Nephrology and Renal Diseases

Review Article

ANG II, ANG-(1-7), ALDO and AVP biphasic effects on Na^+/H^+ transport: the role of cellular calcium

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Abstract

This article reviews the main body of knowledge regarding NHE1 and NHE3 exchangers and their interaction with Angiotensin II, Angiotensin-(1-7), Aldosterone and Arginine Vasopressin, particularly their renal actions. This work addresses the biphasic effects of different hormonal doses on NHE1 or NHE3 in proximal tubule in Wistar, SHR (hypertensives) and their control WKY (normotensives) rats or MDCK cells (which share similarities with the collecting duct). The hormones were applied alone, with their inhibitors or plus agents that change the $[Ca^{2+}]i$. The data are compatible with hormonal stimulation of these exchangers by increases of $[Ca^{2+}]i$ in lower range, and inhibition at high $[Ca^{2+}]i$. In MDCK cells and Wistar rats, low doses of ANG II, ALDO or AVP stimulated the exchangers, while high doses inhibited them. ANG–(1-7), in Wistar or WKY rats has inverse, dose-dependent effects. In SHR rats, the biphasic effects of ANG-(1-7) were similar to the effects of ANG II, ALDO or AVP in Wistar rats. The interactions between these effects may represent a mechanism that regulates extracellular volume. In hypertensives animals, a high plasma level of ANG-(1-7) inhibited NHE3 in the proximal tubule, which mitigated hypertension. Figure 6 shows a schematic model to describe these biphasic hormonal effects.

Introduction

NHE

The Na^+/H^+ exchanger (NHE) is a ubiquitous membrane protein that is present as a number of isoforms in living organisms. In mammals, the NHE1 and NHE3 isoforms catalyze the electroneutral exchange of Na^+ and H^+ to control their respective concentration gradients, a process that is essential for numerous physiological processes, including controlling cell volume, pH, and systemic electrolyte levels, and acid-base and fluid volume homeostasis. In addition, NHE activity facilitates the progress of other cellular events, such as adhesion, migration, and proliferation. So far, ten different mammalian NHE isoforms (NHE1-10) that share 25-70% amino acid identity have been identified and characterized [1-3]. These isoforms have a common predicted secondary structure that consists of 12 conserved membrane-spanning segments at the amino-terminus and a more divergent, cytoplasmically oriented carboxy-terminus. These isoforms show considerable heterogeneity in their patterns of tissue/cell expression and membrane localization. Functional studies have revealed further differences in their kinetic properties, their sensitivity to pharmacological antagonists, their regulation by diverse hormonal and mechanical stimuli, and many of their essential physiological functions. In the nephron, individual NHE isoforms have different functions. These functions are reflected in their differential expression along the segments of the nephron, their localization in renal epithelial cells at the basolateral (e.g., isoform NHE1) or apical surface (e.g., isoform NHE3), and their activation in response to distinct agonists [4-13].

NHE1

The NHE1 isoform is ubiquitously expressed in the plasma membranes of virtually all mammalian cells, where it regulates intracellular pH, salt concentration and cell volume [14-16]. Therefore, NHE1 is critical for controlling and maintaining some of the most fundamental processes in cellular physiology, including cell growth and differentiation [14]. NHE1 has two functional domains: an amino-terminal ion translocation domain that consists of ~500 amino acids that catalyzes amiloride-sensitive Na^+/H^+ exchange and contains a built-in modifier site (pH sensor) and a regulatory carboxy-terminal cytoplasmic domain that consists of ~300 amino acids that determines the set point value of the modifier site [17,18]. Depending on the stimulus, NHE1 activation is often associated with one of two mechanisms: 1) phosphorylation, such as by assorted serine/threonine protein kinases [19], p38 mitogen-activated protein kinase (MAPK) [20] and p90 ribosomal S6 kinase [21], and 2) binding to regulatory proteins, resulting in conformational changes. Specifically, phosphatidylinositol 4,5-bisphosphate (PIP2) binds to the juxtamembrane region of NHE1, and actin-binding proteins in the ezrin, radixin and moesin family (ERM) connect NHE1 to the cytoskeleton. Different serine kinases, such as the ERK-regulated kinase p90RSK, the Ste20-like Nck-interacting kinase (NIK) and the Rho-associated kinase p160ROCK, phosphorylate NHE1 near its C-terminus [22]. In addition, Ca^{2+} regulates NHE1 by binding to its juxtamembrane region *via* calcineurin B homologous proteins 1 and 2 (CHP1 and CHP2) [23,24] and tescalcin (CHP3) [25] or by binding to two neighboring sites in its C-terminal regulatory domain *via* calmodulin (CaM) [26]. Associations with its high-affinity CaM domain release an autoinhibitory intramolecular interaction

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Received: December 07, 2016; **Accepted:** January 02, 2017; **Published:** January 05, 2017

that enhances NHE1 activity [11]. Although several other studies have been conducted to clarify how binding between Ca^{2+}/CaM and NHE1 occurs [27-31], and while the structure of the juxtamembrane region of the regulatory domain (503-545) involved in complexes with CHP1 or CHP2 has been determined using nuclear magnetic resonance [32] or X-ray crystallography [33], the binding mechanism by which Ca^{2+}/CaM activates NHE1 still is not well-described. Recently, using small-angle X-ray scattering analysis, Köster and co-workers [1] studied the molecular mechanisms underlying the phosphorylation-dependent regulation of NHE1, and they proposed an extended model to explain how Ca^{2+} regulates NHE1 activity *via* its C-terminal regulatory domain. These authors showed how CaM interacts with both CaM-binding regions in NHE1 and provided insight into how posttranslational modification by phosphorylation affects CaM binding to results in either the stimulation or the inhibition of NHE1 activity. In this model, CaM binding weakened the interaction between the autoinhibitory region and the proton modifier site, allowing protons unhindered access to this site and resulting in the up-regulation of the transport activity of NHE1. These authors [1] also reported that: 1) upon CaM binding, NHE1 is activated by a shift in sensitivity towards an alkaline intracellular pH, 2) the 2.23 Å crystal structure of the NHE1 CaM binding region (NHE1CaMBR) complexes with CaM and intracellular calcium [Ca^{2+}]i, 3) the C- and N-lobes of CaM bind to the first and second helix of NHE1CaMBR, respectively, and 4) both the NHE1 helices and Ca^{2+} -bound CaM become elongated, as confirmed by an analysis of their X-ray structure. More recently, it was demonstrated that 1) NHE1 and CaM are associated *in vivo* through endothelin-dependent signaling pathways [7], and 2) the modulation of NHE1 activity by various activators and inhibitors occurs as a result of the direct binding of these molecules to the lipid-interacting domain (LID), which alters the association between the LID and the plasma membrane [8].

NHE3

The NHE3 isoform is present in the epithelial brush borders of intestinal Na^+-absorptive cells and in renal tubules, where it mediates the majority of gastrointestinal and renal Na^+ absorption [34,35] and renal HCO^{3-} reabsorption [36-38]. NHE3 also influences other brush border transport processes, such as intestinal brush border Cl^-/HCO^{3-} exchanger, a putative anion transporter [39,40], and Cl^- secretion, which is mediated by the cystic fibrosis transmembrane regulator [41,42]. The more consistent characteristics of NHE3 include the following: 1) it is activated under basal conditions, 2) under basal conditions, NHE3 interacts with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), 3) only the γ isoform of this kinase associates with NHE3, 4) the NHE3 C-terminal domain, which is necessary for CaMKII binding under basal conditions, is 586–605 aa long and was predicted using multiple modeling programs to be α-helical, and 5) binding is rapidly reduced under conditions involving elevated physiological levels of Ca^{2+} [13]. However, other proteins associate with NHE3, including NHERF1–4, phospholipase Cγ, and CK2α [43,44]. In addition, the CaMKII-mediated inhibition of basal NHE3 activity is NHERF2-dependent, occurs as a result of changes in the NHE3 turnover number and is associated with the phosphorylation of NHE3. This regulatory effect requires amino acids at its C-terminal to interact with the CaMKII binding domain downstream of NHE3 aa 690 (Ser693, Ser694, and Ser810), which is part of the putative CaMKII phosphorylation consensus sequence [45,46]. Recently, it was demonstrated that 1) NHE3 basal activity is regulated by a signaling complex that is controlled by the sequential effects of two kinases, Akt and GSK-3, which act on a Ser cluster in the same NHE3 C-terminal domain that binds ezrin, and 2) these kinases regulate the dynamic association between ezrin and NHE3 to affect basal NHE3 activity [9]. CaMKII is therefore generally inactive in the presence of basal levels of Ca^{2+}, and active CaMKII is generally created *via* the autophosphorylation and release of the kinase sequence from the CaMKII autoinhibitory domain. These events are followed by autophosphorylation at Thr286/287 (depending on the species being studied) or oxidation at Met281 or Met282 [47], which result in a conformational change in CaMKII that allows high affinity interactions with its target proteins and prevents the inactivation of the kinase by re-association between its catalytic domain with the autoinhibitory domain when Ca^{2+} returns to basal levels. Similarities in the sequence alignment between the CaMKII binding domain of NHE3 and the CaMKII autoinhibitory domain [48] suggest that the catalytic subunit of the activated CaMKII (freed from its autoinhibitory domain) binds to a domain in its substrate, which resembles the kinase autoinhibitory domain but cannot inactivate the catalytic domain, while preventing access to the kinase autoinhibitory domain [49,50]. Therefore, CaMKII constitutively binds to, phosphorylates, and inhibits NHE3 *via* a NHERF2 protein-dependent process [48]. Because it has multiple functions, NHE3 is regulated by a wide variety of agonists in response to physiological conditions [4,13,51].

Angiotensin ii

The kidney is an important source of several components of the Renin-Angiotensin System (RAS) as well as a target organ for their activities. These mechanisms were recently described in a relevant review [52]. Most of the major known effects of the RAS are related to the activity of ANG II. However, a growing amount of evidence indicates that other peptides that have been more recently described, such as ANG-(1-7) and their respective receptors, increase the functional spectrum of the RAS [see below in ANG-(1-7) section]. Angiotensinogen, an α-2-globulin that is constitutively produced and released into the circulation mainly by the liver, is converted to ANG I by Renin, and the mRNAs of both ANG I and Renin are expressed in juxtaglomerular cells and renal tubular cells. Angiotensin-converting enzyme, which cleaves ANG I to form ANG II, is found principally in the lung but is also found in other tissues, including the kidneys. Complete RASs have also been described in other tissues, including the brain, vasculature and adrenal cortex [53,54].

In addition to its presence as a circulating hormone that has strong vasoconstrictive effects on systemic and glomerular hemodynamics, ANG II also has important paracrine effects on cell proliferation and the tubular transport of ions [55]. Under pathological conditions, ANG II has been shown to promote vascular remodeling, cardiac hypertrophic remodeling, and extracellular matrix deposition [56], and it is also implicated in inflammation, endothelial dysfunction, atherosclerosis, hypertension, and congestive heart failure.

Receptors and intracellular pathways

In this section, we briefly overview the available data regarding the signaling and functions of the various ANG II receptors. These topics have been reviewed in more detail in other publications [57,58]. There are two major ANG II receptors, AT1 and AT2. In addition, AT1 receptors are further subdivided into AT1A and AT1B [57]. While AT1 may be the main receptor that mediates the effects of ANG II on blood pressure, AT2 may also be partially involved in the regulation of blood pressure [59]. ANG II regulates blood pressure *via* AT1 receptors in both renal and extrarenal tissues and renal and extrarenal AT1A

receptors contribute almost equally to maintaining baseline blood pressure [60,61]. ANG II exerts a wide variety of functions *via* AT1, including (but not limited to) roles in cardiovascular homeostasis, renal functions, ion flux, protein phosphorylation, gene expression, cell growth, the stimulation of ALDO release, and central effects, such as eliciting thirst and AVP secretion [62].

The AT1 receptor is a 40-kDa protein that consists of 359 amino acids and is a member of the seven transmembrane domain G protein-coupled receptor family.These receptors typically couple with Gq complexes, resulting in the activation of downstream intracellular signaling pathways that lead to the activation of phospholipase C (PLC), phospholipase A2 (PLA2), and phospholipase D (PLD) [63]. The activation of PLC triggers an increase in the formation of 1,4,5-inositol triphosphate (IP3) and diacylglycerol (DAG), which promote the release of calcium from intracellular stores and the activation of protein kinase C (PKC), respectively [58]. In addition to inducing G protein- and non-G protein-related signaling pathways, ANG II, *via* AT1 receptors, performs its functions *via* MAP kinases (e.g., ERK 1/2, JNK, and p38MAPK), receptor tyrosine kinases (e.g., PDGF, EGFR, and insulin receptor), and nonreceptor tyrosine kinases [e.g., Src, JAK/STAT and focal adhesion kinase (FAK)]. The AT1R-mediated activation of NAD(P)H oxidase leads to the generation of reactive oxygen species, which are widely implicated in vascular inflammation and fibrosis. ANG II also promotes associations between scaffolding proteins, such as paxillin, talin, and p130Cas, which leads to focal adhesions and extracellular matrix formation. These signaling cascades lead to contraction, smooth muscle cell growth, hypertrophy, and cell migration, which are events that contribute to both normal vascular functions and disease progression [58]. The mechanisms involved in the regulation of the AT1 receptor functions and the magnitude of local ANG II effects include the regulation of the following: 1) AT1 receptor transcription, 2) AT1 receptor surface expression *via* internalization and membrane recycling and 3) AT1 receptor activity by accessory proteins.

The AT2 receptor is a seven-transmembrane domain protein that consists of 363 amino acids that have a molecular mass of 41 kDa and share 34% homology with AT1 [64]. AT2 receptors are highly expressed in a variety of tissues in developing embryos, but AT2 expression declines after birth [65,66]. Until now, the range of AT2-dependent functions and AT2-linked intracellular signaling events are still not completely understood [67]. In addition, under certain conditions, AT2 receptors form heterodimers with AT1 receptors, which results in the attenuation of AT1-mediated effects on ANG II (1). Several studies confirmed that ANG II binding to AT2 results in an increase in the formation of vasodilator agents, such as NO, prostanoids, and bradykinin [68].

Proximal tubule

The renal proximal tubule reabsorbs approximately 65% of the NaCl that it filters, which contributes to the regulation of plasma volume and blood pressure. Moreover, the proximal tubule reabsorbs approximately 80% of filtered bicarbonate, which plays an important role in the maintenance of systemic acid-base balance [69]. This process is dependent primarily on Na^+, involves the luminal Na^+/H^+ exchanger (NHE3) and the basolateral Na^+-HCO^{3-} cotransporter (NBC) and mediates a majority of sodium-coupled proximal renal reabsorption processes [69,70]. Under normal conditions, ANG II participates in several mechanisms that are involved in the renal acidification process. In their study of the *in vivo* microperfusion of the rat proximal convoluted tubule, Liu and Cogan were the first to show that ANG II increases proximal tubular bicarbonate reabsorption [71]. Another study also using an *in vivo* stationary microperfusion technique to study the rat proximal tubule [72] indicated that ANG II added to luminal or peritubular perfusion fluid stimulates bicarbonate reabsorption [73]. The stimulatory effects of ANG II on proximal NHE3 activity are mediated by both cAMP-dependent [71,74] and cAMP-independent mechanisms [75-77], although these effects may depend on the specific ANG II receptor subtype [78]. In addition, it is recognized that ANG II stimulates proximal Na^+-HCO^{3-} reabsorption by stimulating basolateral Na^+-HCO^{3-} cotransporter activity [79-81]. More recently, other studies have indicated that in IRPTC cells (a SV40-transformed cell line that was derived from rat proximal tubules) long-term exposure to ANG II (10-9 M) resulted in the upregulation of H^+-ATPase activity at least in part because it increased the cell surface expression of B2 subunit. These studies have also demonstrated that this regulatory pathway is dependent on mechanisms involving the activation of tyrosine kinase, p38 MAPK, and PI3K.

Dose-dependent biphasic effects of ANG II in the proximal tubule

It has been widely reported that in the proximal tubule, the effects of ANG II on NHE3 may be biphasic and dose-dependent: at low concentration, ANG II stimulates NHE3, while high concentrations of ANG II inhibit NHE3 [82,83]. Electrophysiological studies of isolated and perfused S2 segments of rabbit renal proximal tubules have confirmed that picomolar concentrations of ANG II stimulate [79] and micromolar concentrations of ANG II inhibit basolateral Na^+-HCO^{3-} cotransport [63]. The effects of ANG II on Na^+/K^+-ATPase in the proximal tubule are also biphasic [79,84,85]. It is likely that the inhibitory effect of high concentrations of ANG II on proximal transport could have some physiological significance because intrarenal concentrations of ANG II are much higher than the concentrations observed in plasma [86].

Although controversial data have been reported concerning the receptor subtype(s) that are responsible for the biphasic effects of ANG II on proximal tubular transport [87,88], some studies have clearly indicated that both luminal and basolateral AT1A receptors mediate the biphasic effects of Ang II on proximal transport [89-91]. It has been shown that at physiological concentrations, binding between ANG II and the AT1 receptor induces the rapid activation of PLC, which results in the release of IP3 and DAG, which are themselves involved in slightly increasing $[Ca^{2+}]i$ by mobilizing Ca^{2+} from intracellular storage and by activating PKC, respectively [92-94]. These processes may ultimately result in the activation of ERK [71,83,90]. At high concentrations, ANG II induces the activation of the phospholipase A2 (PLA2)/arachidonic acid/5,6-epoxyeicosatrienoic acid (EET) pathway and/or the NO/cGMP pathway and the mobilization of intracellular Ca^{2+}, also through mechanisms involving the AT1 receptor [83,86,95,96].

Hence, through these different pathways (which lead to lower or higher levels of $[Ca^{2+}]i$ through changes that are mediated by the AT1 receptor), ANG II exerts a dose-dependent bimodal effect. These findings were confirmed *in vitro* when it was demonstrated that in rat intact proximal tubule cells, ANG II, at low doses, stimulated NHE3 through a G protein–dependent protein kinase C pathway, whereas at higher doses, ANG II inhibited NHE3 activity through a mechanism involving cytochrome P-450–dependent metabolites [83]. In addition, in rabbit proximal tubular cells, physiological concentrations of ANG II stimulated amiloride-sensitive NHE3 activity [97]. In renal cortical

homogenates [98] and cultured renal proximal cells [99], ANG II inhibited adenyl cyclase. More recently, in opossum kidney proximal tubule (OKP) cells, it was confirmed that the activation of NHE3 by ANG II is mediated by IP3 receptor-binding protein, which is released with IP3 (IRBIT) and Ca^{2+}/calmodulin-dependent protein kinase II [100]. These results support the physiological relevance of IRBIT as an NHE3-interacting protein and its critical role in the regulation of NHE3 activity by ANG II, at least *in vitro*.

While the biphasic effects of ANG II on proximal tubular transport have been studied in rats, mice, and rabbits [101-105], little is known about the effects of ANG II on human proximal transport. However, recently, Shirai and colleagues found that in human cells, ANG II, unlike what was observed in other species, induced the dose-dependent and strong stimulation of human proximal tubular transport *via* the activation of AT1-dependent nitric oxide/guanosine 3', 5'-cyclic monophosphate/ERK [106]. Currently, the molecular mechanisms underlying species-specific differences in proximal tubular responses to the NO/cGMP pathway remain unknown [107].

Distal nephron

The effects of ANG II on HCO^{3-} transport in the thick ascending limb are controversial. ANG II inhibits HCO^{3-} absorption *via* a cytochrome P-450–dependent mechanism [108] and stimulates NHE3 [101]. In addition, ANG II stimulates Na^+/H^+ exchange in the early distal tubule and amiloride-sensitive Na+ channels in the late distal tubule of cortical nephrons [104].

Studies using *in vivo* microperfusion have confirmed that a low dose of luminal ANG II stimulates Na^+/H^+ exchange in the early and late distal segments of the rat kidney in addition to vacuolar H^+-ATPase in late distal segments [109]. In the rabbit cortical collecting duct, peritubular ANG II stimulated β cell apical HCO^{3-} secretion through a basolateral AT1 receptor [105]. In intercalated cells of connecting tubules and cortical collecting ducts, ANG II stimulated H^+-ATPase and may have contributed to the regulation of chloride reabsorption and bicarbonate secretion [110]. In the α intercalated cells of the mouse cortical collecting duct, ANG II stimulated the secretion of H^+ into the lumen, which drove Cl^- reabsorption *via* apical Cl^-/HCO^{3-} exchange and generated a more favorable electrochemical gradient for epithelial Na^+ channel (ENaC)-mediated Na^+ reabsorption [111]. ANG II stimulated ENaC in the rat cortical collecting duct *via* a Ca^{2+}-independent PKC pathway that activated NADPH oxidase (NOX), resulting in an increase in superoxide generation. This stimulatory effect of ANG II on ENaC may be partially responsible for blocking the arachidonic acid-induced inhibition of ENaC [112].

Dose-dependent biphasic effects of ANG II on Na^+/H^+ in the distal nephron

In research performed using MDCK cells (a permanent cell line with morphological and physiological similarities to cells in the collecting duct), we demonstrated that ANG II has a dose-dependent biphasic effect on Na^+/H^+ in the distal nephron [113]. In this study, we used fluorescent probes to measure the recovery rate of intracellular pH [(pHi)r] after inducing the acidification of the pHi *via* an NH_4Cl pulse, and we also monitored the $[Ca^{2+}]i$. Figure 1 shows that in the controls, the (pHi)r was 0.088 ± 0.014 pH units/min [114]. The addition of ANG II (10^{-12} M) to the bath caused a significant increase (38%) in the (pHi)r, and in the presence of ANG II (10^{-9} M), this increase was even more significant (123%). However, the addition of ANG II (10^{-7} M) significantly decreased it (77%). Using Atrial Natriuretic

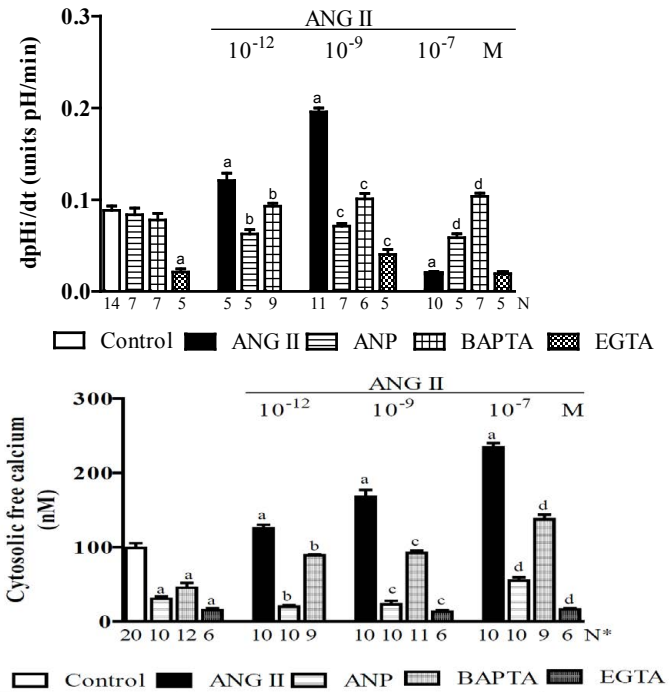

Figure 1. A study of MDCK cells [113] showing the effects of ANG II (at 10-12, 10-9 and 10-6M) alone or plus ANP (10-6 M), dimethyl-BAPTA/AM (5 X 10-5 M) or EGTA (2.5 mM) on the: A - pH intracellular recovery rate and B - cytosolic free calcium concentration. Values are shown as the means ± SE. N= no. of observations. N* = no. of experiments in which the maximum fluorescent signal was averaged for 10 cells. dpH/dt = pH intracellular recovery rate in the first 2 min after a NH_4Cl pulse. a = $P < 0.05$ *vs.* control. b = $P < 0.05$ *vs.* ANG II (10-12 M). c = $P < 0.05$ *vs.* ANG II (10-9 M). d = $P < 0.05$ *vs.* ANG II (10-7 M).

Peptide (ANP) or dimethyl-BAPTA-AM alone did not affect the (pHi) r but impaired both the stimulatory and inhibitory effects of ANG II on (pHi)r. The (pHi)r also decreased significantly when EGTA was applied alone (73%) or in addition to ANG II (10^{-9} M) (71%), but was not significantly altered when EGTA was applied with ANG II (10^{-7} M). Figure 1 also shows that MDCK cells exhibited a mean baseline $[Ca^{2+}]i$ of 99 ± 7 nM [115]. The subsequent addition of ANG II (10^{-12}, 10^{-9}, and 10^{-7} M) increased $[Ca^{2+}]i$ progressively from control values to 130% in a dose-dependent manner. The addition of ANP (10^{-6} M) to the bathing solution led to a rapid and significant decrease in $[Ca^{2+}]i$ (70%), and the subsequent addition of ANG II resulted in the recovery of $[Ca^{2+}]$ I, which reached 50% without exceeding normal baseline values, even in the presence of ANG II (10^{-7} M). This figure also indicates that dimethyl-BAPTA-AM alone led to a significant decrease in $[Ca^{2+}]$ i (50%) and that the subsequent addition of ANG II resulted in the recovery of $[Ca^{2+}]i$ without achieving control values. However, the presence of EGTA in the cell suspension led to a significant decrease in $[Ca^{2+}]i$ (85%), and the subsequent addition of ANG II did not result in the recovery of $[Ca^{2+}]i$. Hence, the results indicate that ANG II operates through different pathways (which lead to either a small or large increase in $[C^{2+}a]i$) and has a dose-dependent bimodal effect on the regulation of (pHi)r by modulating Na^+/H^+ activity. ANP and dimethyl-BAPTA-AM cause a moderate decrease in $[Ca^{2+}]i$ that does not affect (pHi)r but does impair the pathway that causes an increase in $[Ca^{2+}]i$, thereby blocking both the stimulatory and the inhibitory effects of ANG II on this process. In addition, EGTA caused a sharp reduction in $[Ca^{2+}]i$, impaired (pHi)r and blocked both the stimulatory and the inhibitory effects of ANG II on (pHi)r. In this study, experiments that were performed on per meant filter supports indicated that the Na^+/H^+

exchanger, which is responsible for Na^+-dependent (pHi)r, is located on the basolateral membrane. Taken together, these results suggest that $[Ca^{2+}]i$ plays a role in regulating (pHi)r *via* a process that is mediated by NHE1 exchanger and stimulated/impaired by ANG II. To better understand this bimodal effect of ANG II on NHE1 and to support previous findings [11,116], it was suggested that site A of this exchanger (the high-affinity site on CaM) works as an autoinhibitory domain, and a discrete increase in $[Ca^{2+}]i$ (in the presence of low concentrations of ANG II) may induce Ca^{2+}/CaM binding to this region, thereby blocking the inhibitory interaction, resulting in the activation of NHE1 (Figure 6). On the other hand, it is possible that site B of the exchanger (the low-affinity site for CaM) binds to Ca^{2+}/CaM only at high $[Ca^{2+}]i$ (in the presence of high concentrations of ANG II). Under these conditions, it inhibits NHE1 activity (Figure 6). To test this hypothesis, we used site-directed mutagenesis to provide some new information regarding how each Ca^{2+}/CaM-binding site in the carboxy-terminus of NHE1 confers responsiveness to ANG II [117]. This study indicated that under control conditions, Ca^{2+}/CaM binding sites do not function to maintain the basal activity of NHE1. However, in the presence of slightly increased $[Ca^{2+}]i$ that was induced by low ANG II concentrations, site A seems to be responsible for the stimulation of NHE1, whereas in the presence of a significant increase in $[Ca^{2+}]i$, which was induced by high levels of ANG II, site B plays an important role in maintaining the basal activity of the Na^+/H^+ exchanger. Therefore, these results showed that both sites play important regulatory roles in the calcium-dependent modulation of NHE1 by ANG II.

In more recent studies, it was demonstrated that in MDCK cells, the regulation of NHE1 and NHE3 activity by ANG II is mediated by the activation of the ANG II type I receptor/phospholipase C/calcium/calmodulin pathway [118].

Angiotensin-(1-7)

Over the past 15 years, our understanding of the RAS has substantially increased, and it is currently accepted that both circulating and tissue RAS are far more complex than previously thought. Thus, in addition to its traditional components (described in section 2.2: ANG II in the present review), the modern concept of the RAS includes the following: a new enzyme (ACE2) [119,120], peptides such as ANG-(1–7) and ANG A [121], the pro-renin receptor [122], Mas receptor [123], and Mas-related G-protein–coupled receptor D [124] and the heptapeptide alamandine [124]. However, although ANG III and ANG IV, the smaller peptide fragments of the RAS, also have biological activity, their plasma levels are much lower than the levels of ANG II or ANG-(1-7) [125,126].

This area of research has been described in several previous reviews [52,127-133].

Mas receptor

The identification of the ACE homolog ACE2, an enzyme that is important for the generation of ANG-(1–7), and the G protein-coupled receptor Mas, which is encoded by the Mas proto-oncogene and is a receptor for ANG-(1–7), allowed researchers to determine that the RAS system contains at least two cascades: i) the ACE2-ANG-(1–7)-Mas axis, which probably act as the counterregulatory portion of ii) the classical RAS axis, or the ACE-ANGII-AT1 and AT2 receptors axis. The Mas gene is expressed in the brain, testes, kidney, heart [134-136] and central nervous system, where it is found in various regions, including cardiovascular regulatory areas [137]. In agreement with the activities that were previously described for ANG-(1–7), Mas-deficient mice exhibited increased blood pressure, impaired endothelial function, decreased NO production, and decreased endothelial NO synthase expression [138,139]. Also in agreement with findings showing the cardioprotective effects of ANG-(1–7), a genetic deletion of the Mas receptor impaired heart function and changed the extracellular matrix to a profibrotic state [140]. After the activation of the Mas receptor, the intracellular signal transduction mechanisms that are involved in the following processes or tissues are poorly understood: i) *in vivo*, in the rat heart, ANG-(1–7) stimulated the phosphorylation of Janus kinase 2 (JAK2), insulin receptor substrate (IRS)-1 and Akt [141], ii) Mas receptor activation led to an increase in NO production *via* the phosphorylation of eNOS, a process that involves the activation of phosphatidylinositol 3-kinase-dependent Akt phosphorylation [142,143], and iii) upon the activation of the Mas receptor, MAPK phosphorylation is inhibited [144,145].

Other receptors

ANG-(1–7) can also bind to the AT1 and AT2 receptors, although only at high hormonal concentrations [146-148]. However, studies indicating that AT2 is involved in ANG-(1-7)-induced vasorelaxation have not produced conclusive data [124,148-150], and the possibility of a physical or functional interaction between Mas and AT2 should be considered [152-155].

In addition, the existence of a new ANG-(1–7) receptor subtype has been suggested [156], and an interaction between ANG-(1–7) and different ANG II receptors has also been proposed [157,158]. In hypertensive animals, ANG-(1–7)–induced vasodilation was restored by acute or chronic AT1 blockade with losartan, suggesting an interaction between AT1 and Mas [157-159]. Furthermore, the contribution of AT2 and bradykinin B2 receptor (BKR) to the vascular effects of ANG-(1–7) should not be disregarded, and they suggest the potential presence of crosstalk between BKR with Mas and AT2 [148,160,161].

Synthesis of ANG-(1–7)

ANG-(1–7) is formed from ANG I and ANG II *via* the activity of ACE, ACE2, and several other enzymes [162]. ACE is the main enzyme that is responsible for the conversion of ANG I into ANG II, and ACE2 then cleaves ANG II into ANG-(1–7) [163]. Furthermore, ACE2 forms ANG-(1–9) from ANG I, and ANG-(1–9) can be converted into ANG-(1–7) by ACE [119]. However, the preferable physiological substrate for ACE2 is ANG II [120,163].

ANG-(1–7) is subsequently metabolized into an inactive fragment, ANG-(1–5), by ACE [164-166]. The half-life of ANG-(1–7) is several seconds, and ACE inhibitors, which inhibit the metabolism of ANG-(1–7) into ANG-(1–5), increase the half-life of ANG-(1–7) [167].

ACE2 is expressed in the heart, kidneys, and testes and, to a lesser extent, the liver, lungs, small intestines and brain [168-170]. In the kidney, ACE2 gene expression has been observed in glomeruli, the vasa recta and all nephron segments except for the thick ascending limb [171]. Additionally, relatively high amounts of ACE2 were detected in the apical brush-border membrane of proximal tubule epithelia, where it colocalized with ACE [171].

Actions of Ang-(1–7) in the kidney

Several studies have demonstrated that the kidney is an additional target of ANG-(1–7) activity. However, the renal effects of this heptapeptide are incompletely understood. In isolated, perfused rat

kidneys and anesthetized animals [172,173], ANG-(1–7) has been shown to increase sodium and water excretion and the glomerular filtration rate (GFR) without affecting renovascular resistance. Hence, it is also possible that ANG-(1–7) induces diuresis and natriuresis by inhibiting renal tubular Na+/K+-ATPase [174,175]. However, ANG-(1–7) also induced antidiuresis in water-loaded rodents (258) and increased renal tubular sodium reabsorption in rats [176]. In addition, in normal and hypertensive rats [177] and in water-loaded [178] or virgin female rats [179], ANG-(1–7) had an antidiuretic effect that was mediated, at least in part, by the Mas receptor. Furthermore, in Sprague-Dawley rats, the administration of ANG-(1–7) resulted in antidiuresis that was associated with an upregulation in renal aquaporin-1 [180], and in isolated rat inner medullary collecting duct cell suspensions, ANG-(1–7) (10^{-9} M) increased cAMP production [181], a response that was attenuated by Mas receptor antagonists and the pharmacological blockade of the AVP V2 receptor. ANG-(1–7) also caused afferent arteriolar vasodilation and antagonized the vasoconstrictor effects of ANG II [114].

Actions in the proximal tubule: In the proximal tubular segment, the actions of ANG-(1-7) are often conflicting. In the proximal tubule of anesthetized animals, ANG-(1–7) increases urinary flow rates and sodium excretion. This effect was abolished by the Mas receptor antagonist A779 in rats [175], but in dogs, this hormonal effect was partially blocked by the AT1 receptor antagonist EXP 3174 but not by the AT2 receptor antagonist PD123319 [182]. In isolated rat proximal straight tubules, similar to ANG II, ANG-(1–7) exerts a biphasic effect through AT1 receptors. At physiological levels (10–12 M), ANG-(1–7) increases fluid and bicarbonate reabsorption, while at high concentrations (10–8 M), ANG-(1–7) decreases fluid reabsorption [183]. In rabbit proximal tubular cells, ANG-(1–7) inhibits sodium reabsorption by activating phospholipase A2 [184]. In addition, in isolated basolateral membranes of kidney proximal tubules [175,185], ANG-(1–7) modulated the activity of Na^+/K^+-ATPase, and in isolated pig kidney inner cortical membranes [186], it inhibited Na^+-ATPase activity *via* an effect that was reversed by an AT2 receptor antagonist. Furthermore, in isolated proximal tubules, i) ANG-(1–7) stimulated the release of arachidonic acid [184], and ii) the inhibition of prostaglandin release that resulted from COX inhibition attenuated the ANG-(1–7)-induced increase in urine flow and sodium excretion [187]. Taken together, these results suggest that in the proximal tubule, ANG-(1–7) affects natriuresis and diuresis by activating Mas receptors. However, AT1 and AT2 receptors may also be involved.

Dose-dependent biphasic effect of ANG-(1–7) on proximal Na^+/H^+ exchanger: Because i) the nature of the mechanism underlying the effect of ANG-(1–7) on proximal nephron bicarbonate reabsorption has not yet been clearly defined [175,182-184], ii) apical NHE3 mediates most NaCl, $NaHCO^{3-}$, and fluid reabsorption by the renal proximal tubule and is critical for the normal maintenance of extracellular fluid volumes, blood pressure, and acid-base balance [188], and iii) under physiological conditions, plasma concentrations of ANG-(1–7) are in the picomolar range but can increase under conditions involving extracellular volume expansion [189,190], the purpose of a recent study by our laboratory [191] was to determine the acute direct effects of different doses of ANG-(1–7) on net bicarbonate reabsorption ($JHCO^{3-}$) *via* the NHE3 exchanger *in vivo* in the proximal convoluted tubules (S2 segment) of normal Wistar rats. The results were evaluated using stationary microperfusions *via* H ion-sensitive microelectrodes. Figure 2 shows that the control $JHCO^{3-}$ was 2.82 ± 0.078 [192] nmol/cm^{-2}.s^{-1}. ANG-(1–7), at a low hormonal dose (10^{-12} or 10^{-9} M), significantly

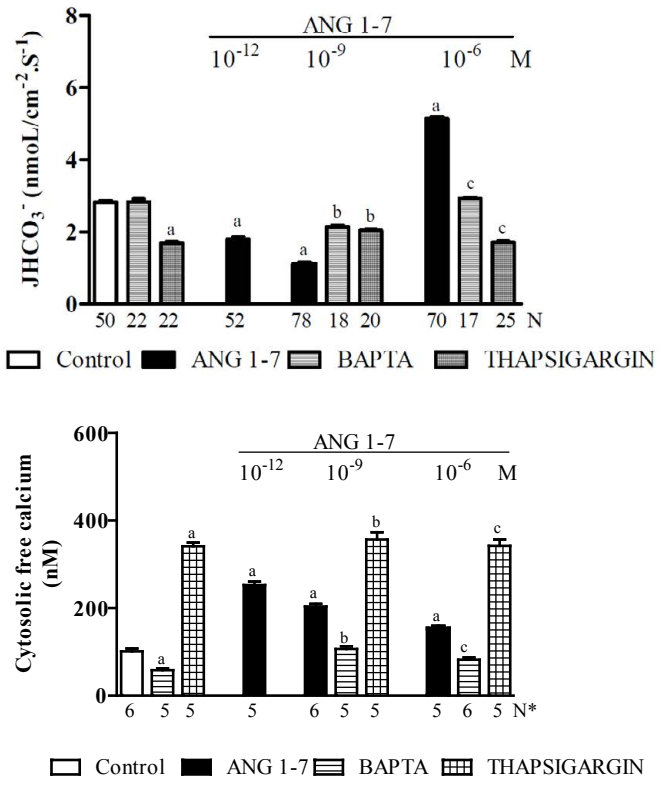

Figure 2. An *in vivo* study of the proximal convoluted tubules of normal Wistar rats [191] showing the effects of ANG-(1-7) (10-12, 10-9 or 10-6 M) alone or plus dimethyl-BAPTA/AM (5 X 10-5 M) or Thapsigargin (10-5 M) on: A - net bicarbonate reabsorption and B – the cytosolic free calcium concentration. Values are shown as the means ± SE. N= no. of observations. N* = no. of experiments in which the maximum fluorescent signal was averaged for 10 cells. $JHCO_3^-$ = net bicarbonate reabsorption. a = $P < 0.05$ *vs*. control. b = $P < 0.05$ *vs*. ANG-(1-7) (10-9 M). c = $P < 0.05$ *vs*. ANG-(1-7) (10-6 M).

inhibited (37% or 61%, respectively) while a high hormonal dose (10^{-6} M) stimulated (90%) the NHE3 exchanger. This biphasic action of ANG-(1–7) on Na^+/H^+ was the reverse of the biphasic action that was described for ANG II, *i.e.,* as previously discussed, it is widely accepted that ANG II stimulates Na^+/H^+ exchange at low doses and inhibits it at high doses (Figure 1). Figure 2 also shows that dimethyl-BAPTA-AM alone did not affect the $JHCO^{3-}$ concentration but did impair both the inhibitory and the stimulatory effects of ANG-(1-7) on it. In addition, Thapsigargin alone inhibited $JHCO^{3-}$ and impaired both the inhibitory and stimulatory effects of ANG-(1-7) on it. Figure 2 also shows that the proximal convoluted tubule exhibited a mean baseline $[Ca^{2+}]i$ of 101 ± 2 nM [164]. The addition of ANG-(1-7) (10^{-12}, 10^{-9}, and 10^{-6} M) increased $[Ca^{2+}]i$ (by 124%, 100% and 78%, respectively). The addition of BAPTA led to a significant decrease in $[Ca^{2+}]i$ (by 42%), and the addition of ANG-(1-7) resulted in a recovery of $[Ca^{2+}]i$ without achieving baseline values. Thapsigargin alone or with ANG-(1-7) (10^{-9} or 10^{-6} M) increased $[Ca^{2+}]i$ (by approximately 350%). The data in that study also showed that the biphasic effect of ANG-(1–7) on the Na+/H+ exchanger occurred *via* the Mas receptor and that A779 (a Mas receptor antagonist) abolished this hormonal biphasic effect. These results are also consistent with previously described findings in the literature that indicated that the renal effects of ANG-(1–7) are dose dependent and mediated, at least in part, by the Mas receptor [193-196]. Nevertheless, in isolated rat proximal straight tubules (S3 segment), it was shown that, like ANG II, ANG-(1–7) exerts a biphasic effect through AT1 receptors: at physiological levels, ANG-(1-7) increases fluid and

bicarbonate reabsorption, while at high concentrations, ANG-(1-7) decreases these parameters [183]. This finding is in contrast to previous results described from *in vivo* experiments involving rat proximal convoluted tubules (S2 segment) [191]. Thus, whether one or more different signaling systems are involved in mediating the effect of ANG-(1–7) on NHE3 activity in the S2 and S3 proximal segments remains to be established. In addition, the renal effects of ANG-(1–7) may also involve AT1 and AT2 receptors [182,184,186] and V2 receptors [181], indicating the existence of a cross-talk mechanism between the Mas receptor and the ANG II and AVP systems. Furthermore, in a study of isolated rat proximal straight tubules [183], the actions of ANG-(1–7) were blocked by an AT1 antagonist, suggesting a different site of action than the one that was assessed in the *in vivo* rat proximal convoluted tubules study [191]. However, the results of the *in vivo* rat proximal convoluted tubules study were consistent with the hypothesis presented in the current literature [128,197], which suggests that both ANG-(1–7) and ANG II have opposite effects on several cardiovascular mechanisms and that at physiological doses, ANG-(1–7) is a vasodilator and ANG II is a vasoconstrictor. In addition, the results of *in vivo* experiments on rat proximal convoluted tubules [191] were compatible with the notion that the NHE3 exchanger is stimulated by a moderate increase in $[Ca^{2+}]i$ in the presence of a high dose of ANG-(1–7) (10^{-6} M) and that the inhibition of this exchanger largely increases the $[Ca^{2+}]i$ that was induced by low doses of ANG-(1–7) (10^{-12} or 10^{-9} M). These findings are in opposition to those described in previous studies of ANG II [113,198]. Additionally, the experiments in the *in vivo* rat proximal convoluted tubules study used BAPTA or thapsigargin [191] and confirmed the role of cytosolic calcium in the regulation of NHE3. Thus, the interaction between the opposing dose-dependent effects of ANG-(1–7) and ANG II on the renal proximal Na^+/H^+ exchanger and $[Ca^{2+}]i$ levels may represent an important physiological regulatory mechanism that controls extracellular volume and/or changes in pH. However, this is a complex mechanism, and additional factors need to be investigated. In addition, other factors must be considered. For example, in the *in vivo* rat proximal convoluted tubules study [191], the effects of luminal perfusions of ANG-(1–7) were examined using a stopped-flow microperfusion technique that is not affected by glomerular filtration rates and allows the researcher to measure tubular acidification on line [72]. Moreover, the experimental conditions were such that the measurements were performed after pre-determined amounts of hormone were added to the *in vivo* tubules. Therefore, the hormonal effects were local and did not cause systemic hemodynamic changes because the amount of hormone that was introduced into the tubules was insignificant. This result was confirmed by the observation that there were no changes in urine flow and osmolality, Na+ excretion, or systemic acid-base values during the experiments in which luminal hormonal microperfusions were performed [191]. Furthermore, in this study, the luminal perfusion solution for ANG-(1-7) contained raffinose (a nonresorbable molecule), which was added to achieve isotonicity. This prevented fluid reabsorption from being induced by the hormone. Thus, it is possible that the conflicting and incompletely understood proximal effects of this heptapeptide that are described in the current literature can be explained by the fact that in several studies in which ANG-(1–7) was injected parenterally, induced systemic hemodynamic effects under conditions in which the measurements were not made at determined hormonal levels within the proximal tubule.

Given that i) it is widely recognized that the RAS plays a role in the pathophysiology of cardiovascular and renal diseases and that under such situations, the beneficial effects of ANG-(1-7) are in opposition to the deleterious effects of ANG II [199], and ii) since the plasma concentrations of ANG-(1–7) are higher in hypertensive rats [126,189,190], it is likely that in this situation, the high plasma levels of ANG-(1-7) have an inhibitory effect on NHE3 in the proximal tubule, which should ease hypertension. Hence, the purpose of a current study in our laboratory is to clarify the direct effects of ANG-(1–7) on the NHE3 exchanger *in vivo* in the proximal convoluted tubules of SHR rats (hypertensives) and their controls, WKY rats (normotensives) [unpublished data]. Figure 3 shows that in WKY rats, the control proximal JHCO3- that is maintained by the NHE3 exchanger is 2.41 ± 0.12 (57) nmol/cm^{-2}s^{-1}. ANG-(1–7), when applied at a low dose (10^{-9} M), significantly inhibited (by approximately 30%) while a high dose (10^{-6} M) stimulated (by approximately 70%) the activity of the NHE3 exchanger. This biphasic action of ANG-(1–7) on NHE3 in the WKY rats was similar to the biphasic action that was described for ANG-(1-7) in Wistar rats (see Figure 2). Figure 3 also shows that the proximal convoluted tubule in the WKY rats exhibited a mean baseline $[Ca^{2+}]i$ of 99 ± 3 nM [193]. The subsequent addition of ANG-(1-7) (10^{-9} or 10^{-6} M) increased the $[Ca^{2+}]i$ i (by approximately 100% and 58%, respectively). However, in the SHR rats, the control proximal JHCO3- was 1.98 ± 0.13 [200] nmol/cm^{-2}s^{-1}, and applying ANG-(1–7) at a low dose (10^{-9} M) significantly stimulated (by 57%) while a high dose (10^{-6} M) significantly inhibited (by 38%) the NHE3 exchanger. Hence, the biphasic action of ANG-(1–7) on NHE3 in the SHR rats was similar

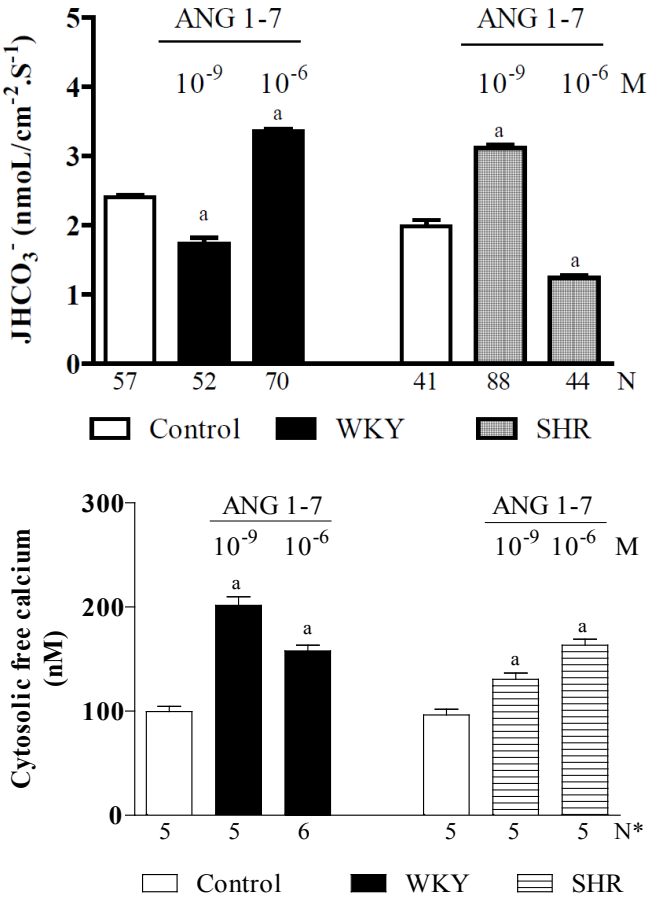

Figure 3. Current studies of *in vivo* proximal convoluted tubules in SHR (hypertensive) rats and their WKY (normotensive) controls, indicating the effects of ANG-(1-7) (10-9 or 10-6 M) on: A - net bicarbonate reabsorption and B – cytosolic free calcium concentrations [unpublished data]. Values are shown as the means ± SE. N = no. of observations. N* = no. of experiments in which the maximum fluorescent signal was averaged for 10 cells. JHCO3− = net bicarbonate reabsorption. a = $P < 0.05$ *vs.* control.

to the effect observed for ANG II in Wistar rats (Figure 1). Therefore, these results indicate that in hypertensive animals, a high plasma concentration of ANG-(1-7) [126,189,190] attenuates hypertension. Figure 3 shows that proximal convoluted tubules in SHR rats exhibited a mean baseline $[Ca^{2+}]i$ of 96 ± 2 nM [195], and the subsequent addition of ANG-(1-7) (10^{-9} or 10^{-6} M) increased it (by approximately 30% and 63%, respectively). The biphasic effects of ANG-(1-7) on the Na+/H^+ exchanger in Wistar, SHR (hypertensives) and controls WKY (normotensives) rats are summarized in Figure 6.

Aldosterone

ALDO is a steroid hormone with mineralocorticoid activity that plays an important role in the maintenance of Na^+, K^+, water and acid-base balance *via* effects on renal electrolyte excretion. ALDO is produced mainly by the adrenal glomerulosa, but there is also evidence showing that it can be synthesized in other tissues. The most convincing evidence has been found in the central nervous system, whereas data suggesting that this hormone is produced in the heart remain controversial. The evidence demonstrating the extra-adrenal secretion of ALDO, coupled with its known non-epithelial actions, has led to speculation that ALDO may act in such tissues in an autocrine or paracrine manner [201,202].

Adrenal-derived ALDO is the principal source of the circulating and locally available supply of this hormone. Several factors regulate ALDO production, including adrenaline, vasoactive intestinal polypeptide, serotonin, ouabain, atrial natriuretic peptide, dopamine, heparin and adrenomedullin. Recently, novel factors that are secreted by adipose tissue have also been shown to stimulate ALDO synthesis *in vitro*. However, the principal regulators of the synthesis and secretion of this hormone are ANG II, the concentration of extracellular potassium and ACTH [203,204].

It was classically accepted that ALDO is synthesized in the adrenal zona glomerulosa and that its lipophilic nature causes it to enter the cell *via* diffusion through the cellular membrane. It then binds to a specific mineralocorticoid receptor (MR) that is located in the cytosol of target cells in epithelial and non-epithelial tissues. The translocation of the resulting steroid receptor complex to the cell nucleus modulates the gene expression and translation of specific ALDO-induced proteins that regulate electrolyte and fluid balance, and consequently blood-pressure homoeostasis. ALDO may also act through alternative receptors in epithelial and non-epithelial tissues in a rapid non-genomic manner that is independent of gene transcription and translation. The modulatory effect of non-genomic signalling responses on the transcriptional effects of MR is an emerging theme, and the relevance of these events to the effects of ALDO on whole-body electrolyte and acid-base homeostasis remains to be determined. In addition, for more than a decade, it has been accepted that an increase in ALDO circulating levels, in combination with a high-sodium diet, induces hypertension and cardiovascular and renal damage that are triggered by, among other factors, MR, epidermal growth factor receptor (EGFR) and oxidative stress independent of the RAS [205].

Regulation of ALDO biosynthesis

Renin–Angiotensin system: ALDO biosynthesis is principally regulated by the RAS, a system that is described above in sections 2.2 ANG II and 2.3 ANG-(1-7) of the present review. The stimulation of Renin release increases the plasma levels of ANG II, which subsequently stimulates the secretion of ALDO from the adrenal glands [206]. The stimulation of the formation of ANG II and ALDO increases sodium reabsorption and consequently increases the sodium content of the body, which, in turn, inhibits Renin gene expression [206-208]. Hence, the *in vivo* effect of ALDO may be an indirect consequence of changes in the sodium content of the body, extracellular volume and/or blood pressure. It is also possible that ALDO exerts a direct positive effect on Renin gene expression at the cellular level, probably by stabilizing Renin mRNA [52]. The adrenal response to ANG II occurs within minutes, a time course that implies that no new protein synthesis is required. The acute, ANG II-mediated release of ALDO may involve its rapid synthesis from intermediate compounds in the steroid genic pathway or its de novo synthesis from cholesterol, possibly as a consequence of Star protein activation, which leads to an increase in the transport of cholesterol to the inner mitochondrial membrane.

Extracellular K^+ concentration ($[K^+]e$): The production of ALDO is acutely sensitive to very small changes in $[K^+]e$. Increased $[K^+]e$ stimulates ALDO secretion, which helps to maintain K^+ homoeostasis. The effects of $[K^+]e$ and ANG II are synergistic, so that the prevailing $[K^+]e$ determines the concentration/effect of ANG II-mediated ALDO production [203,204,206].

ACTH: Despite the opposing effects that have been observed in the presence of acute and chronic ACTH, there is no doubt that this hormone is involved in the normal, physiological regulation of ALDO production [209-211].

Intracellular pathways of ALDO

Important intracellular signalling peptides that are involved in ALDO activities include epidermal growth factor (EGF) and its receptor (EGFR) [212], which participate in signalling events related to the activation of G protein-coupled receptor proteins, growth hormones and cytokines *via* a transactivation mechanism that may play a central role in signal transduction [213]. The interaction between MR and ALDO triggers genomic actions and also promotes the transactivation of the EGFR, an event that is necessary for the non genomic activation of the ERK1/2 cascade. These are mitogen-activated protein kinases (MAPKs), which are a group of serine/threonine kinases that, when stimulated, phosphorylate their substrates at serine and/or threonine residues. These kinases are involved in transducing signals from the cell membrane to the nucleus [214]. The ERK1/2 signalling pathway is activated by RAF-type MAP kinases. When these kinases are phosphorylated, they activate the MAP kinases MEK1 and MEK2, which themselves phosphorylate and activate the ERK1/2 kinases. When activated, the ERK1/2 kinases phosphorylate several effector molecules that are involved in many fundamental cellular processes, such as differentiation, proliferation, apoptosis, survival and metabolism [215]. In the kidneys, the ERK1/2 pathway participates in the hormonal regulation of different ionic carriers along the nephron, and they play an important role in maintaining the homeostasis of extracellular fluids [216].

In addition to the important physiological role of ALDO in the kidney, in recent years, the role of ALDO in the pathogenesis of renal injury has also been extensively studied, and several studies have indicated that ALDO and its classic receptor (MR) are involved in the development of inflammation, oxidative stress, glomerulosclerosis, fibrosis, and apoptosis in renal tissue [217-221]. Additionally, *in vivo* and *in vitro* studies have suggested that ALDO contributes to the occurrence of apoptosis in glomerular, tubular and mesangial cells [219,222,223]. According Briet and Schiffrin [224] and Brown [200], the signaling pathways that potentially involve ALDO in the induction of inflammation, oxidative stress, apoptosis and fibrosis

in the kidney could include the following. i) After entering the cell, ALDO binds to cytoplasmic receptors (MR and/or GR) to promote the transactivation of EGFR and the phosphorylation of c-Src, resulting in the phosphorylation of ERK1/2 and pro-fibrotic responses. In addition, C-Src also stimulates the function of NADPH oxidase (probably NOX 4) and the production of reactive oxygen species (ROS), which participate in the induction of inflammation, oxidative stress, apoptosis and fibrosis in kidney tissues. ii) ALDO binds to a membrane receptor (GPR30) to stimulate the transactivation of EGFR and the phosphorylation of ERK1/2 by triggering the same processes described above. iii) Finally, the translocation of a complex containing MR and/or GR-ALDO into the nucleus promotes the transcription of fibrosis-related (e.g., PAI-1 and TGFb-1) and apoptosis (e.g., BAX and BCL-2) genes.

Classic genomic actions of ALDO

Epithelial actions: Classically, it has been accepted that ALDO acts on epithelial cells, particularly in the renal collecting duct, where it regulates the transport of Na^+, K^+, H^+ and water. The main functions of classic genomic ALDO activity in epithelial cells are: i) Na^+ reabsorption across the apical membrane, which is mediated by the luminal amiloride-sensitive epithelial Na^+ channel (ENaC); ii) transport across the basolateral membrane, which is driven by the ouabain-sensitive Na^+/K^+- ATPase; iii) the secretion of K^+ from the cell into the lumen through luminal K^+ channels; iv) the secretion of H^+ through the luminal vacuolar H^+-ATPase; and v) water transport, which follows the movement of Na^+ across the membrane. Apical Na^+ channel activity is the limiting step in the transport process, and it is likely that ALDO ultimately acts to increase the open time of existing ion channels and/or increase the total number of such channels [225]. However, other protein targets have also been identified, including i) a luminal NHE3 exchanger in the colon [226,227] and the proximal tubule [228] and a basolateral NHE1 in the proximal tubule [229,230], ii) an Na^+/K^+-ATPase in human kidney proximal tubule (HKC11) cells [231], iii) a luminal thiazide-sensitive Na^+/Cl^- cotransporter in the distal renal tubule that appears to mediate Na+ reabsorption in response to volume depletion [232], and iv) a renal proximal H^+-ATPase [233].

The genomic epithelial actions of ALDO operate in early (1 – 6 h) and late (> 6 h) phases. The genomic early phase is mediated by changes in gene expression that activate ion channels and signaling proteins, which then induce electrolyte-transport proteins. The genomic late phase results from both primary and secondary effects on gene expression. One of the early ALDO-induced proteins is Sgk1, a serine-threonine kinase [234,235]. Presumably, Sgk1 binds and phosphorylates the ENaC regulatory protein (Nedd4–2) to reduce its binding to ENaC [236]. A subsequent reduction in ENaC ubiquitination by Nedd4–2 increases ENaC density and stability at the apical membrane, resulting in increased ENaC-dependent Na^+ reabsorption. In addition, during the early phase of genomic ALDO action, the expression of the small, monomeric Kirsten Ras GTP-binding protein-2A (Ki-RasA) is induced and required for the ALDO-mediated effects on Na^+ transport in renal epithelial cells. Ki-RasA appears to have dual contrasting effects on ENaC channels by i) keeping the channel open and ii) decreasing the number of channels in the plasma membrane [235,237,238]. The activity of the lipid kinase PI3K is increased by ALDO in the kidney [235,239], and the inhibition of PI3K reduces both the early and late genomic actions of ALDO [225].

The corticosteroid hormone-induced factor (CHIF) is expressed in the basolateral membranes of epithelial cells in the distal colon and nephron [235,240]. ALDO probably stimulates CHIF expression, and the resulting protein interacts with final effectors to promote ion transport [241,242].

Target tissue specificity: Although it has been almost 60 years since ALDO was first isolated and its physiological effects on renal function identified [243], until the end of the last century, the specificity of ALDO action was believed to be conferred by the abundant presence of high-affinity type 1 MR in the cytosol of ALDO target tissues [244]. This receptor belongs to the nuclear receptor superfamily and is composed of several functional domains that include an N-terminal domain, a highly conserved DNA-binding domain and a C-terminal ligand-binding domain [244]. ALDO binding results in a conformational change and a dissociation of associated proteins followed by dimerization and translocation to the cell nucleus [245] and the subsequent activation or repression of transcriptional activity [246].These effects can be inhibited by actinomycin and cycloheximide, which block transcription and translation, respectively [225].

The MR is distinguishable from the glucocorticoid receptor (GR), which is ubiquitously expressed and exhibits a higher affinity for glucocorticoids. The lack of specificity of the MR and the fact that plasma levels of glucocorticoids are 1000 times higher than those of ALDO suggest that MR should be predominantly occupied by glucocorticoids. Hence, the ability of ALDO to act on MR was partly explained by the fact that the majority of glucocorticoids are bound to proteins in plasma. However, the concentration of free glucocorticoids in plasma is about 100 times higher than the concentration of ALDO, suggesting that there must be an additional mechanism underlying the action of ALDO in the target tissues independent of the MR [225].

Non-epithelial actions: MR is also localized in a number of non-epithelial tissues, especially in the cardiovascular system (CVS) and central nervous system (CNS). However, while the properties of the MR in these tissues are largely similar, the effects that MR mediates are extremely diverse. In the CVS, ALDO promotes cardiac hypertrophy, fibrosis and abnormal vascular endothelial function, while in the CNS, it appears to regulate blood pressure, salt appetite and sympathetic tone [247-250].

Crosstalk between ALDO and ANG II also plays a role in regulating the migration of vascular smooth muscle cells *via* interactions with c-Src-regulated redox-sensitive RhoA pathways [251], which emphasizes the benefits of dual therapy [252]. In addition, the subcommissural organ is involved in the central regulation of ALDO secretion and sodium homoeostasis, while an amygdala MR has been implicated in the control of salt appetite [253].

Non-genomic actions of ALDO

Since the end of the last century, in addition to its classic genomic activities, ALDO has been demonstrated to induce rapid cellular non-genomic effects. On the basis of several lines of evidence [254-257], it has been proposed that non-genomic ALDO actions i) are mediated by a distinct and specific membrane receptor that is different from the classical intracellular MR, ii) are insensitive to classical MR antagonists, such as canrenone or spironolactone, iii) are based on a high affinity for ALDO but not for glucocorticoids. The physiological and clinical relevance supporting these rapid effects remains unclear, but their existence has been described in various target organs and cells, including amphibian skin and urinary bladders [258,259], vascular smooth muscle cells and endothelial cells [260], skeletal muscle cells [261], human mononuclear leukocytes [262], cardiac myocytes [263],

skin fibroblasts from MR-knockout mice [256], colonic epithelial cells [264] and isolated colonic crypts [265]. Several sites in the kidney, particularly cultured kidney cells, have been shown to be sensitive to non-genomic ALDO action [266], including principal cells that were freshly isolated from rabbits [267], the human distal colon [268], *in vivo* renal proximal tubules (S2 segment) [228], isolated renal proximal tubules (S3 segment) [229,233], medullary thick ascending limbs [269] and renal collecting duct cells [270]. Its non-genomic actions include effects on signal transduction pathways and ion transporters, such as the epithelial Na^+ channel [267], the Na^+/H^+ exchanger [228,229,271,272] and the vacuolar H^+-ATPase [230,233,258,259,273].

While the non-genomic actions of ALDO influence electrolyte homeostasis, pH and cell volume in classical MR target organs, these activities also contribute to pathophysiological effects in the renocardiovascular system that cause endothelial dysfunction, inflammation and remodeling [274]. However, the molecular mechanisms underlying the nongenomic actions of ALDO on electrolyte transporters and signaling enzymes and the consequences of these nongenomic actions on whole-body acid-base balance remain unknown [275].

The non-genomic receptor: The nature of the receptor that initiates rapid non-genomic ALDO-induced signaling remains unknown. However, evidence supporting its existence comes from several sources. Data in the literature indicate the following: i) the initiation involves MR in most instances or ii) the existence of a plasma membrane-binding site for ALDO. Although the presence of a high-affinity membrane-associated receptor that is insensitive to MR antagonism and is unable to bind glucocorticoids has been detected in the vascular endothelium [276], a full structural characterization of this non-genomic receptor has yet to be achieved. Some studies have indicated that rapid non-genomic responses are mediated by a variety of receptor types that are associated with the plasma membrane or its caveolae components, and these receptors may include a membrane-associated nuclear receptor [277]. The intermediate steps that couple the ALDO-MR interaction with the nongenomic activation of specific protein kinases are not fully characterized; however, the transactivation of EGFR is a crucial step in transducing this activating signal to various downstream signaling intermediates that are responsive to ALDO [278]. In addition, rapid ALDO effects also involve a multitude of signaling molecules and include cross-talk with genomic ALDO effects as well as with the ANG II receptor and EGFR [274]. More recent results in studies of human and mouse endothelial cells have indicated that striating (a calmodulin-binding protein) is a mediator for nongenomic mechanisms of ALDO and estrogen effects on pERK and phosphorylated eNOS, respectively, suggesting a unique level of interaction between the ALDO receptor and the estrogen receptor in the cardiovascular system [279]. Finally, it has been shown that the functions of non-genomic ALDO effects may be to modulate other signalling cascades, with mechanisms depending on the surrounding milieu [274].

Additionally, it has been shown that through both genomic and non-genomic mechanisms, ALDO stimulates Na^+ reabsorption, K^+ secretion [280], Na^+/H^+ exchange [230] and H^+-ATPase [233] in the kidney, and Na^+/H^+ exchange in vascular smooth muscle cells [281].

Dose-dependent biphasic effect of ALDO on Na+/H+ exchanger

Approximately one decade ago, it was shown that i) ALDO rapidly increases Na^+/H^+ exchanger activity in a variety of cells, including distal colon and renal epithelial cells [255,282], and ii) ALDO also rapidly inhibits the Na^+/H^+ exchanger and HCO^{3-} reabsorption in medullary thick ascending limbs [283]. Hence, the activation/inhibition of Na^+/H^+ exchanger by ALDO needed to be more clearly defined, because it was possibly that similar to ANG II [83,113,198], ALDO had a dose-dependent biphasic effect on the Na^+/H^+ exchanger (with low doses stimulating and high doses inhibiting the exchanger). In addition, it was also shown that an elevation in $[Ca^{2+}]i$ has the following effects: i) it serves as a second messenger in the ALDO-mediated initiation of nongenomic Na^+/H^+ exchanger activation [271], and ii) it is a pre-requisite for the genomic action of ALDO, and strong evidence indicates that pregenomic hormonal responses can influence genomic processes [284]. Therefore, we performed a study to clarify the genomic and nongenomic effects of ALDO on the Na^+/H^+ exchanger and the role of $[Ca^{2+}]i$ in these processes [230]. The experiments were performed using isolated rat proximal straight tubules (S3 segments) and fluorescent probes to measure pH intracellular recovery rates (pHi)r after the acidification of the pHi was induced by a NH_4Cl pulse. We then monitored the $[Ca^{2+}]i$. Figure 4 shows that in the controls, the (pHi) r was 0.191 ± 0.006 [137] units pH/min and that administering ALDO at 10^{-12} M after 2 min, 15 min or 1 h of preincubation (pi) resulted in an increase in the (pHi)r (by approximately 50%, 25% and 70%, respectively). However, administering ALDO at 10^{-6} M after 2 min, 15 min or 1 h pi significantly decreased the (pHi)r (by approximately 25%, 48% or 38%, respectively). ALDO, when administered at 10^{-12} M or 10^{-6} M, at 2 min, 15 min or 1h pi therefore had a dose-dependent stimulatory effect on $[Ca^{2+}]i$. Results that are not shown indicated that at ~1 min after the addition of ALDO (at either 10^{-12} or 10^{-6} M) to the

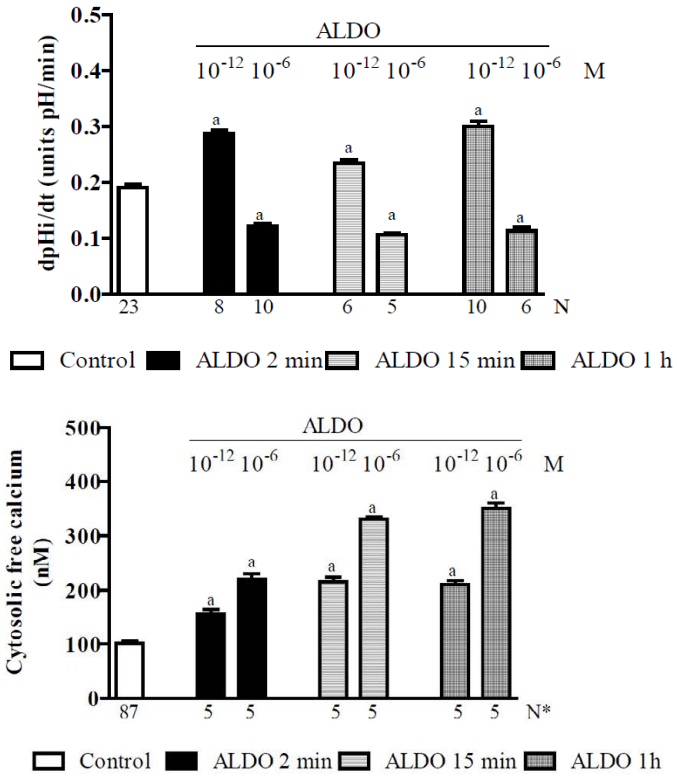

Figure 4. Research using isolated proximal S3 segments from normal Wistar rats [230] showing the effects of ALDO (10-12 or 10-6 M) after 2 min, 15 min or 1 h of preincubation on: A - pH intracellular recovery rates and B - cytosolic free calcium concentrations. Values are shown as the means ± SE. N = no. of observations. N* = no. of experiments in which the maximum fluorescent signal was averaged for 10 cells. dpH/dt = pH intracellular recovery rate in the first 2 min after a NH_4Cl pulse. Pi = preincubation. a = $P < 0.05$ *vs.* control.

bath, there was a transient and dose-dependent increase in the $[Ca^{2+}]$ i. At 15 min after the addition of ALDO, the $[Ca^{2+}]$i was significantly higher, mainly for ALDO at 10^{-6} M. After 1 h of hormone addition, the $[Ca^{2+}]$i remained high and was not different from the values found after 15 min. These results also indicated that adding actinomycin D (an inhibitor of gene transcription) or cycloheximide (an inhibitor of protein synthesis) did not influence the effects of ALDO (administered at either 15 or 2 min pi) but did inhibit the effects of ALDO (1h pi) on the (pHi)r and the $[Ca^{2+}]$i (data not shown). Hence, this study indicated that ALDO had a dose-dependent biphasic effect on the exchanger that was mediated by nongenomic (15 or 2 min pi) and genomic (1 h pi) pathways. These data were compatible with results showing that the Na^+/H^+ exchanger was stimulated when cellular calcium was increased by a lower range of ALDO (at 10-12 M) and inhibited when cellular calcium was increased by high levels of ALDO (at 10^{-6} M). Hence, this biphasic action of ALDO on Na^+/H^+ is similar to the biphasic action described for ANG II (Figure 1). The genomic ALDO-induced regulation of the exchanger seems to be a mineralocorticoid-specific effect because the ALDO receptor antagonist spironolactone significantly inhibited the activity of the exchanger (results not shown). In addition, our data show that in the presence of HOE 694 (a specific inhibitor of basolateral NHE1), administered alone or with ALDO (10^{-12} or 10^{-6} M, 2 min pi), the (pHi)r was completely inhibited. However, in the presence of S3226 (a specific inhibitor of apical NHE3) alone, the (pHi)r was not different from control values, and S3226 also failed to prevent both the stimulatory effect of ALDO (10^{-12} M, 2 min pi) or the inhibitory effect of ALDO (10^{-6} M, 2 min pi) on the (pHi)r. Hence, these results indicated, for the first time, that ALDO modulates the mechanism regulating pHi *via* the NHE1 exchanger in the proximal tubule. The results of this research also indicated that MR and probably GR participate in the genomic and nongenomic effects of ALDO on $[Ca^{2+}]$i and on the Na^+/H^+ exchanger. These data are in agreement with previous findings using RT-PCR that indicated the presence of these receptors in proximal S3 segments [230].

Arginine vasopressin

The Arginine vasopressin (AVP), or human antidiuretic hormone (ADH), is a neurohypophyseal cyclic octapeptide (1,099 D) with a 3-amino acid tail that plays an important role in water homeostasis and vasoconstriction and has functions in the kidney that have been well documented and will be the focus of this section of this Review. However, it has widely been recognized that AVP also has various nonpressor and nonantidiuretic activities that were reviewed in detail in a recent study [285]. The main site of AVP production is the hypothalamus. However, smaller amounts are also produced locally by many others tissues. Thus, AVP has endocrine, autocrine and paracrine effects. In normally hydrated persons, circulating AVP levels are very low (~ 1 pg/ml) because it is rapidly metabolized by the liver and excreted by the kidneys [286]. Hence, its half-life is 15 to 20 min. Because AVP is a small peptide, it is easily filtered through the glomerulus, but it is not metabolized in the kidney but is instead excreted in an unchanged form in the urine. Under normal physiological conditions, the very low concentration of AVP in plasma makes it difficult to measure. However, copeptin (the C-terminal part of the pre-prohormone that is released with AVP) is a marker of AVP secretion because it is secreted along with AVP in equimolar amounts, and it is easier to monitor because its half-life is longer and it is a more stable protein [287].

Control of AVP synthesis and release

The gene encoding AVP is expressed in the large-diameter neurons of the supraoptic (SO) and paraventricular (PV) nuclei of the hypothalamus. This gene encodes a prohormone that must undergo specific proteolytic processing to produce the active AVP hormone. Thus, the AVP gene encodes three peptides: i) the 9–amino acid peptide arginine vasopressin, ii) a carrier protein called neurophysin-2 and iii) a small glycoprotein called copeptin.

Osmoreceptors are located in the organum vasculosum laminae terminalis and subfornical organ of the hypothalamus. These two areas breech the blood-brain barrier and are able to sense changes in plasma osmolality [288], and they have therefore been shown to be involved in controlling body water content. They respond to elevations in plasma osmolality by increasing the activity of mechanosensitive cation channels that are located in their cell membranes [289,290]. This results in significant membrane depolarization that increases the frequency of action potentials. These osmosensitive neurons project to the large-diameter neurons in the SO and PV nuclei of the posterior hypothalamus, and these neurons then synthesize AVP as a prohormone that is packaged in granules. By binding to the carrier protein neurohypophysin, these granules are transported along hypothalamic–neurohypophyseal tracts to the axon terminals of magnocellular neurons in the neurohypophysis, where the AVP is then stored. When stimulated by the osmosensitive neurons, these magnocellular neurons release stored AVP into the neurohypophysis – an area that also lacks a blood-brain barrier – and AVP enters the general circulation. Hypothalamic–neurohypophyseal tracts also innervate other areas of brain and the spinal cord, which enables AVP to exert its actions not only systemically but also locally in the brain and spinal cord.

Principal determinants of AVP plasma levels

The two major factors that control AVP release are osmotic and non-osmotic stimulation [291]. However, the osmotic and non-osmotic pathways independently enter the same AVP neurons in the SO and PV nuclei [292]. Nevertheless, the baro- and osmoreceptor pathways do not function in isolation because a reduction in plasma volume increases the sensitivity of osmoreceptors.

Osmotic AVP release: The most sensitive stimulus of the secretion of AVP is plasma osmolality. Under normal circumstances, the low concentration of AVP in the plasma is very sensitive, and it increases rapidly and linearly in response to very small changes in plasma osmolality [286,289]. However, this sensitivity is influenced by the nature of the solute, the rate of the change in plasma osmolality, and the age and alcohol intake of the individual [293]. A normal level of plasma osmolality is ~ 293 mOsm/kg, and when it increases by about 1% of this value, there is a rise in the: i) synthesis of AVP in the hypothalamus, ii) secretion of AVP from vasopressinergic nerve endings in the neurohypophysis, and iii) release of the AVP that was previously stored in the neurohypophysis into the general circulation. Recently, in a transgenic rat line expressing an AVP-eGFP fusion gene, the *in vivo* molecular processing of the AVP-eGFP fusion gene and the secretion of AVP that was stimulated by osmotic stimulation were demonstrated for the first time [294]. In addition, high plasma osmolality triggers thirst. However, the increased renal reabsorption of water free of solutes in response to AVP lowers plasma osmolality, reducing the stimulus for AVP secretion and thirst. The mechanisms that ensure water homeostasis and the fundamentals of water balance disorders were reviewed in detail in a recent publication [289].

Nonosmotic AVP release: the secretion of AVP is also influenced by changes in isotonic plasma volume (IPV) and blood pressure. Decreases

in circulatory IPV and arterial blood pressure diminish the sensitivity of high-pressure and low-pressure (left atrial) baroreceptors and are potent nonosmotic stimuli for AVP release. Afferent fibers from arterial baroreceptors terminate in the nucleus of the tractus solitaries (NTS) of the dorsomedial medulla oblongata [295]. A1 adrenergic neurons in the ventrolateral medulla are involved in the afferent pathway from the NTS to the magnocellular neurons of the SO and PV nuclei in the hypothalamus, which synthesize AVP [296,297]. Furthermore, the baroreceptor-mediated afferent pathway for AVP release is activated by other factors, such as low cardiac output, left atrial distension, atrial tachycardia and hypoxia [291]. Nonosmotic AVP responses require larger variations than changes in plasma osmolality [298]. Therefore, reductions of 5% to 10% in IPV have little effect on AVP secretion, whereas reductions of 20% to 30% in IPV increase AVP plasma levels, causing them to reach 50 to 100 pg/ml [299]. At these high levels, AVP has potent V1a-mediated vasoconstrictor effects [300]. In the kidney, sympathetic activation induces i) arteriolar vasoconstriction, ii) a reduction in the glomerular filtration rate, iii) an increase in proximal Na+ and water reabsorption, with reduced distal delivery of water and Na+, and iv) an increase in distal Na+ and water reabsorption. In addition to baroreceptor activity, neurohumoral activation through ANG II increases the amount of AVP that is released in response to any given plasma osmolality [301]. Thus, a decrease in the effective circulatory blood volume impairs baroreceptor sensitivity, leading to an increase in the activity of the sympathetic nervous system, the activation of the RAS system and the nonosmotic release of AVP. Furthermore, AVP release is also stimulated by nausea and pain *via* central nervous input [302].

AVP Receptors

AVP acts *via* V1a, V1b, and V2 receptors that are located in the kidney and others regions.

V1 receptors: V1a receptors, which mediate the vasoconstrictor effect of AVP, are present in many tissues, including the smooth muscle cells of vessels and the brain, adrenal cortex, adipose tissue, and liver cells [285]. In the kidney, V1a receptors are localized in the renal vasculature, juxtaglomerular apparatus, macula densa cells, connecting tubule, collecting duct [303,304,305] and vasa recta [306]. AVP binding to the V1a receptor activates Gq/phospholipase C-b, resulting in an increase in $[Ca^{2+}]i$ levels and the activation of PKC, which causes vasoconstriction, platelet aggregation and the growth of smooth muscle cells [307,308]. V1b receptors are mainly present in the anterior pituitary, adrenal medulla, islet cells of Langerhans, and white adipose tissue [285]. Although the expression of the V1b receptor in the kidney has been described, its renal localization and functions are not well understood [309,310].

V2 receptors: As previously mentioned, the V2 receptor acts in the renal principal cells of the distal convoluted tubule, connecting tubule, collecting tubule and duct [305,311], and thick ascending limb of Henle [312]. It has been demonstrated that the human V2 receptor cDNA encodes 371 amino acids and a protein that has seven transmembrane domains, which is characteristic of G-protein-coupled receptors [314]. Thus, the V2 receptors have physiological functions that are mediated largely by the heterotrimeric G-protein Gs. These induce the activation of adenylyl cyclases to increase the intracellular level of cAMP [314] which activates PKA and is catabolized by cAMP-dependent phosphodiesterase. In turn, the phosphorylation of PKA mediates AVP cellular signaling to the aquaporin 2 water channel. This leads to i) the translocation of aquaporin 2 water channels from the membranes of cytoplasmic vesicles to the luminal tubular membrane, ii) an increase in the water permeability of these membranes, and iii) an increase in the expression of this water channel.

AVP Renal Actions on Na^+reabsorption

AVP effects on Na^+ reabsorption in the cortical collecting tubule: AVP up-regulates Na^+ reabsorption in the cortical collecting tubule by stimulating ENaC channels [315,316]. This rapid AVP effect is the result of membrane trafficking of ENaC *via* the regulation of the ubiquitin ligase Nedd4-2 [317]. However, it is probable that AVP is also involved in the long-term regulation of ENaC in this renal segment [318].

AVP receptors involved in distal nephron HCO3- transport:

The cortical distal tubule of the mammalian kidney plays an important role in the renal control of HCO^{3-} reabsorption and H^+ secretion. Processes such as electroneutral Na^+/H^+ exchange (*via* the basolateral NHE1 isoform or the apical NHE3 isoform) and electrogenic H^+ secretion (*via* H+-ATPase) in addition to H+/K+-ATPase and Cl^-/HCO^{3-} exchange and Cl–channels play key roles in determining the rate and direction of bicarbonate transport in this nephron segment [319]. Although the major function of AVP in the kidney is to increase water permeability in the cortical collecting duct (CCD) and inner medullary collecting duct (IMCD) [320], AVP has also been shown to be involved in tubular fluid acidification. In the rat kidney, AVP inhibits bicarbonate reabsorption in the thick ascending limb [321,322] and stimulates proton secretion (or bicarbonate reabsorption) in the distal tubule [321] and CCD [321,323]. However, in A6 cells (an amphibian distal nephron cell line), adding AVP at either low (10-10 M) or high (10^{-6} M) concentrations inhibited basolateral NHE1 activity [192]. Studies in isolated perfused mouse medullary thick ascending limbs showed that AVP stimulated basolateral NHE1 while simultaneously inhibiting apical NHE3 [324]. Thus, it is possible that the difference in AVP responses in distal nephron HCO3- transport may vary according to the cell membrane type that is being studied. Since the concentration of AVP in luminal fluid may exceed 1,000-fold its concentration in plasma [325] and considering the potential existence of functional luminal AVP receptors [326,327], a study was conducted *in vivo* in early distal (ED) and late distal (LD) segments of the rat kidney to determine whether luminal AVP (10^{-9} M) regulates acid-base transport in the rat cortical distal tubule [115]. The results of this study indicated that AVP has a direct effect that stimulates net HCO3- reabsorption in the ED and LD segments by activating VI receptors at the luminal membrane because this stimulatory effect was prevented by simultaneous luminal perfusion with the V1 antagonist [(d (CH2)5, Tyr (Et)2) arginine vasopressin]. In addition, it was observed that luminal AVP exerts effects on $[Ca^{2+}]i$ in the medullary thick ascending limb [328] and CCD [329]. So, taken together, these findings suggest that luminal AVP might play a significant role in the regulation of HCO^{3-} reabsorption in the distal convoluted, intercalated and initial collecting duct segments *via* V1 receptor-mediated mechanisms.

The possible physiological role of luminal AVP in distal nephron activities should also be considered. The concentration of AVP in the luminal fluid may be higher than that in plasma, even under physiological conditions. Data from kinetic studies and from studies of renal clearances have suggested that the tubular secretion of AVP into the distal nephron is an important component of renal clearance of this hormone in humans [325], dogs [330,331] and rats [332]. In humans, AVP clearance is in the range of 0.1 to 2 ml/min/kg, and urine AVP levels range from 5 to 500 p Mol [329]. In addition, it was

reported [326] that water permeability stimulated by basolateral AVP was inhibited by luminal AVP within this concentration range and that luminal AVP at a concentration of 100 pMol increased $[Ca^{2+}]i$ [329]. These data collectively suggest that luminal AVP might play a significant role in regulating the tubule transport of water and ions, especially when the AVP concentration is high.

Most studies have analyzed AVP activity after it has been applied at the distal nephron basolateral surface. It's activity is then mediated by V2 receptors *via* the adenylate cyclase/cAMP-protein kinase A signalling system, and this pathway, at high-AVP concentrations, is thought to inhibit the Na^+/H^+ exchanger [333]. However, V1 receptors have been detected in both apical and basolateral membrane domains, where they have been shown to mediate AVP activity *via* phospholipase C-IP3-calcium signalling [329,334]. However, it is known that PKC, when phosphorylated, may stimulate the Na^+/H^+ exchanger [92]. In addition, in MDCK cells, the stimulatory effect of AVP on NHE1 is mediated by the activation of V1 receptors that are located on the basolateral cell membrane surface, while basolateral V2 receptors have a dose-dependent inhibitory effect [335].

Thus, because the response to AVP involving distal bicarbonate reabsorption may vary with the hormonal dose being studied and the type of receptor that is present on the distal cell membrane surface, a study was performed [336] to determine whether perfusing AVP into peritubular capillaries at either low (10^{-11} M) or at high (10^{-9} M) concentrations would regulate bicarbonate transport *in vivo* in ED and LD segments in rats. In these experiments, the kinetics of HCO^{3-} reabsorption were evaluated using an *in vivo* stopped-flow tubular microperfusion technique which it is not affected by the glomerular filtration rate [72]. During this research [336] the systemic alterations that are caused by microperfusing hormones into peritubular capillaries were avoided, as confirmed by an absence of changes in urine flow, Na^+ excretion, and systemic acid-base alterations. Another advantage of using *in vivo* peritubular capillary AVP perfusion is that it allows the researcher to ensure that measurements of acidification kinetics are performed at well-determined hormonal levels because it has been shown that some peptides have a short half-life when injected parenterally. This is important because the intact structure of AVP is required for it to binding to its receptor [337]. To detect which specific basolateral receptor AVP regulates distal bicarbonate reabsorption, this study [336] examined the effects of the following V1 and V2 receptor-specific antagonists: 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP; a membrane-permeant cAMP analog) and deamino-Cys1,d-Arg8 vasopressin (dDAVP; a V2-selective agonist), respectively, and dDAVP (an AVP analog that specifically binds to adenylyl cyclase-coupled V2 receptors). In conclusion, this work [336] was the first to indicate that: 1) peritubular AVP (10^{-11} or 10^{-9} M) significantly stimulated HCO^{3-} reabsorption in ED and LD segments by activating basolateral V1 receptors, 2) basolateral V2 receptors functions in a dose-dependent inhibitory effect that was mediated by cAMP, and 3) endogenous AVP stimulated bicarbonate reabsorption in ED and LD segments through a basolateral V1 receptor-mediated process.

AVP dose-dependent biphasic effect on Na+/H+ exchanger: Figure 5 demonstrates the effect of AVP (10^{-12}, 10^{-9} or 10^{-6} M) on (pHi)r and $[Ca^{2+}]i$ in MDCK cells [335]. In control cells, the (pHi)r was 0.101 ± 0.005 pH units/min [79]. The addition of AVP (10^{-12} or 10^{-9} M) to the bath caused a significant increase in the (pHi)r (by 77% or 31%, respectively), but a lower concentration of AVP (10^{-6} M) resulted in a significant decrease (by 69%). ANP alone or dimethyl-BAPTA-AM alone did not affect the (pHi)r but impaired both the stimulatory and the inhibitory effect of AVP on it. Figure 5 also shows that MDCK cells exhibited a mean baseline $[Ca^{2+}]i$ of 99 ± 5 nM . The subsequent addition of AVP (10^{-12}, 10^{-9} and 10^{-6} M) progressively increased $[Ca^{2+}]i$ from control values to 340 ± 4 nM (25) in a dose-dependent manner. The addition of ANP alone to the bathing solution led to a rapid and significant decrease in $[Ca^{2+}]i$ to 40 ± 4 nM [121], and the subsequent addition of AVP (10-12, 10^{-9} and 10^{-6} M) resulted in a recovery of $[Ca^{2+}]i$ that reached 80% without exceeding normal baseline values. The figure also shows that dimethyl-BAPTA-AM alone led to a significant decrease in $[Ca^{2+}]i$ (by 55%), and the subsequent addition of AVP (10^{-12}, 10-9 and 10-6 M) resulted in a recovery of $[Ca^{2+}]i$ that did not achieve the respective control values. Hence, ANP and dimethyl-BAPTA-AM, which caused a moderate decrease in $[Ca^{2+}]i$, did not affect the (pHi)r but impaired the pathway that caused an increase in $[Ca^{2+}]i$, thereby blocking both the stimulatory and the inhibitory effect of AVP on this process. So, these studies are compatible with stimulation of the NHE1 exchanger by increases of $[Ca^{2+}]i$ in the lower range (that is, 10^{-12} or 10^{-9} M AVP; mediated by basolateral V1 receptors) and inhibition at high $[Ca^{2+}]i$ levels (10^{-6} M AVP; *via* activation of basolateral V1 receptors). They also are compatible with inhibition of the exchanger at high cell cAMP levels (at 10^{-6} M AVP, mediated by basolateral V2 receptors).

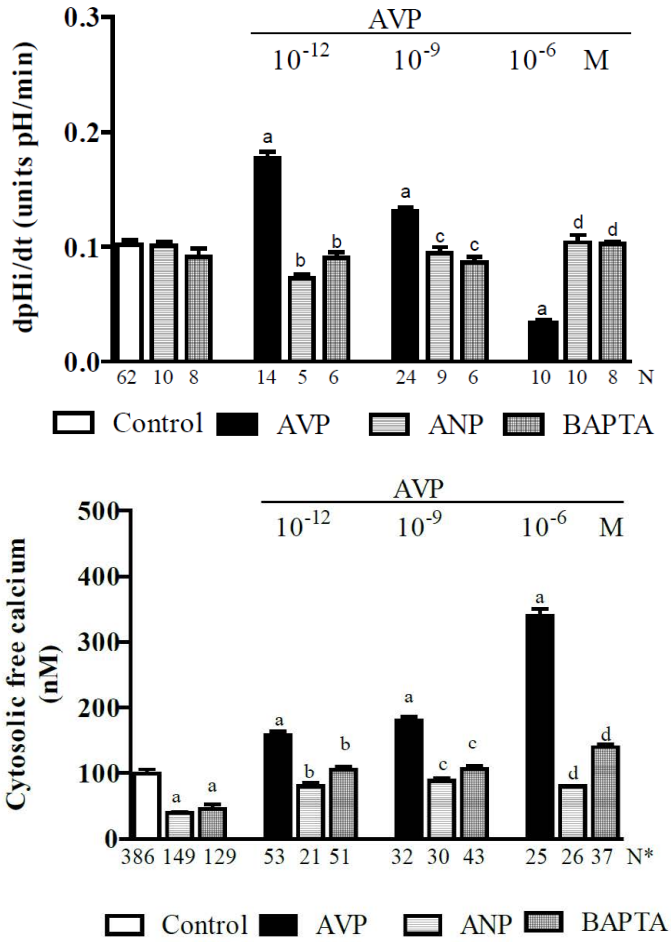

Figure 5. Research in MDCK cells [335] indicating the effects of AVP (10-12, 10-9 or 10-6 M) alone or plus ANP (10-6 M) or dimethyl-BAPTA/AM (5 X 10-5 M) on: A - pH intracellular recovery rates and B - cytosolic free calcium concentrations. Values are shown as the means ± SE. N = no. of observations. N* = no. of experiments in which the maximum fluorescent signal was averaged for 10 cells. dpHi/dt = pH intracellular recovery rate in the first 2 min after a NH_4Cl pulse. a = $P < 0.05$ *vs*. control. b = $P < 0.05$ *vs*. AVP (10-12 M). c = $P < 0.05$ *vs*. AVP (10-9 M). d = $P < 0.05$ *vs*. AVP (10-6 M).

Therefore, the biphasic action of AVP on the Na^+/H^+ exchanger is similar to the biphasic action described for ANG II (Figure 1) and ALDO (Figure 4).

This dual regulation of the Na^+/H^+ exchanger (or bicarbonate reabsorption) by AVP may represent a relevant regulatory mechanism that prevents blood alkalinization under conditions involving volume depletion because it is known that plasma AVP levels are usually 20–30 times greater than normal when the blood volume is reduced by 20–30% (in rats, dogs, and humans) or the plasma osmolality is increased by 10% [338].

Summary and conclusions

Figure 6 summarizes some of the data produced by our laboratory that have been discussed in this Review. These data have been combined in a schematic model that describes the biphasic effects of ANG II, ALDO, AVP and ANG-(1-7) on the calcium-dependent regulation of the Na+/H^+exchanger (NHE) by CaM. In the presence of basal activity, CaM binds at two sites to the cytosolic tail of the NHE: region A (a high affinity site that works as an autoinhibitory domain of the exchanger) and region B (a low affinity site that acts as an autostimulatory domain of the exchanger). A discrete increase in $[Ca^{2+}]i$ (caused by different hormonal concentrations, as indicated in Figure 6) may induce Ca^{2+}/CaM to bin to region A, thus blocking the inhibitory interaction and activating the NHE. On the other hand, region B of the exchanger binds to Ca^{2+}/CaM only when there has been a higher increase in $[Ca^{2+}]i$ (induced by various hormonal concentrations, as indicated in Figure 6), and under these conditions, binding inhibits the NHE. This scheme is based on theoretical studies [81,90] and on experimental studies using site-directed mutagenesis [18] it is known that the biphasic hormonal effects on the calcium-dependent regulation of NHE3 are dependent on calmodulin (CaM). Briefly, the data indicate that CaM binds to NHE1 at two sites in the cytosolic tail: region A (a high affinity site that contains residues 637-657) and region B (a low affinity site that is comprised of residues 657-700) [26]. Despite the fact that extensive research has been conducted to determine how binding between Ca^{2+}/CaM and NHE1 occurs and the structure of the regulatory domain that is involved in its complex with CHP1 or CHP2 using nuclear magnetic resonance [32], X-ray crystallography [33] and, more recently, small angle X-ray scattering analysis [1], the binding mechanism by which Ca^{2+}/CaM activates NHE1 is not easy to understand and is not yet satisfactorily or fully described. In section 2.1 NHE - NHE1 in this review, there is a presentation of the main proposals that are currently available. Additionally, under basal conditions, NHE3 is active, and CaMKII binds to the NHE3 C-terminal autoinhibitory domain (aa 586–605). This binding is rapidly reduced by a discrete increase in [Ca2+]i, which causes a conformational change in CaMKII that allows high-affinity interactions with target proteins and prevents the inactivation of the kinase by re-association between the catalytic domain and the autoinhibitory domain after Ca^{2+} returns to basal levels [13,47,48]. Other proteins that associate with NHE3 require this NHE3 domain, and these include NHERF1–4, phospholipase Cγ and CK2α [43,44]. The CaMKII-mediated inhibition of basal NHE3 activity is NHERF2-dependent, modifies NHE3 turnover rates, and causes the phosphorylation of NHE3. In addition, this regulatory effect requires amino acids in the C-terminal-binding autostimulatory domain that are downstream of NHE3 to interact with CaMKII (aa 690) [45,46]. Hence, CaMKII constitutively binds to, phosphorylates, and inhibits NHE3 *via* a NHERF2 protein-dependent process [48]. More details of these mechanisms are provided in section 2.1 NHE – NHE3 in the present Review. Therefore, the present results indicate stimulation of these exchangers by increases of $[Ca^{2+}]i$ in the lower range, and inhibition at high $[Ca^{2+}]i$ levels. In support of this idea, we have provided the following results: i) ANP and dimethyl-BAPTA-AM, by causing a moderate decrease in $[Ca^{2+}]i$, do not affect the (pHi)r but impair the pathway that causes an increase in $[Ca^{2+}]i$, thereby blocking both the stimulatory and the inhibitory effect of ANG II (Figure 1) or AVP (Figure 5) in this process; ii) EGTA alone causes a dramatic decline in $[Ca^{2+}]i$, impairs the (pHi)r and blocks both the stimulatory and the inhibitory effect of ANG II on it (Figure 1); iii) Dimethyl-BAPTA-AM alone does not affect the $JHCO^{3-}$ but impairs both the inhibitory and the stimulatory effect of ANG-(1-7) on it (Figure 2); and iv) thapsigargin alone causes a dramatic increase in $[Ca^{2+}]I$, inhibits the $JHCO^{3-}$ and impairs both the inhibitory and the stimulatory effect of ANG-(1-7) on it (Figure 2). Determining whether modifications of the $[Ca^{2+}]i$ represent an important direct mechanism for altering exchanger activity or a side-effect of other signalling pathways will require additional studies.

In conclusion, taken together, the data described in this review are compatible with the hormonal stimulation of NHE1 or NHE3 being induced by increases in $[Ca^{2+}]i$ at lower concentrations and inhibited at

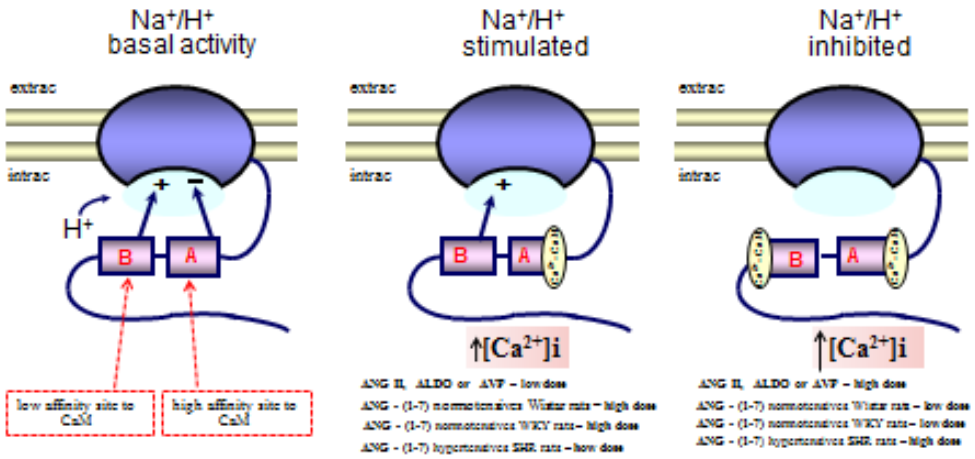

Figure 6. Schematic model indicating the biphasic effects of ANG II, ANG(1-7), ALDO and AVP on the calcium-dependent regulation of the Na+/H+ exchanger by Calmodulin. CaM = Calmodulin. $[Ca^{2+}]i$ = cytosolic free calcium concentration. ↑ $[Ca^{2+}]i$ = discrete increase in $[Ca^{2+}]i$. ↑$[Ca^{2+}]i$ = high increase in $[Ca^{2+}]i$. ▬ = inhibition. + = stimulation. Extrac = extracellular. Intrac = intracellular. The details of the mechanisms involved are discussed in sections 2.1 NHE and 3. Summary in this Review.

high $[Ca^{2+}]i$ levels. In MDCK cells or in the proximal tubule in Wistar rats, ANG II, ALDO (*via* genomic and nongenomic effects) or AVP stimulate these exchangers at low doses and inhibit them at high doses. However, ANG–(1-7), in Wistar or WKY (normotensives) rats, has inverse dose-dependent effects. Moreover, in SHR (hypertensive) rats, the biphasic effect of ANG-(1-7) is similar to that of ANG II, ALDO or AVP in Wistar rats. The interactions between these hormonal effects may represent a regulatory mechanism of extracellular volume in normotensives animals. Accordingly, in hypertensives animals, a high plasma level of ANG-(1-7) inhibits NHE3 in the proximal tubule, which mitigates hypertension.

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