Cellular and functional aspects of the renal kallikrein system in health and disease

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The kallikrein kinin system is a tissue-derived system with potent renal and cardiovascular effects. Within the kidney, the components of the kallikrein kinin system (kallikrein, kininogen, kinins, kininases, kinin receptors and mediators/modulators) originate from or are located in discrete segments of the nephron in highly specialized cells which determine its physiological effects. The kallikrein system acts on the kidney in a paracrine fashion in two anatomical microenvironments where the system regulates glomerular function, renal hemodynamics, and salt and water excretion. Impairment of the renal kallikrein system contributes to the development of hypertension, in particular to the salt-sensitive hypertension, and other pathologies like diabetes. There are several links between the vasodepressor kallikrein system and the vasopressor renin system which are relevant to normal renal function and to the pathophysiology of hypertension and renal diseases. Local induction of kininase II or angiotensin converting enzyme in the kidney could be a novel mechanism contributing to the renal damage in hypertension and other renal diseases. This review evaluates cellular and functional aspects of the renal kallikrein system with emphasis placed on the cellular localization of its components along the nephron, the links to other vasoactive systems, and the contribution of the system to the pathogenesis of hypertension.

Key terms: *angiotensin I-converting enzyme, aprotinin, bradykinin receptors, cyclooxygenases, hypertension, kallikrein*

INTRODUCTION

Cumulative evidence substantiates the kallikrein kinin system (KKS) as a tissue-derived vasoactive system with potent renal and cardiovascular effects. The renal KKS is composed of: (i) kallikrein, the bradykinin generating enzyme; (ii) kininogen, the substrate; (iii) bradykinins, the effector hormones; (iv) kininases, the metabolizing enzymes; and (v) an unknown number of enzyme activators and inhibitors. The final effects of bradykinin (BK) are a result of its local production determined by the interaction between the active enzyme and the substrate; its half-life determined by the balance between production and degradation, and finally by the presence of BK receptors and associated transduction signal mechanisms. The formation of BK requires the presence of the various components of the KKS at the same or

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closely related, accessible sites. With the conviction that the evaluation of the renal KKS critically depends on the precise localization of its components within the kidney, we have focused our studies on the morphofunctional bases of the KKS.

Since several aspects of the KKS are addressed by prominent authorities of the field in this issue of Biological Research (12, 18, 20, 40, 61, 74, 85, 98, 100), this review will be restricted only to a few selected cellular and functional aspects of the kallikrein system.

As homage to one of the many outstanding features of Dr Héctor R Croxatto, namely his enthusiasm to recruit students to science, in particular to the field of vasoactive agents, this review was written in collaboration with a group of students currently working in our laboratory.

RENAL KALLIKREIN

Kallikrein (EC 3.4.21.35) is a serine protease (27-40 kDa, pI ≈ 4) codified by the true kallikrein gene or *KLK1*, which message encodes a preprokallikrein and the proenzyme becomes fully active by the removal of the zymogen peptide by an unknown yet proteolytic enzyme. The active enzyme cleaves its physiological substrate, kininogen, to release lys-BK or BK (18).

The connecting tubule cells: the site of origin of renal kallikrein.

The anatomical localization of kallikrein has been a subject of much controversy for many years since it has been described in proximal tubules, distal convoluted tubules, glomeruli, peripolar cells, outer medullary and papillary ducts (reviewed in 119). Nowadays, there is broader agreement on the localization of renal kallikrein within the connecting tubule cells (CNTc), product of concurrent results obtained from microdissected nephron segments (73, 89) and immunolocalization (37, 117), yet the site of expression of the kallikrein gene is still controversial (17, 25, 42, 125) (*vide infra*). With ultrastructural immunohistochemistry, we demonstrated the presence of kallikrein exclusively in the CNTc (117). This cell type –together with the intercalated cell (Ic)– are the components of the connecting tubule (CNT), a novel nephron segment located between the distal convoluted tubule and the cortical collecting duct (64, 65) (Fig 1). The structural features

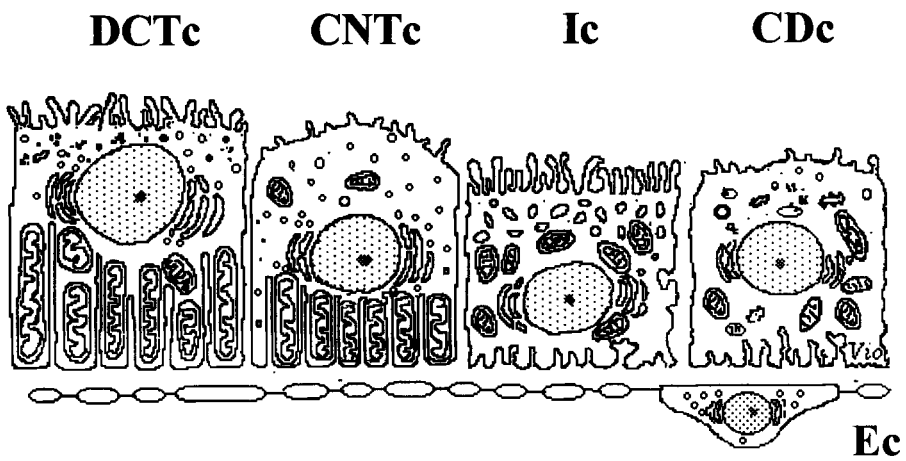

Fig 1. Cellular components of distal nephron post-macula densa. The distal convoluted tubule contains a single type of cell (**DCTc**); the connecting tubule, connecting tubule cell (**CNTc**) and intercalated cell (**Ic**); the cortical collecting duct (**CCD**), collecting duct cell (CDc, formerly principal cell) and shares Ic with the connecting tubule. Altogether, connecting tubule and collecting duct contain main components of kallikrein system (kallikrein, kininogen, kininases and BK-B2 receptors), have similar morphology at the light microscope and can be differentiated via specific markers (see Fig 3B). **Ec**, endothelial cell.

of CNTc are a well developed biosynthetic apparatus containing kallikrein, potassium secretory channels in the luminal plasma membrane (77) and abundant Na,K-ATPase in the basal plasma membrane (60). Since kallikrein is found in the biosynthetic apparatus, we proposed that kallikrein is synthesized in a unique cell type in the rat kidney, the CNTc (117) (Fig 2). A similar distribution is present in normal human kidneys (118).

Remarkably, the kallikrein-containing cells establish a close and extensive anatomical contact with the afferent arteriole of the juxtaglomerular apparatus (118) (Figs 3A and 4). This anatomical relationship between tubular and vascular structures containing kallikrein and renin, observed in rat and human kidneys (4, 105), calls for a physiological function and is consistent with a paracrine function of the KKS in the regulation of renal and glomerular hemodynamics, and renin release (119). It is

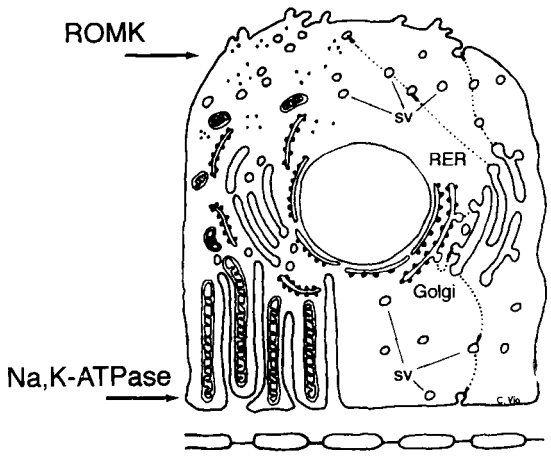

Fig 2. Intracellular processing pathway of kallikrein in connecting tubule cell. Left side, ultrastructural features: smooth luminal surface bearing K^+ secretory channels (**ROMK**); well developed Golgi apparatus and trans Golgi network around lower part of nucleus; vesicles with electron lucent content near to Golgi and close to plasma membrane; basal plasma membrane, with abundant **Na,K-ATPase**, forms prominent basal infoldings containing rod-shaped elongated mitochondria, in close contact with fenestrated endothelium from capillaries. The presence of ROMK in apical membrane and of Na,K-ATPase in basolateral membrane confers to this cell type a prominent role in K^+ secretion. Right side, subcellular distribution of kallikrein and hypothetical intracellular processing pathway: rough endoplasmic reticulum (RER), Golgi complex and trans Golgi network, and secretory vesicles (sv) trafficking to luminal and basal plasma membranes.

interesting to note that both renin and kallikrein systems are linked up at several levels, as will be discussed later.

In contrast to the kallikrein localization restricted to the CNTc, *in situ* hybridization methods have shown the presence of kallikrein mRNA at the vascular pole of the glomeruli and, to a lesser degree, in the distal tubular cells of rat kidneys (125), whereas kallikrein mRNA has been shown in distal tubules, collecting ducts, loops of Henle and juxtaglomerular cells of human kidneys (17). Given this discrepancy, we re-examined kallikrein gene (*KLK1*) expression and renal kallikrein localization in rat kidney by using *in situ* hybridization and immunohistochemistry. With gene-specific oligonucleotides and cRNA *rKLK1* probes and antibodies, we demonstrated *rKLK1* gene expression and renal kallikrein localization exclusively in the CNT, and lack of both gene expression and enzyme localization in glomeruli, loops of Henle, collecting ducts or arterioles (115). Therefore, kallikrein mRNA reported previously on other nephron segments or glomeruli may represent cross-hybridization with another member of the kallikrein gene family.

Intracellular distribution, polarity and sorting of kallikrein.

The intracellular distribution, sorting and polarity of the secretory pathway of kallikrein concern with important cell biology issues, since they deal with "how" and "from where" the enzyme exits from the CNTc. Although these issues are of obvious importance to understand the regulation of the enzyme, they have received little attention in the past.

Based on the ultrastructural subcellular distribution of kallikrein, we hypothesized that the enzyme is sorted to both the apical and basolateral poles of the CNTc, where could exit the cell and/or remain as a membrane-bound enzyme. This hypothesis was in agreement with the enzymatic activity observed in subcellular fractions (11, 123, 126), as well as with the results of studies on isolated perfused kidneys showing kallikrein in both urinary and

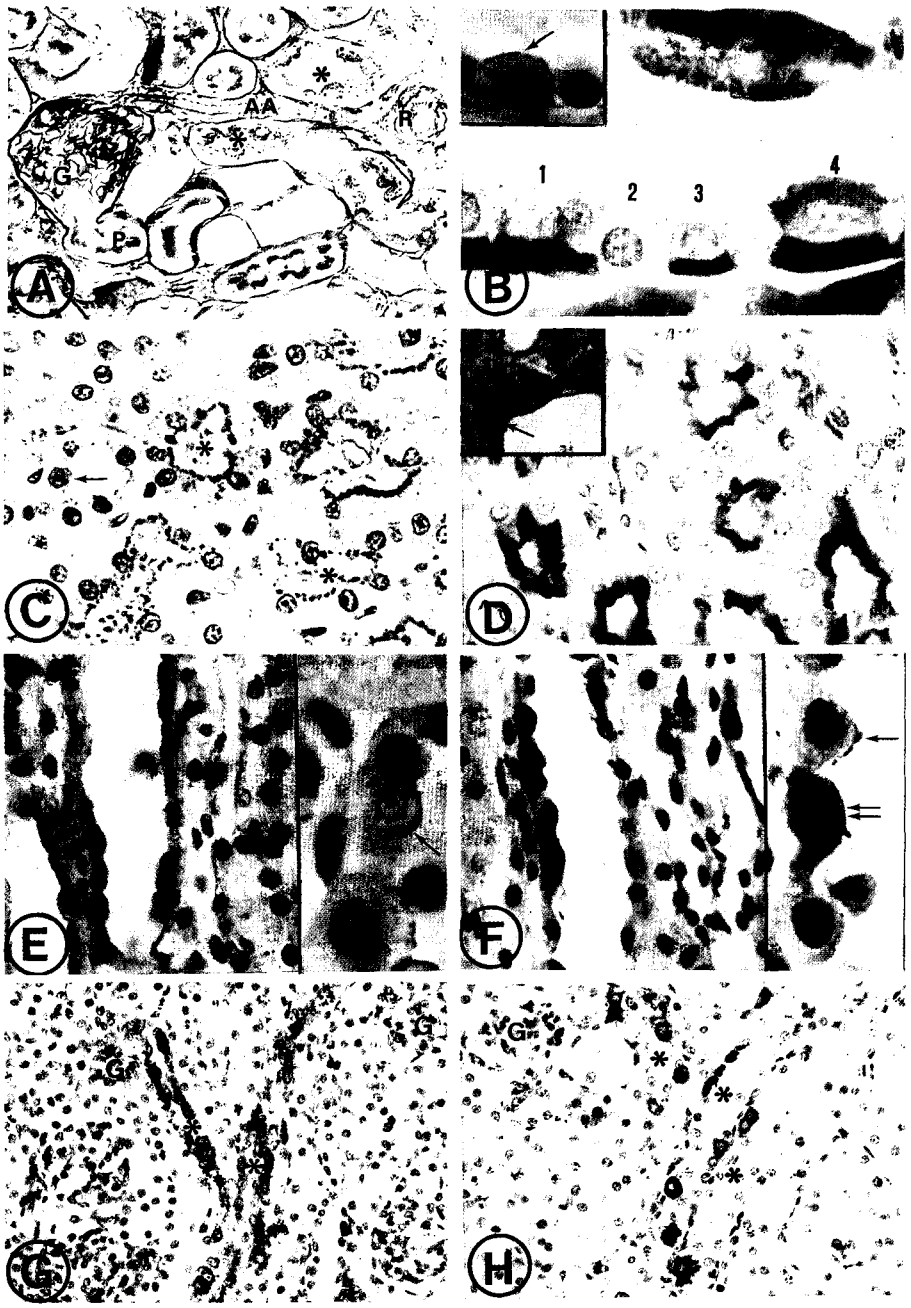

Fig 3. Cellular distribution of components of kallikrein system and mediators in the kidney. **A.** Axial section of radial cortical artery (R), afferent arteriole (AA), corresponding glomerulus (G) and proximal tubule (P) at urinary pole of glomerulus. Connecting tubule immunostained for kallikrein (asterisks) establishes close contact with AA. **B.** Cellular characteristics of distal nephron and differential distribution of cell markers. Four cell types marked with arabic numbers: 1, DCT cell containing Na,K-ATPase in basal portion; 2, Ic devoid of NA,K-ATPase and kallikrein; 3, CD cell with Na,K-ATPase in basal portion but smaller in size than DCT cell; 4, CNTc with kallikrein in cytoplasm and apical pole, and Na,K-ATPase in basal portion; in addition, CNTc contains ROMK in luminal membrane (inset). Note that different cell types are intermingled in this segment of the nephron, making it difficult to differentiate them without cell markers. **C.** Distribution of exogenous aprotinin in kidney. Aprotinin concentrated in endocytotic pathway of proximal tubules (asterisks) and in connecting tubule cells (arrows), the latter distribution partially explaining the renal effects of aprotinin, whereas the former one suggests metabolism in proximal tubules. **D.** ACE in kidney. ACE distributed in apical pole of proximal tubule cells, and also induced in tubule interstitial space in hypertensive kidneys (arrow in inset). **E-F.** Cellular distribution of BK-B2 receptor (E) and of bradykinin antagonist HOE-140 (F). BK-B2 receptors concentrated in collecting duct (E), mainly in luminal pole of cells (arrow in inset). HOE-140 (injected in vivo) displays similar distribution in collecting ducts (F), but its cellular distribution is heterogeneous: present at luminal pole (single arrow) in some collecting cells, while in intracellular space in other collecting cells, suggesting internalization of the antagonist. Within the cell, HOE-140 remains for up to 4 h, indicating sequestration of the molecule, an observation which may account for long-lasting effect of the antagonist *in vivo*. **G-H.** Distribution of COX-2 in postnatal (G) and adult (H) kidneys. Cyclooxygenase-2 present exclusively in TAL segment of young and adult rats, but more abundant in postnatal period (G) than in adult stage (H), a difference suggesting an important role of COX-2 in postnatal development.

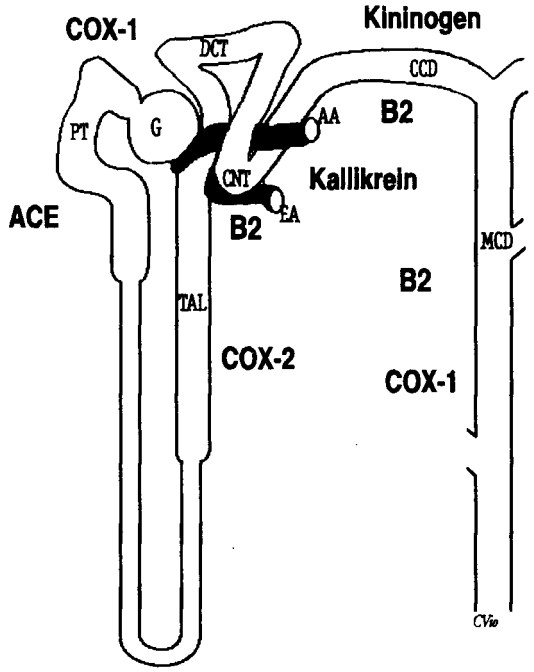

Fig 4. Scheme of nephron pointing to a novel tubulo-vascular relationship relevant to the function of kallikrein system. Anatomical relation between connecting tubule and juxtaglomerular apparatus, compatible with observations in human and rats showing that connecting tubule consistently establishes close contact with afferent arteriole, thus providing anatomical bases for interaction between kallikrein and renin systems, and to contribution of kallikrein system to glomerular function and renal hemodynamics. The other nephron microenvironment related to excretory function of kallikrein system is CNT-CCD, which provides support for role of the system in sodium and water excretion. **AA**, afferent arteriole; **EA**, efferent arteriole; **G**, glomerulus; **PT**, proximal tubule; **TAL**, thick ascending limb of Henle's loop; **DCT**, distal convoluted tubule; **CNT**, connecting tubule; **CCD**, cortical collecting duct; **MCD**, medullary collecting duct; **COX-1**, cyclooxygenase 1; **COX-2**, cyclooxygenase 2; **B2**, type 2 bradykinin receptor. Along the nephron, main localization of kallikrein (CNT), kininogen (CCD), BK-B2 receptors (AA, CCD, MCD), kininase II/ACE (PT), COX-1 (G, CCD, MCD), COX-2 (TAL), their functional implications being discussed in the text.

venous effluents (10, 78, 94, 113), and in the renal lymph (90). The hypothesis has been confirmed recently in Madin Darby canine kidney (MDCK) distal tubule cells (1), a well established model for studying the polarity and sorting of secretion. Abe *et al* (1) described the synthesis of prokallikrein in MDCK cells transfected with rat kallikrein cDNA and its secretion into both the apical and basolateral poles in a 4:1 ratio. Although extrapolation to secretion *in vivo* should be done cautiously, this secretory ratio suggests that a considerable amount of kallikrein could be thus released to the interstitial space and circulation.

Like the secretion, the activation of kallikrein appears to be polarized, since the venous outflow of isolated kidneys contains 90% of prokallikrein, whereas the urine contains only 40% of prokallikrein, suggesting a differential activation of the enzyme (10, 94). In any event, kallikrein exiting the cell through the luminal or basal side would be critically located to act on tubular or interstitial kininogen generating BK, which in turn is able to influence renal function via nitric oxide (NO) and arachidonic acid metabolites (105).

Other important issue not yet elucidated for renal kallikrein is whether its secretion follows the constitutive or the regulated pathway. Proteins secreted constitutively are released in a continuous fashion commensurate to the rate of synthesis and turnover, whereas the regulated proteins are packaged in a concentrated form into secretory granules and are released only upon stimulation of that particular cell. The most prominent morphological elements of the latter pathway are the secretory granules themselves, and both CNTc (containing kallikrein) and collecting duct cells (containing kininogen) lack the classical secretory electron-dense granules, suggesting that the secretion of kallikrein and kininogen in the kidney is constitutive. This observation raises an apparent contradiction with the multiple factors known to regulate the release of kallikrein (*vide infra*). However, the commonly referred to as "constitutive" pathway is tightly regulated and can be modulated by extracellular stimuli (63). Finally, kallikrein has been characterized as a membrane-bound enzyme, since it is found in purified membrane fractions and detergents are required for its solubilization (126), but its anchorage to the membrane remains largely unknown.

Some of the points previously mentioned illustrate the fact that, despite the enormous progress made in the molecular genetics and functional aspects of kallikrein, there still exist large gaps in the basic cell biology of this enzyme.

Regulation of renal kallikrein: role of potassium and therapeutic potential.

Renal kallikrein is regulated by sodium and potassium balance, and by hormones like adrenocortical steroids, insulin, catecholamines and thyroid hormone (6), as reviewed in this issue by Margolius (74), Clements (18) and Katori (61).

Among the many factors involved in the regulation of kallikrein, the role of dietary potassium has been underestimated, receiving lesser attention than the others. This factor deserves serious consideration not only because it seems to be a key regulator of kallikrein, but also for its therapeutical implications.

As mentioned before, the presence of potassium secretory channels (ROMK) at the luminal plasma membrane (77) and abundant Na,K-ATPase in the basal plasma membrane (60) (Fig 3 B) of the CNTc –the cell of origin of kallikrein– confer to this cell type a prominent role in the secretion of potassium by the distal nephron (62, 65). Consistent with this, high potassium intake stimulates CNTc, producing an overall cell hypertrophy including its biosynthetic-secretory apparatus resulting in increased excretion of kallikrein and potassium (48, 116). Under this condition, the CNTc size correlates with the excretion of kallikrein and potassium, and a positive correlation is also observed between the urinary excretion of kallikrein and potassium (116). A similar stimulating effect of high potassium has been observed in renal cortical slices and isolated perfused kidneys (6). In humans, kallikrein excretion varies directly with potassium intake (51). Moreover, the analysis of the association of urinary potassium with urinary kallikrein within statistically inferred kallikrein genotypes of 769 individuals in 58 Utah pedigrees demonstrated a significant statistical interaction between urinary potassium and the inferred major gene for kallikrein (53).

In a clinical study, Valdés *et al* (110) supplemented potassium intake to a group of low-kallikrein untreated hypertensive patients in a crossover, double-blind, randomized fashion. Together with the increase in urinary potassium, a significant increase in kallikrein excretion and decreases in systolic, diastolic and mean blood pressures were observed. Again, this study revealed a positive correlation between urinary excretions of kallikrein and potassium (110). The stimulating effect of potassium on renal kallikrein contributes to explain some of its beneficial effects on blood pressure (68, 109).

The therapeutical implications in the management of hypertensive patients, in particular in salt-sensitive ones (*vide infra*), are of obvious importance. Partial substitution of sodium salts by potassium salts in the diet would have the double benefit of reducing sodium intake and stimulating kallikrein. This dietary change on salt intake is along with current recommendations of non-pharmacological management of hypertension, and has already proven to reduce blood pressure and decrease the number of deaths from both stroke and ischemic heart disease in large scale population studies in Finland (59).

We do not know yet the mechanism underlying the effect of potassium on kallikrein, but probably the interaction of several hormonal regulators results in the observed effect. Since both renal kallikrein and potassium excretion are under multihormonal regulation (6, 36), the activity of Na,K-ATPase represents perhaps the common link. It is well established that an increase in Na,K-ATPase activity mediates the secretion of potassium by CNTc during chronic potassium load, and the factors reported to regulate renal kallikrein show a remarkable similar pattern to those that regulate Na,K-ATPase activity. Thus, both Na,K-ATPase activity and renal kallikrein have been shown to be regulated by sodium and potassium diet, mineralocorticoids, glucocorticoids and insulin (6, 57).

Neural control of kallikrein

The information about the neural control of kallikrein is limited, its secretion seeming to be under sympathetic inhibition. Albertini *et al* (2), with evidence obtained from renal nerve stimulation, central β-adrenoceptor

blockade and peripheral sympathectomy in rats, concluded that normal sympathetic tone in the kidney modulates the release of kallikrein in an inhibitory manner. Further studies *in vitro* have disclosed that the adrenergic control of kallikrein release is mediated by β_1-adrenergic inhibitory mechanisms (46).

KALLIKREIN INHIBITORS

The activity of a potent proteolytic enzyme as kallikrein must be closely regulated by a balance between activators of the proenzyme and inhibitors of the active enzyme. Little information exists about endogenous inhibitors of the KKS, yet there is emerging information about kallistatin –a newly identified serine proteinase inhibitor (serpin)– which binds to kallikrein and inhibits its enzymatic activity *in vitro* (17). To inhibit the kallikrein system, the most widely used agent has been aprotinin. This polypeptide, of bovine origin, is an effective inhibitor of kallikrein (and of other serine proteases) *in vivo* and *in vitro*, and has been used to study the possible actions of the KKS in various physiological and pathological conditions (23, 41). The administration of aprotinin affects, at the glomerular level, the glomerular filtration rate and renal plasma flow, the tubulo-glomerular feedback mechanism, renin secretion and proteinuria, whereas at the tubular level, it affects the excretion of kinins, sodium, potassium, water and prostaglandins (15, 82, 99). Despite the large number of studies using exogenous aprotinin, very little information was available on its cellular distribution in the kidney (58). We have recently demonstrated the cellular and subcellular distribution of aprotinin in the rat kidney, the polypeptide displaying a restricted distribution to proximal, connecting and some collecting tubules (120) (Fig 3C). In proximal tubules, aprotinin is present in the endocytotic pathway, where it might undergo intracellular metabolism. In connecting tubule cells, aprotinin is observed co-localized with kallikrein, providing an anatomical base for an explanation of some of the effects of aprotinin over renal function. In collecting ducts, aprotinin is present in the basal portion of the cells, the functional meaning of this localization being largely unknown (120).

KININOGENS

Kininogens exert their function by interacting with proteinases as substrate for kallikrein, binding to coagulation proenzymes and assembling them on the contact phase (98), and inhibiting cysteine proteinases by forming inactive enzyme-inhibitor complexes (81). There are two forms of kininogen: the high molecular weight (HMW, 88-114 kDa) and the low molecular weight (LMW, 50-68 kDa). In humans, LMW kininogen is cleaved by tissue kallikrein. In rats, two LMW kininogens have been characterized, one of them being considered to correspond to the human LMW kininogen, and the other –the T-kininogen– as an acute-phase protein and not a substrate for kallikrein (6). Kininogens, which are abundant in plasma, are derived entirely from the liver; yet kininogen has been demonstrated in the kidney, located at the distal and collecting tubules (91), and –close to kallikrein– in the principal cells of the collecting duct in transition segments between connecting and cortical collecting tubules (39). Furthermore, the presence of kininogen mRNA in the human distal nephron indicates that kininogen is synthesized in the kidney (50). Although kininogen is present in urine (91) and renal lymph (90), additional studies are required to elucidate the mechanism of secretion of renal kininogen by the collecting tubules and its relative contribution to the circulating and urinary kininogens.

Regardless of whether kininogen originates from the blood stream or also in part from the kidney, the interstitial generation of kinins is possible at the basolateral membrane of the CNTc or in the interstitium, as demonstrated by direct measurement with microdialysis technique (106). Moreover, interstitial kinins and

mediators (prostaglandin E_2 and cGMP) are under the regulation of physiological stimuli such as sodium diet (104). Once kinins are formed within the lumen or in the interstitium, structures further downstream (preglomerular arterioles, medullary circulation and collecting ducts) would be the targets of kinins in a paracrine fashion. Locally generated kinins can regulate glomerular filtration and hemodynamics (55), without affecting distal tubular excretory function, or can regulate papillary blood flow and sodium excretion (75), without affecting cortical blood flow. These examples support the local actions of kinins on discrete anatomical microenvironments like those composed by the CNT-juxtaglomerular apparatus (Figs 3A and 4) or the connecting tubule-collecting duct (CNT-CD) (Figs 3E-F and 4). For a full description of the paracrine actions of the kallikrein system on these renal microenvironments, see Vio *et al* (119).

KININASES

Once BK is generated, several peptidases participate in its metabolism. Among them are kininase I, kininase II/angiotensin converting enzyme (ACE), neutral endopeptidase 24.11 (NEP), endopeptidase 24.15 and aminopeptidase P. The most active metabolizing kinin enzymes in the kidney seems to be kininase II/ACE and NEP. Both are present in the kidney in the brush border of proximal convoluted tubules and also in urine (26, 28). In addition, kininase II is extensively found in endothelial cells, where NEP is almost absent. The main BK products by the action of kininases are: BK-(1-7), formed by the action of either NEP or kininase II; BK-(1-5), formed by further hydrolysis by kininase II or EP 24.15; and des-Arg^9-BK, formed by the action of kininase I.

In recent years, enormous progress has been achieved in the study of the molecular properties and genetic structure of kininase II/ACE, reviewed in this issue by Costerousse *et al* (20). This enzyme is anchored to the plasma membrane by a C-terminal hydrophobic segment and exists in two isoforms: somatic and germinal. The somatic ACE form, a 170 kDa glycoprotein, is expressed in vascular endothelial cells and at the brush border of renal proximal convoluted tubule, jejunal villus and epididymal duct epithelia. *In situ* hybridization studies have documented the presence of somatic ACE mRNA in renal tubule epithelium, jejunal enterocytes and epididymal epithelium, demonstrating an inverse correlation between the levels of ACE mRNA and the enzyme it encodes in a given epithelium. Low mRNA levels together with high levels of the enzyme in the kidney indirectly suggest that ACE is a stable membrane protein with a low turnover rate (102).

In addition to the somatic ACE form, a smaller isoform (90 kDa), or germinal form has been detected in the testis, being expressed uniquely in germinal cells with a precise stage-specific pattern, from round spermatids to spermatozoa. The significance of the ultraselective expression of germinal ACE and of its specific messenger RNA at a very precise stage of spermatogenesis remains uncertain, but its importance has been highlighted by the finding that male homozygous mice ACE -/- mouse (ACE knock out) display significantly reduced fertility (30).

The molecular cloning of human endothelial enzyme revealed two very similar domains, each of which bears a functional active site. These two active sites display highly similar kinetic parameters for the natural substrates, except that the carboxyl-terminal active site –but not the amino-terminal site– is activated by chloride (56). Among its natural substrates, BK is the one for which the enzyme displays the most favorable kinetic parameters. Indeed, the K_m (Michaelis constant) is 88 times lower for BK than for angiotensin I (0.18 μM *vs* 16 μM), and the k_{cat}/K_m is 24 times higher for BK than for angiotensin I, indicating that BK is the preferred substrate for the enzyme (56). This finding is specially relevant, owing to the similar levels of angiotensin I and BK found in renal tissue (13).

CONTRIBUTION OF BRADYKININ TO THE EFFECT OF CONVERTING ENZYME INHIBITORS AND OTHER LINKS WITH THE RENIN SYSTEM

Angiotensin I-converting enzyme inhibitors (CEI) are used to prevent the vasoconstrictor and fibrogenic influence of the activated renin-angiotensin system; however, they were discovered by Ferreira (31) as BK potentiator peptides in snake venoms. Current data support the proposal that the effects of CEI are due to both inhibition of angiotensin II formation and reduced BK degradation. The exact extent to which inhibition of angiotensin II formation and BK degradation contribute to the effects of CEI is still under study. Accumulating evidence indicates that some of the cardiovascular and renal effects of CEI are due to reduced BK degradation, with resultant increased local BK levels, and thus they should be considered as "bradykinin potentiating agents" (7, 45). Renal effects of BK upon stimulation of B2 receptors are mediated by intrarenal prostaglandin E_2 and NO generation, as demonstrated by Siragy *et al* (105). The prevention of BK degradation BK by CEI's increases two-fold the renal levels of BK and decreases BK-(1-7), indicating that ACE/kininase II plays an important role in BK metabolism, and that increased BK mediates –in part– the renal effects of ACE inhibition (14). In addition, the existence of a local KKS in the vascular wall (85) provides support for locally generated kinins contributing to the acute vasodilator actions of ACE inhibitors.

It is interesting to note that both renin and kallikrein systems are linked at several levels: they share the common enzyme ACE/kininase II (20, 27), BK influences renin secretion (49), and kallikrein converts prorenin to renin *in vitro* (101). Furthermore, recent data suggest that –during sodium depletion– the renin-angiotensin system tonically stimulates renal BK production and cGMP formation via a non-AT1 angiotensin receptor (107). The most unexpected recent finding, in regard to the links between kallikrein and renin systems, is the discovery that a novel hormone of the renin system (angiotensin 1-7) is both a substrate and inhibitor for ACE, and is also a vasodilator agent acting via a kinin-mediated release of NO, as reviewed in this issue by Brosnihan *et al* (12).

The kallikrein system is likely to play an important role in counterbalancing the renal effects of the renin-angiotensin system. An impairment of the KKS, as observed in the Bk2r-/- mice or in the BN-Ka mutant rats (*vide infra*), results in an increased sensitivity to angiotensin II (70, 72). Thus, an impaired KKS could contribute to increase blood pressure levels by leaving the activity of vasoconstrictor agents unbalanced. The postnatal development represents another situation of imbalance between kallikrein and renin systems; thus, the increasing values of kallikrein observed during the renal postnatal maturation (111), together with the decreasing values of renin occurring in the same period (47), may contribute to explain the decline of vascular resistance and the increase in blood flow which occurs in the kidney after birth.

BRADYKININ RECEPTORS AND MEDIATORS

Two types of BK receptors has been identified so far: B1 and B2. They have different structure, regulation and are activated by different agonists: des-Arg9-BK for B1, and BK and lys-BK for B2 (93). The BK-B1 type of receptor is expressed in smooth muscle cells and lung fibroblasts in pathological states in response to a variety of inflammatory stimuli and it is regulated by the transcription nuclear factor *kappa*B (NF-*kappa*B) (84, 97).

Most of the vascular and epithelial effects of BK are mediated through the B2 type receptor. The genomic structure of the rat B2 receptor codifies for a predicted protein sequence of 366 aminoacids (41.7 kDa), homologous to members of the seven transmembrane G protein-coupled receptors family. Its mRNA is present in the kidney, heart, lung, brain, uterus and vas deferens (76). For a detailed description of the localization of B2 receptors, see Figueroa and Müller-Esterl (40). With antibodies

directed against specific aminoacidic domains of the B2 receptor, this was described as widely distributed in rat kidney structures, namely, straight portions of proximal tubules, distal straight tubules, connecting tubules, collecting ducts and in smooth muscle cells of cortical radial arteries and afferent arterioles. Furthermore, in tubular cells, the B2 receptor was observed in the basal infoldings and luminal membranes (38). More recently, with a novel approach to the identification of receptors, we infused *in vivo* the B2 antagonist HOE-140 and followed the time-course of its cellular distribution in the kidney with specific antibodies against HOE-140. The specific binding to the B2 receptor was then visualized by its co-localization with antibodies against intracellular and extracellular domains of the B2 receptor (121). In this study, the B2 receptors were observed with a more restricted localization, mainly to the luminal plasma membranes of distal tubules and collecting ducts. The antagonist HOE-140 was present along the endocytotic pathway of proximal tubules, which are devoid of B2 receptors; consequently, this localization corresponds to a site of renal metabolism of the antagonist. In addition, HOE-140 was observed along distal tubules and collecting duct of the medulla and papilla, in cells containing the B2 receptor, and was absent in intercalated cells lacking B2 receptors, indicating the binding of the antagonist to the receptor. Although HOE-140 was located in the luminal plasma membranes of many cells, the antagonist was observed intracellularly in other cells, suggesting internalization of the antagonist. This was not an artifact due to the thickness of the tissue section (7 μm), since it was confirmed in thin (0.5 μm) sections. Interestingly, it has also been reported that angiotensin II and angiotensin II antagonists –saralasin and Dup753– are internalized with the AT_1 receptor (19). The sequestration of the antagonist is consistent with the long lasting effect of HOE-140. Furthermore, this internalization of HOE-140 is dependent on microtubules, since it is decreased by pretreatment with taxol or colchicine (Nazal & Vio, unpublished).

The regulation and function of B2 receptors are currently being studied in cells expressing the receptor. Recent studies are representative examples. One of them demonstrated that BK action in mesangial cells results in tyrosine phosphorylation of cellular proteins, suggesting a role for tubulin and mitogen-activated protein kinase (MAPK) in the signaling cascade of BK leading to altered mesangial function, alteration that could be relevant to those observed in diabetes (54). The other study, in human keratinocytes, on the stimulatory effect of BK on phosphorylation of proteins at tyrosine residues, suggests that MAP kinase, actin, paxillin and the epidermal growth factor (EGF) receptor were the most likely candidates for BK-induced tyrosine phosphorylation. These effects in keratinocytes might be associated with events related to mitosis, adhesion and variation in cell shape (100). Furthermore, in an elegant work to elucidate the desensitization of B2 receptor, Pizard *et al* (87) transfected Chinese hamster ovary (CHO-K1) cells with the human BK-B2 receptor cDNA, and established a cell line that expressed stably and at high density a receptor exhibiting B2 receptor properties, in terms of coupling to cell signaling, desensitization and internalization. In these cells, equilibrium kinetic analysis and studies of the effects of receptor occupancy by agonists or antagonists on the kinetics of BK-receptor complex dissociation revealed features typical of negative cooperative binding, suggesting that this phenomenon can participate in the desensitization process.

Mechanisms of action of bradykinin: nitric oxide vs eicosanoids

The BK receptor is coupled to G protein and the interaction of BK with its receptor leads to the activation of two signaling enzyme systems, namely, phospholipase C and phospholipase A2 (6). Activation of phospholipase C by BK, with activation of the phosphoinositide second-messenger system, leads to NO production, which then activates the soluble guanylate cyclase

generating cGMP. Following activation of phospholipase A2, arachidonic acid is released and becomes a substrate for the three enzymatic pathways of arachidonic acid metabolism: the cyclooxygenase pathway, leading to formation of prostaglandins (PGs) and thromboxanes; the lipooxygenase pathway, leading the formation of leukotrienes; and the cytochrome P-450-mediated oxygenation of arachidonic acid, leading to formation of epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acid (HETE) isomers. The arachidonic acid metabolites exert biological actions on renal cells when they are released locally in discrete anatomic microenvironments along the nephron, in a paracrine fashion.

The relative contribution of NO *vs* arachidonic acid products to the renal effects of BK waits for further work to be understood. Intrarenal infusions of BK significantly increase renal blood flow, diuresis, natriuresis and kaliuresis, being these BK effects abolished by the inhibition of NO synthesis, whereas the administration of a precursor of NO (L-Arg) prevents the inhibitory effect of NO synthesis on the renal vasodilator and excretory responses to BK (67). While this work suggests that the renal vasodilator and excretory responses to BK are largely dependent on NO, studies in isolated kidneys have shown that –although the inhibition of NO generation reduced the vasodilator responses to BK– arachidonic acid metabolites derived from cyclooxygenase and cytochrome P450 pathways account for up to 70% of the vasodilator effect of BK (43). Both mediators of BK actions are also able to interact; in fact, NO directly activates cyclooxygenase to cause an increase in the enzymatic activity (96).

CYCLOOXYGENASES IN THE KIDNEY: COX-1 *VS* COX-2

Prostaglandins are synthesized by the prostaglandin G/H synthase or cyclooxygenase (COX). Until recently, it was believed that the cyclooxygenase was a single enzyme responsible for the generation of prostaglandins; however, a second form of the enzyme was identified that is induced by pro-inflammatory cytokines and growth factors, and is specifically associated with cells and tissues involved in inflammation (66). The current hypothesis about the role of constitutive *vs* induced COX isozymes is that –under normal homeostatic conditions– COX-1 is the constitutive enzyme present in most cells and tissues, whose activity accounts for the continuous production of physiologically important prostaglandins, while COX-2 is induced at the site of inflammation and produces pro-inflammatory prostaglandins. This hypothesis has been challenged recently by the demonstration of a constitutive, glucocorticoid-insensitive COX-2 in a subset of cells of the thick ascending limb (TAL) of Henle's loop of normal kidneys (112). This pool of constitutive COX-2 observed in TAL does not coexist with COX-1 in the kidney, since the latter is present in arterial vascular endothelial cells, medullary and cortical collecting ducts, and medullary interstitial cells (108). The physiological role for COX-2 in TAL is still unknown, but an obvious function is the generation of prostaglandins, in particular PGE_2. Cortical and medullary TAL cells synthesize PGE_2 and contain PGE_2 receptors (9, 29). In this segment, PGE_2 inhibits NaCl reabsorption, effect that is mediated via inhibition of Na,K-ATPase activity (122). It should be stressed that the TAL segment has a crucial role in salt and water homeostasis, because it reabsorbs an important fraction of the NaCl filtered load, participates in the generation of hyperosmolarity in the medullary interstitium –required for the operation of the countercurrent mechanism–, and is the target of furosemide, the prototype of the most potent class of diuretics. Since this segment of the nephron is so far devoid of BK receptors, PGE_2 production here should be independent of BK, and one candidate for its hormonal regulation is angiotensin II (32, 33).

Evidence for a very important role of COX-2 in kidney development has emerged from mice with COX-2 gene disruption (24, 79). They develop severe renal

abnormalities weeks after birth; such abnormalities are not detectable at birth and were evident only with the increasing age, suggesting an important role of COX-2 during renal postnatal development. Concordant with this, we have observed high amounts of COX-2 present in TAL cells during early postnatal development, with a progressive decline to adult levels after weaning (Figs 4E-F) (92). The presence of this constitutive COX-2 in normal animals –also observed in humans (86)– may have important therapeutical consequences, since COX-2 selective inhibitors were designed to spare the kidney (and gastrointestinal tract), assuming that the enzyme was absent in the normal kidney and was only induced in pathological conditions. Since that assumption has proven to be incorrect, the therapeutical indications of selective COX-2 inhibitors require a re-evaluation, to asses whether they are sufficiently renal sparing to warrant its use in patients requiring long term therapy.

PATHOPHYSIOLOGY OF THE KALLIKREIN SYSTEM

Pathophysiological aspects of the kallikrein system in diabetes, renal and cardiovascular diseases are reviewed in this issue by Margolius (74), Costerousse (20) and Katori (61). We will briefly comment the alterations of the KKS in salt-sensitive hypertension and the local induction of ACE as a pathogenic mechanism of the progression of hypertension and renal diseases.

Salt-sensitive hypertension: a case of impairment of the kallikrein system.

As discussed before, one of the main functions of the kallikrein system is related to sodium excretion. Consistent with this, it can be hypothesized that a deficiency (or inhibition) of any main component of the system would result in an impairment of sodium handling and hypertension.

As reviewed in this issue by Katori (61), the Brown Norway Katholiek (BN-Ka) rats –due to a point mutation of alanine^{163} to threonine in the common chain of kininogens– are unable to secrete kininogens from the liver, resulting in negligible levels of HMW and LMW kininogens in blood stream and almost undetectable urinary levels of kinins (22). The BN-Ka rats have normal blood pressure, despite the impairment of the kallikrein system, as long as they are fed with a low (0.3%) sodium diet (71). However, after administration of 2% NaCl in the diet, BN-Ka rats excrete less sodium and water than BN-Ki (normal) rats and develop hypertension. Supplementation of LMW kininogen during 2% NaCl ingestion restored the kinin level in urine, increased urinary volume and sodium excretion, and restored blood pressure to normal levels (71).

The blood pressure response to chronic salt loading has been also evaluated in a rat strain inbred for low urinary kallikrein excretion. Low-kallikrein rats showed higher systolic blood pressure values than control rats (130 *vs* 114 mm Hg) on normal sodium diet, and their blood pressure further increases after high sodium diet (153 *vs* 112 mm Hg). Moreover, chronic infusion of kallikrein to low-kallikrein rats prevents the hypertensive effect of high sodium diet, indicating that the deficiency in kallikrein can account for the salt-sensitivity to hypertension (69).

Additional evidence originates from mutant mice with disruption of the gene encoding for the bradykinin-B2 receptor (B2-KO). The B2-KO mice placed on a long-term high sodium diet develop higher blood pressure, reduced renal blood flow and increased renal vascular resistance, as compared to wild type mice (3). Other study directed to characterize the cardiovascular phenotype of the B2-KO (70) described –under basal conditions– higher blood pressure and heart weight in Bk2r-/- than in the wild-type Bk2r+/+. Chronic blockade of B2-receptors or inhibition of nitric oxide synthase (NOS) increased blood pressure of Bk2r+/+ to the levels of Bk2r-/- mice. Again, long-term high sodium diet increases blood pressure of Bk2r-/- and Bk2r+/- mice, whereas it was ineffective in Bk2r+/+ animals.

Further evidence was obtained from Dahl salt-sensitive and salt resistant rats.

Thus, human kallikrein gene delivered to Dahl salt-sensitive (Dahl-SS) rats fed to a high-sodium diet has a protective effect on salt-induced hypertension, renal damage and cardiac hypertrophy (16). These rats express human kallikrein in several tissues (heart, kidney, lung, liver, adrenal gland) and, following gene injection, they respond with diuresis and increased urinary levels of sodium, kinins and cGMP. This response and the reduced blood pressure are consistent with activation of the KKS. Mukai *et al* (80) found that Dahl salt-resistant (SR/Jr), whose blood pressure is not increased when fed with a high salt diet, had significantly high blood pressure when concomitantly treated with BK-B2 antagonist HOE-140, suggesting that kinin activation of BK-B2 receptors contributes to mechanisms conferring resistance to increase blood pressure on exposure to a high sodium diet.

An animal model with targeted disruption of the kallikrein gene, not available yet, will be useful to further explore the hypothesis discussed above. In the meantime, it is known that BN-Ki (normal, non-mutant) rats under kallikrein inhibition with aprotinin develop hypertension when placed on a long-term sodium diet (71). Thus, the experimental evidence available so far supports the hypothesis stating that an impairment of the kallikrein system contributes to salt-sensitive hypertension.

Several clinical studies have also addressed the issue of salt-sensitivity to blood pressure in normotensive (8) and hypertensive patients (34, 35), in relation to kallikrein. Urinary excretion of active kallikrein is significantly lower in salt-sensitive than in salt-resistant patients. Also, plasma atrial natriuretic peptide levels are higher in salt-sensitive than in salt-resistant hypertensive patients, and a significant correlation between urinary kallikrein and plasma atrial natriuretic peptide is observed in salt-sensitive hypertensive patients (34). Moreover, when active and inactive urinary kallikrein excretion rates were evaluated, it was observed that the active/total kallikrein ratio decreased in salt-sensitive patients, suggesting an impairment of inactive to active kallikrein conversion during NaCl loading as a new mechanism in human salt-sensitive hypertension (5).

As mentioned before, the therapeutical implications in the management of hypertensive patients, in particular in salt-sensitive subjects, are of obvious importance. Low sodium diet would prevent the development of hypertension in low-kallikrein patients, and the partial substitution of sodium by potassium intake would stimulate the kallikrein system.

Local induction of angiotensin I converting enzyme as a pathogenic mechanisms in hypertension.

Induction of ACE gene expression occurs normally in somatic and germinal cells during their differentiation and maturation process, as well as in tissue sites during pathological processes. Thus, local induction of ACE occurs in the heart during remodeling, in macrophages of the activated cardiac interstitium and in the hypertensive aorta (20).

The presence of ACE in the activated cardiac interstitium contributes to fibrous tissue formation through local generation of angiotensin II or BK degradation. The contribution of angiotensin II to fibrosis in the heart and kidney has been well established (95, 124); however, the role of BK has been underestimated. Cardiac fibrosis after myocardial infarction and in chronic hypertension involves increased synthesis and deposition of collagen within the myocardium. Angiotensin-converting enzyme inhibitors limit hypertrophy and fibrosis; their mechanism of action remains controversial, although kinins have been assumed to play a role. Recently, a BK-induced reduction in collagen type I and III gene expression –mediated via prostacyclin PGI_2– has been demonstrated in cardiac fibroblasts (44). This study supports the argument that stabilization of endogenous kinins enhances prostacyclin production and results in attenuation of collagen gene expression, and modulation of collagen synthesis and deposition within the myocardium.

As in the cardiovascular system, we (114) have recently demonstrated a widespread induction of ACE occurring concomitantly in tubular epithelial, vascular endothelial and interstitial cells in the hypertensive (contralateral) kidney of Goldblatt hypertensive rats (Fig 3D). This increased ACE in the hypertensive kidney may contribute to the local elevated levels of angiotensin II, which in turn contribute to the abnormal hemodynamic and tubular reabsorptive function described in this model (83, 88). The defective sodium handling by the contralateral kidney in the 2K1C renovascular hypertensive rat model has been well documented, and can be a consequence of intrarenal changes in angiotensin II and kinins levels. Thus, elevated levels of angiotensin II promote sodium retention (83, 88), whereas increased kinin degradation would decrease sodium excretion (103, 127), resulting in both cases in a sodium excretory defect. Furthermore, CEI treatment promotes sodium excretion and restores normal sodium handling in this contralateral kidney (52, 88). The increased angiotensin II content observed in the contralateral kidney of renovascular hypertension, in the face of decreased local renin, may be due at least in part to the increased availability of ACE in the renal circulation interstitium and tubules. The presence of ACE in renal interstitial fibrous tissue provides support for its contribution to fibrous tissue formation, through local generation of angiotensin II or BK degradation (103, 127). The observation of ACE induction in vessels, tubules and interstitium of the hypertensive rat kidney could be extended in the future to other types of renal diseases, and may provide a pathological basis for the putative deleterious effect of ACE in the diseased kidneys, and the beneficial effect of ACE inhibitors.

CONCLUDING REMARKS

We dedicate this review to Prof Héctor R Croxatto, since he called the attention about kallikrein deficiency as a possible pathogenic factor in hypertension in 1970 (21). He has witnessed since then the enormous progress in studies about the cellular origin, molecular biology, target tissues and actions of components of the kallikrein kinin system –and related systems– in health and disease. In addition to the kidney, local generation and paracrine actions of BK have been proposed in other tissues, such as the heart and vessels, pituitary, brain, colon, reproductive tract, *etc.*

Don Héctor has made many contributions –and will continue to do so– to the broad field of peptide hormones, but –equally important to his scientific contributions– he has also transmitted to many us his enthusiasm and devotion to science.

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