

Functional characterization of (pro)renin receptor in association with V-ATPase

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1. ABSTRACT

The (pro)renin receptor [(P)RR] is a unique molecule that binds prorenin and renin in tissues, not only leading to their activation, but also inducing intracellular signaling. As a key player in the local renin-angiotensin system, (P)RR activation plays an important role in the development of cardiac fibrosis and proteinuria in hypertension and diabetes. Intriguingly, the fragment (P)RR is also called ATP6AP2 because it has been shown to be associated with vacuolar-type H⁺-ATPase (V-ATPase). The V-ATPase is a multi-subunit proton pump involved in diverse and fundamental cellular processes, including receptor-mediated endocytosis, processing of proteins and signaling molecules, membrane sorting and trafficking, and activation of lysosomal enzymes. The role of (P)RR in the function of the V-ATPase is implicated in the previous findings and vigorously investigated in the recent studies. Furthermore, the novel function of the (P)RR as an adaptor protein between the Wnt receptor complex and the V-ATPase was discovered. Thus, the (P)RR is a multi-functional molecule that shows the complex structure and behaviour. This review highlights the current insights and the future perspectives in research regarding the (P)RR and mammalian V-ATPase.

2. INTRODUCTION

The (pro)renin receptor [(P)RR] was first identified as a receptor for renin and prorenin in 2002 (1). The (P)RR bound renin and prorenin, not only exert their enzymatic activation, but also cause an angiotensin II independent intracellular signaling. It has been demonstrated that the (P)RR plays a key role in the local renin-angiotensin system (RAS) and its own intracellular signalling plays an important role in the development of cardiac fibrosis and proteinuria in hypertension and diabetes (2-5). Recent studies revealed the (P)RR is an integral component of mammalian vacuolar-type H⁺-ATPase (V-ATPase) which is a multi-subunit proton pump involved in diverse and fundamental cellular processes, including receptor-mediated endocytosis, processing of proteins and signaling molecules, membrane sorting and trafficking, and activation of lysosomal enzymes (6). Moreover, the (P)RR works as an adaptor protein between the Wnt receptor complex and the V-ATPase (7). Thus, the (P)RR is a multi-functional molecule that shows the complex structure and behaviour. This review particularly focuses on the roles of (P)RR on the function of mammalian V-ATPase, and address the issues that should be solved in the future studies.

3. (PRO)RENIN RECEPTOR UNDER PATHOLOGICAL CONDITIONS

Renin is produced from its inactive proenzyme prorenin. Plasma prorenin levels are elevated in patients with diabetes mellitus compared with healthy subjects. Moreover, higher levels of prorenin are present in the plasma of diabetic patients with microalbuminuria compared with those without incipient microalbuminuria (8-10). The cause of the increase in prorenin levels is unclear, but may reflect increased prorenin gene expression and/or decreased prorenin clearance. The prorenin propeptide consists of a 43 amino acid sequence at the N-terminus. This is thought to cover the enzyme active site ('closed' formation) and prevent access of angiotensinogen. Prorenin can be activated proteolytically or non-proteolytically. The main physiological pathway for the activation of renin is proteolytic cleavage of the propeptide, which opens up the active site for angiotensinogen and its subsequent cleavage to angiotensin I. *In vivo*, proteolytic activation of prorenin occurs almost exclusively in the juxtaglomerular cells within the kidney. Non-proteolytic activation of prorenin is a reversible process and can be viewed as the propeptide unfolding from the enzymatic cleft. Non-proteolytic activation can be induced *in vitro* by low temperatures or low pH (optimal at pH 3.3) (11). The (P)RR was identified as a molecule that induces non-proteolytic activation of prorenin (1). The (P)RR bound prorenin becomes enzymatically active as a result of a conformational change without cleavage of the prosegment and thereby producing the local angiotensin I and subsequently generating angiotensin II. Therefore, this molecule provides reasonable explanations of the significance and enzymatic role of the prorenin in end organ damages in diseases associated with the local activation of the RAS, such as hypertension, diabetes, and preeclampsia (2-5, 12). Moreover, (P)RR possesses its own intracellular signaling. Activated (P)RR induces the mitogen activated protein (MAP) kinases extracellular signal-regulated kinase (ERK) 1/2 and p38 pathways, leading to upregulation of profibrotic and cyclooxygenase-2 genes independent of angiotensin II generation, which plays an important role in the end organ damage as well. It has been demonstrated that (P)RR blockade mitigates the cardiac fibrosis and proteinuria in hypertensive and diabetic models (2-5). Based on these findings, the genetic ablation of (P)RR had been expected to be protective under the pathologic conditions.

4. SIGNIFICANCE OF THE (P)RR IN EMBRYONAL DEVELOPMENTS

Previous reports have suggested the essential role of the (P)RR in the function of V-ATPase. Mouse embryonic stem cells deficient for the (P)RR failed to generate chimeras when injected into blastocysts, in contrast to the successful birth of RAS component knockout models, such as renin, angiotensinogen, and angiotensin type 1 receptor knockout mice (13-15). Mutant zebrafish for (P)RR display early developmental abnormalities, including eye and body hypopigmentation, as well as neuronal cell death, and die early in

development, implicating the critical function in the melanocytes and neurons (16). Zebrafish embryo mutants for V-ATPase subunits as well as (P)RR share similar phenotypes (16), indicating a possible link between the two proteins. Similar phenotypes such as small heads, shortened tails, and defects in melanocyte and eye pigmentation were also observed in *Xenopus*, when morpholino antisense RNA against (P)RR was injected into embryos (7). A exonic splice enhancer mutation of the human (P)RR is associated with a family with X-linked mental retardation and epilepsy, allowing us to speculate an important role of the (P)RR in the synaptic vesicles (17, 18). Collectively, these phenotypes observed in the mutation or deletion of the (P)RR suggested the essential roles of the (P)RR in development.

Several groups have yet to generate (P)RR knockout mice despite vigorous attempts, possibly because (P)RR deleted embryonic stem cells do not form chimeras after blastocyst injection (19). To circumvent the embryonic lethality, the tissue specific (P)RR knockout mouse was generated (6). Surprisingly, the cardiomyocyte specific (P)RR knockout mice developed fulminant heart failure and inevitably died early. The (P)RR depleted cardiomyocytes displayed the perinuclear accumulation of numerous vacuoles, including multi-vesicular bodies and autophagolysosomes, which appeared to be caused by RAS independent mechanisms. Since a C-terminal (P)RR fragment was identified as vacuolar adenosine triphosphatase (V-ATPase), H⁺-transporting, lysosomal accessory protein 2 (ATP6AP2) (20), which plays an essential role in controlling intracellular vesicular acid environment (21), the investigation for the underlying cellular mechanisms accounting for this incredible phenotypes observed in the (P)RR ablated cardiomyocytes was involved in the role of (P)RR on the function of V-ATPase, and disclosed that (P)RR is an integral component as an accessory subunit for the function of the mammalian V-ATPase, in particular for the stability and assembly of V_O subunits. According to our observations, the expression levels of the (P)RR in each tissue were closely correlated with the other V-ATPase subunits in mice (Figure 1), implicating that the essential function of the (P)RR is a mammalian V-ATPase related protein. Since the level of the (P)RR in the kidney is relatively low compared to the other V-ATPase subunits, other accessory subunits or unknown isoforms of the (P)RR could be coupled with the V-ATPase.

5. POSSIBLE PHYSIOLOGICAL FUNCTION OF THE (P)RR BASED ON THE V-ATPASE

V-ATPase is a large multi-subunit, membrane-associated protein complex that carries out the active transport of protons across the membrane, thereby creating the acidic environment of intracellular compartments and of the extracellular space. The V-ATPase is expressed in virtually all kinds of cells, and widely distributed in various subcellular compartments such as the trans-Golgi network, endosomes, lysosomes, secretory granules, melanosomes, synaptic vesicles, and endocytic vesicles. V-ATPase-dependent acidification of organelles facilitates protein

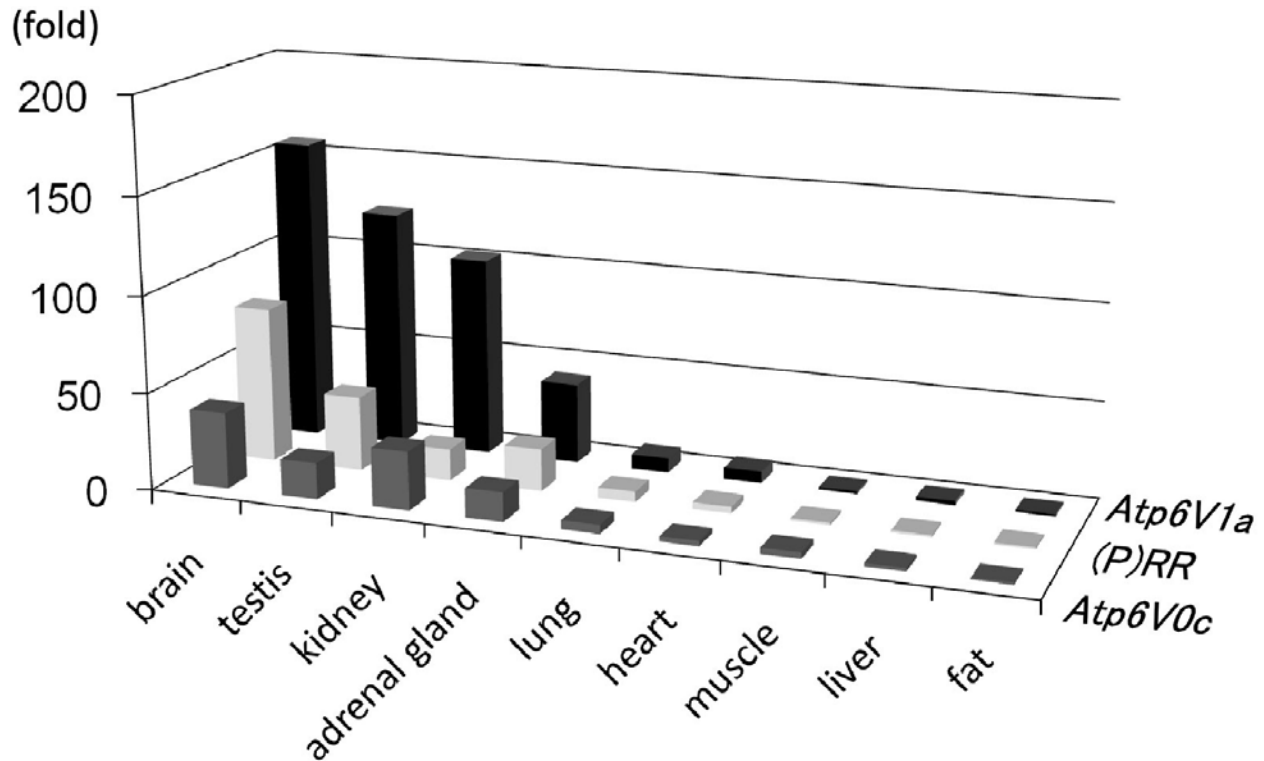


Figure 1. The expression levels of the vacuolar-type H^+ -ATPase subunit genes and the (pro)renin receptor gene in mice. The gene expression levels in each tissue were calculated as compared to the expression levels in the fat tissue as a baseline.

sorting, membrane trafficking and fusion, receptor-mediated endocytosis, and lysosomal protein degradation (22). For instance, hydrolases responsible for protein degradation in the lysosome have an optimal activity at a low pH. It is also involved in diverse cellular processes such as phagocytosis, virus entry, metastasis, and embryonic left-right patterning (22, 23). In intracellular vesicles, the V-ATPase also serves as a pH sensor to regulate trafficking (24). The (P)RR might also sense the acidity levels of the intracellular compartments and accordingly regulate V-ATPase activity (6). Mutations in V-ATPase components, including the (P)RR, or pharmacological inhibition of V-ATPase activity leads to the disruption of pH homeostasis, results in an accumulation of membrane proteins in endocytic compartments, and in some cases blocks transport between the late endosome and the lysosome, ending in lethality in various organisms (6, 25). Moreover, specific V-ATPase complexes participate in highly differentiated cellular and tissue functions, including urine acidification in renal intercalated cell, bone resorption in osteoclasts. They are also abundantly present in the endocrine tissues, such as beta cells in pancreas and chromaffin cells in adrenal glands, participating in the exocytosis in the regulated secretion in an acidification independent manner (26, 27). This ubiquitous and specific distribution of V-ATPase suggests that the enzyme is required for diverse fundamental cellular processes, and underscores the significance for the cell survival. Since the accumulation of

the intracellular vesicles in the (P)RR ablated cardiomyocytes was reproduced by the V-ATPase pharmacological inhibitor bafilomycin, at least several physiological roles of the (P)RR would be attributed to the functions of the V-ATPase.

V-ATPase is evolutionally conserved beyond species including plants, yeasts, and mammals (28). V-ATPase is integrated into V_1 and V_o sectors (Figure 2). In the mammalian counterpart, the V_1 sector contains eight different types of subunits (A-H) and is responsible for ATP hydrolysis. The V_o sector contains seven different subunits including a, c, c', d, e, accessory subunit Ac45, and (P)RR, which form a membrane embedded component, and is required for the translocation of the proton across the membrane. Several subunits (a, d, B, C, E, G, and H) contain isoforms, rendering a variety of the tissue and intracellular specific localization of the V-ATPase (29). Furthermore, the redundancy provided by the isoforms may compensate for the function of other isoforms (30). Of note, the two accessory subunits Ac45 and (P)RR have not been shown to have any yeast homologues.

The biogenesis of V-ATPase requires the coordinated associations that have not been fully elucidated. The V_o subunits are synthesized and assembled on the ER membrane, and then targeted from the ER towards each subcellular destination, where it associates the V_1 sector to assemble into the final V-ATPase complex.

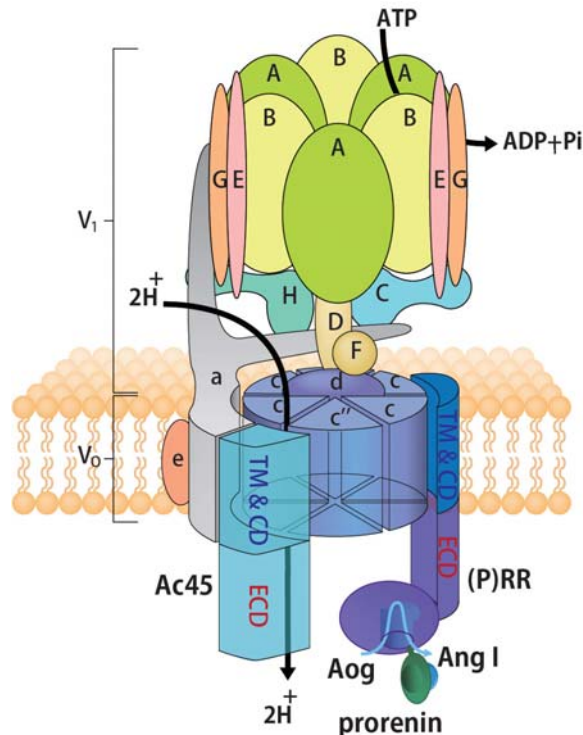


Figure 2. Putative structure of the mammalian vacuolar-type H^+ -ATPase and the (pro)renin receptor. The (pro)renin receptor bound prorenin undergoes the non-proteolytic activation, generating the angiotensin I. Both the (pro)renin receptor and the Ac45 have the furin cleavage sites between transmembrane domain and extracellular domain. TM & CD, transmembrane and cytosolic domain; and ECD, extracellular domain; Aog, angiotensinogen; Ang I, angiotensin I.

Studies in yeast cells have shown that the loss of a V_1 subunit has little effect on the stability of the remaining V_1 subunit, while the loss of any single V_0 subunit affects the stability and assembly of the remaining V_0 subunits (31, 32). In mutant yeast cells lacking a V_0 complex, the V_1 subunits are stable but present in the cytosol and are unable to assemble onto the vacuolar membrane. In yeast, several additional genes (*Vma12p*, *Vma21p*, and *Vma22p*) that are required for V-ATPase assembly have been identified for the V_0 subcomplex (33). These assembly factors are localized in the endoplasmic reticulum membrane and cells lacking any single assembly factor were unable to assemble V-ATPase. All the V_1 subunits were present but localized in the cytosol and thus not associate with the vacuolar membrane (34). The V_0 subunits were detected at greatly reduced levels in the mutant cells that lacked these assembly factors, an effect that is similar to that observed after the loss of a V_0 subunit (34). The previous studies demonstrated that the yeast V_1 sector subunits are able to be exchanged with the mammalian V_1 subunits (35, 36). However, there has not been any study showing the successful rescue of the yeast V_0 mutants by the mammalian V_0 counterparts (37). Since

the mammalian V_0 sector possesses the additional accessory subunits Ac45 and (P)RR, it would be impossible to replace V_0 complex without these mammalian specific subunits. In fact, the (P)RR selectively affects the stability and assembly of the V_0 subunits in murine cardiomyocytes and embryonic fibroblasts (6). Further experiments are warranted to clarify whether the accessory subunits are important for mammalian V_0 subunits in any types of cells.

6. THE (P)RR FUNCTIONS DEPENDENT ON ITS STRUCTURE AND CELLULAR LOCALIZATION

In humans, the (P)RR gene is located on the X chromosome at locus p11.4. The messenger RNA is 2034 base pair long and no alternative splicing product. The protein is composed of 350 amino acids and a predicted mass of 37 kDa. The (P)RR contains four different domains: an N-terminal signal peptide, an extracellular domain, a single transmembrane domain and a short cytosolic domain (Figure 3) (38). The extracellular domain is responsible for binding with renin and prorenin, whereas the transmembrane and cytosolic domain is associated the V-ATPase. Unlike other components of the RAS, the (P)RR gene is highly conserved among species, and (P)RR orthologues are found in many species, including rat, mouse, chicken, as well as in more primitive species such as frog, zebrafish, *Caenorhabditis elegans*, and *Drosophila melanogaster* (19), in which the role of RAS would be unlikely to be meaningful. The degree of homology of the (P)RR between human, rat, and mice is about 95% for the nucleotide sequence and over 80% at the amino-acid level (39). Thus the (P)RR is an evolutionally conserved protein despite no yeast orthologues. The highest homology is found in the transmembrane and cytoplasmic regions, corresponding to the ATP6AP2 discovered from the bovine chromaffin granule (20). This corroborates the most fundamental function of the (P)RR works as a subunit of the V-ATPase. Meanwhile, the extracellular domain displays a high amino acid sequence identity exclusively in vertebrates (19). Since the RAS emerged later in evolution, the ATP6AP2 protein may have acquired renin and prorenin binding properties (40). Consequently, the RAS could affect the intracellular vesicular environment by modulating the V-ATPase function via (P)RR.

The (P)RR has been considered to be present mainly on the plasma membrane. The recent study revealed, however, that the most of the (P)RR exists in the intracellular, particularly perinuclear distribution, which would be in line with the subcellular localization of the V-ATPase (41, 42). In fact, sequence analysis disclosed that the cytosolic domain of the (P)RR owns the endosome and the lysosome sorting signal as well as the endoplasmic reticulum retention motifs (19). The previous studies have also demonstrated that (P)RR has a predilection in the trans-Golgi network, where it undergoes cleavage by proteases such as proprotein convertase furin and ADAM19 to generate a 28-kD N-terminal soluble fragment, which is secreted outside the cell, along with a 10-kD C-terminal residue that likely represents the ATP6AP2 (43-45). Consistent with this, N-terminal fragment (P)RR is present in plasma and urine (43).

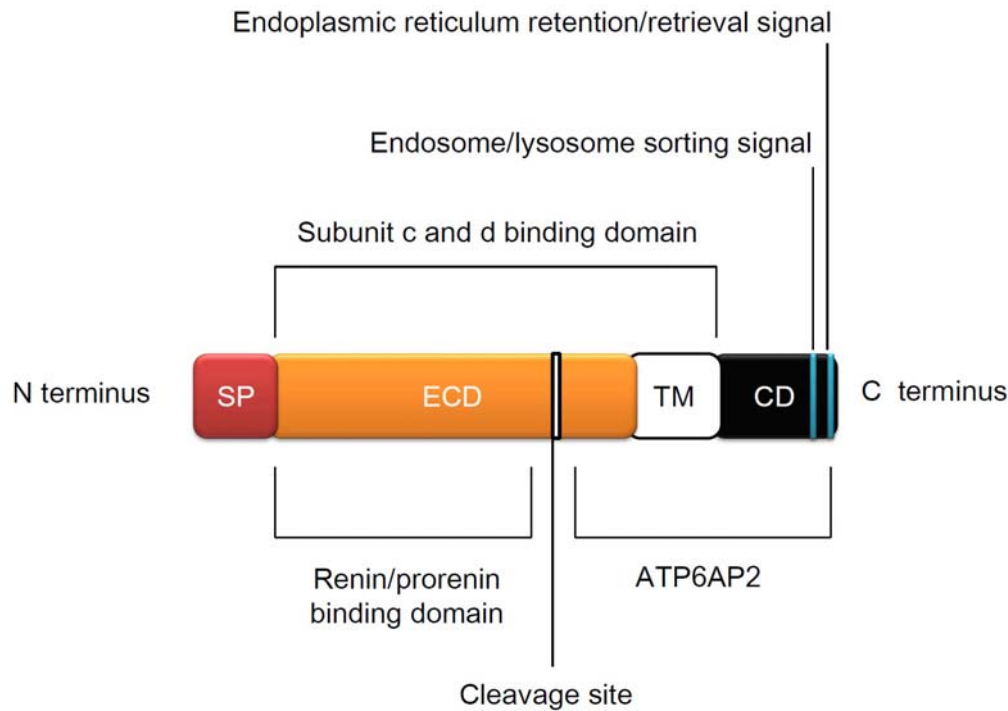


Figure 3. The structure of the (pro)renin receptor. SP, signal peptide; ECD, extracellular domain; TM, transmembrane domain; CD, cytosolic domain

However, there are still many problems to be solved; whether the V-ATPase demands the full-length (P)RR or allows the truncated (P)RR; whether the (P)RR always accompanies the V-ATPase or sometimes exists alone; whether the amount of the soluble (P)RR reflect the intracellular environment; and to what extent the soluble (P)RR contribute to the local RAS. The function of the (P)RR could be determined by its cellular localizations and its forms. The future study must address these issues and unravel the complex behavior of this molecule.

The recent study confirmed that (P)RR binds to the V-ATPase V_O sector subunit c and d but not to control transmembrane proteins (7). It was demonstrated that the transmembrane and the extracellular domain of (P)RR are required for binding to the subunit c. This results are incredibly unexpected and conflicting with the fact that the transmembrane and cytosolic domains of the (P)RR exists with the final V-ATPase complex (20). If this observation is the case with the mammalian V-ATPase, it would be extremely difficult to distinguish between the RAS related function and V-ATPase associated function of the (P)RR. The exact domain of the (P)RR directly contacting V-ATPase remains elusive.

7. INSIGHT FROM ANOTHER V-ATPASE ACCESSORY SUBUNIT: AC45

Interestingly, another accessory subunit of the V-ATPase Ac45 shares some features with the (P)RR. The previous study demonstrated that Ac45 is also copurifying

with the V-ATPase from chromaffin granules (46). The gene of the Ac45 is single-copied and located in a chromosome Xq, consisting of 10 exons spanning up to 8 kb in the mouse and human genomes. Ac45 is also a substrate of furin, and the cleavage of Ac45 by furin has shown to be required for intragranular acidification (47). Cytosolic terminus of Ac45 contains elements necessary for its proper interaction with V_O domain subunit a3, c, c', and d (48). In contrast to the (P)RR, however, the gene structure of Ac45 is poorly conserved in its invertebrate orthologs of *Caenorhabditis elegans* and *Drosophila melanogaster*, encompassing only six and four exons extending over 4.1 and 2.1 kb, respectively. Furthermore, the overall degree of amino acid sequence identity between the mammalian and invertebrate Ac45 proteins is very low, except for a surprisingly highly conserved putative targeting motif in the carboxy-terminal region (49). Notably, Ac45 has been recently demonstrated to represent the regulator of the proton pump and control V-ATPase-mediated granular acidification that is necessary for efficient prohormone processing (50). Similar to the (P)RR, whether the Ac45 functions as a receptor remains established.

8. SIGNAL TRANSDUCTION, V-ATPASE DEPENDENT ACIDIC ENVIRONMENT, AND THE (P)RR.

Recently, the novel function of the (P)RR as an adaptor protein between the Wnt receptor complex and the V-ATPase was discovered in *Drosophila* and *Xenopus*

using genome wide siRNA screening (7, 51, 52). Wnt signaling is involved in virtually every aspect of embryonic development and also controls homeostatic self-renewal in a number of adult tissues, and tumor formation (53). Wnts bind seven-pass transmembrane receptors Frizzled, which enables Frizzled to cooperate with a single-pass transmembrane molecule of LRP5 and LRP6. Currently, three different pathways are believed to be activated upon Wnt receptor activation: the canonical Wnt/ β -catenin cascade, the noncanonical planar cell polarity (PCP) pathway, and the Wnt/ Ca^{2+} pathway (53). Since the Wnt signalling was disturbed by the inhibition of the V-ATPase function through the knock down of either the (P)RR or other V-ATPase subunits along with the pharmacological inhibitors of V-ATPase, the (P)RR seems to mediate the internalization of the receptors and the subsequent proper signal transduction as a hinge molecule between the Wnt receptor and the V-ATPase. Furthermore, it has been shown that V-ATPase is also required for the activation of the Notch receptor in the physiological as well as pathological settings (54, 55). Therefore, the acidification of an intracellular compartment could be crucial factor for a wide variety of signal transductions. Indeed, this appears to be also applicable to the (P)RR-mediated signaling itself. The inhibition of V-ATPase with bafilomycin attenuates ERK 1/2 phosphorylation induced by renin and prorenin in collecting duct/distal tubule lineage Madin-Darby canine kidney cells (41). However, the ERK 1/2 phosphorylation could be explained by the effects of other intracellular signal transductions rather than the direct influence of (P)RR-mediated signaling. The V-ATPase-mediated vesicular acidification is necessary for the proper signal transduction by controlling receptor-mediated endocytosis and receptor recycling. Therefore, bafilomycin might have disturbed the granular acidification, thereby nonspecifically suppressed various signal transductions. The future studies should address the important issue on whether ERK 1/2 phosphorylation is attributed to the (P)RR specific intracellular signaling or ligand nonspecific signaling.

9. FUTURE DIRECTIONS

The (P)RR is an accessory subunit of the mammalian V-ATPase in addition to the receptor for the renin and prorenin. Although there has not been any explanations why a subunit of the V-ATPase acquired the function as a receptor for the renin and prorenin, it would be a tempting story that the tissue RAS and the intracellular environment are interconnected and regulated with each other through the (P)RR. In addition, we need to know exactly to what extent the intracellular signaling of the (P)RR contributes to pathogenesis of hypertension and diabetes by distinguishing each function of the (P)RR in the future experiments.

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Key Words: Renin-Angiotensin System, Signal Transduction, V-ATPase, Vesicular Acidification, Review

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