et al. 2013). In spinal primary afferents, glutamate burst by asynchronous release is mediated by TRPV1, which is a Ca^{2+}-permeable non-selective cation channel (Peters et al. 2010). Interestingly, different components of SNARE complex contribute to the exocytosis as like in synchronous mode of release. In hippocampal neurons, double C2 domain (DOC2) family of proteins acts as a Ca^{2+}sensor that selectively maintains asynchronous release (Yao et al. 2011). Most strikingly, the isoforms of synaptotagmins such as Syt1 and Syt7 also mediate Ca^{2+}-dependent asynchronous vesicle release. The C2 domains (C2A and C2B) of Syts possess differential affinity for Ca^{2+}, and they have independent role in neurotransmission release. The C2B domain is highly required for Syt1-dependent asynchronous release, and binding of Ca^{2+} to C2A domain inhibits asynchronous release, while Ca^{2+} binding at C2A domain of Syt7 is required during asynchronous release (Bacaj et al. 2013; Yoshihara et al. 2010). VAMP4 is an important SNARE component of vesicles to maintain Ca^{2+}-dependent asynchronous neurotransmission. Molecular studies found that VAMP4 directly forms complexes with syntaxin 1 and SNAP25 without interaction with complexins or Syt1, which are important proteins of synchronous neurotransmission (Raingo et al. 2012).

The spontaneous release of neurotransmitter can also be observed in all neurons, which is an autonomous process independent of presynaptic actions (Kaeser and Regehr 2014). This mode of release is predominant in pyramidal synapses of neurons of hippocampal CA1 region as well as in inhibitory synapses. Emerging evidences show the differential mechanism of Ca^{2+} influx during spontaneous neurotransmitter release at excitatory and inhibitory synapses. In neocortex, Ca^{2+} transients are required for spontaneous release of glutamate, which is mediated by

bulking of extracellular Ca^{2+} and G protein-coupled Ca^{2+}-sensing receptor (CaSR) activation, instead of Ca^{2+} channel-mediated influx of Ca^{2+} (Vyleta and Smith 2011). However, the spontaneous release of GABA at neocortical inhibitory synapses is driven by various types of VDCC, and it is currently unclear what type of VDCC is mainly involved in spontaneous Ca^{2+} influx (Williams et al. 2012). In experimental study, it has been shown that R-type channel has an important role in spontaneous glutamate release from hippocampus neurons, although stochastic opening of P/Q and N-type VDCC has influence on spontaneous glutamate release (Ermolyuk et al. 2013). In contrast, a study in caudal tract nucleus found that Ca^{2+}-dependent TRPV1 channel promotes spontaneous glutamate release under physiological temperature (Shoudai et al. 2010). The neural vesicle SNARE, VAMP7 and the plasma membrane protein SNAP25 are implicated in vesicle docking to the plasma membrane (Bal et al. 2013). The Ca^{2+} sensors such as Syt1 and DOC2b play important role in spontaneous fusion of vesicles and release. However, Syt1 only acts as a clamp for vesicle release (Kavalali 2015). A molecular study revealed that the cofactor of Syt, complexin, modulates the timing and releasing activity of Syt, and thereby it regulates kinetics of vesicle fusion and subsequently controls evoked and spontaneous fusion (Jorquera et al. 2012). A single spontaneous release event can globally influence neuronal excitability by eliciting electrical activity as well as Ca^{2+}-dependent signals. For example, a single glutamate release event can activate NMDA receptor activity at rest. Similarly, the induction of metabotropic glutamate receptors such as mGluR1 and mGluR transduces Ca^{2+}-dependent neuronal signalling in response to spontaneous glutamate release.

4.3 Ca^{2+} Homeostasis and Neurodegenerative Diseases

The Ca^{2+} signalling play a central role in CNS function, which emphasizes its potential relevance for the development and progression of neurological problems, in particularly neurodegenerative diseases. The neurodegenerative diseases are a heterogeneous group of disorders characterized by gradual losing of a particular or group of neuronal system. Regardless of the spectrum of causes of all these diseases, the most common phenomenon related to molecular pathogenesis of these disorders is alteration of Ca^{2+} homeostasis and its associated signalling pathway. The dysregulation of Ca^{2+} homeostasis differentially affects neuron functions and survival depending upon the cell type and degree of modulations in Ca^{2+} channel activities, sensitivity of Ca^{2+} sensors and Ca^{2+} buffering systems. In ageing neurons, the gradual impairment of Ca^{2+} balance by consecutively active release from intracellular store by $InsP_3R$ and RyR, increased influx through L-type VDCC, increase of Ca^{2+}-dependent K^+ channel-mediated slow trace hyperpolarization and/or elevation of calcineurin and calpain activities leads to overaccumulation of intracellular Ca^{2+}. This leads to perturbation of energy metabolism and evokes Ca^{2+} stress to mitochondria as well as ER, which triggers neuronal cell death and progressive loss of neural function. In this section, we provide the connection between Ca^{2+}

homeostasis disruption, neural loss and development/progression of neurodegenerative diseases.

4.3.1 Ca^{2+} Signalling and AD

AD is the most common form of age-related dementia, which is clinically characterized by progressive deterioration in cognitive functions including memory, behaviour, reasoning and language. The development of AD involves degenerative loss of neurons in hippocampus, basal forebrain, amygdala and cortex regions (entorhinal cortex, frontal cortex and inferior parietal cortex), which are integral part of memory and learning. Most of the AD cases are sporadic and idiopathic in which clinical symptoms emerge during advancement of age (<60 years). However, a small percent (<5%) of people are affected by genetically linked or familial AD (FAD). Regardless of causes, the common clinical feature of AD is accumulation of amyloid β (Aβ) peptide and extracellular deposition of diffuse Aβ plague as well as neurite plaques. This accumulation is either due to overproduction of Aβ, altered processing or lack of clearance. Normally, a lower amount of Aβ42 (about 10%) is produced along with Aβ40 (90%) under physiological condition and released during synaptic activity. At lower physiological concentrations, Aβ42 plays an important role in promoting growth factor expression and adult neurogenesis, assisting modulation of neuron-astrocyte signal LTP and memory recording and storage. But, missense mutations in the amyloid precursor protein (APP) or mutations of presenilin-1/2 (PS1/2), a transmembrane protein having catalytic submit of the APP-cleaving α-secretase complex, lead to increase of hydrophobic Aβ42 oligomer fragments, which induces plague deposition, neuronal toxicity and loss of synapses in AD patients (Supnet and Bezprozvanny 2010a, b). The histopathological analysis of autopsy samples from AD patients showed that intracellular neurofibrillary tangles (NFTs) due to hyperphosphorylated tau (τ) protein deposits and shrinkage of the cerebral cortex along with extracellular Aβ aggregates are pathological hallmarks of this neurological disorder.

There is a casual link between Aβ accumulation and drastic changes in intracellular Ca^{2+} signalling, which is an initiative factor of neuronal cell death and cognitive decline in AD. Aβ accumulation induces oxidative stress and inflammatory process, which is the primary route cause to hasten Ca^{2+} overload-induced neural cell death in AD patients. Emerging evidences reveal that the Ca^{2+} signalling has positive influence on Aβ production, and thus there is a bidirectional relationship between Ca^{2+} signalling and Aβ in the pathogenesis of AD. In experimental studies, increase of cytosolic Ca^{2+} using RyR agonist (caffeine) and Ca^{2+} ionophore agonist (A23187) promoted Aβ production from APP. In addition, the influx of Ca^{2+} through L-type VDCC increases neuronal Aβ42 production. In contrast, inhibition of SERCA pump with thapsigargin reduced the Aβ generation because APP processing is partly carried out in ER compartments. These findings indicate that the increase of cytosolic Ca^{2+} either from internal or external stores upregulates APP

processing, and the ER Ca^{2+} signalling particularly is required in regulating the APP processing (LaFerla 2002; Supnet and Bezprozvanny 2010a).

On the other side, the progressive accumulation of Aβ fractions and disturbance of APP processing machineries promote Ca^{2+} mishandling by ER and mitochondria, which are central players in Ca^{2+} dynamics. The oligomeric form of Aβ can itself act as an ionophore for Ca^{2+} by insertion into plasma membrane. The Aβ binding is facilitated by flipping of phosphatidylserine (PtdS) from inner plasma membrane due to mitochondrial impairment (ATP depletion)-induced plasma membrane depolarization (Supnet and Bezprozvanny 2010a). In intracellular locations, Aβ fractions stimulate Ca^{2+} release from ER by activating IP3R and RyR2 receptor. Apart from this, the mutation of PS2 increases Ca^{2+} accumulation in ER, but this mutation promotes the expression and functions of RyR2/3. In physiological conditions, PS is an intrinsic protein localized to the ER membrane, which also regulates SERCA activity in addition to its role in the generation of Aβ fractions. In AD patients, the mutation/impairment of PS activity modulates SERCA functions and reduces Ca^{2+} restoration to ER, which, in turn, leads to sustained increase of cytosolic Ca^{2+} level. Apart from this, the uncontrolled Ca^{2+} can trigger the upregulation of expression of plasma membrane $Ca_v2.1$, an L-type calcium channels (LTCC) in the hippocampus, and provoke Ca^{2+} influx. Although $Ca_v2.1$ is essential for the long-term potentiation (LTP), spatial memory and synaptic plasticity of hippocampus, its over-activation is detrimental to hippocampal cells. The G protein-coupled CaSR of plasma membranes is an important Ca^{2+} homeostasis protein in neural cells. In astrocytes, Aβ42 interacts with CaSR and suppresses its activity, which consequently leads to oversecretion of Aβ from neighbouring cells (LaFerla 2002; Shoudai et al. 2010; Supnet and Bezprozvanny 2010a). NCX is another Ca^{2+} homeostasis protein involved in Ca^{2+}efflux by exchanging Na^+. However, the binding of aggregated Aβ fractions to the hydrophobic region of NCX alters its transport properties. In the late stage of AD, the two isoforms of NCX (NCX2 and NCX3) expression are highly altered in positive terminals of parietal cortex, in which NCX3 expression is reduced. This is mainly due to Aβ overexpression, which induced hike of intracellular Ca^{2+} level and activation of calpain (Ca^{2+}-dependent non-caspase protease)-mediated cleavage pathway (Atherton et al. 2014). In addition, the Aβ-induced activation of calpain promotes hyperphosphorylation of tau, which has been implicated in neuronal apoptotic cell death (LaFerla 2002). The excessive accumulation of Ca^{2+} facilitates MCU and mitochondrial RyR-dependent influx of Ca^{2+} into mitochondrion. This leads to impairment of mitochondrial energy metabolism and mitochondrial pore opening and consequently activates apoptotic cell death pathway. In addition, the loss of mitochondrial ATP production also alters plasma membrane potential, which favours Ca^{2+} influx by turning on glutamate-dependent receptors such as metabotropic glutamatergic receptors (mGluR1/5) and N-methyl-D-aspartate receptor (NMDAR) (Magi et al. 2016; Supnet and Bezprozvanny 2010a). These receptors trigger efflux of Ca^{2+} from ER by increasing the production of InsP3. In AD patients, the impairment of glutamatergic system is also one of the important factors contributing to Ca^{2+} imbalance. The glutamate-dependent synaptic activation of NMDARs or mGluRs is required for the long-term synaptic depression (LTD), which plays

crucial role in learning and memory in situation like cognitive demands that need a flexible response. Normally, glutamate is removed from extracellular space after glutamatergic synapse, and excitatory signal is terminated by Na^+-dependent glutamate transporter (EAAT)-mediated clearance of glutamate. Aβ overaccumulation disrupts neuronal glutamate clearance and enhances long-term synaptic depression (LTD) by reducing the expression of EAAT family members (EAAT1 and EAAT2) in hippocampus and frontalis medius regions, while the expression kainate-type glutamate receptors are upregulated in the same region in AD patients. However, the expression of LTP-type ionotropic NMDA and AMDA receptors is decreased (Magi et al. 2016). Thus, the drastic alterations of Ca^{2+} homeostasis system by aggregated Aβ proteins trigger oxidative stress and Ca^{2+}-dependent (calpains) activation of cell death signalling in neurons, which leads to loss of neurons, resulting in cognitive impairments in AD patients (Fig. 4.1).

4.3.2 Ca^{2+} Signalling and HD

HD is a slowly progressive inherited autosomal dominant neurodegenerative disease caused by the expansion of CAG repeats in exon-1 of gene encodes for huntingtin protein (Htt) that yields a high number of polyglutamine (PolyQ) in the N-terminal of Htt protein. Normally, the human HTT gene possesses 6–35 CAG repeats. But there are >40 CAG repeats, and it stretches up to 180 repeats in HD patients. In most patients, the slower progression of HD development is resulting from an intermediate number (Prell et al. 2012, 2013; Quik et al. 2012; Raingo et al. 2012; Rcom-H'cheo-Gauthier et al. 2014) of CAG repeats. The onset of HD begins between the age of 35 and 50 years, and patients die after 15–20 years of onset due to inevitable progression of this disorder (Bano et al. 2011; Bezprozvanny and Hayden 2004; Giacomello et al. 2013). Currently, the physiological role of Htt is largely unknown. Several experimental studies with transgenic mice revealed their importance in the development of the brain, in particular, forebrain formation. In fact, complete deletion of Htt leads to early embryonic lethality by increasing apoptosis. Some studies found that Htt functions in the neuronal cell maintenance (Reiner et al. 2003; Zeitlin et al. 1995). The normal Htt protein offers protection to neural cell against various toxic stimuli including excitotoxicity (Cattaneo et al. 2005). However, mutation to Htt leads to aggregation and accumulation of mutant proteins inside the cell as like in various other forms of neurodegenerative disorders such as AD and PD and causes destructive neurological problems. In HD patients, the pathological changes are predominantly seen in the striatum along with significant alterations in the cerebellar cortex and thalamus regions. Among different neuronal cells affected by HD disease, GABAergic medium spiny neurons are more sensitive to degenerative process induced by mutant Htt (mHtt). The other cells of striatum such as caudate nucleus, globus pallidus and putamen are susceptible to HD-induced neuronal loss (Bezprozvanny and Hayden 2004; Giacomello et al. 2013). The general symptoms of HD include neuropsychiatric defects such as progressive chorea, dementia seizures and motility impairment (Bano et al. 2011; Giacomello et al. 2013).

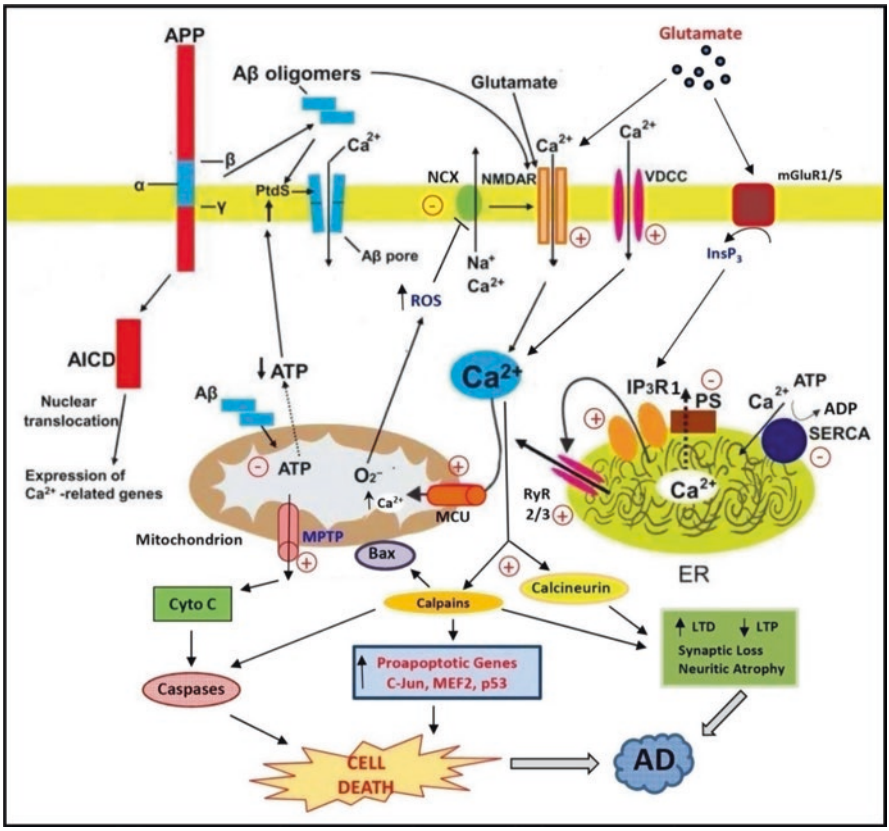

Fig. 4.1 Mechanisms of Ca^{2+} dysregulation in Alzheimer's disease (AD). The sequential cleavage of mutant β-amyloid precursor protein (APP) produces amyloid β peptide (Aβ). Aβ forms oligomers and passes into plasma membrane by binding to phosphatidylserine (PtdS). The PtdS is flipping from inner plasma membrane due to membrane depolarization induced by ATP depletion caused by mitochondrial impairment. Aβ acts, either directly or indirectly, on mitochondrial system to cause increased ROS production, Ca^{2+} overload and decreased ATP production. The function of membrane ion-motive ATPases (NCX) is impaired by increased oxidative stress (excessive ROS production). In addition, the membrane depolarization leads to the opening of glutamate receptor channels (NMDAR) and voltage-dependent Ca^{2+} channels (VDCC) and promotes influx of bulk amount of Ca^{2+} into the cytoplasm. Presenilins (PS) function as an ER Ca^{2+} leak channel, and PS mutations result in excessive accumulation of Ca^{2+} in the ER. Aβ positively modulates ryanodine receptor (RyR2/3) and IP_3 receptor (IP_3R) channels, while it inhibits smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) to alter ER Ca^{2+} release and uptake. The activation of glutamate-dependent mGluR1/5 receptors increases production of InsP3- and InsP3R-mediated Ca^{2+} release. The elevated cytosolic Ca^{2+} activates calcineurin and calpains and leads to facilitation of abnormal LTD and LTP, modification of neuronal cytoskeleton, synaptic loss and neuritic atrophy. In mitochondria, the excessive Ca^{2+} is taken up through mitochondrial Ca^{2+} uniporter (MCU), which eventually leads to opening of mitochondrial permeability transition pore (MPTP), release/activation of pro-apoptotic factors. Calpains also directly activate expression/activity of many pro-apoptotic factors (C-jun, MEF2, p53, Bax, caspases, etc.). This eventually leads to neural loss by apoptotic cell death and onset AD (Adapted and Modified from Magi et al. (2016))

The mitochondrial and ER dysfunction and Ca^{2+} dyshomeostasis are important contributors in the molecular aetiology of HD. In striatal neuronal cells, the extended polyQ of mHtt can be cleared by various caspases and calpains and other proteases. These cleaved products form aggregate due to their strong tendency of polymerization. Thus, the proteolytic cleavage of mHtt is key event of Htt toxicity in HD patients. The mHtt is mainly localized to cytoplasm, and it can translocate to subcellular compartments such as nucleus, mitochondria, ER and Golgi apparatus (De Mario et al. 2016; Schulte and Littleton 2011). The intracellular Ca^{2+} dysregulation is one of the fundamental mechanisms of neuronal loss in HD disease. The aggregates of mHtt fragments alter Ca^{2+} handling machineries by both directly binding with them and influencing the expression of Ca^{2+} homeostatic genes (Tang et al. 2003; Zuccato et al. 2010). The alterations of mitochondrial Ca^{2+} buffering and handling activities lead to defect in mitochondrial respiratory chain, which plays central role in the onset and late stages of HD (Bossy-Wetzel et al. 2008). Several mechanisms have been implicated in the increase of cytosolic Ca^{2+} in neurons of HD patients. The overstimulation of extrasynaptic NMDAR is one of the primary routes for the Ca^{2+} influx of Ca^{2+} and glutamate-mediated excitotoxicity in striatal neuronal cells. The mutant Htt activates NR1 and NR2B subtypes of NMDAR, which are abundantly expressed by medium spiny neurons of striatum. Molecular studies suggest that the weak interaction of Htt protein with PSD95 promotes the Src kinase-mediated hyperphosphorylation of NR2 subunits of NMDAR, which increases NMDAR currents (Bezprozvanny and Hayden 2004; Song et al. 2003). Dopamine (DA) is an important neurotransmitter for motor and cognitive function. In striatum, it can modulate glutamatergic-mediated neurotransmission by acting on DA receptor type 1 and type 2 (D1R and D2R) in medium spiny neurons. These two receptors have differential affinity for DA and act through different pathways. D1R increases Ca^{2+} influx by cAMP-PKA-dependent phosphorylation and activation of both AMPR and NMDAR receptors. In addition, cAMP-PKA pathway activation by D1R also stimulates opening of L-type VDCC channels. In contrast, D2R activation negatively regulates cAMP and inhibits NMDAR. But D2R increases Ca^{2+} by PLC-dependent production of InsP3 and activation of InsP3 receptor of ER. In the early stage of HD, DA neurotransmission is increased by both increase of DA and DA receptor expressions. Thus, the crosstalk between DA and glutamate synergistically activates synaptic plasticity and causes excitotoxicty in HD patients at early stage. But, the expression of D1R and D2R is dramatically decreased in late stage of HD (Gardoni and Bellone 2015). $InsP_3R1$ is the predominant isoform of neuronal Ca^{2+} pump, which is sensitized to $InsP_3$ by mHtt either by direct binding or through HAP1A-mediated ternary complex (InP_3R1-HAP1A-Htt). Interestingly, normal Htt does not associate with $InsP_3R1$ (Tang et al. 2003). It is well known that InP_3R1 is involved in the efflux of Ca^{2+} from ER; therefore, the activation of mHtt-mediated activation of InP_3R1 provokes the Ca^{2+} release from ER. The activation of metabotropic glutamate receptor (mGluR5) fuels InP_3R1 activity by generating InsP3 in HD neurons; however, its neuroprotective signalling is desensitized by

mHtt-mediated reduction of PKC-dependent mGluR5 phosphorylation (Tang et al. 2003). The pharmacological inhibition of NMDAR (NR1/NR2B) and mGluR5 remarkably blocks mHtt-induced loss of mitochondrial membrane potential and cell death, which confirmed their role in accumulation of Ca^{2+} in HD neural cells (Ribeiro et al. 2014). Apart from this, Htt also activates a variety of VDCC by directly binding with them. Htt possesses binding motifs to interact with a2/d accessory subunits of VDCC as well as pore-forming submit of CaV2.2 (N-type VDCC), and this interaction can trigger Ca^{2+} influx to intracellular space (Bezprozvanny 2010). In this context, the elevation of intracellular Ca^{2+} leads to overaccumulation of Ca^{2+} in mitochondria. This eventually leads to opening of mitochondrial permeability transition pore (MPTP) and release of pro-apoptotic factors and cell death.

Currently, it is unknown whether mHtt is altering mitochondrial activity by directly interacting with mitochondrial Ca^{2+} transporter systems (MCU and NCX), but it binds with mitochondrial outer membrane and change membrane polarization state (Bezprozvanny 2010). The mitochondrial defect in HD is directly linked to the interaction of mHtt protein with transcriptional factors (CREB, p53 PGC-1α, etc.) involved in expression/activity of many mitochondrial proteins including ROS scavenger superoxide dismutase (SOD1 and 2), nuclear respiration factor (NRF1 and 2), cytochrome C, mitochondrial transcription factor (mTfam), thermogenic uncoupling protein (UCP) and respiratory chain complex components (Bossy-Wetzel et al. 2008). Apart from this, mHtt alters mitochondrial integrity by modulating mitochondrial dynamics (mitochondrial fission and fusion). mHtt enhances mitochondrial fragmentation process by directly binding with Drp1 (a key fission protein) and stimulating its enzymatic activity, while mHtt inhibits fusion process by strong binding with mitochondrial fusion protein 1 (Mfn1) and interferes the function of this pro-survival factor (Wang et al. 2009). Thus, the mHtt-induced disintegration of mitochondrial dynamic network and excessive mitochondrial fragmentation is detrimental to cell function and that consequently turns on cell death cascade signalling (Costa and Scorrano 2012; Wang et al. 2009). In fact, Drp1 also is a pro-apoptotic factor in caspase-dependent and caspase-independent pathway by physically interacting with several apoptotic molecules such as p53 and Bax in HD neural cells. In addition, Ca^{2+}-induced activation of calcinuerin (a protein phosphatase) positively modulates Drp1 activity by dephosphorylation, which is the active form of Drp1 (Costa and Scorrano 2012). Interestingly, the inhibition of Drp1 or overexpression of Mfn1/OPA1 attenuated the mitochondrial fragmentation and rescued mitochondrial function from mHtt toxicity in both experimental animals and cell culture model of HD (Costa and Scorrano 2012; Wang et al. 2009). Collectively, these findings reveal that parallel events of mitochondrial defect and intracellular Ca^{2+} dysregulation initiate the neural cell loss by triggering cell death signals in HD patients (Fig. 4.2).

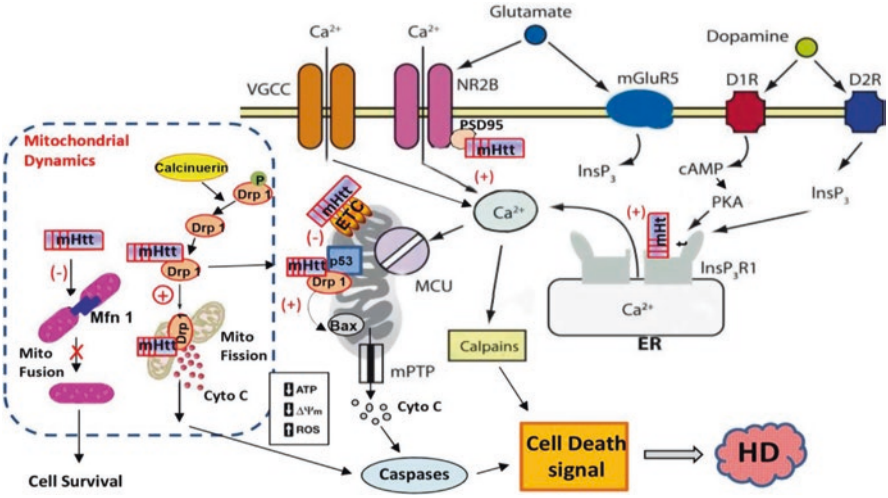

Fig. 4.2 Mechanism of Ca^{2+} dyshomeostasis in HD. The mutant huntingtin (mHtt) causes cytosolic and mitochondrial Ca^{2+} overload and apoptosis by directly or indirectly binding with various Ca^{2+} handling machineries. In the early stage of HD, the increased release of dopamine (DA) and expression of its receptor (D1R and D2R) promote NMDAR and InsP3R1-mediated Ca^{2+} influx, by increasing cAMP and PKA activation, which is a positive modulator of glutamate-induced Ca^{2+} signal. D2R is directly involved in $InsP_3R$ activation by acting as upstream for $InsP_3$ production. mHtt binds with submits of NMDA receptor (NR1A/NR2B) either directly or through PSD95 and increases their activity. mHtt also strongly binds with $INsP_3R1$ and positively modulates Ca^{2+} release. mHtt impairs mitochondrial function by inhibiting electron transport chain (ETC) component expression/activity, mitochondrial membrane polarization (mPTP) and release of pro-apoptotic factors. mHtt directly affects mitochondrial dynamics. It increases mitochondrial fragmentation by binding with Drp1 (mitochondrial fission protein), while it blocks mitochondrial fusion through direct interaction with Mfn1 (mitochondrial fusion protein). It also promotes Drp1 pro-apoptotic activity by increasing dephosphorylation of Drp1 (active form) through Ca^{2+}-dependent activation of calcineurin. mHtt also facilitates binding and translocation of Drp1 and p53 to mitochondria, which consequently activates cell death pathways. Increased cytosolic Ca^{2+} activates calpains which cleave huntingtin as well as stimulating activation of many apoptotic factors. This eventually leads to neuronal striatal neural cell death and onset of HD. *MCU* mitochondrial calcium uniporter, *L-type VDCC* L-type voltage-dependent calcium channel

4.3.3 Ca^{2+} Signalling and PD

PD is the second most common incurable neurodegenerative disease that affects aged population of above 65 years. PD is clinically characterized by the deficiency of dopamine in striatum due to progressive loss of dopaminergic neurons within the substantia nigra pars compacta (SNc-DA) and aggregation of α-synuclein (α-Syn), a protein expressed in presynaptic terminals of dopaminergic neurons. The aetiology of PD development is sporadic and largely unknown. However, the genetic forms of PD are linked to mutations of several genes (α-Syn, Parkin, DJ-1, PINK1 and LRRK2). The striatum is primarily susceptible to PD, but pathological changes can also be seen in cortex region at later stages. The clinical symptoms of PD

include resting tremor, rigidity, gait difficulty, bradykinesia and stooped posture (Cali et al. 2014; Ganguly et al. 2017). The abnormalities in ubiquitin-proteasome system (UPS) along with oxidative stress and mitochondrial dysfunction are key pathogenic events in the development of PD.

The altered Ca^{2+} homeostasis due to alterations in the activity/expression of Ca^{2+} handling machineries is one of the crucial factors involved in the loss of neurons. Given the fact that DA is essential for the fine-tuning of excitatory and inhibitory synapses in spiny GABAergic neurons in striatum, the loss of SNc-DA leads to the lack of inputs in this region and onset of PD. SNc-DA is a distinct type of neuron that can autonomously generate action potentials without synaptic input. Such pacemaking activity of SNc-DA is vital to maintain basal dopamine (DA) level in innervated regions like striatum (Cali et al. 2014; Rivero-Rios et al. 2014). Unlike other neurons, SNc-DA neurons rely on L-type Ca^{2+} channel with $Ca_v1.3$ pore-forming submit, for the pacemaking activity. This channel opens relatively at hyperpolarized potential to allow Ca^{2+} entry (Cali et al. 2014; Rivero-Rios et al. 2014). The weakest feature of SNc-DA is the low Ca^{2+} buffering capacity compared to other types of pacemaking cells. Thus, the large burden of Ca^{2+} handling makes SNc-DA neurons easily susceptible to neurodegenerative process during the development of PD (Rivero-Rios et al. 2014).

The reliance of autonomous pacemaking activity on Ca^{2+} influx through L-type Ca^{2+} channel imposes a sustained metabolic stress (ATP production) on mitochondria, which naturally accelerate cellular ageing and death. In both idiopathic and familial PD, the defect in protein folding process leads to increase of unfolded protein response and causes ER stress in SNc-DA. Given that ER is the major Ca^{2+} restoration centre of all cells, the impairment of ER integrity obviously favours steep rise in cytosolic Ca^{2+}. The defective proteostasis and fragmented Golgi apparatus have been observed in neurons from PD patients. The Ca^{2+}-calmodulin-dependent protein kinases are involved in this fragmentation process (Thayer et al. 2013). PARKIN, PINK1 and DJ1 have pleiotropic functions in SNc neurons. These proteins are actively involved in processing and degradation of misfolded proteins by ubiquitin-dependent proteasome system (UPS). The dysfunction of this system due to genetic mutation or PD-related environmental toxins (paraquat, MPTP and rotenone) accumulates α-Syn and other proteins, which consequently causes ER stress and ultimately leads to activation of cell death pathways (Cali et al. 2011). PARKIN, PIN1 and DJ also play essential role in mitochondrial homeostasis and Ca^{2+} handling. DJ1 protects mitochondria from Ca^{2+} stress by activating mitochondrial uncoupling response through expression of uncoupling proteins (UCP4 and UCP5), which are required to attenuate the ROS-induced increase of inner mitochondrial membrane potential (Guzman et al. 2010). PINK1 inhibits Drp1, a mitochondrial fragmentation inducer, by blocking the activity of calcinuerin, which is a Ca^{2+}-dependent phosphatase. In PD patients, the lack of PINK1 favours Ca^{2+}-calcinuerin-Drp1-induced mitochondrial fission process (Sandebring et al. 2009). In aged SNc-DA, the raised Ca^{2+} level and oxidative stress also contribute to the aggregation of α-Syn, which causes toxicity to SNc-DA. Recent studies found that Ca^{2+} directly binds with α-Syn and promotes the formation of oligomers. Emerging

evidences suggest that α-Syn directly interacts with membrane phospholipids (Burre et al. 2010), and thus the aggregates of α-Syn can promote Ca^{2+} influx by forming a pore-like structure on membrane (Cali et al. 2011). Similarly, α-Syn aggregate raises mitochondrial Ca^{2+} transients, which in turn aggregate the oxidative stress (Cali et al. 2014; Rcom-H'cheo-Gauthier et al. 2014). Currently, the mechanism of pore formation is remaining unknown. Thus, the defective proteasomal pathway and aggregation of α-Syn together with uncontrolled accumulation of Ca^{2+} causes oxidative stress, mitochondrial impairment and ER stress in SNc neurons, which eventually leads to cell death and functional loss of neural cell striatum in PD patients.

4.3.4 Ca^{2+} Signalling and ALS

ALS is a fatal neurodegenerative disease, which selectively affects upper and lower motor neurons of the brain stem, cerebral cortex and spinal cord. In familial ALS, the genetic mutation of Cu and Zn superoxide (SOD1), a superoxide scavenging enzyme, is responsible for the development of this disorder. However, the aetiology of sporadic ALS is multifactorial, and the pathogenic mechanisms remain largely unknown. ALS is clinically characterized by failure of muscle activities such as loss of muscle force, breathing difficulties, swallowing difficulties and limb spasticity. The ALS patients die within 3 to 4 years after onset of disease, mainly due to muscular atrophy and paralysis (Grosskreutz et al. 2010; Jaiswal 2014). The excitotoxicity by AMPA/kainate receptors, oxidative stress, mitochondrial dysfunction and Ca^{2+} dyshomeostasis are major interrelated pathways, which account for the motor neural loss in ALS patients. Emerging evidences reveal that increased activity of Ca^{2+}-permeable AMPA receptors, lowered Ca^{2+} buffering capacity and Ca^{2+}-dependent aggregation of proteins (particularly SOD1) are crucial pathogenic events in ALS (von Lewinski and Keller 2005).

The high availability of glutamate and abundance of Ca^{2+} glutamate-dependent AMPA receptor play crucial role in the bulk entry of Ca^{2+} into motor neurons. In ALS patients, the defective clearance of glutamate from the synaptic cleft due to the selective loss of glutamate transporter 1 (GLT1) in the astroglial cells of motor cortex and spinal cord leads to elevation of glutamate in the extracellular space. Thus, high availability of glutamate switches on the Ca^{2+}-permeable AMPA receptors and causes excitotoxicity. AMPA receptor normally consists of at least one GluR2 submit, which has a low Ca^{2+} permeability capacity. However, the lack of GluR2 submits in the motor neuron increases Ca^{2+} permeability capacity of AMPA receptor, which is clearly seen in motor neurons of ALS patients. The soluble factors expressed from astrocytes can upregulate the expression of GluR2 in motor neurons; however, the mutant SOD1 aggregation interferes the production and secretion of these factors, and that could be reason for the suppression of GluR2 mRNA expression in ALS patients (Kawahara et al. 2003; Van Damme et al. 2005).

The uncontrolled influx of Ca^{2+} and loss of counterbalance by failure of Ca^{2+} sequestration machineries further elevate the accumulation of Ca^{2+} in cytoplasm. In

ALS neurons, a very early loss of expression of Ca^{2+}-binding proteins such as calbindin and parvalbumin causes burden to mitochondria, which acts as a calcium-buffering intracellular organelle (Alexianu et al. 1994). Currently, the contribution of VDCC in Ca^{2+} influx in motor neurons, particularly during ALS condition, is unclear. However, several experimental studies indicate that VDCC is also activated in ALS neurons. In a mouse model of ALS, N-type Ca^{2+} is highly expressed in cortex motor neuron and similar expression pattern also observed in cultured cortical neurons (Pieri et al. 2013). In contrast to this, a progressive loss of expression/function of $Ca_v1.1$, an L-type Ca^{2+} channel, is found in muscle fibres of ALS mouse. As the skeletal muscle $Ca_v1.1$ is required for the muscle force and mass, the defect in $Ca_v1.1$ signal might be responsible for the dissolution of neuromuscular junction, causing muscular atrophy in ALS patients (Beqollari et al. 2016). Apart from this, the hyper-activation of unfolded response by overaccumulation of mutant SOD1 causes ER stress and consequently affects ER mitochondrial Ca^{2+} cycle (Prell et al. 2012). In ER, the Ca^{2+}-binding proteins such as calreticulin and calsequesterin are required to ensure the steady state of Ca^{2+} level in ER, and those proteins also play a vital role in folding and export of proteins. Calreticulin activity is also required for store-operated Ca^{2+} influx (SOCI) as well as for ER-specific IP3R receptor expression. In ALS neuron, the suppression of calreticulin expression and activity is directly linked to the aggregation of mutant SOD1 and activation of Fas/NO pathway. The decrement of Ca^{2+} buffering action and blockage of Ca^{2+} release from ER lead to increase of Ca^{2+} level in ER, which makes cells become vulnerable to death (Prell et al. 2013). Thus, dysregulation of Ca^{2+} plays central role in the mitochondrial oxidative stress, ER stress and functional impairment, which perpetuate the neuron degeneration in ALS.

4.4 Summary

With respect to calcium ion, the neurotransmitter concentrations are consistent in the subjects that are vulnerable. Taken together, the visible pictures from the study of the neurodegenerative disorders appear awfully complex, with many doubts and gaps to fill. The steps outlined in this chapter will help to correct the malfunctions of VDCC and imbalances of calcium that leads to neurological disorders. Further research can be done in the field of regulatory function of calcium and VDCC's influence in neurodegenerative disorders.

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Voltage-Dependent Calcium Channels: From Physiology to Diseases

5

Abstract

"Knowing our genes help knowing our enemy and knowing our therapy". Ion channels are expressed in every living cell that belongs to a group of membrane proteins that are important for in and out flux of ions of the cells. A mutation in the encoding sequences of calcium channel causes channelopathies. It can be either congenital or acquired. Mutation of ion channels can alter activation, ion selectivity and abnormal gain of function in humans. Acquired forms of channelopathies cause autoimmune disorders or drugs administration. Human Genome Project data revealed that several hundreds of different ion channel genes can be existent. This specific ion channel subunit which is encoded by specific genes assembling with other subunits to form an active membrane pore for selective ions. These disorders are very rare and therefore physicians, patients and scientists are usually not familiar with all aspects of the clinical symptoms. This chapter deals the current knowledge of different calcium channelopathies and also discusses the recent updates in the calcium ion deficit diseases.

Keywords

Channelopathies • Calcium flux • Channel subunits • Hereditary diseases • Mutations • Voltage-gated calcium channels

5.1 Introduction

Ca^{2+} is indisputably a fundamental part of many important cellular processes. A network of ion channels, calcium-sensing receptors and intracellular molecules carry out the initiation and regulation of Ca^{2+} signalling (Chakravarti et al. 2012; Rajagopal et al. 2015a). A tight regulation of Ca^{2+} homeostasis is important for the normal physiological function. Any disturbance/defect in Ca^{2+} signalling cascade and calcium handling machineries can affect Ca^{2+} homeostasis and eventually leads to the

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development of many pathological conditions as mentioned in previous sections. It is quite interesting in the aspect that it activates particular signalling cascade at microdomains in the multifaceted intracellular environment. This is achieved by Ca^{2+} gradient-dependent response of various Ca^{2+} calcium sensors and transporters. The excitable cells such as glial cells, neuronal cells and muscle cells having the calcium channels such as voltage-dependent calcium channels (VDCC), which is one of the key gateways for the calcium influx. The alternative splice variation gives rise of different isoforms of Ca^{2+}channel with distinct voltage threshold of activation and functions (Pidasheva et al. 2005; Riccardi and Martin 2008; Ward and Riccardi 2012). The identification of variation in native calcium currents has increased our knowledge about the role of VDCC in various physiological processes. However, their pathophysiological role remains largely unknown. In recent years, the studies on naturally occurring channelopathies in human and targeted gene deletions in experimental animal models have uncovered their role in the pathophysiology of various disorders, but still we are in the beginning stage to comprehend their contribution in the pathogenesis. Many laboratories have observed that genetic alterations or a small perturbation in VDCC function causes a wide variety of abnormalities in mammalian developmental, physiological and behavioural functions. At least in those instances wherein the channelopathies can be attributed to gain-of-function mechanisms, the data point towards new therapeutic strategies for developing the specific calcium channel inhibitors (Adams and Snutch 2007).

5.2 Calcium Channels Types and Their Functions

Calcium channels (Ca_v) are multisubunit protein complexes composed of a pore-forming α1 subunit and smaller auxiliary β, α2/δ and γ subunits. These channels are classified into Ca_v1 (L-type), Ca_v2 (2.1 P-/Q-type, 2.2 N-type and 2.3 R-type) and Ca_v3 (T-type) families primarily based on the sequence homology of the α1 subunits (Rajagopal et al. 2014, 2015b; Zamponi et al. 2015). Four genes encode L-type calcium channels, while the neuronal P-/Q-, N- and R-type and T-type calcium channels are coded by three genes. All these genes are localized on different chromosomes in human (Fig. 5.1) (Dolphin 2006). In general, the members of Ca_v1 family are involved in heart functioning, whereas members of Ca_v2 family are involved in neurotransmitter release, and their activity is modulated by several second messengers, including G-protein βγ subunits, calmodulin and PKC isozymes (Fang et al. 2006; Kamatchi et al. 2004). T-type channels have fast kinetics and low voltages of activation and inactivation properties that can specifically affect how and when cells reach action potential threshold. Therefore, these channels are critical regulators of excitability since these channels are unique among VDCCs (Adriano et al. 2012).

The functional impairment of L-type Ca^{2+} channel is responsible for the mood disorders, Parkinson's disease, cardiovascular disease and hypertension. The blocking agents of this family of receptors, in particular, $Ca_v1.3$ channel can prevent/arrest the onset and/or progression of these disorders (Zamponi et al. Zamponi et al.

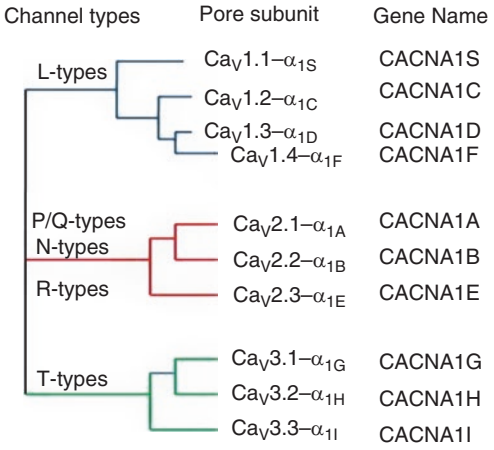

Fig. 5.1 A typical calcium channels classification

2015). Similarly, L-type Ca^{2+} channel such as $Ca_v1.2$ and $Ca_v1.3$ enhance the secretion of insulin from pancreatic β-cells through a mechanism of excitation-secretion coupling. The blocking agents of these L-type Ca^{2+} channels are useful to control the congenital hyperinsulinemia (Muller et al. 2004; Shanbag et al. 2002). The T-type Ca^{2+} channel is functionally important for the cardiovascular system and renin-angiotensin system and also for the release of catecholamines. The enhancement of T-type, in particular, $Ca_v3.2$ activity contributes to pain hypersensitivity (Francois et al. 2014) and pressure overload-induced hypertrophy (Chiang et al. 2009). The antagonists of this T-type calcium channel are beneficial in these patients. In this context, the selectivity of calcium channels is important to get a better outcome without any adverse effects. The renal vascular and tubular cells express all subtypes of voltage-gated calcium channels (L, T, N and P/Q type). Thus, the usage of a general calcium channel blocker for hypertension can affect the microcirculation and haemodynamic of the renal tissue (Koichi et al. 2007).

Molecular studies found that L-type calcium channels are only present in afferent arterioles, while N- and T-type channels are present in both efferent and afferent arterioles of the renal tissue. The usage of L-/N- or L-/T-type calcium channel blockers exert renoprotective effect by reducing glomerular pressure and proteinuria in chronic kidney disease patients, although they have no effect on systolic or diastolic blood pressure. This reveals the long-term benefits of selective calcium channel blockers (Ando 2013; Thamcharoen et al. 2015). The chronic inflammatory and neuropathic pain are regulated by N-type calcium channels. The potent selective inhibitors such as Ziconotide are useful to compromise the pain in these patients (Miljanich 2004). Thus, a detailed understanding of specific role of different isoforms of calcium channel and their splice variants is essential to develop a potential selective therapeutics.

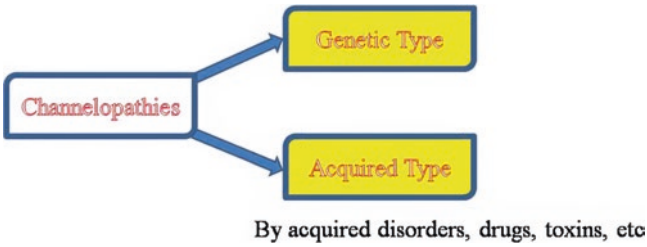

Fig. 5.2 Two main types of channelopathies

5.3 Channelopathies

The dysfunction of ion channels located in the membranes of cells and many cellular organelles leads to a heterogeneous group of disorders, which are known as channelopathies. The field of channelopathies is expanding rapidly in this decade by utilizing current methods and techniques in molecular genetics and electrophysiology (Fig. 5.2) (Catterall and Swanson 2015; Kim 2014). VDCCs are mainly regulating intracellular processes such as contraction, secretion, and neurotransmission and gene expression by mediating calcium influx in response to membrane depolarization. Over the past decade, mutation analysis of VDCCs has increased our understanding of gating functions of VDCCs, and that linked to a heterogeneous group of genetic diseases called "calcium channelopathies" (Dolphin 2006; Fang et al. 2006; Kamatchi et al. 2004; Rajagopal et al. 2008b).

Calcium channelopathies comprise muscular, neurological, cardiac and vision syndromes. Recent investigations suggest that calcium channelopathies occur may be due to electrophysiological defects or abnormalities in $\alpha 1/Ca_V$ subunit protein processing, including folding, posttranslational modifications, quality control and trafficking defects (Biel et al. 2009; Dworakowska and Krzysztof 2000). Taken together, the experimental studies with mutated VDCCs offer a depth understanding of the corresponding human disorders and providing important new insights into VDCCs' function (Table 5.1).

5.4 From VDCCs to Channelopathies

Calcium enters into the cells through VDCCs, a unique route, and thereby controlling a wide variety of physiological processes. The local changes in the membrane potential serve as the primary integrator of numerous ionic channel inputs in excitable cells such as neuronal, muscle cells, etc. The $\alpha 1/Ca_V$ protein has a number of active forms due to alternative splicing in the main subunit of VDCC. In the past decades, mounting number of studies have greatly contributed to identify the key mechanisms that generate multiple forms of VDCC activity (Doyle and Egan 2007; Habermann et al. 2003). The mutations in genes encoding VDCCs have been linked to the development of genetic and non-genetic disorders in human, in particular,

Table 5.1 Dysfunction of ion channels in organ systems

Systems	Diseases	Types of calcium channels involved
Central nervous system [CNS]	Epileptic syndromes	CACNA1H
	Ataxia syndromes	CACNA1A
	Familial hemiplegic migraine	CACNA1A
Peripheral nervous system [PNS]	Pain syndromes and neuropathies	TRPA1
Heart	Long and short QT syndromes	CACNA1C
	Brugada syndromes	CACNA1C
	Catecholaminergic polymorphic ventricular tachycardia (CPVT)	RYR2
Skeletal muscle diseases	Periodic paralysis	CACNA1S

Adapted from (Imbrici et al. 2016)

muscular and neurological abnormalities. These genetic diseases are designated as "calcium channelopathies". The alterations of electrophysiological status of cells are believed to be a primary cause of channelopathy. However, emerging evidences indicate that channelopathy has multiple etiologies of several different aspects of VDCC processing and function can be affected (Jessica and Zivkovic 2011).

5.5 Calcium Channelopathies Associated with L-Type VDCCs

The expression of $Ca_V1.1$ (CACNA1S) and $Ca_V1.4$ (CACNA1F) are restricted to skeletal muscle. The mutations in the skeletal muscular CACNA1S gene (HypoPP1 or HOKPP1) causes hypokalemic periodic paralysis type 1 and that was the first calcium channelopathy found in human. Molecular studies revealed that there are missense mutations of arginine residues within the S4 voltage sensor segment of domains II and IV of CACNA1S (Matthews et al. 2009). These mutations cause a reduction in the density of calcium current and mild changes in the gating properties of CACNA1S in heterologous expression systems (Simms and Zamponi 2014). Another mutation within the $Ca_V1.1$ channel also causes malignant hyperthermia susceptibility type 5 (MHS5).

Identifications of number of mutations in novel calcium channel gene: CACNA1F, which causes genetic incomplete X-linked congenital stationary night blindness type 2 (CSNB2), a recessive nonprogressive retinal disorder due to the defective gene of $Ca_V1.4/\alpha1F$. In fact, CACNA1F gene shares strong homology with other dihydropyridine-sensitive (L-type) calcium channels Ca_V1 proteins. The other missense mutations in CACNA1F would theoretically lead to functional changes, but their impact in biological system yet to be identified (George 2004). A variety of alterations occur in $Ca_V1.4$ channels expressing these missense mutations. Some of these mutations cause an apparent reduction in Ca^{2+} current density (Strom et al.

1998), while some others display altered channel activity despite their expression at the protein level is not significantly altered (Hoda et al. 2005).

Timothy syndrome (TS) is a multi-organ disorder that occurs in children. The characterizations of this disease are severe electrophysiological defects in cardiac system and sudden death, and other important features of this syndrome are syndactyly, immune deficiency, intermittent hypoglycaemia, cognitive abnormalities and autism. This genetic disorder is predominantly due to mutations of $Ca_V1.2$ subunit gene named as CACNA1C gene. TS appears as a sporadic trait in all but one family (Sepp et al. 2017). Recent studies found that de novo missense mutation (G406R and G402S) in exon 8A of the $Ca_V1.2$ subunit of L-type channels (CACNA1C) is responsible for the development of TS (Ornoy et al. 2016) and these mutations cause different clinical features in patients (Kawaida et al. 2016). TS mutations lead to complete dysregulation of L-type calcium channel in different expression systems, resulting in irregularities in inward calcium currents during depolarization. In the heart and brain, exon 8 is the dominant splice variants although 8A also codes for the S6 segment of domain I of L-type channels. In TS condition, there are many phenotypic abnormalities appear due to the lack of channel inactivation, which results in persistent calcium channel activation and increased calcium entry. For example, L-type channel activation is predicted to delay action potential repolarization and consequently to favour long QT syndrome (LQT8), which is observed in TS (Landstrom et al. 2016).

5.6 Channelopathies Associated with Ca_v 2.0 Family

The neuronal P-/Q-type VDCCs are associated with channelopathies, and the mutations which occur in this channel lead to complex multigenic syndromes such as migraine, seizure and ataxia syndromes. CACNA1A is the gene that encodes alternate splice isoforms of the $Ca_V2.1$ subunit, which generates the P- and Q-type channels (Adams et al. 2009). Neuronal and neuroendocrine cells have P-/Q-type channels, and these channels are preferentially located at presynaptic terminals. These channels play a central role in neurotransmitter release, especially for the release of excitatory neurotransmitters, as well as in neuronal excitability of somatodendritic cells (Cao and Tsien 2005).

The "neuronal" VDCCs (or Ca_V2) are encoded by three different genes, which generate N, P/Q and R types of VDCC. However, few mutations in CACNA1A have been identified and linked to disorders associated with these channels. Ophoff et al. found that familial hemiplegic migraine type 1 (FHM1) and episodic ataxia type 2 (EA2) are caused by mutations in these genes (Ophoff et al. 1996). Zuhlke and his colleagues found that human CACNA1A gene possesses a polymorphic CAG repeat (Zühlke et al. 2003) and provides a molecular basis for spinocerebellar ataxia type 6 (SCA6) (Fig. 5.3).

This schematic secondary structure diagram indicates the location of many (but not all) mutations in FHM1 (●), EA2 (○) and SCA6 [CAG]. The mutations leading

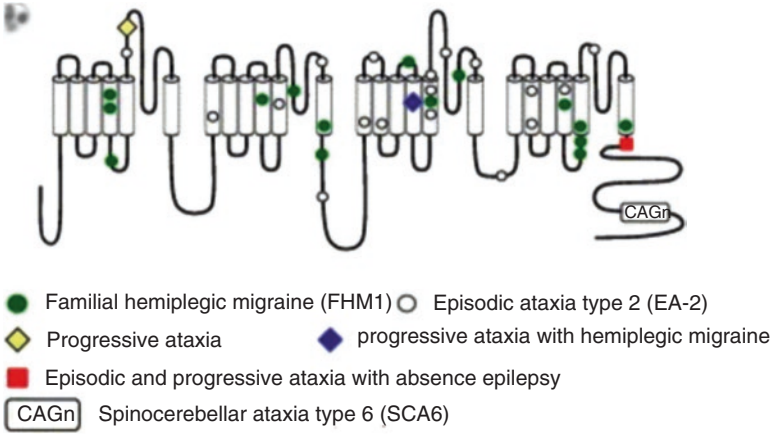

Fig. 5.3 Human spontaneous mutations in the $Ca_V2.1/\alpha1$ subunit of the P-/Q-type calcium channel

to intermediate phenotypes, such as progressive ataxia [◆] and absence epilepsy (■), are mentioned.

Familial hemiplegic migraine type 1 (FHM1) is characterized by recurrent attacks of disabling headache and progressive cerebellar atrophy with a rare autosomal dominant form of migraine with aura. The first missense mutations linked to FHM1 were described by Ophoff et al. (Ophoff et al. 1996), and several others have been reported (up to ten). Taken together, the electrophysiological characterizations of FHM1 mutant $Ca_V2.1$ channels in heterologous expression systems reveal a complex biophysical characteristics of this channel, both in terms of recovery from inactivation (Angelita et al. 2005) and single-channel gating (Wappl et al. 2002). Kaja et al. generated a knock-in mouse mutant bearing the human R192Q CACNA1A mutation and they found that an increase in channel activity due to FHM1 mutations (Kaja et al. 2015). They observed an increase in $Ca_V2.1$ current density and a hyperpolarizing shift in the voltage dependence of the current activation in mutation of R192Q CACNA1A gene in mice cerebellar granule cells. In addition, this mutation enhances the neurotransmitter release and increases susceptibility to cortical spreading depression.

Episodic ataxia type 2 (EA2) is an autosomal dominant paroxysmal cerebellar disorder, which has very unique characterizations such as ataxia, migraine-like symptoms, interictal nystagmus and cerebellar atrophy. In clinical conditions, these symptoms are fully controlled using acetazolamide, a carbonic anhydrase inhibitor. There are more than 20 mutations in CACNA1A channels, which are associated with EA2 occurrence. Among those mutations, the majority of them are nonsense mutations (Yafang et al. 2013), and few others are missense mutations (Wan et al. 2011). The $Ca_V2.1$ proteins are led to truncate due to these nonsense mutations, which would be incorrectly folded. These mutations significantly affect the $Ca_V2.1$ proteins' function. The truncated EA2 mutant produces a dominant negative effect

by significantly decreasing P-/Q-type current, while it is expressed in heterologous system along with wild-type $Ca_V2.1$ protein (Schorge and Rajakulendran 2012). This may be due to either defective biosynthetic processing or sequestering of the regulatory β subunits. The other mechanisms such as unfolded protein response could lead to the accumulation of misfolded EA2 mutants in the endoplasmic reticulum (ER) and trigger an ER-mediated translation inhibition (Isabelle et al. 2006). These "suicide subunits" may be a hallmark of the various P/Q-type channel defects in EA2. The phenotypes lethargic, ducky and stargazer are linked to mutations in the regulatory subunits, β4, α2δ2 and γ2, respectively. The neuronal channelopathies linked to CACNA1A mutations, migraine, ataxia and seizures present significant comorbidity and suggest shared pathophysiological mechanisms (Verrottia et al. 2011).

5.7 $Ca_v3.0$ Family Association with Channelopathies

Autism spectrum disorder (ASD) is the name for a group of developmental disorders, and the study of molecular mechanisms of ASD has not yet been discovered clear. Recent functional studies of T-type channel ($Ca_V3.2$) mutations and other discoveries linked to ASD indicate that uncommon calcium signalling due to mutations in VDCCs could contribute to the development of ASD.

The P-/Q-type and T-type channels are very important contributors to seizure genesis, which is one of the most prominent neurological disorders (Marino et al. 2010). The VDCC mutations provide exciting molecular tools to elucidate the contribution of various VDCCs to normal physiology and development of pathological conditions. It is well known that the mutant VDCCs cause a wide range of monogenic diseases, which indicate that they are likely to be an important factor to polygenic diseases, in particular, in neurological disorders. Indeed VDCC mutations are now implicated in some complex multigenic neuropsychiatric disorders, such as ASD (Breitenkamp et al. 2015). Although these calcium channel genes probably reflect only a minority of the relevant etiological candidates in neurological disorders, they warrant special interest as they represent convenient drug targets for novel therapeutics.

5.8 Protein Kinase C (PKC) Modulation on Calcium Channel's Physiology

The PKC isozymes play a fundamental role in the insertion of different ion channels to cell membrane in response to different agonist. The selectivity of PKC isozymes to target sites on different ion channels indicate the possibility of modifying the action of specific members of the calcium channel family using more specific PKC isozyme inhibitors (Rajagopal et al. 2008a). Studies using mutated PKC βII found that it is involved in phosphorylation of C-terminal sites of α1 2.2 subunits. Both PKCβII and PKCε can modulate the stimulatory Thr-422 in the I–II linker of Ca

channels, but PKCε is better at regulating the inhibitory Ser-425 site. The weak effects of PKCβII and PKCα on the inhibitory site could be due to their indirect effects on PKCε function. Hence, the differential activation of PKC isozymes could selectively regulate the activities of different members of Ca channel family. Even within one Ca channel type, the different combination of activated PKC isozymes could allow the graded levels of activation or inhibition and susceptibility or resistance of the channel to subsequent stimulatory events (Rajagopal et al. 2009).

Ca_v channel subunits especially $Ca_v\beta$ belong to the guanylate kinase family, and this subunit contributes scaffolding multiple signalling pathways around the channel. The tridimensional structure of this subunit supports the above statement as it has large space for the interaction with putative partners. PKCα may be one such partner. Insulin secretion is the result of interaction between $Ca_v\alpha1$ subunits, their Ser/Thr sites and $Ca_v\beta$ and PKC isozymes. The identification of the roles of these components will not only be significant for the understanding of the intricacies of the insulin secretion but also for the ion channel physiology (Rajagopal et al. 2014).

5.9 Future Perspectives

Available information about calcium channelopathies has yielded an important pattern which includes (Adams et al. 2009) how the interaction between calcium channel subunit occurs (Adams and Snutch 2007) and how the functional loss or gain of ca channel activity could occur. The developments in the last two decades reveal that the large phenotypic variability due to mutations in calcium channel genes does not simply reflect electrophysiology-related gain or loss of channel activities; it causes more subtle variations in the biophysical parameters. In addition, the identification of the role of VDCC's subunits will not only be significant for the understanding of the intricacies of the channelopathies but also essential for the ion channel physiology.

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Channelopathies: Application of Natural Products Using Nanotechnology

6

Abstract

Ca^{2+} channels have fundamental role in numerous physiological functions, by regulating intracellular Ca^{2+} homeostasis, in all organs and tissues including the heart, muscle and brain. There are different types of Ca^{2+}channels, which mediate specific cellular functions in these tissues depending on their sensitivity to Ca^{2+} gradient. They have critical role in many pathological conditions such as hypertension, neurodegenerative diseases, pain, muscle dysfunctions, etc. Thus, targeting Ca^{2+}channels gives great relief from many disorders. There are many naturally available Ca^{2+} blockers/agonists, which have been demonstrated for their effectiveness in experimental system, and some of them have been under clinical trial for various pathological conditions. However, their clinical effectiveness is mainly hindered by poor pharmacokinetics and low bioavailability at target sites. Nanotechnology-based delivery systems offer a promising solution for the above-mentioned problems. The Food and Drug Administration (FDA) has approved several nanomedicines to use them as a first line of therapeutics, and many of them are under consideration. The nanosystem-based therapeutic strategies could help to improve the efficiency of drugs in treating channelopathies, and it strengthens the efforts of the translation of natural products to utilize them in clinical application.

Keywords

Calcium channels • Nanocarriers • Nanomedicine • Natural products • Phytomedicine

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6.1 Introduction

Calcium is an integral part of many physiological functions including muscle contraction, regulation of heartbeat, neurotransmission, cell division, immune functions and saliva production. It contributes to the production and activities of hormones involved in digestion and energy and fat metabolism. The Ca^{2+}-dependent system is vital for the transportation of nutrients and other substances across cell membranes (Abrams 2007; Houston and Harper 2008; Peacock 2010). Thus, a tight regulation of Ca^{2+} level in both intracellular and extracellular milieus is important for proper functioning of organ and tissues. The calcium channels' structure and its functions are closely associated. Any variation in channel structure due to genetic defect or pathological insults results in malfunctioning of those channels, and it is linked to the development and progression of many disorders. The disease associated with dysfunction of such channel system is termed as channelopathy, and the study of diseases associated with channel defect is called as channelomic studies. The channelomic study provides potential therapeutic agents by examining their binding affinities with calcium channels and helps to screen their effectiveness at channelopathies (Camerino et al. 2007; Doyle et al. 1998; Waxman 2007).

The advancement of screening and validation techniques has offered a scientific evidence for the use of various plants and marine products as well as bioactive chemical constituents from them as therapeutics for various disorders and diseases. Globally, the people have great interest on plants and other natural product-based therapeutics because they have fewer side effects and are cost-effective and mostly available from their dietary sources. The molecular framework of natural products is an inspiration for synthesis of new feasible compounds, and they provide an innovative solution to address important challenges in drug designing. In fact, the majority of therapeutic drugs currently available in market are natural products (secondary metabolites) or derivatives of natural compounds from plant, microbes or marine species. Quinine from cinchona bark (antimalarial), paclitaxel from Pacific yew tree (anticancer), plitidepsin from the Mediterranean tunicate (anticancer), ziconotide – a peptide toxin from cone snails (analgesic) – and apomorphine, a derivative of morphine from poppy seeds (Parkinson's disease), are fewer examples for natural product-based drugs (Dias et al. 2012). The pleiotropic action of many natural products such as omega-3 PUFA, curcumin, resveratrol and α-lipoic acid on various signalling molecules and receptors lead to the study of their clinical values, and extensive clinical trials are conducted with these compounds. In addition, the synergistic effect of natural products with pharmaceutical drugs is providing encouraging outcomes in clinical trials, to utilize them in clinical settings (Bulaj et al. 2016). The advancement of pharmacological sciences such as smart screening methods, advanced separation methods, structural analysis and metabolic engineering and synthetic biology has offered robust system for the drug discovery from natural sources (Table 6.1).

Table 6.1 Diet sources for calcium

Types of food	Sources	Consumption of calcium per serving
Vegetables	Green leafy	200 mg
	Collard greens	325 mg
	Bok choy	82 mg per 1 ounce
	Beet greens	86 mg per ½ cup
	Almonds	90 mg per ½ cup
	Pinto beans	96 mg per ½ cup
	Kale	102 mg per ½ cup
	White beans	106 mg per ½ cup
	Tahitian taro root	122 mg per ½ cup
	Cowpeas	116 mg per ½ cup
	Nopales	112 mg per ½ cup
	Spinach	123 mg per ½ cup
	Turnip greens	125 mg per ½ cup
	Edamame	131 mg per ½ cup
	Amaranth leaves	138 mg per ½ cup
	Broccoli	200 mg
	Mustard green	142 mg per ½ cup
	Collard greens	188 mg per ½ cup
	Tempeh	184 mg per one cup
	Stinging nettles	214 mg per ½ cup
	Sesame seeds	273 mg per 1 ounce
	Tofu	861 mg per ½ cup
Sea food	Fish	125–325 mg
	Sardines	
	Salmon	
	Shrimp	
Dairy	Skimmed ricotta	300–355 mg
	Low-fat plain yogurt	
	Milk	

6.2 Commonly Recommended Calcium Supplements

1. Calcium carbonate is the least expensive and has the most elemental calcium.
2. Calcium citrate is the most easily absorbed.
3. Calcium phosphate is also easily absorbed and does not cause constipation.
4. Calcium supplements are available in liquid, tablet and chewable forms.
5. It is important to note that some medications could interact negatively with calcium supplements. These medications include blood pressure beta-blockers like atenolol, which may increase the amount of aluminium absorbed into the blood;

Table 6.2 |According to the National Institutes of Health (NIH), the daily allowances are:

Age group	Stage	Daily recommended dietary allowance (RDA)
0–6 months	Children	200 mg
7–12 months	Children	260 mg
1–3 years	Children	700 mg
4–8 years	Children	1000 mg
9–18 years	Teenagers	1300 mg

cholesterol-lowering bile acid sequestrants such as colestipol, which may increase the loss of calcium in the urine; and oestrogen medications, which can contribute to an increase in calcium blood levels (Table. 6.2).

6.3 Natural Products as Calcium Channel Modulators

Many naturally available agents can modulate the pathological alterations of calcium homeostasis. In particularly, they act on calcium channels to control the influx/efflux of Ca^{2+} ion. Mounting number of experimental studies demonstrates the potential use of Ca^{2+} channel modulators in various Ca^{2+} dysregulation-associated disorders. The edible plants like Indian mulberry (*Morinda citrifolia*) (Gilani et al. 2010) and turpeth (*Operculina turpethum L.*) (Sharma et al. 2016) can inhibit Ca^{2+} movement by blocking VDCC, which is linked to their anti-colic, antidiarrheal and antihypertensive effects. The phytochemicals from abundantly available plant sources, such as vincarosine (Wu et al. 2014), quercetin (Hou et al. 2014), hesperetin (Liu et al. 2016) and vanillin, and its analogs exert antihypertensive effect by promoting vascular relaxation through antagonizing L-type Ca^{2+} channel and inhibition of Ca^{2+} influx. Similarly, resveratrol can block L- and T-type Ca^{2+} channel, and this blockage favours insulin secretion from native pancreatic β cells (Jakab et al. 2008; Raffai et al. 2015). The inhibition of L-type Ca^{2+} is beneficial to attenuate vasoconstriction in aorta of the heart. A phytoactive compound from the heartwood of *Rhus verniciflua* stokes (fisetin) improves blood circulation by attenuating aorta constriction through a mechanism of inhibition of L-type Ca^{2+} channel-mediated extracellular Ca^{2+} entry as well as store-operated Ca^{2+} entry (Park 2014). Similarly, the conotoxins from marine cone snail are effective for the chronic pain due to their inhibitory action on voltage-gated ion channels. A synthetic form of conotoxin MVIIA (ziconotide), an N-type VDCC blocker, is approved by FDA to treat patients with chronic neurological pain (Sakai et al. 2011). The increased expression/activation of Ca^{2} release-activated Ca^{2+} channels (CRAC) is one of the mechanisms responsible for the increase of intracellular Ca^{2+} in immune cells that causes inflammatory response by activating immune cells. The dietary phytoactive compounds, curcumin and caffeic acid phenethyl ester, attenuate CRAC current by blocking expression of Orai and STIM proteins, which are key molecular components of CRAC (Shin et al. 2012). In chronic inflammatory disease like Behcet's disease, hyperforin from St. John's wort can attenuate the development and

progression of BD by blocking cytosolic increase of Ca^{2+} through VDCC and TRPM2 channels in neutrophil, which is the mechanism of Ca^{2+}-dependent neutrophil activation (Nazıroğlu et al. 2014). Some natural constituents target non-selective Ca^{2+}-permeable cation channels. The naturally available chemical constituents such as α-fatty acid, gallic acid and resveratrol can block transient receptor potential (TPR) channels. Galangin is a natural flavanol from ginger family that selectively inhibits the Ca^{2+} influx through inhibition of a C subfamily member of TRP (TPRC5). Similarly, kaempeferol and quercetin can also block TPRC5 (Naylor et al. 2016). As the TRPC5 is closely associated with many pathological conditions including epilepsy, seizure, cancer (metastasis) and proteinuric kidney disease (Phelan et al. 2013; Schaldecker et al. 2013), these naturally available TRPC5 blockers are useful to treat these disorders.

In opposite to this, the activation of Ca^{2+} is one of the effective strategies to alleviate the disorders such as cancer, atherosclerosis and gastro-oesophageal reflux disease (GERD). The natural phenolic compounds such as ellagic acid (Liang et al. 2016), caffeic acid (Chang et al. 2013), thymol (Hsu et al. 2011) and esculentin (Chang et al. 2016) increase intracellular Ca^{2+} level by stimulating intracellular Ca^{2+} stores, which promotes cell death in malignant cells. Similarly, the flavonoids such as apigenin and genistein (isoflavone compound) act as anticancer drugs due to their nature of stimulating TRPV4 and/or TRPC5, which is one of the strategies of chemoprevention (Naylor et al. 2016). Quercetin and myricetin act as a vasorelaxant by activating L-type Ca^{2+} channel in vascular smooth muscle cells of the coronary artery. This property of these two compounds provides a vital support to their antiatherogenic activity by eliciting coronary dilation without affecting the heart contractility and relaxation (Fusi et al. 2005). In addition, L-type channel-mediated influx of Ca^{2+} and activation of calmodulin are required for the contractile function of a variety of smooth muscles including oesophageal (ESO) and lower oesophageal sphincter (LES). A defect in this channel operation is associated with the development of GERD. Bombesin (a peptide) from amphibian skin has a potential therapeutic value in GERD patients due to its positive effect on L-type VDCC-mediated Ca^{2+} influx (Tsai et al. 2015). Many naturally available compounds have proved their efficacy in experimental conditions to use them as a Ca^{2+} channel activator/inhibitor (Table 6.3). Despite a great improvement is made in the screening, discovery and identification of natural product-based drugs, the major obstacle for translating those bioactive natural products into a clinically useful therapeutic agent is their low bioavailability, difficulties to reach target sites and loss of efficacy in biological metabolism (Atanasova et al. 2015; Patwardhan and Vaidya 2010; Potterat and Hamburger 2006). For example, Prialt (conotoxin peptide, ziconotide) is an FDA-approved analgesic drug (N-type Ca^{2+} blocker) that must be administered intrathecally to the spinal cord due to their inability to cross blood-brain barrier. This limits its potential use in patients. Thus, an effective strategy that can bypass these limitations is required to achieve maximum/optimum response without off-target effects of natural products. It is widely accepted that combining phytomedicine with nanotechnology can yield a significant benefit. In recent years, the use of nanotechnology has shown immense success in the field of drug delivery (Jain et al. 2015).

Table 6.3 List of some potential naturally occurring Ca^{2+} channel modulators

Type of Ca^{2+} channel	Bioactive compound names	Function
Ca^{2+}channel blockers		
L-type channel	Vincarosine, quercetin, hesperetin, vanillin	Antihypertensive
T-type channel	Resveratrol	Hormone (insulin) secretion
N-type channel	Conotoxin MVIIA	Neurological pain
Transient receptor potential (TPR) channels	Galangin, kaempeferol, quercetin	Epilepsy, seizure, cancer (metastasis) and proteinuric kidney disease
Ca^2 release-activated Ca^{2+} channels (CRAC)	Curcumin and caffeic acid phenethyl esters	Anti-inflammation
TRPM2	Hyperforin from St. John's wort	Neutrophil inactivation in Behcet's disease
Ca^{2+}channel activators		
Intracellular Ca^{2+} store efflux channels	Ellagic acid, caffeic acid, thymol, esculentin	Anticancer
TRPV4/TRPC5	Apigenin, genistein	Anticancer
L-type channel in aorta	Quercetin, myricetin	Vasorelaxant and anti-atherogenesis
L-type channel in oesophagus	Bombesin from amphibian skin	Improve oesophagus and LES function in GERD

6.4 Nanosystem-Based Therapy

Nanotechnology is the greatest revolutionary advancement of the modern era that has made a significant positive impact in many fields including medicine. In recent years, the nanosized therapeutics has been used for the treatment of diabetes, allergy, cancer and neurodegenerative diseases. The nanosystem-based therapeutics has several advantages such as lower dose, improved activity and site targeted action with greater bioavailability. Moreover, the field of nanotechnological systems has developed several delivery systems with highly biocompatible, non-immunologic and higher degree of biodegradability (Bonifacio et al. 2014). In particular, the growing interest of utilizing nanodelivery system in phytomedicine is due to improved stability, pharmacokinetics and enhanced intracellular penetration observed in many experimental systems. The bioconjugation of stevioside (FDA-approved natural antidiabetic medicine) with biodegradable pluronic F-68-based PLA nanoparticles remarkably improved its intestinal absorption and bioavailability (Barwal et al. 2013). Likewise, the nanoformulation or nanosystem-mediated delivery of curcumin and quercetin has showed the powerful benefits of nanomedicine, by improvement in their pharmacokinetics and increased bioavailability, and all the above nanoparticle-guided specific binding and internalization into targeted tumour cells (Nam and Lee 2016; Yallapu et al. 2012). Currently, several clinical trials are underway with phytochemicals loaded with nanodelivery systems, which shows the feasibility of this method, and providing hope to utilize this method for the natural product-based therapeutics (Bonifacio et al. 2014; Ganesan et al. 2017).

6.5 Nanodelivery System

The successful and efficient delivery of plants extracts and bioactive compounds using nanodelivery system mainly depends on choosing a correct carrier, which depends on the nature of phytochemicals, route of administration and type of disease and target location. Some type of nanosized delivery system can easily disperse and release in a free form by their soluble nature (hydrophilic) in the biological fluids. Although some of them are non-soluble (hydrophobic) in biological fluids, they deliver the drugs at cellular/subcellular levels. The nanodelivery system is basically categorized as polymeric nanoparticles (hydrophilic, hydrophobic or mixed type) or lipid-based nanoparticles (hydrophobic). There are different types of nanomaterials (natural, synthetic or semisynthetic) are used to prepare nanocarriers, and the nanomaterials have their advantages and certain limitations in the biological system. The nanoparticles release the therapeutic agents by three main mechanisms: (1) the hydration-induced swelling of nanocarrier polymers and followed by diffusion of the drug, (2) the dissociation of therapeutic agent from the polymer-drug complex by swelling of nanoparticles and changes in their structure in biological fluids and (3) the release of therapeutic agents from central core mediated by enzymatic cleavage or degradation of nanocarrier at the delivery site (Han et al. 2015; Paliwal et al. 2014).

The lipid-based nanocarriers are spherical vesicles that composed of one or more lipid bilayer structures covering an aqueous core. Liposome is a lipid-based hydrophobic carrier that is mainly composed of phospholipid and cholesterol. One of the prominent features of liposome is self-assembly. They form bilayer membranes to enclose an aqueous core in which hydrophilic therapeutic agents can be incorporated. In addition, liposomes are also useful to deliver hydrophobic compounds. Based on its size, liposomes are classified into small unilamellar vesicles, large unilamellar vesicles, multilamellar vesicles and lamellarity. Liposomes mainly protect encapsulated drugs from degradation by biological fluids such as saliva. Recently, phytosome is developed to deliver phytoconstituents (plant extracts and phytochemicals), which vastly improved their absorption and bioavailability. It is composed of phospholipids incorporated with phytoconstituents and produces a lipid compatible molecular complex. Phytosomes have advantage over liposomes due to their distinct feature of each phospholipid molecule bound with therapeutic agent, while there is no chemical bond between lipids (phosphatidylcholine) and phytoconstituents in liposomes (Ajazuddin 2010; Isacchi et al. 2017). Given the fact that majority of bioactive phytoconstituents are water-soluble molecules, the phytosomes are efficient delivery system for phytoactive constituents as well as plant extracts. The polymeric nanocarriers are submicron-sized vesicles used to transport materials.

The nanospheres and nanocapsules are shell-like structures constituting polymeric matrix and polymeric wall, respectively, which have loaded with drugs in the inner space. This type of system is useful for protecting drugs from the surrounding environment as well as controlled release of drug. They differ in the way of holding the ingredients. In nanosphere, the phytoconstituents are dispersed throughout the

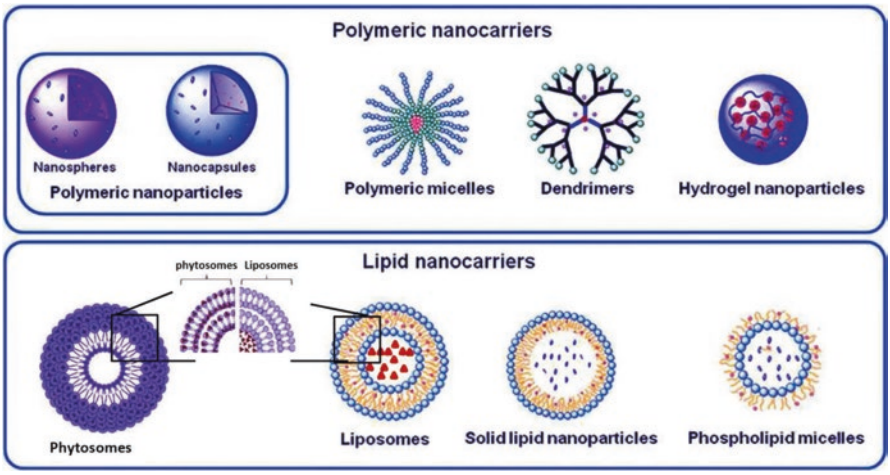

Fig. 6.1 Diagrammatic structures of most common nanocarriers for drug delivery (Adapted and Modified from Conniot et al. 2014)

particles, while therapeutic agents are at the centre of the core surrounded by polymeric membrane (Isacchi et al. 2017). Dendrimer is another type of nanostructure, which is a highly branched polymer with a well-organized three-dimensional structure enclosing a central core. Among different nanocarrier system, dendrimer is considered as the most versatile form due to its capability to accommodate >100 terminal groups. In this, the surface possesses hydrophilic nature, and the central core has hydrophobic property. Micelle is a self-assembling carrier generally used to deliver hydrophobic therapeutic agents. It is generated by conglomeration of amphiphilic molecules such as cholesterol. In aqueous environments, the hydrophilic groups are in outside, and hydrophobic groups are assembled inside the shell structure. A reverse occurs in a nonaqueous environment. Micelles can accommodate only hydrophobic drugs in the central core, and they are useful for intravenous administration of hydrophobic drugs (Ajazuddin 2010). In recent years, hydrogel nanoparticles show most promising effect in drug delivery system due to their high hydrophilicity. The naturally available chitosan and alginate can be used to prepare hydrogel nanoparticles. In addition, the synthetic hydrogel carrier polyvinyl alcohol, polyethylene oxide, poly-ethyleneimine, etc. are used depending on the characteristic features of the drug (Dalby et al. 2013) (Fig. 6.1).

6.6 Methods of Preparation of Nanotherapeutics

The selection of nanocarrier material and the method of nanoparticle preparation enormously depends on the physiochemical properties of the drug and the therapeutic target location in the biological system. In the preparation of nanocarriers for therapeutic applications, the biodegradable synthetic polyesters or natural polymers

are used. The materials used for the nanocarrier should be biocompatible in terms of being non-toxic, non-allergic, non-carcinogenic and easily biodegradable. The most widely used natural polymeric substances are chitosan, gelatin, sodium alginate and albumin. The phospholipids and phosphatidylcholine are main composition of lipid-based carriers. Apart from them, many biodegradable synthetic polymers such as polylactide (PLA), polylactide-co-glycolide (PLGA), poly-e-caprolactone (PCL) and poly(alkyl cyanoacrylate) (PACA) are used for the nanoparticle preparation (Ganesan et al. 2017; Isacchi et al. 2017; Paliwal et al. 2014). The functionalization of nanoparticles is important to gain the full efficiency of nanosized delivery system. Some polymers required copolymerization with polyethylene glycol (PEG) or thermo-reversible gelatin polymers (TGPS) to avoid the recognition and destruction by mononuclear phagocytic system. It also enhances their half-time in circulation. Similarly, tagging of nanoparticles with target-recognizing molecules such as folic acid is much efficient for targeted delivery. In addition, tagging with specific target-based antibodies or antigens helps the nanocarriers to reach target sites (Conniot et al. 2014; Paliwal et al. 2014).

Generally, the methods like high-pressure homogenization, complex coacervation, coprecipitation, salting out, nanoprecipitation or solvent displacement, solvent emulsification-diffusion method, supercritical fluid method and self-assembly method are most commonly used to prepare the nanosystem-based therapeutics. In high-pressure homogenization method, a very high shear stress due to the samples pushed with a pressure results in the disruption of particles and producing in a size of nanometre range. This method is more consistent to produce nanostructured lipid carriers, in particular, to generate lipid drug conjugate, solid lipid nanoparticles and nanoemulsions in a large scale. The homogenization is carried out in hot or cold temperature, which depends on the solubility of the drugs. For lipophilic drugs, homogenization is carried out in a temperature above the melting point of lipids. In melted form, the drug-loaded phospholipid and aqueous phase are mixed at a high shear pressure and continuously kept at the same temperature until the drug is completely loaded. Then, the lipid nanoparticles are formed by cooling them to room temperature. This method is unsuitable for temperature-sensitive phytoactive constituents. So, cold homogenization is another approach for the lipid nanoparticle preparation. In this method, a high-pressure grinding of lipid with active constituents/drugs generates a transient heat, which melts the lipids, forming lipid microparticles by quickly solidification. The resulting microparticles are converted into nanoparticle by further homogenization in a high-speed stirrer under cold (below room temperature) aqueous surfactant environment. In both hot and cold methods, temperature formed during emulsification, pressure, homogenizing instrument and time length are important factors that affect the nanoparticle size and quality (Isacchi et al. 2017; Paliwal et al. 2014).

In complex coacervation method, the nanoparticle is prepared on the basis of interaction of two oppositely charged polyelectrolytes in an aqueous phase. In this method, the emulsion of carriers is prepared in volatile solvents by a high-speed homogenization or ultrasonication, and then the emulsion of polymers is subjected to evaporation, under reduced pressure or continuous stirring at room temperature,

to get the polymeric nanoparticles. Then the nanoparticles are purified by suspending them in distilled water and ultracentrifugation. A simple coacervation can be done by using electrolyte system (sodium alginate-$CaCl_2$) and a water-miscible non-solvent or dehydration agents (gelatin-isopropanol sodium sulfonate) (Mora-Rodríguez et al. 2015; Reis et al. 2006). Co-precipitation is a modified method of complex coacervation, which offers a great stability to drugs with poor water solubility properties. This method is mainly used to prepare core-shell particles in nanosize (Isacchi et al. 2017). By using the technique like emulsion-diffusion method, it is possible to prepare nanoparticles of both lipophilic and hydrophilic phytoactive substances. For lipophilic active components, the polymer of nanocarrier is dispersed in organic solvent phase along with target compound, oil and water. In this method, the organic phase containing polymeric material and active constituent is emulsified with water (aqueous phase) by vigorous agitation, and then continuous addition of water at slower rate leads to the diffusion of solvents from nanovesicles to external space, resulting in a nanocapsule with active drug substances (Mora-Rodríguez et al. 2015). The nanoprecipitation or solvent displacement method of nanocapsule preparation needs both solvent (organic) and non-solvent (aqueous) phases of sample treatment. In this method, the nanocapsular structure is obtained as a colloidal suspension by slowly adding the solvent mixed nanocarriers to the aqueous phase with moderate stirring. In this method, ethanol, acetone or hexane is mostly used as a solvent, and water is used as an aqueous phase (Mora-Rodríguez et al. 2015). In supercritical fluid method, a nanosized phytoconstituents are prepared by forcing them with a liquid or gas (mostly water or carbon dioxide) above its thermodynamic critical point of pressure and temperature. This method is advantageous than others due to mild temperature and avoidance of organic solvent system in the preparation process (Paliwal et al. 2014). In self-assembly method, the components and polymers organize into a nanostructure based on their physical and chemical properties without any external forcing techniques (Isacchi et al. 2017).

6.7 Nanosized Ca^{2+} Channel Blockers from Phytomedicine: Current Status

Ca^{2+} channel dysregulation is the fundamental mechanism of many disorders ranging from food allergy to neurodegenerative diseases like AZ and PD. One of the major challenges in the treatment of disorders associated with Ca^{2+} dysregulation is its ubiquitous distribution in all type of cells and tissues. Thus, specifically targeting Ca^{2+} channels of pathological locations and reaching the target site without any hindrance are crucial for the effective therapy. The nanosystem-based therapeutics has offered a solution to overcome all these hurdles. The recent advancement of nanotechnology in the field of medicine greatly improved the nanocarrier system in several aspects including crossing over blood-brain barrier (BBB) and doing their function without eliciting any unwanted response. In particular, the trespassing ability of nanocarriers is much helpful to treat a majority of Ca^{2+} dyshomeostasis-associated disorders of CNS. Currently, several studies demonstrate the incredible

efficiency of therapeutic agent-loaded nanoparticles in Ca^{2+}-associated pathological conditions. S100A1 is a Ca^{2+}-binding protein, which is under clinical trial for therapy in HF patients. S100A1 can prevent loss of cardiomyocyte contractile and cell death by blocking spontaneous opening of RyR2 and increase of intracellular Ca^{2+}. However, the major obstacle to use S100A1 in clinical setting is its inability to cross the plasma membrane of cardiomyocytes. Recent identification of N-acetylglucosamine (N-GlcNac)-coated polyketal nanoparticles has provided solution for this by specifically internalizing S100A1 into cardiomyocytes. In this, N-GlcNac has played a vital role to specifically guiding nanoparticles to bind with CM (Maxwell et al. 2015). Similarly, the chemotherapeutic failure due to emergence of multidrug resistance phenotype in cancer cell can be conquered by iron oxide nanoparticle (IONP)-mediated delivery of verapamil, a Ca^{2+} channel blocker along with cancer chemotherapeutics (Mahmood et al. 2014). Lomerizine is an L- and T-type Ca^{2+} channel blocker that protects secondary neurodegeneration caused by neurotraumatic injury. But its insoluble nature in physiological fluids is the major limitation of its therapeutic value. In experimental study, poly(glycidyl methacrylate) nanosphere-mediated intracellular delivery of lomerizine remarkably improved its therapeutic function (Benjamin et al. 2014).

Recently, extensive researches have improved the therapeutic efficacy of phytoactive compounds by integrating nanotechnology, which also provided some promising strategy to deliver the Ca^{2+} blockers from natural sources. Curcumin is an L-type Ca^{2+} blocker who's circulating longevity and efficiency are increased by nanoformulation with liposomes and phytosomes as well as encapsulation by solid lipid nanoparticles (Ajazuddin 2010). In particular, PLGA-based curcumin nanoformulation has a significant therapeutic value in a variety of disorders including AZ, PD and inflammatory diseases (Yallapu et al. 2012). Quercetin has a dual role in L-type Ca^{2+} channel activity in aorta and vascular smooth muscle cells that shows vasorelaxant activity. The nanoprocessing methods such as nano-emulsification, phytosome formation and liposomal loading highly increase its therapeutic activity (Ajazuddin 2010). The bioavailability, cellular internalization and therapeutic potential of resveratrol, a T-type Ca^{2+} channel, are remarkably increased by nanopreparation using polymers of poly(epsilon-caprolactone) or poly(D,L-lactic-co-glycolic acid)-poly(ethylene glycol) (Bilia et al. 2017). Apigenin acts as an activator of TRPV4 and TRPC5 channels. Its bioavailability is reduced by its low water and lipid-soluble nature. However, the solubility can be improved by solid dispersion with carbon nanopowder, which is an inert nanomaterial with large surface area that has the capability to reduce aggregation (Watkins et al. 2015). Likewise, loading of ellagic acid and eugenol in PLGA nanoparticle increased their therapeutic potential by increasing bioavailability and controlling their release (Watkins et al. 2015). A natural polysaccharide (chitosan)-based nanoparticle is a promising nanocarrier for vanillin, which acts as an L-type Ca^{2+} channel blocker with antihypertensive activity (Li et al. 2016).

6.8 Summary

Despite very few studies are available to demonstrate the advantage of integrating nanotechnology methods to process naturally available Ca^{2+} modulators, these reports together with vast number of other studies in various disease models provide an undoubtful trust to utilize nanomedicine methods to improve the effects of phytoactive drugs. In addition, the intense growth and advancement of nanotherapeutics encourage the identification/development of new drugs from various natural sources to alleviate Ca^{2+}-associated disorders.

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