Review

Elucidation of the Physiological Functions of Membrane Proteins as Novel Drug Target Candidate Molecules

Atsuhiko Ichimura

Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University; Kyoto 606–8501, Japan.
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For pharmaceutical research focused on identifying novel drug target candidate molecules, it is essential to explore unknown biological phenomena, elucidate underlying molecular mechanisms and regulate biological processes based on these findings. Proteins expressed on the plasma membrane and endoplasmic reticulum (ER) membrane play important roles in linking extracellular environmental information to intracellular processes. Stimulating membranous proteins induces various kinds of changes in cells, such as alterations in gene expression levels and enzymatic activities. However, the physiological functions and endogenous ligands of many G-protein-coupled receptors (GPCRs) have not been determined, although GPCRs already constitute a large class of drug-target membrane proteins. Furthermore, the precise physiological roles played by many ER membrane proteins have not been elucidated to date. In this review article, I summarize the results of our recent studies, including the observations that the lipid sensor FFAR4/GPR120 controlled systemic energy homeostasis and that the ER membrane monovalent cation channel trimeric intracellular cation (TRIC)-B and the plasma membrane divalent cation channel transient receptor potential melastatin 7 (TRPM7) regulated bone formation. I further describe the therapeutic significance of these membranous protein-related biological processes.

Key words  G-protein-coupled receptor; free fatty acid; ion channel; intracellular Ca\(^2+\); bone formation; chondrocyte

1. INTRODUCTION

Many proteins are located on the cell surface membrane and on the endoplasmic/sarcoplasmic reticulum (ER/SR) membrane, which transduce extracellular signals into changes in key physiological processes. Membrane proteins, including G-protein-coupled receptors (GPCRs) and ion channels, sense various chemical and physical changes in the surrounding environment and trigger intracellular signals to adapt to the environment. Since dysfunction of these membrane proteins compromises cell functions and causes various diseases, 1,2 functional analysis of membrane proteins is useful for the development of novel drugs. Elucidating the pathophysiological mechanisms regulated by membrane proteins is essential for the development of new therapies.

GPCRs are also called seven-transmembrane receptors based on their structure in the cell membrane. In eukaryotes, GPCRs are the largest family of signaling receptors, including approximately 800 kinds of GPCRs in humans. 3,4 Activated by external signals through coupling to different classes of heterotrimeric (G\(\alpha\beta\gamma\)) guanosine 5'-triphosphate (GTP)-binding proteins (G proteins) and/or arrestins, GPCRs mediate intracellular signaling cascades and thus modulate cell and tissue physiology. GPCRs have been implicated in many diseases, such as cancer, depression, and type 2 diabetes. Hence, these receptors present a wide range of therapeutic targets for various diseases. 5,6 Of the approx. 800 human GPCRs, approximately 350 different types of non-olfactory GPCRs have been characterized in the human genome. 7,8 Although at least 165 of these GPCRs have been validated as drug targets, the endogenous ligands of approx. 140 GPCRs remain unidentified. 9 These GPCRs are known as orphan GPCRs, which may represent an abundant source of drug targets.

Ion channels are also distributed on the plasma and endoplasmic/sarcoplasmic reticulum membrane. The human genome encodes more than 200 pore-forming subunits of plasma membrane ion channels. 10,11 Ion channels selectively and rapidly transport ions, such as Ca\(^2+\), K\(^+\), and Na\(^+\), across biological membranes in response to various stimuli and thus contribute to intracellular ion homeostasis to maintain cellular functions. Since ion channels regulate important biological processes, such as action potential firing, membrane potential, and mitochondrial function, dysfunctions and/or altered expression of ion channels lead to many diseases, such as cancer, neural disorders, musculoskeletal disorder, cardiovascular diseases and kidney diseases. 1,2,5,6,14 Therefore, ion channels also represent attractive drug target candidates; approx. 19% of current U.S. Food and Drug Administration (FDA)-approved drugs are ion channel modulators. 15,16 Hence, the identification of novel molecular mechanisms regulated by ion channels might contribute to the discovery and development of new drugs.

In this review article, I describe the physiological and pathophysiological functions and underlying molecular mechanisms by which GPCRs sense free fatty acids (FFAs) to modulate systemic energy homeostasis, and I outline the novel functions of ion channels controlling bone mineralization and outgrowth. Given recent important advances in this field, I further discuss the therapeutic potential of chemical modula-

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e-mail: ichimura.atsuhiko.2r@kyoto-u.ac.jp
tors targeting these membrane proteins.

2. FFA RECEPTOR GPR120 AND ENERGY HOMEOSTASIS

Since several groups previously reported a series of GPCRs whose endogenous ligands are FFAs, FFAs have been recognized not only as essential energy sources but also as signaling molecules. Currently, there are four FFA receptors (FFARs), specifically two long-chain fatty acid receptors (GPR40/FFAR1 and GPR120/FFAR4) and two short-chain fatty acid receptors (GPR41/FFAR3 and GPR43/FFAR2). Many studies have clearly indicated that FFARs mediate various biological processes and therefore play important roles in physiological functions, such as peptide hormone secretion, adipocyte differentiation, anti-inflammatory effects, nerve activation and immune responses. Of these FFARs, GPR120 was dephosphorylated in 2005. GPR120 is activated primarily by long-chain FFAs, \( \omega-3 \) FAs (such as \( \alpha \)-linolenic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)). GPR120 is highly expressed in the intestine, immune cells, lung, taste buds and adipose tissue. Hirase et al. originally showed that the activation of GPR120 by FFAs stimulates the secretion of incretin glucagon-like peptide 1 (GLP-1) from enteronecrotic L-cells. In adipocytes, GPR120 regulates adipogenic processes, such as adipocyte development and differentiation. In 2010, Oh et al. showed that GPR120 is a functional \( \omega-3 \) FA receptor on proinflammatory macrophages and mediates the anti-inflammatory effect of \( \omega-3 \) FAs in tissue macrophages. As chronic macrophage-mediated tissue inflammation is a mechanistic feature of obesity-related insulin resistance, these researchers further showed that activation of GPR120 by \( \omega-3 \) FAs inhibits inflammation in macrophages and reverses insulin resistance and other symptoms of metabolic syndrome in obese mice. The study elucidated the mechanism governing the anti-inflammatory effect of \( \omega-3 \) FAs and highlighted the functional role played by GPR120, especially in the inflammatory state associated with obesity. In 2012, we generated GPR120-deficient mice to directly examine the role played by GPR120 in lipogenesis and glucose and energy homeostasis. GPR120-deficient mice exhibited more severe high fat diet (HFD) (60% kcal fat)-induced obesity than wild-type (WT) mice. Histological analyses demonstrated that adipocyte size in both epididymal and subcutaneous fat of HFD-fed GPR120-deficient mice was significantly increased in comparison with those of WT mice. Gene expression analysis in white adipose tissue and liver tissue showed down-regulation of insulin signaling-related genes, such as Insr, Irs1 and Irs2. Genome-wide gene expression analysis in HFD-fed GPR120-deficient mice further indicated reduced expression of adipogenic and lipogenic genes in white adipose tissue and enhanced expression of lipogenesis-related genes in the liver. These results suggested that GPR120 was required for normal adipogenesis and that GPR120 deficiency might compromise systemic lipid homeostasis. In fact, lipidomic analysis in white adipose tissue, the liver and plasma indicated that major lipid clusters of HFD-fed GPR120-deficient mice were significantly changed compared with those of WT mice. Taken together, the results of our study in GPR120-deficient mice consistently demonstrated that GPR120 acts as a sensor of dietary fat and regulates systemic energy homeostasis. Next, we further attempted to examine the association of GPR120 with the development of obesity in humans. Exon sequencing of GPR120 in obese and lean European subjects successfully identified two nonsynonymous mutations, p.R270H and p.R67C. Of these two mutations of GPR120, we found that p.R270H associated with obesity in human. In vitro functional analyses of mutated GPR120 demonstrated that the p.R270H mutant receptor did not have the ability to transduce intracellular Ca\(^{2+}\) signals. Hence, our study demonstrated that dysfunction of GPR120 leads to obesity, not only in mice but also in humans. Recently, further efforts to clarify novel physiological roles of GPR120 demonstrated several important molecular mechanisms regulated by the receptor (Fig. 1). For instance, Quesada-López et al. reported that stimulation of GPR120 activated brown fat activity and the browning of white fat in mice through the induction of fibroblast growth factor-21, suggesting that regulation of thermogenesis is mediated by mice. Furthermore, hepatic triglyceride content was significantly increased in HFD-fed GPR120-deficient mice. These observations consistently indicated that GPR120 deficiency caused not only severe HFD-induced obesity but also adipose tissue inflammation and hepatic steatosis. Next, we examined glucose homeostasis in HFD-fed GPR120-deficient mice. HFD-fed GPR120-deficient mice showed higher levels of fasting blood glucose and insulin than did WT mice. Insulin tolerance tests showed that HFD-induced insulin resistance was more prominent in GPR120-deficient mice than in WT mice. In white adipose tissue and liver tissue of HFD-fed GPR120-deficient mice, insulin-induced phosphorylation of Akt (on Ser473) was not observed. These observations were consistent with the more severe obesity-associated insulin resistance observed in HFD-fed GPR120-deficient mice. Microarray analysis and subsequent real-time PCR analysis and Western blot analysis in white adipose tissue and liver tissue showed down-regulation of insulin signaling-related genes, such as Insr, Irs1 and Irs2. 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**Biography**

Atsuhiko Ichimura completed his B.Sc., M.Sc., and Ph.D. from Kyoto University Graduate School of Pharmaceutical Sciences under the supervision of Prof. Gozoh Tsujimoto. He subsequently worked in the laboratories of Prof. Toshio Miyata at Tohoku University Graduate School of Medicine in 2011 as an Assistant Professor. In 2014, he returned to the Laboratory of Biological Chemistry, Kyoto University Graduate School of Pharmaceutical Sciences, and began working under Prof. Hiroshi Takehama on the functional analysis of proteins expressed in plasma and the endoplasmic reticulum membrane. Throughout his career, he has consistently investigated the physiological function of membrane proteins, such as GPCRs and ion channels, and has attempted to obtain new basic knowledge to contribute to the development of new therapeutic methods.
GPR120,25) Yore et al. showed that branched FAs signal through GPR120 to enhance insulin-stimulated glucose uptake in adipocytes.26) Moreover, we demonstrated that 10-hydroxy-
cis-12-octadecenoic acid (HYA), an initial linoleic acid-related
gut-microbial metabolite, activates GPR40 and GPR120 and
promotes GLP-1 secretion.27) Supplementation with HYA at-
tenuates HFD-induced obesity in mice without eliciting ara-
chidonic acid-mediated adipose inflammation. We have also
previously described the pathological characteristics of OI and
bone fragility and other connective tissue manifestations. We
generated Tric-b knockout mice and investigated the bones of the
Tric-b knockout mice to elucidate the underlying pathophysiologi-
cal mechanisms of OI patients carrying TRIC-B mutations.39)
Since all of these mutations may result in functional loss of TRIC-B, counterion channel activity was predicted
to be required for normal bone formation. Hence, we gener-
ated Tric-b knockout mice and investigated the bones of the
Tric-b knockouts to elucidate the underlying pathophysiologi-
cal mechanisms of OI patients carrying TRIC-B mutations.39)
We determined the pathological features of Tric-b knockout
bones and subsequently isolated and cultured primary osteoblasts. Our results demonstrated that TRIC-
B is essential for active osteoblasts to produce a sufficient
amount of collagen for normal bone mineralization. Although
the systemic bone structure of Tric-b knockout mice was mac-
roscopically normal, a series of histological analyses showed
that Tric-b knockout bone exhibited less mineralization and
reduced deposition of extracellular matrix components, such
as collagen and calcium. Further analysis using electron mi-
croscopy demonstrated that a majority of collagen-producing
active osteoblasts from Tric-b knockout mice exhibited ultra-

3. COUNTERION CHANNEL TRIC-B AND BONE
FORMATION

Ca\(^{2+}\)-permeable channels expressed on the ER/SR mem-
brane, such as inositol trisphosphate receptors (IP3Rs) and
ryanodine receptors (RyRs), were identified and characterized
during the 1980s and 1990s.29,30) These ion channels mediate
the release of Ca\(^{2+}\) from intracellular stores, which are rap-
idly activated, and the release lasts for several milliseconds.
Since the mobilization of divalent cations quickly generates
the membrane potential to inhibit Ca\(^{2+}\) release, sustained
Ca\(^{2+}\) release would be possible if other ion movements pro-
vided countercurrents to suppress membrane potential genera-
tion.31,32) However, real channels/transporters, which mediate
counterion movement, have not been identified for many
years. In 2007, Yazawa et al. reported that two trimeric in-
tracellular cation (TRIC) channels regulate countermovement
of cations across the ER/SR membrane to buffer the transient
negative membrane potential arising due to intracellular Ca\(^{2+}\)
release.33) The TRIC family consists of two members, TRIC-A (encoded as TMEM38A) and TRIC-B (encoded as TMEM38B).
Although the TRIC subtypes have 42% amino acid sequence
identity, TRIC-A and TRIC-B have different expression pat-
terns, indicating that TRIC subtypes contribute different
biological responses. TRIC-A is predominantly expressed in
tissue, such as skeletal muscle and cardiac muscle,
and is distributed in the SR membrane and nuclear envelope.
TRIC-B is ubiquitously expressed throughout tissues and
both cell types. Both TRIC-A and TRIC-B form homotrimeric
complexes and act as functional monovalent cation-selective
channels. Genetic studies in humans have found that recessive
mutations in TRIC-B but not TRIC-A cause osteogenesis im-
perfection (OI). OI is a group of rare divergent hereditary dis-
ases characterized by low bone density, leading to increased
bone fragility and other connective tissue manifestations. We
previously described the pathological characteristics of OI and
their classifications in another review article.34) The first case
of OI caused by homozygous deletion mutations in the TRIC-
B locus was reported in individuals in Arabia. Different kinds
of point mutations or deletion mutations of the TRIC-B gene
were also reported in Bedouin, Albanian and Chinese OI pa-
tients.35–38) Since all of these mutations may result in function-
al loss of TRIC-B, counterion channel activity was predicted
to be required for normal bone formation. Hence, we gener-
ated Tric-b knockout mice and investigated the bones of the
Tric-b knockouts to elucidate the underlying pathophysiologi-
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Fig. 1. Schematic Diagram of Ligand Specificity, Signaling Pathways and Associated Biological Outcomes for GPR120/FFAR4

GPR120/FFAR4 is activated by long-chain fatty acids. Known signaling path-
ways downstream of GPR120/FFAR4 and main cell types and tissues expressing
GPR120/FFAR4 are illustrated. Illustrations were modified from the resources
distributed in the Togo picture gallery (http://g86.dbcls.jp/~togoriv/), licensed under
CC-BY 4.0 Togo picture gallery by the Database Center for Life Science (DBCLS),
Japan. (Color figure can be accessed in the online version.)
structural defects, such as dilated rough ER, smaller Golgi body and decreased secretory vesicles. As the ultrastructure of Tric-b knockout osteoclasts was relatively normal, it was predicted that Tric-b function was primarily required for collagen production in osteoblasts. By using isolated primary cultured osteoblasts, we found that Tric-b deficiency inhibited collagen secretion from the cells. Therefore, the amount of intracellular collagen was significantly increased, whereas extracellular collagen deposition and mineralization were significantly reduced, in Tric-b knockout osteoblasts. Abnormally accumulated collagen deposits were located in the ER of Tric-b knockout osteoblasts; therefore, the ER was roughly dilated. As described above, TRIC-B acts as a counterion channel implicated in the maintenance of intracellular Ca\(^{2+}\) stores and therefore Ca\(^{2+}\) signaling. Hence, we investigated intracellular Ca\(^{2+}\) handling in primary cultured Tric-b knockout osteoblasts. Our results demonstrated that Tric-b deficiency impaired the IP\(_3\)-mediated release of Ca\(^{2+}\) from the ER, resulting in the overloading of Ca\(^{2+}\) stored in the ER. In addition, the transient expression of Tric-b rescued both impaired Ca\(^{2+}\) handling and ER morphology with abnormal collagen accumulation. Taken together, our results demonstrated that TRIC-B plays an essential role in the production of collagen from osteoblasts. Hence, both Tric-b deficiency in mice and loss of functional TRIC-B in humans might cause compromised collagen production and decreased extracellular mineralization, resulting in OI-like bone formation failure (Fig. 2). Recently, Kasuya et al.\(^{40}\) and Yang et al.\(^{45}\) reported the crystal structure of the TRIC-B channel from prokaryotes and Caenorhabditis elegans. Moreover, Wang et al. reported high-resolution crystal structures of vertebrate TRIC-B channels in both Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free states.\(^{42}\) Based on these structural characteristics of TRIC-B, a novel modulator of TRIC-B will be developed, which may contribute to new treatment strategies for bone-related diseases.

4. DIVALENT CATION CHANNEL TRANSIENT RECEPTOR POTENTIAL MELASTATIN 7 (TRPM7) AND BONE OUTGROWTH

Intracellular Ca\(^{2+}\) acts as one of the most important and ubiquitous second messengers. As described in Introduction, eukaryotic cells equip a variety of ion channels and pumps to transfer ions across the membrane. These membrane proteins tightly regulate intracellular Ca\(^{2+}\) concentrations. During cellular differentiation and maturation, ion channels contributing to Ca\(^{2+}\) influx across the plasma membrane and Ca\(^{2+}\) efflux from intracellular stores constitute Ca\(^{2+}\)-handling machinery that regulates specific cellular responses. Moreover, elucidation of the precise regulatory mechanism governing intracellular Ca\(^{2+}\) homeostasis is essential for a better understanding of cell type-specific functions. We recently focused on Ca\(^{2+}\) handling in growth plate chondrocytes. In the growth plates of long bones, chondrocytes proliferate, mature, secrete extracellular matrix, and eventually undergo apoptosis.\(^{44,45}\) Since Ca\(^{2+}\) homeostasis maintained by a variety of membrane proteins was largely uncharacterized in chondrocytes under physiological conditions in vivo, we developed a new method for Ca\(^{2+}\) imaging in slices of living bones isolated from mouse embryos.\(^{46}\) By using this method, we found that the resting intracellular Ca\(^{2+}\) concentration spontaneously fluctuated in growth plate chondrocytes, which were freshly prepared from developing femoral bones. Pharmacological inhibitory experiments revealed that Ca\(^{2+}\) fluctuations were generated by Ca\(^{2+}\) entry through plasma membrane channels but not by Ca\(^{2+}\) release from intracellular stores. Therefore, we next attempted to identify cell-surface Ca\(^{2+}\)-permeable channels responsible for Ca\(^{2+}\) fluctuations. Pharmacological screening and subsequent genome-wide gene expression analysis using a microarray successfully identified TRPM7 channels as candidates. TRPM7 forms a divalent cation-permeable channel, and its gating is stimulated and suppressed by various factors, such as intracellular Mg\(^{2+}\) and Mg\(^{2+}\)-nucleotide complexes, extracellular...
pH, and extracellular oxygenation.\(^{47,48}\) We pharmacologically assessed whether TRPM7 predominantly generated spontaneous Ca\(^{2+}\) fluctuations. Spontaneous Ca\(^{2+}\) fluctuations were significantly attenuated by treatment with FTY720 and NS8593, which are widely used as small molecule inhibitors of TRPM7. Conversely, naltriben and NNC550396, which are reported to activate the TRPM7 channel, significantly enhanced spontaneous Ca\(^{2+}\) fluctuations. These results indicated that TRPM7 may serve to modulate spontaneous Ca\(^{2+}\) fluctuations in growth plate chondrocytes. Further analyses with chemical modulators concluded that TRPM7 may coordinate with large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels and phospholipase C (PLC) to generate and maintain Ca\(^{2+}\) fluctuations in growth plate chondrocytes. To further confirm the responsibility of TRPM7 on spontaneous Ca\(^{2+}\) fluctuations, we generated Trpm7 conditional knockout (cKO) mice by using the Cre-LoxP system, as Trpm7 global knockout mice die at an early embryonic stage.\(^{59}\) We crossed Trpm7\(^{fl/fl}\) mice with 11Enh-Cre mice, which express Cre recombinase under the control of the Col11a2 promoter and enhancer and thus delete loxP-flanked genes in a chondrocyte-specific manner.\(^{59}\) Chondrocyte-specific knockout of Trpm7 resulted in significantly weakened spontaneous Ca\(^{2+}\) fluctuations with reduced autophosphorylation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), which is one of the major Ca\(^{2+}\)-dependent enzymes that triggers downstream signals.\(^{51–53}\) (Figs. 3A, B). Furthermore, chondrocyte-specific Trpm7-deficient mice exhibited systemic retardation of long bone outgrowth. These mice survived postnatal development for at least 3 weeks and indicated generalized growth failure (Fig. 3C). These findings clearly demonstrated that TRPM7-mediated spontaneous Ca\(^{2+}\) fluctuations are essential for growth plate chondrocytes and thus contribute to bone outgrowth. Our results provide the novel insight that bone outgrowth can be controlled by the modulation of intracellular Ca\(^{2+}\). There are several peptide hormones that are known to enhance bone outgrowth. Our data further suggested that intracellular Ca\(^{2+}\) mobilization might be associated with the functional activity of these peptide hormones, such as growth hormone and c-type natriuretic peptide.\(^{54,55}\) Further research may determine the precise functional roles played by intracellular Ca\(^{2+}\) in bone outgrowth and characterize signaling cascades and their associated molecules.

5. CONCLUSION

Accumulating evidence obtained from various studies, including those described above, supports the notion that a variety of physiological responses are mediated by membrane proteins. Nutrient sensing GPCRs contribute to systemic energy homeostasis through regulation of various physiological processes. Both counterion channels expressed in the ER membrane and divalent cation channels expressed in the plasma membrane play important roles in bone development through the regulation of signal cascades involving intracellular Ca\(^{2+}\). Our results revealed pathophysiological mechanisms associated with dysfunction of membrane proteins, which are involved in human diseases, such as obesity and OI. Therefore, selective modulators of these GPCRs and ion channels may represent promising targets for new drug development for treating human diseases. Further investigations may uncover more detailed molecular networks regulated by membrane proteins and thus open new avenues of research in drug development.

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