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Bioregulation of Kinins: Kallikreins, Kininogens, and Kininases*

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I. Overview and Perspectives

KALLIKREINS are a group of serine proteases that are found in glandular cells, neutrophils, and biological fluids. In the very first review of kallikreins by Frey et al. (1950), there is a wealth of knowledge, much of which has since been rediscovered. The kallikreins are divided into two main groups: tissue and plasma (Fiedler, 1979; Movat, 1979). The two types differ in their molecular weight, pI, substrate specificity, immunological characteristics, type of kinin released (Webster and Pierce, 1960; Webster, 1970; Fritz et al., 1977), and functional importance (reviewed by Bhoola et al., 1979; Schachter, 1980; Fuller and Funder, 1986; Kaplan and Silverberg, 1987). A single gene codes for plasma kallikrein (Seidah et al., 1989), whereas tissue kallikrein is a member of a multigene family that shows different patterns of tissue-specific gene expression (Mason et al., 1983; Ashley and Macdonald, 1985; Baker and Shine, 1985; Wines et al., 1989; Richards et al., 1989; Qin et al., 1991).

By means of enzymic action, kallikreins release the vasoactive peptides, kinins, from endogenous substrates called kininogens (Müller-Esterl, 1989). Enzymes that possess the capacity to release kinins from kininogens are collectively called kininogenases, a term first introduced by Eugene Werle (1960). This generic name includes enzymes such as plasma and tissue kallikreins, trypsin, plasmin, and snake venom (*Borthrops jararaca*) proteases. Although the primary function of tissue kallikreins may be to form kinins, an additional function is processing enzymes for protein precursor molecules, enzymes, and hormones and has received considerable attention in recent years (Lazure et al., 1983; Seidah et al., 1986, 1988; Drinkwater et al., 1988). Questions for the future are the precise enzymic determinants for the nonkininogenase substrates and whether such functions are physiologically important. An additional question concerns the molecular relationship and biochemical characteristics of tissue kallikreins that release kallidin (Lys-bradykinin) and those that form bradykinin.

In mammals, three types of kininogens have so far been described: HK§ (a high molecular weight form present in blood), LK (a low molecular weight substrate that occurs in blood and localises in various tissues) (Habermann, 1963; Suzuki et al., 1967; Jacobsen, 1966), and an acute phase protein called T-kininogen that occurs only in the rat (Okamoto and Greenbaum, 1983). Kininogens are multifunctional proteins involved in cascade reactions during clotting and inflammation and as inhibitors protecting cells from damage by cysteine proteases (Müller-Esterl, 1989). It is generally accepted that tissue kallikrein (except the rat and mouse enzyme) forms kallidin (Lys-bradykinin) from LK, and plasma kallikrein forms bradykinin from HK. Nevertheless, in vitro, tissue kallikrein is considered to release kinins from both HK and LK (Iwanaga et al., 1977; Girolami et al., 1986). Formation of kallidin by tissue kallikrein involves the cleavage of a Met-Lys bond at the NH_2 terminus, as well as the COOH terminal Arg-Ser bond in the kinin sequence of the kininogen molecule (Iwanaga et al., 1977; Pisano et al., 1978a; Fiedler, 1979). The spatial relationship between the cellular localisation of tissue kallikrein and kininogen in the kidney (Figueroa et al., 1988) and sweat glands (Poblete et al., 1991) must be of functional significance.

The structure of kinins formed by kallikreins are illustrated in fig. 1. Kinins are vasoactive peptides that influence a number of biological processes. They are hypotensive, increase vascular permeability, are potent pain-producing autacoids, contract smooth muscle of the bronchopulmonary tree, intestine and uterus, and increase sperm motility. At the cellular level, kinins promote chloride and glucose transport, release known transmitters from neurones, activate phospholipase A_2, and stimulate osteoclasts. Two receptors (BK2 and BK1), linked to specific G protein-coupled second messengers, modulate the cellular actions of kinins. In addition to recruiting all of the known second messenger systems, a particular new advance has been the partici-

§ Abbreviations: HK, H-kininogen; LK, L-kininogen; CPN, carboxypeptidase N; CPM, carboxypeptidase M; KI, kininase I family; KII, kininase II family; ACE, angiotensin I-converting enzyme; NEP, neutral endopeptidase 24.11; 5-HT, 5-hydroxytryptamine; HF, Hageman factor; HFa, activated HF; HFf, HF fragment; PMN, polymorphonuclear leukocyte; HAE, hereditary angioneurotic oedema; PSA, prostate-specific antigen; cDNA, complementary DNA; SMG, submaxillary gland; EGF, epidermal growth factor; EGF-BP, EGF-binding protein; mRNA, messenger RNA; NGF, nerve growth factor; KBP, kallikrein-binding protein; SBTI, soya bean trypsin inhibitor; ANF, atrial natriuretic factor; AVP, arginine vasopressin; SHO, spontaneously hypertensive Okamoto; PGE and PGF, prostaglandin E and F; CNT, connecting tubule; EDRF, endothelium-derived-relaxing factor; CSF, cerebrospinal fluid; IL, interleukin; TNF, tumour necrosis factor; pI, isoelectric point; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine 5′-monophosphate; DCT, distal convoluted tubule; CD, collecting duct; CCD, cortical CD; ATP, adenosine triphosphate; WKY, Wistar-Kyoto; RIA, radioimmunoassay; TAMe, *p*-toluene sulphonyl-*l*-arginine methyl ester; fMLP, formyl-Met-Leu-Phe; pA_2 logarithmic measure of the potency of an antagonist.

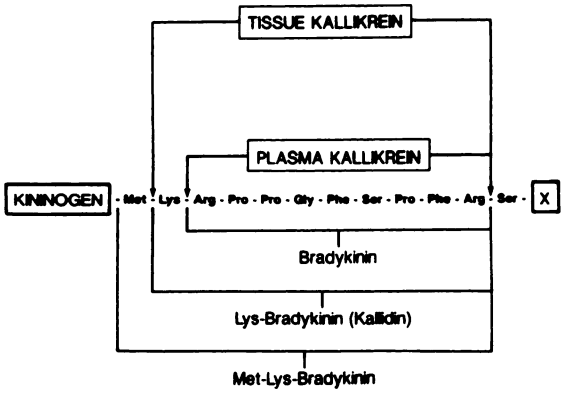

FIG. 1. Formation of kinins by the enzymic action of plasma and tissue kallikreins on kininogens. Ser-X, extended sequence in the light chain of kininogen commencing from the COOH terminus of the kinin moiety.

pation of nitric oxide in the signal transduction events of kinins on neurones and blood vessels.

When produced, the different kinins are inactivated rapidly by peptidases called kininases that are found in the blood and tissues. The two main families of kinin-inactivating peptidases are called KI and KII (Erdös, 1990). The KI family includes plasma KI-CPN (Erdös and Sloane, 1962; Erdös, 1979) and the cell membrane enzyme, KI-CPM (Skidgel et al., 1984; Johnson et al., 1984), both of which remove Arg^9 from the kinin molecule. The KII group of enzymes, KII-ACE (Skeggs et al., 1956; Yang and Erdös, 1967; Vane, 1969; Erdös, 1979) and KII-NEP (Almenoff and Orlowski, 1983; Gafford et al., 1983), liberate the dipeptide Phe^8-Arg^9. KII-ACE further degrades the remaining COOH terminus by cleaving the dipeptide Ser^6-Pro^7 (Erdös, 1979; Guder and Hallbach, 1988).

The physiological relevance of each component of the kallikrein-kinin system continues to unfold with important advances in the geneology and gene characterisation of kallikreins, kininogens, and kininases, molecular biology of the kinin receptor, cell-specific signal transduction events modulated by kinins, and cloning of the kinin BK2 receptor. The recent discovery of a tissue kallikrein in the neutrophil (Figueroa and Bhoola, 1989) and the attachment of LK, HK, and plasma prekallikrein to the external surface of the neutrophil membrane (Figueroa et al., 1991b; Henderson et al., 1992) ensures a unique role for kinins in inflammatory diseases. The design of new drugs that will specifically inhibit kallikreins in inflamed tissue is a challenge for the future.

Research into induction of latent kallikrein genes in tumour tissue will be another growth area. Gene expression of tissue kallikrein and the subsequent formation of kinins could stimulate proliferation of tumour cells and, by increasing vascular permeability, enhance metastases (Roberts, 1989). The observation of increased kinin receptor expression due to oncogenic transformation (Roberts and Gullick, 1989) gives further importance to a mitogenic role for kinins in tumour tissue. A major advance has been the synthesis of kinin antagonists (Vavrek and Stewart, 1985). With the advent of new kinin antagonists (Hock et al., 1991; Wirth et al., 1991) and superagonists (Stewart and Vavrek, 1991), it will be possible to discern the functional importance of kinins in arthritis, asthma, allergic rhinitis, algesia, and septic and endotoxic shock. But, the question whether any of the functions attributed to each component of the system can be considered to be a primary factor in the cause of a specific disorder or disease remains to be established. In this regard, we look to selective antagonists, leading to the development of new drugs, for further insights.

II. Historical Reminiscences

The first reference to a substance resembling glandular (tissue) kallikrein (urohypotensine) appeared in the publications of Abelous and Bardier (1909). The authors showed that an alcohol-insoluble fraction of human urine caused hypotension when injected intravenously into the anaesthetised dog. A similar hypotensive action of human urine was observed by Pribram and Hernheiser (1920) on the blood pressure of the rabbit. Later, Frey (1926) also demonstrated the presence of this substance in human urine. The compound responsible for the hypotensive property was isolated and shown to be a non-dialysable, thermolabile substance of high molecular weight (Frey and Kraut, 1928; Kraut et al., 1928). Subsequently, Werle and his colleagues extensively studied this biologically active material, and in a series of publications demonstrated its presence in blood, pancreas, and the salivary glands (Frey et al., 1932, 1950). They assumed that the hypotensive factor in urine had originated from the pancreas and, therefore, by derivation from the Greek word, kallikreas for pancreas, named it kallikrein (Kraut et al., 1930a; Werle, 1934). Because of its marked hypotensive action and possible functional role in regulating blood flow, they called kallikrein Kreislaufhormone (Schmidt and Fritz, 1989) and drew attention to the analogous renin-angiotensin system.

In 1937, Werle and his colleagues discovered that when the kallikreins were incubated with serum they enzymically released a smooth muscle-contracting substance from an inactive precursor (kininogen); the new activity in the mixture gradually increased and then disappeared slowly. The new biologically active molecule was called substanz DK (meaning "darmkontrahierende substanz"; Werle and Grunz, 1939) and, unlike the kallikreins, it was dialysable and thermostable, contracted the isolated guinea pig ileum, and showed marked hypotensive activity. Later, Werle and Berek (1948) renamed substanz DK and called it kallidin and the precursor, kallidinogen. About this time, Rocha e Silva and colleagues (1949) reported that incubation of venom extracts of *B. jararaca* or trypsin with the globulin fraction of dog plasma resulted in the formation of a substance that produced a slow, delayed contraction of the isolated guinea pig ileum.

The response interval for this new substance was seven times greater than that obtained with histamine or acetylcholine. Using Greek nomenclature, the authors called it bradykinin (*kinin* meaning movement and *brady* meaning slow).

A number of peptides closely resembling kallidin and bradykinin in their pharmacological properties have since been discovered in insect venoms. Discovery of the first nonmammalian kinin in wasp venom was made by Jaques and Schachter in 1954. They drew attention to the similarity of the contractile responses of the isolated guinea pig ileum to kallidin, bradykinin, and an extract of wasp venom, assayed in the presence of inhibitors to acetylcholine and histamine (Schachter, 1970). After further studies, Schachter and Thain (1954) gave these substances the generic name of kinins. At the end of the 1950s, working in Mel Schachter's laboratory, Bhoola observed the presence of a kinin-like peptide in the venom of hornets which, in addition, contained high concentrations of acetylcholine, 5-HT, and histamine (Bhoola et al., 1960). Of particular interest and fascination has been the question of why kinins in insect venoms coexist with bioactive substances that regulate neurotransmission in mammals and when and why was the kinin moiety inserted into the kininogen molecule. Another intriguing question is whether kinin receptors exist in insects and whether the same ancestral gene codes for both insect and mammlian kinin receptors.

III. Genealogy, Chemical Structure, Properties, and Functional Importance

A. Kallikreins

1. Plasma kallikrein. Notably, plasma kallikrein participates in surface-dependent activation of blood clotting, fibrinolysis, regulation of vascular tone, and inflammation. Molecular events in blood clotting involve a number of proenzymes, namely, factor XII (HF), factor XI (plasma thromboplastin antecedent), and prekallikrein (Fletcher factor), in which HK is essential for the activation cascade. After the cascade is triggered, clotting occurs to initiate thrombus formation together with the concomitant release of kinins on endothelial and subendothelial surfaces. For the historical evolution of the subject, consult previous reviews (Movat, 1979; Müller-Esterl et al., 1986; Kaplan and Silverberg, 1987).

Genetic analysis of the plasma prekallikrein-kininogen components of this system has revealed some inherited deficiencies: Fletcher factor (prekallikrein deficiency; Wuepper, 1973), Fitzgerald trait (HK deficiency; Saito et al., 1975; Donaldson et al., 1976), Tachibana trait (HK deficiency with reduced levels of LK and prekallikrein; Nakamura et al., 1985), and Flaujeac, Williams, and Fujiwara traits (absence of total kininogen with reduced amounts of prekallikrein; Lacombe, 1975; Colman et al., 1975; Oh-Ishi et al., 1981).

a. SYNTHESIS AND CHEMICAL PROPERTIES. Plasma prekallikrein is encoded by a single gene and synthesised in the liver. It is secreted by hepatocytes as an inactive molecule (plasma prekallikrein) that circulates in plasma as a heterodimer complex bound to HK (Mandle et al., 1976). The prekallikrein molecule is a single-chain glycoprotein (pI 8.9) that exists in two forms with molecular masses of 85 and 88 kDa, both of which are present in human plasma (Mandle and Kaplan, 1977; Hojima et al., 1985; Veloso and Colman, 1991).

The mature plasma kallikrein molecule (EC 3.4.21.34) has been purified to homogeneity from human, guinea pig, porcine, and rat plasma. Minor species differences in molecular weights are accounted for by the carbohydrate chains (Yamamoto et al., 1980; Seidah et al., 1988, 1989; Paquin et al., 1989). The mature human enzyme contains 619 amino acids with 371 amino acids from the NH_2 terminus linked to a catalytic chain of 248 residues. Hydrolysis of a single Arg-Ile bond (in positions 371 and 372) results in the formation of a two-chain proteinase molecule held together by a disulphide bridge (Seidah et al., 1989). Both molecular forms are cleaved by HFa in such a manner that the heavy chain is linked through the disulphide bridge to a light chain of two differing molecular weights, depending on the initial molecular form involved (Bouma et al., 1980). The heavy chain comprises four domains arranged in sequential tandems of 90 to 91 residues. Each of the four domains are bridged by 6 half-cysteine residues, except the last one which carries two additional half-cysteine residues to link together the heavy and light chains. The amino acid sequences of the repeat segments show considerable homology with factor XI (Fujikawa et al., 1986; Chung et al., 1986). The active site of the enzyme resides in the light chain (35 or 38 kDa) in a catalytic triad consisting of His-415, Asp-464, and Ser-559 (Mandle and Kaplan, 1977; Van der Graaf et al., 1982). An aspartate at 559 provides specificity for substrate binding and the preferential cleavage of the COOH terminus of basic amino acids.

Plasma kallikrein releases bradykinin from HK by hydrolysis of Lys-Arg and Arg-Ser bonds to give a nonapeptide with arginine at both NH_2 and COOH terminals. Although LK is a poor substrate for plasma kallikrein, it will form bradykinin in the presence of neutrophil elastase. The mechanism appears to be as follows: neutrophil elastase cleaves a fragment from LK, from which plasma kallikrein readily releases bradykinin (Sato and Nagasawa, 1988). Such an important mechanism may exist in vivo for kinin generation from LK, particularly because LK has been localised on the external membrane of the neutrophil (Figueroa et al., 1991b). This serine protease is also considered to participate in the conversion of prorenin to renin (Sealey et al., 1978; Rumpf et al., 1980; Derkx et al., 1979) and in the in vitro processing of prohormones (Seidah et al., 1988). The inability to find plasma kallikrein mRNA transcripts in

any tissue other than the liver suggests that plasma kallikrein does not normally function as a processing enzyme cleaving selected pairs of basic amino acids in prohormones in vivo (Seidah et al., 1988).

b. ACTIVATION CASCADE: FACTOR XII, PREKALLIKREIN, AND FACTOR XI. Studies of the evolution in mammals of the zymogens of contact activation suggest that prekallikrein and factor XII have evolved at the same rate (Veloso et al., 1986). Both prekallikrein and coagulating factor XI circulate bound to HK through domain 6 (Mandle et al., 1976; Thompson et al., 1977; Wiggins et al., 1977; Sugo et al., 1980). Thus, following vascular damage, HK settles on the endothelial and tissue surfaces, to which it becomes anchored through the positively charged, histidine-rich region of domain 5 and, simultaneously through its adjacent domain 6 site, combines with prekallikrein or factor XI. It orientates both molecules toward HF, so that, once activated, HF can form active kallikrein and factor XI (fig. 2) (Kaplan and Silverberg, 1987).

The heavy chain of the prekallikrein molecule is considered to contain the region that interacts with HFa (Veloso et al., 1987). Although the two segments of the prekallikrein heavy chain may act as functionally independent units, the whole molecule is required for the activation cascade. The tandem repeats in the molecule may themselves play a role in promoting clotting and aggregation of neutrophils by plasma kallikrein (Schapira et al., 1982b; Van der Graaf et al., 1982; Colman et al., 1985). A monoclonal antibody directed to the prekallikrein-binding site on the HK molecule (residues 565 to 595) inhibits dextran sulphate-mediated activation of prekallikrein and HK-dependent activation of clotting in normal plasma (Reddigari and Kaplan, 1989a). Although one or more of the repeat tandems in the prekallikrein heavy chain are also responsible for its binding to domain 6 in the light chain of HK, the precise site on the prekallikrein molecule involved in this binding is still uncertain (Hock et al., 1990).

i. Hageman factor. Small amounts of HF become activated either (*a*) by exposure to an activating macromolecular anionic surface, (*b*) autocatalytically (because of cleavage of HF by preformed plasma kallikrein, a small amount of HFa is normally present in the circulation; when as a result of intrinsic activity of HFa it becomes bound to HK, the essential cofactor, autocatalytic activation commences) or (*c*) feedback action of plasma kallikrein on native HF thereby producing further amounts of HFa and HFf (Kaplan and Austen, 1970; Cochrane et al., 1973; Schiffman et al., 1975; Griffin and Beretta, 1979; Miller et al., 1980; Silverberg et al., 1980; Kaplan et al., 1983). HFf seems to be a degradation product of HFa, but it is still capable of activating prekallikrein (Kaplan and Austen, 1970; Revak et al., 1978). Further acceleration of the conversion of HF to its active enzymic form is achieved by zinc ions (Shimada et al., 1987). When activated, the HF molecule is reoriented so that HF (clotting factor XII), which is a single-chain β-globulin of 80 kDa (Cochrane and Wuepper, 1971), is converted into a two-chain enzyme with a heavy chain of 50 kDa linked through a disulphide bridge to a light chain of 28 kDa (Revak et al., 1977). The light chain contains the active site, and the heavy chain possesses the binding site for attachment to the surfaces (Revak and Cochrane, 1976; Kaplan and Silverberg, 1987).

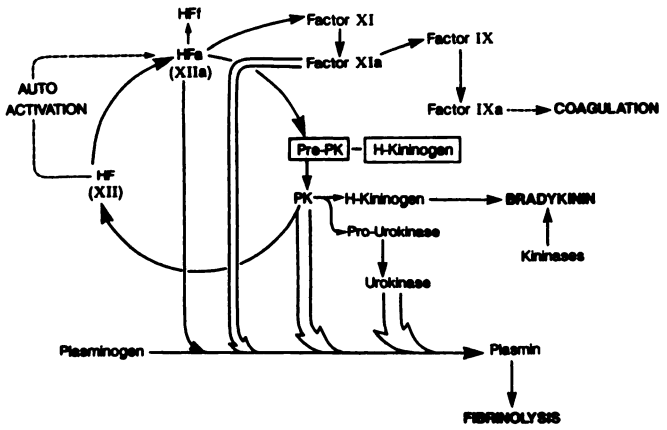

FIG. 2. Schematic representation of the activation cascade involving HF and plasma prekallikrein (Pre-PK). PK, plasma kallikrein.

The light chain domain of guinea pig HF (β-HFa) when injected intradermally caused an increase in vascular permeability by locally activating prekallikrein (Yamamoto et al., 1988), thereby confirming one of the earliest studies demonstrating this effect of the enzyme on the guinea pig skin (Bhoola et al., 1960). Clearly, kallikrein is responsible for inducing HF-dependent vascular permeability changes. Microbial proteinases (56 kDa) cause an acute liquefactive necrosis in guinea pig cornea and enhance vascular permeability in guinea pig skin by activating the HF-dependent pathway to release kinins (Matsumoto et al., 1984; Kaminishi et al., 1990; Molla et al., 1989). Furthermore, bacterial proteases prolong the inflammatory process by degrading members of the family of serine protease inhibitors, termed serpins, which include α_1-antitrypsin, α_2-macroglobulin, c_1 inhibitor, α_2-antiplasmin, and antithrombin III (Maeda and Molla, 1989).

Activation of the HF cycle may be important in drug-induced hypotension. Whereas intravenous dextran rarely causes hypotension in man, it causes a profound decrease in blood pressure in rats (Briseid and Briseid, 1983). Ionic contrast media and acetylcysteine apparently prevent the dextran-evoked activation of factor XII and prekallikrein in rats.

ii. Factor XI (plasma thromboplastin antecedent). Activation by HFa of factor XI, the second major plasma substrate of HFa, requires HK to which factor XI is linked through the amino acid residues 556 to 613 of domain 6. For the cycle to proceed, active factor XIa

is formed and the coagulation cascade continues (Ratnoff et al., 1961). Conversion of plasminogen to plasmin by plasma kallikrein, factor XIa, HFa, and HFf promotes fibrinolysis (Mandle and Kaplan, 1977; Goldsmith et al., 1978). Of these enzymes, plasma kallikrein appears to be the most active, even though it is many thousand-fold less potent than urokinase (Miles et al., 1983). More recent reports indicate that plasma kallikrein itself can activate circulating prourokinase (Hauert and Bachmann, 1985; Huisveld et al., 1985). Which of these is primarily responsible for the endogenous activation of plasminogen is not certain. Clearly, plasma kallikrein forms part of an important pathway in the clotting process.

c. EFFECTS ON POLYMORPHONUCLEAR LEUCOCYTES. Plasma kallikrein has significant effects on PMN leucocytes, which may be of relevance in inflammation. Kaplan et al. (1972) reported that plasma kallikrein showed chemotactic activity for PMN leucocytes. Purified plasma kallikrein also caused marked aggregation of these cells with a similar potency to that of the chemotactic peptide N-fMLP (Schapira et al., 1982a). These actions seemed to be specific effects of plasma kallikrein attributable to the active form of the enzyme, because plasma prekallikrein or plasma kallikrein preincubated with SBTI appeared to be ineffective.

d. INHIBITORS. The present view is that, during formation, plasma kallikrein is rapidly inactivated by C1 inhibitor, which alone has the capacity to inhibit with high affinity both plasma kallikrein and HF (Cameron et al., 1989). Further inhibition could occur with α_2-macroglobulin, antithrombin III, and mutant α_1-antitrypsins. After the the plasma kallikrein-inhibitor complexes are formed, they are rapidly cleared from the circulation. It has been proposed that both plasma kallikrein (Schapira et al., 1981; Schapira et al., 1982a) and factor XIa (Scott et al., 1982) may be protected by HK from inhibition by c1 inhibitor, α_2-macroglobulin, and α_1-antitrypsin, thereby augmenting the plasma half-life of each enzyme.

As already stated, guinea pig plasma enhances vascular permeability when injected into guinea pig skin, but because this effect is short lived it led to the search for a kallikrein inhibitor protein in guinea pig plasma. Historically, at least two circulating inhibitors had been described by Werle and his colleagues (Werle, 1934, Werle and Maier, 1952). The more recently purified kallikrein inhibitor protein, with a molecular mass of 64 kDa, inhibited plasma kallikrein in a dose-dependent manner and formed a complex of 137 kDa. Kallikrein inhibitor protein inhibits trypsin and elastase but not chymotrypsin. It has an inhibitory spectrum that is similar to that of contrapsin in mouse plasma but differs from the profile observed with human plasma kallikrein inhibitor protein (Imamura and Kambara, 1989).

In pathological states such as HAE (Landerman et al., 1962) and inflammatory joint disease (see the section "Arthritis"; Melmon et al., 1967; Kaplan, 1987), activation of either plasma kallikrein or the complement system (C2) or both is believed to be responsible for the symptoms that patients experience. In patients with HAE (an autosomal disorder arising from a reduction in both the amount and efficiency of C1 inhibitor), the clinical state is characterised by localised swellings, laryngeal oedema, and abdominal pain. Some studies support a complement C2-dependent mechanism, but most report bradykinin to be the primary inflammatory mediator formed in HAE, particularly because, during the swelling attacks and concomitant with the formation of bradykinin, there is a decrease in the circulating levels of prekallikrein and HK. Active plasma kallikrein has been detected in suction-induced blister fluids obtained from patients with HAE (Curd et al., 1980). Of particular interest has been the finding that only during pregnancy did the plasma of a patient with HAE show sustained contact activation that resulted in the formation of kallikrein-α_2-macroglobulin and kallikrein-50-kDa HK complexes which disappeared after delivery, with the subsequent return of the intact 120-kDa HK molecule (Chhibber et al., 1989). Replacement therapy with C1 inhibitor during attacks of swelling and oedema results in clinical improvement that is associated with an increase in circulating levels of HK (Kodama et al., 1984).

Another clinical condition in which cascade-formed kinins are considered to be involved is the cutaneous late phase reaction. This reaction is IgE dependent and is caused by the secretion of mediators from activated mast cells. In the early phase of the reaction when wheals and flares occur (appearing in 10 min and disappearing in 60 min), histamine, substance P, prostanoids, and 5-HT may be involved, whereas during the late phase, there is strong evidence for a contribution by kinins (Kaplan et al., 1989a,b).

e. MEASUREMENT OF FACTOR XII, PREKALLIKREIN, AND KALLIKREIN. Values for prekallikrein may be influenced by the in vitro activation procedure, which includes dextran sulphate, kaolin, or ellagic acid and association between the factor XII and active kallikrein (Briseid et al., 1990). Levels of prekallikrein (following activation) and kallikrein may be determined on a selective functional substrate. The rate of hydrolysis of H-D-Pro-Phe-Arg-paranitroaniline (KabiVitrum, S-2302) was determined in a dual beam spectrophotometer at 405 nm. A more sensitive colorimetric assay, developed for the estimation of prekallikrein using S-2302, was performed in a 96-well microplate (De la Cadena et al., 1987). Samples were acidified prior to measurement to prevent kallikrein formed after activation from binding to inhibitors. An excellent correlation was found between the microassay and the standard spectrophometric assay on plasma samples obtained from normal subjects, women receiving oral contraceptives, and patients with liver diseases. The

correlation coefficient was 0.92 for the three populations (De la Cadena et al., 1987). Prekallikrein levels are low in persons with pancreatitis and in cases of thromboembolic disorders but high during late pregnancy (Friberger and Gallimore, 1986). Increased levels of plasma kallikrein were detected in skin chambers and suction blisters of patients with psoriasis (Thomas et al., 1992). The locally formed kinins may participate as inflammation-signaling peptides and as mitogens in the pathogenesis of psoriasis (Poblete et al., 1991).

Determination of HF (factor XII) in human plasma may be accomplished by means of chromogenic peptide substrates either by direct measurement of HFa or by assaying kallikrein generated by HFa. One method is to assay HF in acetone/benzamidine-treated and kaolin-activated, citrated human plasma (Hoem et al., 1989). Measurements are made on the tetrapeptide Bz-Ile-Glu-Gly-Arg-paranitroaniline (KabiVitrum, S-2222).

2. Tissue (glandular, cell) kallikrein. Since the early discovery of kallikrein in the pancreas, salivary glands, and kidney by Werle and his colleagues (Frey et al., 1950), the presence of this enzyme has been extensively studied in many tissues. These studies have established the biochemical, immunological, and enzymatic properties of tissue-specific kallikreins. A characteristic of true tissue kallikrein of all mammalian species should be its ability to release a kinin (kallidin/bradykinin) from kininogen (kininogenase activity). Whereas in most species tissue kallikreins represent a unique class of enzymes that hydrolyse one arginyl and one methionyl bond in the kininogen molecule to release kallidin (decapeptide, Lys-bradykinin), in the rat and mouse the enzyme rather strangely and like trypsin splits one arginyl and one lysyl bond to form bradykinin (nonapeptide).

a. KALLIKREIN GENE FAMILY. Members of the kallikrein gene family (Swift et al., 1982; Mason et al., 1983; Ashley and MacDonald, 1985; Clements, 1989)) are a subdivision of serine proteases that express enzymes of similar molecular structure, mainly with a substrate preference for cleaving arginine residues (Chen and Bode, 1983). Even though they are highly homologous, the proteases coded by these genes show distinct differences in substrate recognition that is reflected in identifiable differences in the amino acid sequence of the enzymes. The different members of this family play diverse enzymic roles by selectively transforming peptide prohormones and growth factors into biologically active molecules (Berger and Shooter, 1977; Bothwell et al., 1979). The active site triad of amino acids considered to be crucial for the catalytic activity of the proteases in this gene family comprise His-41, Asp-96, Ser-189 (the numbering system is based on that of trypsin; some authors prefer the chymotrypsinogen numbering system of His-57, Asp-102, and Ser-195). The three key amino acids are considered to be the primary determinants of the cleavage specificity of true kallikreins. Except for some deviation, one mechanism involved in substrate cleavage appears to be a charge relay system, with Asp-183 (except for Ser-183 in the PSA molecule) as an acceptor and Asp-189 as the recipient sites at the bottom of the substrate-binding pocket, that account for the preference for positively charged P_1 amino acids (notation for the primary substrate residue, according to that described by Schechter and Berger, 1967). Furthermore, Gyl-216 and Ser-206 or Ala-217 serve to accommodate bulky side chains of the substrate (Bode et al., 1983); whereas human, rat, mouse, and porcine kallikreins retain conserved amino acids in key sites (Tyr-99, Trp-215, Asp-189, Gly-216, and Ser- or Ala-226) considered to be primary determinants for kininogenase activity, and divergent residues are observed in small clusters along the external loops of these molecules.

The kallikrein multigene family (reviewed by Fuller and Funder, 1986; Drinkwater et al., 1988; MacDonald et al. 1988; Clements, 1989) comprises closely related clusters of genes that vary in number between the different mammalian species: 24 genes have been identified in the mouse (Richards et al., 1982; Evans et al., 1987), 20 in the rat (Ashley and MacDonald, 1985; Gerald et al., 1986; Chen et al., 1988), 3 in humans (Fukushima et al., 1985; Baker and Shine, 1985; Schedlich et al., 1987; Evans et al., 1988; Richards et al., 1989: Qin et al., 1991), and approximately 3 in the hamster (Howles et al., 1984). Members of this multigene family show different patterns of tissue expression and hormonal control. Except for the mouse, we lack a full genomic map and pattern of expression for the kallikrein gene family in each species. So far, the number of functional genes characterised vary from 14 in the mouse to nine in the rat and only three in humans.

i. Human gene family. Evidence so far suggests that the human kallikrein gene family has three members (Baker and Shine, 1985; Fukushima et al., 1985; Schedlich et al., 1987; Evans et al., 1988), but recently 19 genomic clones that hybridise to a monkey kallikrein cDNA probe have been identified (Murray et al., 1990). The human family comprises the hRKALL, hGK-1, and PSA genes (Table 1) clustered on the long arm of chromosome 19 q13.3–13.4, in a position analogous to the mouse kallikrein locus on chromosome 7 [Evans et al., 1988; Sutherland et al., 1988; Digby et al., 1989; Morris, 1989 (Table 1); Qin et al., 1991]. Of the three distinct alleles that make up the human gene family, hRKALL encodes true tissue kallikrein, a trypsin-like kininogenase, that is expressed in the kidney, pancreas, and SMG. The PSA gene encodes a chymotrypsin-like enzyme that possesses Ser-183 as in chymotrypsin and shows considerable sequence homology with tissue kallikrein and is expressed in acinar and duct cells of the prostate. Increased levels of PSA in serum are used as a marker for prostatic tumours. PSA is believed to cleave semenogelin to produce liquefaction of seminal fluid clots (Watt et

al., 1986; Lundwall and Lilja, 1987; Lilja et al., 1987). Located downstream from the PSA gene is hGK-1 which appears to encode another trypsin-like protease in the prostate (Schedlich et al., 1987, 1988; see table 1). Sequence comparisons of the three clones show a serine instead of aspartic acid in the active site of PSA. Furthermore, the protein product coded by hGK-1 has sequence changes that suggest an enzymic preference for nonkininogen substrates (Qin et al., 1991). Of the three human kallikrein family genes, only hRKALL is expressed in the SMG. Unlike in the mouse, the human SMG appears to lack the genes homologous with those that encode testosterone-inducible γ-NGF- and EGF-processing enzymes in the mouse (Bowcock et al., 1988). Functionally, therefore, the true tissue kallikrein gene in humans may have subsets; therefore, it may, in addition, express enzymes essential for the processing of growth factors (Cohen, 1960, 1962) whose absence may result in degenerative diseases of the brain such as Alzheimer's syndrome (Aoyagi et al., 1990).

ii. Mouse gene family. Clusters of the mouse kallikrein gene family are confined to a single locus on chromosome 7 (Mason et al., 1983; Evans et al., 1987). Of the 24 mouse genes, 14 are functional genes and 10 are pseudogenes which, because of mutations, do not express functional proteins. In spite of the large number of kallikrein-like proteases in the mouse, only two patterns of expression are observed. A single gene, mGK-6, encodes a tissue kallikrein with kininogenase activity in the kidney, pancreas, and SMG. The true tissue kallikrein gene (mGK-6) shows no androgen dimorphism and occurs equally in both male and female tissues (Bhoola and Dorey, 1971; Bhoola et al., 1973). Expression of the rest of the gene family that includes mGK-3 (γ-NGF), mGK-4 (α-NGF), mGK-16 (γ renin), mGK-22 (β-NGF endopeptidase, previously incorrectly described as EGF-BP type A; Woo et al., 1988), mGK-13 (EGF-BP type B; status as a EGF-BP uncertain) and mGK-9 (EGF-BP type C, considered to be the only true EGF-BP) seems to be restricted to the male SMG with little or no expression in any other tissues of the male or female (Fahnestock et al., 1991). Furthermore, the cellular levels of the mRNA encoded by mGK-3 (γ-NGF), mGK-4 (α-NGF), mGK-13 (EGF-BP type B), mGK-9 (EGK-BP type C), and mGK-5 genes are markedly increased by testosterone (Van Leeuwen et al., 1986, 1987). The cellular function of the enzymes coded by the androgen-inducible genes must, therefore, differ from that of true tissue kallikrein. An understanding of the evolutionary deviation or the acquisition of this change by mutation in the mouse presents a fascinating challenge.

The androgen-sensitive kallikrein-like mouse proteins and proteases have been characterised and some of the substrates of these enzymes identified. One member of this family, EGF, isolated from the mouse salivary gland, stimulates mammalian cell differentiation and proliferation (Cohen, 1962). ProEGF, with a molecular mass of 130 kDa, is processed in steps to be finally cleaved at the COOH terminus to a 9-kDa molecule by an EGF enzymic binding protein (Frey et al., 1979). The mature EGF comprises a 74-kDa complex of 53 amino acids that consists of two molecules of EGF (each 9 kDa) linked to two molecules of arginyl esterase (each 29 kDa) known as EGF-BP. Of the so called EGF-BP types B and C, both members of the mouse kallikrein gene family (Drinkwater et al., 1987; Blaber et al., 1987), only type C is considered to associate with EGF to form the high molecular weight complex, EGF-BP type C (Isackson et al., 1987b).

Another androgen-sensitive member of this family, 7S NGF, apparently occurs only in the mouse and is a high molecular weight complex that consists of three polypeptide chains, of which only α and γ are encoded by kallikrein genes (Thomas et al., 1981). Amino acid se-

TABLE 1
Human glandular kallikrein gene family

	hRKALL	PSA	hGK-1
Genes	Similar size, organization: 5 exons; 5–6 kilobase pairs		
Homology	High → 75%	Very high → 85%	← Very high 85%
Localization	All at chromosome 19q13.3–13.4 locus		
Expression	Kidney, pancreas, salivary gland	Prostate*	Prostate
Transcript(s) (kilobase pairs)	1.0	1.6 + minor variants in tumours	1.6
Activity of product	Trypsin-like	Chymotrypsin-like	Trypsin-like
Substrate	Kininogen	Semenogelin	Not known

* Expression fivefold more than in hGK-1.

quence analysis of the γ and α subunits shows a remarkable homology with tissue kallikrein (Bothwell et al., 1979). β-NGF is synthesised as a larger precursor of 33 kDa that is processed at both amino terminals into a 13-kDa fragment. The amino terminus of β-NGF is cleaved at a His^8-Met^9 bond by the serine protease, β-NGF endopeptidase (gene mGK-22), thereby releasing an octapeptide that has the interesting property of inhibiting the NGF endopeptidase (Woo et al., 1988; Fahnestock et al., 1991). In addition to forming NGF_{1-8} from the NH_2 terminus of NGF, β-NGF endopeptidase releases a bradykinin-like peptide from mouse LK (Bothwell et al., 1979). Cleavage of the COOH-terminal end of β-NGF by the γ subunit (gene mGK-3) yields an active molecule with nerve growth activity (Berger and Shooter, 1977; Thomas et al., 1981). α-NGF (gene mGK-4) binds the β subunit but does not cleave it because it is enzymically inactive (Isackson and Bradshaw, 1984; Evans and Richards, 1985); the loss of enzymic activity by the α subunit may be due to mutation within essential sequences required for the active site, resulting in a loss of enzymic function.

iii. Rat gene family. This family may comprise up to 20 closely related genes that show diversity and varied patterns of expression (Ashley and MacDonald, 1985; Gerald et al., 1986; Chen et al., 1988; Shai et al., 1989). Genomic sequences for eight members, rGK-1 to rGK-8, were described by Wines et al. (1989). Major expression of all of these genes occurs in the SMG. Six tissue-specific expression patterns of the genes and the encoding mRNA have been delineated. The mRNA for true tissue kallikrein (rGK-1:PS mRNA) has been identified in parotid, SMG (Ashley and MacDonald, 1985), kidney (Inoue et al., 1989), pancreas (Swift et al., 1982), gastrointestinal tract (Fuller et al., 1989, blood vessels (Scicli et al., 1991), and pituitary glands (Fuller et al., 1985) of the rat. In the pituitary gland, the expression of the rGK-1 and its enzyme activity levels are regulated by oestrogens (Clements et al., 1986; Powers, 1987). Unlike true kallikrein, expression of tonin (S2 mRNA:rGK-2; RSGK-5) is restricted to SMG only. Each of the other mRNAs code for (*a*) unique and novel kallikrein-like enzymes: S1 mRNA (rGK-5) is expressed in SMG, P1 mRNA (rGK-8) in SMG and prostate, and K1 mRNA (RSKG-7) in SMG and kidney; and (*b*) tonin-like enzymes: S3 mRNA in SMG and the prostate. The nature of the encoded kallikrein-like protein expressed by genes rGK-4 and rGK-6 is not known (Ashley and MacDonald, 1985; Wines et al., 1989; Shai et al., 1989). From an evolutionary point of view, the Mastomys rat apparently lacks the γ subunit of NGF (Bowcock et al., 1988), indicative of a mutational deviation in an otherwise closely related genetic kinship to the rat kallikrein gene family (Bell and Fahnestock, 1988; Fahnestock and Bell, 1988). The functional importance of this mutational change needs to be elucidated.

Considerable interest has centred on the kallikrein gene family in the rat kidney so that new insights may be achieved regarding the physiological function of the renal enzyme. True kallikrein and its mRNA PS have been detected in the kidney. Structurally, this gene is composed of five exons and four introns stretching over a length of 4.5 kilobase pairs (Inoue et al., 1989). In addition, a second gene (RSGK-7) coding K1 mRNA of unknown function and enzymic specificity has been identified in the kidney recently. K1 is the dominant kallikrein-related mRNA in the rat kidney and is expressed at about 10 times the level of the true kallikrein mRNA (Brady and MacDonald, 1990; Elmoujahed et al., 1990). The K1 mRNA sequence is encoded by the RSKG-7 gene previously characterised by Chen et al. (1988). In fact, RSGK-7 gene-coded mRNA is expressed at similar levels in the kidney and SMG. However, whereas both mRNA levels are decreased in the SMG by castration and restored by testosterone treatment, no such change is observed in the kidney (Chen et al., 1988; Clements et al., 1988). Although some of the active site and binding residues of true kallikrein have been conserved in the K1 mRNA, Tyr-99 and Trp-215 of the hydrophobic sandwich (formed between Tyr-99 and Trp-215 to accommodate the bulky P2 residues at the cleavage sites for the release kinin from kininogen) have been replaced in tonin by His-99 and Gly-215. This important structural characteristic provides one crucial difference between the enzymic properties of true tissue kallikrein (K1) and tonin (Fiedler, 1987). Such an evolutionary change may have altered the ability of K1 to form kinins with a substrate preference distinct from, but intermediate between, kininogen and angiotensinogen (Brady and MacDonald, 1990). Apart from the true kallikrein gene, there are two additional kallikrein-related genes (rKG-4 and RSKG-7) expressed in the kidney (Chen et al., 1988). The RSGK-4 and RSGK-7 renal genes encode serine proteases that are different from tissue kallikrein and tonin, but unlike rGK-1: PS, they are not expressed in the pancreas. Their functional importance may therefore primarily reside in the kidney. The question of whether these are mutants of the true kallikrein gene (rGK-1) in the rat genome requires investigation, particularly with regard to whether they are expressed in morphologically distinct cells.

The functional importance of tonin in the SMG and a tonin-like enzyme in the prostate remains a mystery. Tonin (rGK-2:S2 mRNA; RSGK-5) produces a single step, slow rate of cleavage of angiotensinogen to form angiotensin II in vitro (Boucher et al., 1974; Lazure et al., 1984). Additional functions attributed to tonin are kininogenase activity, formation of ACTH from proopiomelanocortin and opiate peptides from β-lipoprotein, degradation of substance P, and conversion of prorenin to renin (Seidah et al., 1979a,b; Chrétien et al., 1980; Ikeda and Arakawa, 1984). Of the eight rat kallikrein-

tonin gene family members expressed in SMG, only rGK-2:S3 (tonin-like) and, to a much lesser extent, rGK-8:P1 (kallikrein-like) are selectively expressed in both prostate and SMG. Using monoclonal antibodies, Chao and Chao (1987) localised an esterase A-like enzyme in the prostate that is probably encoded by P1 mRNA and the rGK-8 gene. Of the two proteins expressed in the prostate, whereas P1 mRNA is partially modulated, S3 mRNA shows complete androgen dependence. Prostatic S3 mRNA disappears 8 days after castration and is restored to initial levels by dihydrotestosterone but not by oestradiol benzoate. Rat prostatic cells show no immunoreactivity to either true kallikrein or tonin. The only enzymes expressed by this gene family and detected immunocytochemically are esterase A and an esterase A-like enzyme (Chao and Chao, 1987). Whether S3 mRNA codes for rat esterase A remains to be determined.

iv. Molecular arrangement of kallikrein genes. The distribution and molecular arrangement of some of the kallikrein genes in the mammalian genome have been delineated and studied extensively. They consist of five exons and four introns. Each of the three crucial, active site amino acids are encoded on a separate exon. The gene members are highly conserved, located close to one another, and tightly linked. Individual genes in the mouse are about 5 kilobase pairs in length and approximately 5 kilobase pairs apart (Mason et al., 1983). In contrast, the human genes PSA and hGK-1 are 12 kilobase pairs apart and hRKALL is even further, i.e., 15 kilobase pairs away (Digby et al., 1989). Comparative studies on the evolution of the kallikrein gene family have revealed that the rat and mouse kallikrein genes are more closely related to one another than to the human ones. Mouse and rat exonic regions are similar, but the introns show greater divergence. Because of a greater conservation in the exonic regions that code the functional sequences of the kallikreins, not surprisingly, the human exonic regions are more closely related to those of the mouse and rat than their respective nonexonic clusters. The large number of alleles in the mouse and rat may have occurred as a result of mispairing and unequal crossover during gene duplication.

The intraspecies comparison of the amino acid sequences coded by the kallikrein genes shows a greater homology than a comparison across species. Therefore, whereas the families in the various mammalian species have evolved independently, members within the mouse and rat seem to have coevolved. In contrast, the human kallikrein gene family shows a lesser sequence conservation among its members, suggestive of a greater spatial dispersion within the genome. In the mouse, rat, hamster, and human, only one gene appears to code for true tissue kallikrein. Whereas the kallikrein gene members are a distinct subfamily of serine proteases showing between 60 and 75% homologies, a sequence identity of only 35 to 50% is shared with trypsin, chymotrypsin, and elastase (Fiedler and Fritz, 1981; Mason et al., 1983). The high degree of homology between the kallikrein-like enzymes suggests that they have evolved from a common ancestor through gene duplication.

It must be only a matter of time before the kallikrein gene family will be fully elucidated in several of the mammalian species. The challenge is to determine the cell-specific regulation and expression of the gene, to characterise the new kallikrein-like serine proteases and their substrates, and to identify the precise cell in which these enzymes modeled on kallikrein are located. An important experimental challenge will be to determine in humans the precise relationship between the genes that regulate neurotrophic factors and the true kallikrein gene. Such a study may provide new insights into Alzheimer's disease characterised by premature nerve cell death.

b. ISOLATION AND PURIFICATION. Tissue kallikrein has been characterised in the porcine (Fritz et al., 1967; Fiedler, 1976; Tschesche et al., 1979; Fiedler et al., 1981), rodent, and canine (Hojima et al., 1975) pancreas. Isolation and purification of the enzyme was achieved in the salivary glands of the rat (Brandtzaeg et al., 1976), cat (Moriwaki et al., 1975a), pig (Lemon et al., 1979), mouse (Porcelli et al., 1976), and guinea pig (Fiedler et al., 1983; Mayer et al., 1989). The presence of the enzyme has been reported in vascular tissue obtained from rat mesenteric and tail arteries (Nolly and Lama, 1982; Nolly et al., 1985), in rat and human intestinal tissue (Zeitlin, 1971; Zeitlin et al., 1976b; Moriwaki et al., 1980; Uchida et al., 1980), in rat skeletal (Shimojo et al., 1987) and cardiac (Britos and Nolly, 1981) muscles, in rat and bovine spleen (Lokshina et al., 1976; Swift et al., 1982; Chao et al., 1984), in the rat pineal (Scicli et al., 1984; Chao et al., 1987), and in the porcine, rat, and human anterior pituitary (Powers and Nasjletti, 1982, 1984; Jones et al., 1990). In addition, immunoreactive tissue kallikrein and/or kininogenase activity has been found in the rat ureter (Orfila et al., 1988), adrenal glands (Scicli et al., 1989), and rat aorta (Oza et al., 1990). Human and animal urinary kallikreins have been also isolated and the structures determined.

c. STRUCTURAL CHARACTERISTICS. The true tissue kallikreins (EC 3.4.21.35) are acidic glycoproteins, with apparent molecular masses ranging from 24 to 45 kDa and having pI values close to 4.0 (3.5 to 4.4) (Pisano, 1975; Fiedler, 1979). The active site triad comprises histidine, serine, and aspartic acid, and catalytic reaction steps are similar to enzymes such as trypsin, elastase, and other serine proteases (Stroud, 1974; Pisano, 1975; Kraut, 1977; Fiedler, 1979).

Initial studies concentrated on the isolation and determination of the structure of porcine pancreatic kallikrein (Habermann, 1962; Fiedler and Werle, 1967; Fiedler et al., 1970, 1981; Kutzbach and Schmidt-Kastner, 1972; Tschesche et al., 1979; Kizuki et al., 1982). The enzyme,

isolated from autolysed pancreas, was recovered as a two-chain form called β-kallikrein, and the amino acid sequence was determined (Fiedler and Hirschauer, 1981; Fiedler et al., 1981). Less drastic conditions of isolation yielded a single-chain pancreatic α-kallikrein (Fiedler and Gebhard, 1980). As with other serine proteases, porcine pancreatic kallikrein is synthesised in a "pro" form, and transformation to α-kallikrein occurs by cleavage of the NH_2 terminal heptapeptide (Kamada et al., 1988). Subsequent proteolysis of the molecule at different cleavage sites in the region of the kallikrein loop by enzymes such as trypsin or chymotrypsin produces T-β-kallikrein and C-β-kallikrein, respectively (fig. 3). When a partially degraded form, A-β-kallikrein, was isolated from autolysed pancreas it lacked about seven to eight residues in the region of the kallikrein loop. These different forms of the enzyme showed different kinetic constants on ester substrates and differences in the degree of inhibition by α_1-antitrypsin (Kamada et al., 1990a). Porcine tissue kallikreins from SMG (Lemon et al., 1979) and urine (Fiedler and Gebhard, 1980; Fiedler et al., 1981), with an amino acid sequence probably similar to that of the pancreatic enzyme, were isolated as single-chain α-kallikreins. Both showed similar rates of kallidin release and hypotensive activity, as did the β-kallikrein isolated from the autolysed porcine pancreas (Dittmann and Wimmer, 1979). Human urinary kallikrein is also a single-chain polypeptide that comprises 238 amino acids with Ile at the NH_2 and Ser at the COOH terminus (Geiger et al., 1979; Takahashi et al., 1988). The structure was identical with that derived from a human genomic DNA library but differed by one amino acid at position 162 when compared with deduced sequences for pancreatic or kidney cDNA sequences (Lu et al., 1989).

The number and position of glycosylation sites seem to vary according to species and the synthesising cell. Rat and mouse kallikreins have only one glycosylation site per mole of kallikrein. Porcine pancreatic kallikrein has two or possibly three glycosylation sites (Fritz et al., 1967; Fiedler et al., 1981; Tomiya et al., 1988; Kamada et al., 1990a). Human urinary kallikrein has three asparagine-linked glycosylation sites (Takahashi et al., 1988; Lu et al., 1989), and three additional oxygen glycosylation sites linked to two serine and one threonine residues (Kellermann et al., 1988). Additionally, the total carbohydrate content of porcine SMG (24%, w/w; Lemon et al., 1979) and human urinary kallikrein (20%, w/w) is relatively higher than that of the porcine pancreatic enzyme (12%, w/w; Moriya et al., 1983).

The carbohydrate content of tissue kallikrein confers to the molecule a heterogenous mobility on polyacrylamide gels, leading to the ready separation of the different forms of kallikreins (Fiedler and Hirschauer, 1981; Takada et al., 1985). Sialic acid seems to partially influence the thermal stability of tissue kallikrein because asialo forms of the enzyme are less stable than is the intact molecule (Moriya et al., 1983). Normal microheterogeneity due to the linking of different sialic acid residues to the parent protein has been described for porcine

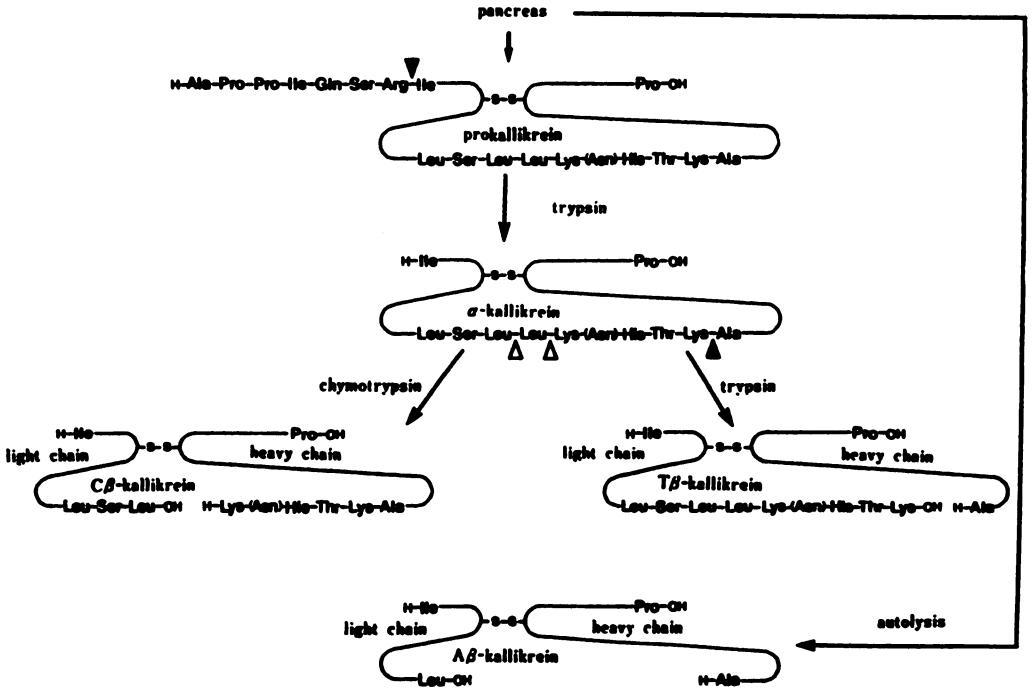

FIG. 3. Conversion of porcine pancreatic prokallikrein to a single-chain α-kallikrein, and the subsequent proteolytic transformation into different molecular forms (C-β- and T-β-kallikreins). Autolysis results in the formation of A-β-kallikrein. Arrowheads, site of tryptic (▲) and chymotryptic (Δ) cleavages. Undetermined amino acid is indicated in parentheses. From Kamada et al. (1990b) and used with permission.

pancreatic and SMG and human urinary kallikreins (Fritz et al., 1967; Lemon et al., 1979; Geiger et al., 1980; Moriya et al., 1981, 1983). The finding, therefore, of increased amounts of anionic kallikreins in the saliva of patients with rheumatic disorders suggested either a primary genetic abnormality or a posttranscriptional change resulting in a modification of the site and nature of glycosylation of the enzyme (Greaves et al., 1989). The attachment of novel acidic carbohydrate moieties to the kallikrein molecule in the Golgi apparatus may provide an insight into our understanding of the primary molecular defect in connective tissue disorders.

i. Prokallikrein. Human urinary tissue kallikrein seems to be synthesised bound to a signal peptide of 17 amino acids that is finally cleaved off to produce an inactive precursor. Four amino acids (Arg-Asn-Asp-Ala) are interposed between the pre-pro- and pro-peptide forms as deduced from the nucleotide sequences. The NH_2 terminal amino acid of the pro segment, Ala^1, is linked by a short, seven-amino acid activation peptide to the primary sequence of tissue kallikrein (Lottspeich et al., 1979; Takada et al., 1985; Takahashi et al., 1988; Takenobu et al., 1990). In the case of human urinary and porcine pancreatic prokallikreins, hydrolysis of the Arg^7-Ile^8 bond results in the release of the NH_2 terminal activation peptide Ala-Pro-Pro-Ile-Gln-Ser-Arg (Baker and Shine, 1985; Fukushima et al., 1985; Takahashi et al., 1986), whereas for rat pancreatic and urinary and mouse SMG prokallikreins, hydrolysis of the Arg^7-Val^8 bond releases the NH_2 terminal septapeptide Ala-Pro-Pro-Val-Gln-Ser-Arg (Mason et al., 1983; Takahashi et al., 1987; Takenobu et al., 1990).

In addition to the active enzyme seemingly occurring primarily in salivary glands, most tissues and body fluids contain in varying proportions the inactive pro form. Although in vitro activation of the proenzyme together with the release of the septapeptide has been performed mainly with trypsin (Frey et al., 1950; Fiedler, 1979; Kamada et al., 1988), it can be achieved also with thermolysin, a bacterial metalloprotease that cleaves substrates at the NH_2 terminus of hydrophobic amino acids. It has been reported that thermolysin is about four times more effective than trypsin in activating the proenzyme from human urine (Noda et al., 1985). Two endogenously occurring serine proteases (arginine esterases: I, identical with tonin; II, similar to esterase B) with prokallikrein activation properties have been identified in the rat SMG (Kamada et al., 1990a). The presence of such converting enzymes in the SMG raises the possibility of salivary tissue kallikreins being synthesised in a pro form but rapidly converted to the active state within the duct cell. Apparently, the rat kidney contains at least three activating proteases, one of which is a thiol proteinase with a molecular mass of 57 kDa and is immunologically identical with one of the rat SMG esterases (Nishii et al., 1989).

ii. Kallidinogen substrate. The primary physiological substrate for tissue kallikrein is kininogen from which it forms kinins. The kinetics, and timing and sequence of hydrolysis, of the two peptide bonds involved in the release of kinin from the kininogen molecule are not yet clearly understood. By examining the hydrolysis of a number of di- and tripeptide methyl esters by porcine pancreatic kallikrein, Fiedler (1987) demonstrated that, in addition to its primary requirement of an arginine moiety in the P1, the residue in P2 confers further specificity for the enzyme. The best substrates contained phenylalanine or leucine as P2 residues (Fiedler, 1987). Release of kallidin from the kininogen molecule requires the hydrolysis Met-Lys in the Leu-Met-Lys (at the NH_2 terminus of kallidin) and Arg-Ser in the Phe-Arg-Ser (at the COOH terminus of kallidin). The efficiency, kinetics, and temporal relationship of the hydrolysis of the two bonds by tissue kallikrein has been the subject of a recent study by Fiedler and Hinz (1992). The inability of tissue kallikreins of most mammalian species to form bradykinin is due to their inability to accommodate the Lys-Arg-Pro sequence for hydrolysis of the Lys-Arg bond in the NH_2 terminus of the peptide. Indeed, an important determinant of specificity for the tissue kallikreins that form kallidin (Lys-bradykinin) is a hydrophobic sandwich of Tyr-99 and Trp-215 that binds the side chains of the P2 amino acids (Leu-Met and Phe-Arg) in the kinin sequence of the kininogen molecule (Fiedler, 1987).

iii. Inhibitors. Inhibitors of tissue kallikrein were first described by Werle and colleagues in many bovine organs, including lung, pancreas, SMGs, lymph nodes, and the kidney (Kraut et al., 1930b). Most of them were small proteins about 5 to 20 KDa in size. They inhibit tissue kallikrein and a number of other proteases including trypsin (Werle and Vogel, 1973; Hochstrasser et al., 1974). More recently, a new inhibitor of tissue kallikrein has been identified in serum and extracts prepared from liver, lung, salivary gland, and liver of the rat (Chao et al., 1986; Chao and Chao, 1988a; Chao et al., 1990a). This inhibitor binds specifically to active tissue kallikrein but not to the inactive or active site-blocked forms. Additionally, it binds to elastase and chymotrypsin but not to plasma kallikrein, urokinase, or collagenase. The kallikrein-binding molecule is an acidic protein (pI 4.2 to 4.6) with a molecular mass of 60 kDa and five possible glycosylation sites. The enzyme and the inhibitor form a 1:1 stoichiometric, heat-stable complex of 92 kDa. The complex is emiocytosed by hepatocytes, a mechanism by which it is cleared from the circulation. The KBP shows a high degree of homology to α_1-antitrypsin, antithrombin III, plasminogen activator, and α_1-antichymotrypsin and, therefore, is considered to be a member of the serpin superfamily (Chao et al., 1990a). Of functional importance is the finding that, whereas liver mRNA values for KBP are lower after acute inflammation, circulating levels of KBP are significantly reduced in spontaneously

hypertensive rats. Furthermore, levels of KBP are higher in males than females, indicative of androgen dependence of the KBP (Chao et al., 1990a).

Another KBP has been isolated from human serum. Like the rat KBP, the human KBP forms a heat-stable 92-kDa complex with the enzyme. Heparin inhibited the complex between kallikrein and the KBP but not that between kallikrein and α_1-antitrypsin (Chao et al., 1990b). The half-maximal binding of kallikrein with α_1-antitrypsin was greater than 6 h (Vogel, 1979; Geiger et al., 1981), whereas that with the KBP was about 30 min. The order of potency in inhibiting the kininogenase activity of tissue kallikrein was as follows: first KBP, next α_1-antitrypsin, and to a much lesser extent, if at all, α_2-macroglobulin. The precise functional importance of KBP remains to be elucidated. An in vitro inhibitor of kallikrein is aprotinin (trasylol). Substitution of one of the amino acids essential for the inhibitory action of aprotinin has resulted in a molecule, [Val 15]aprotinin, that selectively inhibits both plasma kallikrein and neutrophil elastase (Wenzel et al., 1986).

Peptide analogues resembling the amino acid sequence on the COOH terminus of the kinin moiety within the kininogen molecule appear to be effective inhibitors for the active site of the kallikrein molecule (Burton et al., 1987; Burton and Benetos, 1989). The most potent of this new generation of inhibitors is Ac-Ser-Pro-Phe-Arg-Ser-Val-Gln-NH_2, an analogue designed around the COOH terminus of the kinin sequence as it resides within domain 4 of the kininogen molecule, the endogenous substrate for the kallikreins. The Stewart and Vavrek kinin antagonists inhibit the amidolytic activity of human and rat urinary kallikreins with K_i values in the micromolar range. The molecular features promoting such inhibition are the COOH-terminal arginine and a replaced bulky D-amino residue in position 7 of the kinin molecule (Spragg et al., 1988).

d. CELLULAR LOCALISATION. During the last three decades, the cellular storage and synthesis sites of tissue kallikrein have been determined in a number of tissues by the use of techniques such as differential centrifugation, immunocytochemistry, and in situ hybridisation. The concept that tissue kallikrein may be sequestered in subcellular organelles arose from the experiments of Siebert at al. (1955). Clear evidence that tissue kallikrein is sequestered in intracellular organelles was first provided by Bhoola and Ogle (1966) and Erdös et al. (1968) (reviewed in detail by Bhoola et al., 1979). With the advent of specific antibodies and in situ hybridisation techniques, tissue kallikrein was localised in the granular tubules and striated duct cells of SMG (Orstavik et al., 1976a; Schachter et al., 1978; Simson et al., 1979; Orstavik, 1980; Coghlan et al., 1984; Simson et al., 1988), in the acinar (Orstavik, 1980) and β cells (Pinkus et al., 1983) and in the rat transplantable acinar cell carcinoma of the pancreas (Berg et al., 1985a), in the CNT cells of the kidney (Tomita et al., 1981; Proud et al., 1982; Omata et al., 1983; Figueroa et al., 1984a,b), in the colonic mucous cells (Schachter et al., 1983), in the epithelial cells lining the lumen of the coagulating gland and prostate (Schachter et al., 1978; Kumamoto et al., 1989), in the glands of the trachea (M. Schachter, personal communication; Bhoola et al., 1989) and nasal mucosa (Baumgarten et al., 1989), in the prolactin-secreting cells of the anterior pituitary (Vio et al., 1990; Jones et al., 1990), in the ependymal cells lining the third ventricle as well as in the cell bodies of arcuate, supraoptic, paraventricular, and ventromedial nuclei of the brain (Simson et al., 1985), and in the sarcoplasmic reticulum and granules of rat atrial myocytes (Xiong et al., 1990b). The exciting new discovery of tissue kallikrein in the neutrophil and attachment of HK, plasma prekallikrein, and LK on the external surface of its membrane provides new insights for the involvement of kinins in the diapedesis of neutrophils through capillary cell junctions and their participation in the inflammatory process (Figueroa et al., 1991b). The intracellular sequestration granules and storage sites of the enzyme vary from one cell type to another and may relate to the function of the enzyme in each tissue.

e. MEASUREMENT OF TISSUE KALLIKREIN. The ability of kallikrein to either directly (dog intestine) or indirectly (guinea pig ileum and guinea pig and rabbit uterus, contracted by kallidin released from kininogen) produce contraction of isolated smooth muscle preparations was first described by Frey, Werle, and colleagues (Frey et al., 1950). Later, some authors reported that rat glandular kallikrein contracted the rat uterus through a direct combination of the enzyme with receptors on the uterine muscle; however, the results of Figueiredo et al. (1990) indicate that the rat uterine contractions were produced by bradykinin formed within the uterus by tissue kallikrein. For at least five decades, measurement of tissue kallikrein has dependent on the formation of kinins from a variety of kininogen preparations; the released kinins (kininogenase activity, Bhoola et al., 1962) were then assayed on one or more of three preparations: (*a*) the isolated guinea pig ileum or rat uterus (Erspamer, 1948; Beraldo et al., 1956), (*b*) lowering of the systemic blood pressure of the dog or rabbit Frey et al., 1950, 1968), and (*c*) peripheral vasodilation (Binia et al., 1963). The early assays for tissue kallikrein were reviewed in detail by Carretero et al. (1982).

Present day methods used to determine the amount of kallikrein in tissue extracts and in biological fluids can be divided into several groups. As already stated, measurements of tissue kallikrein may be made with methods that involve a kininogenase assay. The kinins released from purified kininogens as substrates may be estimated by bioassay (qualitative identification of kinins, using Gaddum's index of discrimination; Bhoola et al., 1962), RIA (Miwa et al., 1968; Talamo et al., 1969; Shimamoto

et al., 1978), or enzyme immunoassay (Ueno et al., 1981; Geiger and Miska, 1986), and by high performance liquid chromatography (HPLC) (Powers and Nasjletti, 1982; Gau et al., 1983; Kato et al., 1985; Fiedler and Geiger, 1988).

The enzymic activity of tissue kallikrein is also measured on a peptidyl-nitroanilide substrate, H-D-Val-Leu-Arg-paranitroaniline (Amundsen et al., 1979), in the presence of SBTI. Peptide amides of 7-amino-4-methylcoumarin have proved to be more sensitive than the chromogenic substrates, because the coumarin reaction product is highly fluorescent; Pro-Phe-Arg-methylcoumarin is a good substrate for tissue kallikrein (Morita et al., 1977). Furthermore, anti-tissue kallikrein antiserum or the IgG fraction of antibodies can be used to specifically inhibit the amidase activity of tissue kallikrein. To obtain an estimate of the proportion of proenzyme in a sample, kininogenase or synthetic substrate assays are performed before and after trypsin or thermolysin activation, thereby providing values for both the active and the inactive components. However, all of the enzymic methods are subject to interference by inhibitors and trypsin-like enzymes present in the tissue extracts and body fluids. A more specific assay, in which tissue kallikrein-specific IgG was coated to wells on a microtitre plate, was described recently by Witzgall et al. (1991). When samples are added to the plate the antibody recognises and traps tissue kallikrein. The subsequent addition of a selective chromogenic substrate permits the determination of enzymic activity. Greater sensitivity is obtained if kininogen is used as substrate and the released kinin measured by RIA (Witzgall et al., 1991).

Values can also be determined by methods based on the use of specific antibodies such as in RIA (Mann et al., 1980a,b; Rabito et al., 1982; Fink and Güttel, 1978; Bagshaw et al., 1981, 1984; Shimamōto et al., 1980, 1984) and enzyme immunoassays (Hoffman and Geiger, 1983; Sakamoto et al., 1985). The polyclonal antibodies produced against tissue kallikrein usually recognise both active and pro forms of the enzyme (Rabito et al., 1982; Bagshaw et al., 1984). It has been suggested that the septapeptide of the proenzyme may inhibit binding of the antibody to the enzyme (Takahashi et al., 1986), but binding of aprotinin to the active site of tissue kallikrein does not affect immunological detection. By combining specially characterised polyclonal and monoclonal antibodies, Ando et al. (1986) developed an RIA for the measurement of active rat tissue kallikrein in biological fluids and tissue homogenates.

f. LEVELS IN BIOLOGICAL FLUIDS. The use of RIAs, enzyme immunoassays, and synthetic substrates has revealed the presence of tissue kallikrein in a variety of body fluids, namely, saliva, urine, bile, plasma (Fink et al., 1978; Nustad et al., 1978; Fink et al., 1979b; Lawton et al., 1981; Mann et al., 1980a,b; Bagshaw et al., 1981; Rabito et al., 1982; Shimamoto et al., 1984; Masferrer et al., 1985), sweat (Mann et al., 1980b; Hibino et al., 1988b; Mayfield et al., 1989), cerebrospinal (Scicli et al., 1984) and synovial (Selwyn et al., 1989; Worthy et al., 1990a) fluids, and bronchoalveolar lavage fluid (Christiansen et al., 1987).

Apparently, in the rat SMG, immunoreactive tissue kallikrein is released continuously into the circulation (Berg and Nustad, 1986; Nustad et al., 1978a). The physiological relevance of this finding requires elucidation. More recently, another source was identified. Circulating human neutrophils contain a tissue kallikrein (Figueroa et al., 1989; Figueroa and Bhoola, 1989). Degranulating neutrophils could contribute significantly to circulating levels of tissue kallikrein. It has been suggested that in vivo the liver is the main organ that clears tissue kallikrein from the circulation. About 5×10^5 receptors/cell were estimated on isolated rat liver cells in binding studies using tritiated horse urinary kallikrein. On the other hand, experiments in which a perfused rat liver system was used seemed to indicate that porcine pancreatic kallikrein was recognised by a liver mannose receptor, and horse urinary kallikrein was recognised by a galactose receptor (Kouyoumdjian et al., 1989).

g. FUNCTIONAL IMPORTANCE. The diverse sites of occurrence of tissue kallikrein has led to the suggestion that the functional role of this enzyme may be specific to cell types (Schachter, 1980). Apart from its kininogenase activity, tissue kallikrein has been implicated in the processing of growth factors and peptide hormones (Bhoola, 1971; Bothwell et al., 1979; Mason et al., 1983). This idea has been supported by the finding of immunoreactive tissue kallikrein in the β cells of the pancreatic islets (Pinkus et al., 1983) and in the lactotroph cells of the rat (Vio et al., 1990) and human anterior pituitary (Jones et al., 1990). Tissue kallikrein in the prolactin-containing cells (Hatala and Powers, 1989; Jones et al., 1990) and in the coagulating gland cells of the guinea pig is present mainly in the Golgi cisternae, a localisation consistent with its enzyme-processing role. Human pancreatic kallikrein has been reported to sequentially cleave proinsulin to form the active molecule (Ole-Moiyoi et al., 1979). Furthermore, tissue kallikrein hydrolyses, at least in vitro, other biologically important peptides and proteins such as low-density lipoprotein (Cardin et al., 1984), the precursor of ANF (Currie et al., 1984), ANF (Briggs et al., 1984), prorenin (Sealey et al., 1978; Derkx et al., 1979, 1987), vasoactive intestinal peptide (Mutt and Said, 1974), procollagenase (Eeckhout and Vaes, 1977; Tschesche et al., 1989), and angiotensinogen (Arakawa and Maruta, 1980). The ability of kallikrein-like enzymes to form angiotensin II requires enzymic specificity for the Phe-His bond at a pH optimum in the acidic range (Ideishi et al., 1987). For the processing enzyme type of activity of tissue kallikrein, the concentrations required to produce cleavage of the precursor molecules are in the higher K_m range. The physiological significance of these

actions, therefore, needs careful assessment. Kallikrein in each cell type may possess single or multiple functions, common or unique, but for the present, release of a kinin (kallidin/bradykinin) should be considered to be its primary effect.

Tissue kallikrein has been implicated in organ-specific disorders, e.g., the extracellular release of glandular (tissue) kallikrein and trypsin into the circulation in acute pancreatitis (Thal et al., 1963; Ryan et al., 1965; Lasson and Ohlsson, 1984). Such patients show, simultaneously with the onset of hypotension and shock, a decrease in circulating levels of kininogens and plasma prekallikrein, but both components normalise as the patient recovers (Uehara et al., 1989). The pathological effects of kinins formed in the acute phase of pancreatitis may be reversed by the therapeutic use of the new potent kinin antagonists.

Because enterally administered enzyme reached the circulation, a therapeutic role for it was conceived (Moriwaki et al., 1973, 1980; Fink et al., 1978, 1979a,b, 1980, 1989a; Overlack et al., 1983; Miska et al., 1991). Even though virtually all of the enzyme in the circulation was bound to plasma components, it retained the capacity to form kinins. However, because only a small proportion of the administered dose reached the circulation, cumulative dosing may be required for the proposed clinical use in sperm defects (Schill, 1983) and hypertension (Overlack et al., 1981). Prolonged use may result in the formation of antibodies to the enzyme. In fact, autoantibodies to tissue kallikrein have been identified in normal human, guinea pig, rat, and mouse sera (Chao et al., 1988a). The question whether such autoantibodies to tissue kallikrein are important in organ-specific diseases merits study.

i. Homeostatic control of blood pressure. The formation of kinins in organs to increase local blood flow or in the circulation to reduce peripheral resistance and lower the blood pressure is a concept that is still controversial. However, the presence of tissue kallikrein in human (Mann et al., 1980b; Bagshaw et al., 1981, 1984; Shimamoto et al., 1984), porcine (Fink et al., 1978, 1979a,b), and rat plasma (Nustad et al., 1978; Lawton et al., 1981; Rabito et al., 1982) is now well established. Tissue kallikrein in the blood is believed to circulate in multiple forms: enzymatically active (Geiger et al., 1980; Masferrer et al., 1985), as a proenzyme, and bound to α_1-antitrypsin (Fink et al., 1979b; Lawton et al., 1981; Kizuki et al., 1989).

Little is known about the source of circulating tissue kallikrein. Apparently, in the rat SMG, the enzyme is released continuously into the circulation (Berg and Nustad, 1986). Furthermore, when the release was enhanced by adrenergic stimulation and when plasma kininases were inhibited with captopril, the mean blood pressure decreased by 43 mm Hg. This hypotensive effect was blocked by preadministration of antibodies to tissue kallikrein or kinins but not by nonimmune serum (Orstavik et al., 1982).

Evidence suggests that the kidney plays a pivotal role in the control of systemic blood pressure, because it possesses both pressor (renin-angiotensin) and depressor (kallikrein-kinin) components that may be important in the genesis of hypertension. Malfunction in the humoral regulation of arterial blood pressure could result in hypertension, either from an excess or from a deficiency of vasoactive molecules in the circulation. Therefore, a more important probable source of the circulating enzyme is the kidney. Tissue kallikrein was identified in the venous effluent of the isolated rat kidney perfused with an artificial medium (Roblero et al., 1976; Vio et al., 1983). This finding was confirmed in additional experiments in which a renal perfusion pressure-dependent release of the enzyme was observed; of the many secretagogues tested, only potassium and AVP proved to be effective (Lauar and Bhoola, 1986). Increased sympathetic tone may cause the release of renin from the afferent arteriole and inhibition of kallikrein secretion from CNT cells (see IV.A.1.c.iii: "Mechanisms Postulated for the Release of Renal Tissue Kallikrein: Neural Control"). Although such a hypothesis is attractive and may be a factor in essential hypertension, a different mechanism must apply to binephrectomised renal transplant patients in whom hypertension develops.

More recently, additional sources of circulating tissue kallikrein have been identified. A kininogenase has been isolated from rat arteries (Nolly and Lama, 1982; Nolly et al., 1985). Vascular smooth muscle cells in culture express tissue kallikrein, kininogen, and kininase activity, thereby supporting the concept of an intrinsic kinin system modulating vascular tone. In addition, circulating human neutrophils at the end of their life cycle could release tissue kallikrein as they disintegrate (Figueroa et al., 1989). These findings raise the possibility of an active role for the enzyme in the regulation of arteriolar resistance and local blood flow.

Inhibitors based on the amino acid sequence of kininogen around the cleavage site have been used recently to investigate the role of tissue kallikrein in blood pressure regulation. The substrate analogue inhibitor KKK-7, when infused into rats receiving a diet containing a normal quantity of sodium, produces an increase in blood pressure, prevents the vasodepressor action of exogenous kallikrein, and partially reverses the hypotensive action of KII-ACE inhibitors (Burton et al., 1987; Burton and Benetos, 1989). Studies with KII-ACE inhibitors appear to suggest an important physiological role of kinins in maintaining blood pressure. The administration of KII-ACE inhibitors to sodium-deficient animals decreases blood pressure to an extent greater than the reduction achieved with renin inhibitors alone (Blaine et al., 1985). In young SHO rats, long-term KII-ACE inhibition corrects both the low urinary excretion of tissue kallikrein

and the arterial blood pressure (Ader et al., 1986). Chronic treatment of adult SHO rats with KII-ACE inhibitors reverses the development of hypertension and complications such as strokes. Of interest was the linking of a significantly lower ACE activity in the kidney to a reduction in blood pressure observed even 8 weeks after discontinuing treatment (Ikemoto et al., 1986). The increase in renal kinins caused by captopril may be partly responsible for the increase in urinary excretion of PGE_2 which, through its natriuretic effect, could potentiate the hypotensive action of kinins. Evidence in humans further supports this view. A similar significant hypotensive effect of kinins is observed in patients with low renin in whom no further reduction in angiotensin II levels occurs with KII-ACE inhibitors. Furthermore, acute administration of KII-ACE inhibitors shows a better correlation with circulating kinin levels than with a decrease in angiotensin II levels (Iimura et al., 1986). The precise molecular mechanism by which kinins produce systemic arteriolar dilation is now considered to involve the release of EDRFs (Toda et al., 1987), in particular, nitric oxide (Moncada et al., 1991).

3. T-kininogenase. The discovery of T-kininogen by Okamoto and Greenbaum (1983) arose from the early observation that kallikrein did not release bradykinin from rat and guinea pig blood, whereas trypsin did (Fasciolo et al., 1963). During experiments in which kininogens from various species were incubated with large amounts of trypsin, an excessive amount of kinins was generated. Analysis of the data revealed the formation of a new kinin which was named T-kinin (Okamoto and Greenbaum, 1983; Greenbaum, 1986). The obvious question to answer concerned the nature of the enzyme responsible for the formation of T-kinin (Ile-Ser-bradykinin) in vivo. The search for such an endogenous enzyme led to the isolation of an acid protease (pH optimum 4.0) from a Murphy-Sturm lymphosarcoma with the ability to release T-kinin (Bedi et al., 1983b). A different T-kinin-containing peptide (T-kinin-Leu) was released by a cathepsin D isolated from granulomatous tissue induced by carrageenin-evoked inflammation in rats (Sakamoto et al., 1988). In contrast, an endogenous neutral protease able to form T-kinin was isolated from rat and mouse SMG, rat saliva, and rat peritoneal white blood cells (Damas and Adam, 1985). It is optimally active at pH 8.0, increased 10-fold by thiol radicals, and inhibited mainly by leupeptin but to a lesser extent by aprotinin (Barlas et al., 1987, 1989). About this time, Kato et al. (1987) isolated an enzyme from the rat SMG with a molecular mass of 30.4 kDa which they called protease B. This enzyme had the property of extensively degrading T-kininogen. The extent of similarity between protease B and endopeptidase K (Gutman et al., 1988) in the SMG, both with a capacity to release T-kinin, remains unanswered.

One of the T-kininogenases said to localise in the granular convoluted tubule and the striated duct cells of the rat SMG has been purified to homogeneity (Xiong et al., 1990a). The enzyme has an apparent molecular mass of 28 kDa when detected using sodium dodecyl phosphate-polyacrylamide gel electrophoresis. Treatment with dithiothreitol splits the enzyme into a heavy chain of 22 kDa and a light chain of 6 kDa. The T-kininogenase has 74 to 84% identity with rat kallikrein-tonin-related enzymes and seems to be indistinguishable from the thiol-activated molecule described by Barlas et al. (1987). However, it is different from endopeptidase K (Gutman et al., 1988) and from an acid proteinase partially purified from rat granulomatous tissue (Sakamoto et al., 1987). Endopeptidase K, unlike the T-kininogenase purified by Xiong et al. (1990a), produced no change in blood pressure even when large amounts were injected systemically (Gutman et al., 1988).

The concentration of T-kininogenase in the male gland is 2.4 times greater than that found in the female, whereas its circulating substrate, T-kininogen, is about 3.9 times greater in females than in males (Chao et al., 1989). Treatment with oestradiol increases the plasma and liver levels of T-kininogen significantly in both sexes, but testosterone decreases the levels in female rats and has no effect in the males (Oh-Ishi et al., 1989b). Some of the bioregulatory enzymes in the rat appear to be induced by androgens, whereas the associated substrates are modulated by oestrogens. During inflammation, T-kininogenase, unlike T-kininogen, shows no acute phase response (Chao et al., 1989).

All three enzymes (T-kininogenase, endopeptidase K, and the acid proteinase) release T-kinin from T-kininogen. In contrast, kallikreins and snake venom kininogenases are unable to release a kinin from T-kininogen because of the Met-Met-Ile-Ser sequence that precedes the NH_2 terminus of the bradykinin moiety. An important still unanswered question is the precise relationship between the genes that regulate T-kininogenase and the tissue kallikrein-tonin family in the rat SMG.

B. Kininogens

1. Kininogens. a. HISTORICAL. A role for kininogens as potential precursor molecules of kinins was first suggested by Werle et al. (1937). They observed that kallikrein itself failed to contract isolated guinea pig ileum or rat uterus, but this was achieved when serum was added. Werle easily realised that the relationship between kallikrein and serum resembled that of an enzyme and its substrate. The challenge to determine the nature of the kallikrein substrate and the cleavage site for the formation of kinins provided the impetus for the ensuing concerted effort in obtaining pure preparations of kininogens. The 1960s and 1970s were therefore dedicated to purifying and, in some instances, elucidating, the partial amino acid sequences of human (Webster and Pierce, 1963; Jacobsen, 1966; Spragg and Austen 1971, 1974;

Adam et al., 1985), bovine (Suzuki and Kato, 1977; Kato et al., 1981), and rabbit (Wuepper and Cochrane, 1971) kininogens. More recently, the rat (Okamoto and Greenbaum, 1983) and guinea pig (Yamamoto, 1987) kininogen molecules have been isolated and the constituent proteins purified.

b. EVOLUTION AND GENE KINSHIPS. The genealogy of mammalian cysteine proteinase inhibitors shows that kininogens and related proteins have been derived from an ancestral gene which at the first duplication gave increase to stefins and cystatins (Müller-Esterl et al., 1985a; Nakanishi, 1987; Müller-Esterl, 1989). Next, the fetuin family, including α_2-HS glycoprotein (the human analogue of bovine fetuin), was formed from the cystatin gene by duplication. The biological role of α_2-HS is obscure, but it is believed to be involved in brain development and bone modeling. The finding of two cystatin-like domains in fetuins and three in kininogens has led to the view that these proteins have arisen from an ancestral gene by two successive gene duplications (Müller-Esterl et al., 1985a; Kellermann et al., 1987; Rawlings and Barrett, 1990). The various patterns of gene expression have indicated that during evolution successive duplications had occurred. The primordial stefin-type molecule was first extended to form a cystatin block. Thrice repeated blocks of cystatins, recognised as repeats in the genomic segments, provided the essential homology with cysteine proteinase inhibitors and clue toward understanding the coding sequences of the heavy chain of the kininogen molecule. Recently, a novel member of the mammalian fetuin family was identified, the rat tyrosine kinase inhibitor. The sequence identity (percentage of residues in identical positions) among rat tyrosine kinase inhibitor, human α_2-HS glycoprotein, and bovine fetuin was found to be 60 and 62%, respectively (Haasemann et al., 1991b).

The organisation of the human kininogen gene was characterised by isolating the genomic clones containing the corresponding genes, and a structural map of the gene was computed. Detailed analysis of the cDNA data indicated a single K gene for H- and L-prekininogens in the bovine (Kitamura et al., 1983) and human (Kitamura et al., 1985) genomes. The HK and LK molecules, coded by the single K gene, are produced by alternate splicing of the gene transcript. The human kininogen gene consists of 11 exons and includes about 27 kilobase pairs (Kitamura et al., 1985). The nine exons located at the 5′-terminal region of the kininogen gene encode the common sequence of HK and LK mRNA. Exon 10 consists of the common sequence that codes for kinins (kinin exon) and the immediately following unique sequence for HK mRNA (HK exon). Exon 11 is located 91 nucleotides downstream from exon 10 with a small intervening sequence and a segment that specifies precisely the sequence unique to LK mRNA (Nakanishi et al., 1985). Consensus sequences for the donor and acceptor sites of RNA splicing have been identified at the 5′ end of HK exon and flanking the 5′ end of exon 11 (Nakanishi, 1987). Thus, the mature LK mRNA can be produced by splicing of the HK exon together with its flanking 90-kilobase pair nucleotide sequence. On the other hand, the HK mRNA can be generated by including the HK exon but not the exon 11 sequence. The same structural organisation of exons 10 and 11 has been reported for the bovine kininogen gene (Kitamura et al., 1983).

In the rat, gene duplications have resulted in one K gene coding for the HK and LK proteins identical with the human and bovine kininogen molecules and another T gene that codes for the T-kininogens. The K gene codes for two distinct types of mRNAs, one each for the HK and LK. But duplications of the T gene has given increase to two functional genes, T-I and T-II, and an allelic variant (Enjyoji et al., 1988). The T genes show an extensive homology of about 90% with the K gene in the coding as well as the noncoding mRNA regions (Kakizuka et al., 1990). The precise arrangement of the loci for mammalian kininogens and their gene-related proteins on the genome remain to be elucidated.

c. MOLECULAR STRUCTURE. **i. General.** The kininogens are single-chain glycoproteins. They possess an amino-terminal heavy chain, a COOH-terminal light chain with the kinin moiety interleafed between the two polypeptides that are bridged by a single disulphide loop (Kellermann et al., 1987). In association with HF, factor XI, and plasma prekallikrein, the intact kininogen molecule triggers contact activation of blood clotting, for which it is an essential cofactor. During formation of the single chain or following the release of the kinin molecule, the important cysteine protease inhibitory function subserved by the heavy chain becomes apparent. The realisation that this function may reside in the heavy chain was given credence when the cDNA for α_2-thiol proteinase inhibitor produced LK and that of the α_1-thiol inhibitor sequenced HK. Analysis of the cDNA base sequences for human α_2-thiol protease inhibitor showed that it was identical in chemical structure with that of LK (Ohkubo et al., 1984; Müller-Esterl et al., 1985b,c).

So far, the chemical properties and the molecular mass of bovine, human, rat, guinea pig, porcine, and avian (ornithokininogen, Kimura et al., 1987) HK and LK have been elucidated. The finding of Hyp^3-bradykinin and -kallidin in human urine and ascites fluid (Mindroiu et al., 1986; Kato et al., 1988; Maeda et al., 1988) and human plasma (Kato, 1991) suggested the possibility that the hydroxylated proline moiety within the kinin sequence resided in the kininogen molecule. Somewhat surprisingly, only human and monkey kininogens, albeit in variable proportions, possessed a hydroxylated kinin moiety. Such a transformation in the kinin moiety is almost absent from guinea pig, rat, mouse, rabbit, and bovine kininogens (Enjyoji et al., 1988). The genetic and functional significance of these findings are not known.

The two kininogens differ in their structure, size, and susceptibility to cleavage by plasma and tissue kallikreins (Jacobsen, 1966; Komiya et al., 1974; Kerbiriou et al., 1980). HK, a glycoprotein composed of 626 amino acids, has a molecular mass of 88 to 120 kDa, depending on the species of origin (Kato et al., 1976, 1981; Hayashi et al., 1985a; Yamamoto, 1987; Tani et al., 1987; Schmaier et al., 1988a). On the other hand, LK consists of 409 amino acids and varies from 50 to 68 kDa in size (Kato et al., 1976; Müller-Esterl et al., 1985a). Avian (chicken) plasma contains mainly HK, whereas in chicken egg white both LK (66 kDa) and HK (about 90 kDa) have been identified, with the pI approximately 4.5 to 5.5 (Kos and Turk, 1991). Chicken kininogens are strong inhibitors of cysteine proteases (Kos and Turk, 1991).

The mRNA coding for each of the human, bovine, and rat kininogens was determined by molecular cloning and sequence analysis of the matching cDNA (Nawa et al., 1983; Furuto-Kato et al., 1985; Kakizuka et al., 1988, 1990). After the molecular structure and the cDNA sequences of HK and LK were determined, several additional functions were discovered (Müller-Esterl et al., 1986). In fact, each of the kininogens contain domains that perform distinct functions. Enzymic action of plasma kallikrein on human HK follows three phases: the first results in transformation of the substrate into two polypeptide chains (heavy and light) linked together by disulphide bridges, the second is the release of kinin or a larger segment consisting of the intact kinin and the F-1.2 fragment, and the third is the removal of a 10- to 14.5-kDa peptide, namely, the F-1.2, histidine-rich fragment from the amino-terminal region of the light chain (Schiffman et al., 1980; Mori and Nagasawa, 1981; Scott et al., 1984). Dissection by limited proteolysis has made it possible to construct a model for the molecular architecture of these proteins (fig. 4). The model predicts that HK has six domains, whereas LK and T-kininogen possess five (Kato et al., 1981; Janin and Chothia, 1985; Müller-Esterl et al., 1986; Salvesen et al., 1986). Molecular changes resulting from the cleavage of LK by tissue kallikrein require more detailed study.

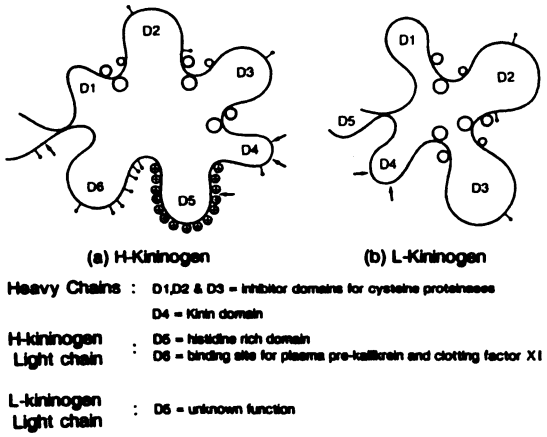

FIG. 4. Molecular architecture of human kininogens, indicating the domains of HK and LK. a, High molecular weight kininogen; b, low molecular weight kininogen. *, putative reactive site; O, potential disulphide loop; 🏌, carbohydrate attachment site; ⊕, histidine-rich region; ↓, cleavage site for kallikrein. From Müller-Esterl et al. (1986) and used with permission.

ii. Heavy chain (cysteine protease inhibitor). Genetic selection has strongly conserved the ability of the heavy chain to inhibit cysteine proteinases. The heavy chains of all three kininogens have the same basic structure, together making up about 64 kDa. The subtypes of T-kininogen, however, have an estimated molecular mass of about 69 kDa (Okamoto and Greenbaum, 1986). The upstream cDNA clone for the heavy chain contains three cystatin-like repeat sequences (Higashiyama et al., 1986) that form by gene duplication domains 1, 2, and 3 (Salvesen et al., 1986). The thiol protease inhibitory potential of domains 2 and 3, with the reactive site pentapeptide sequence of Gln-Val-Val-Ala-Gly repeated on each of the two internal repeat sequences in the molecule (Sueyoshi et al., 1985), is a novel function and was first conceived because of the amino acid homology of the domains with cystatins, inhibitors of cysteine proteases. The function of domain 1 is unknown, but recent evidence indicates that its amino-terminal portion contains a Ca^{2+}-binding site (Higashiyama et al., 1987).

The ability of kininogens to inhibit with high affinity thiol proteinases, such as the cathepsins B, H, and L, ficin, papain, and platelet-derived calpains I and II provides an important bioregulatory function (Ohkubo et al., 1984; Sueyoshi et al., 1985; Müller-Esterl et al., 1985b; Schmaier et al., 1986a). Divalent cations, particularly calcium, are essential for the activation of calpains (cytosolic calcium-activated cysteine proteinases, I and II) released during tissue damage and haemolysis of red blood cells (Ishiguro et al., 1987). During inflammation, calpains perform a dual function; they release kallidin from kininogens and thereby dissociate the heavy chain to inhibit the proteolytic activity of calpains coming from damaged tissues (Higashiyama et al., 1986). During inhibition of platelet calpain, HK, by acting as a substrate for this enzyme, may itself become consumed. Although the heavy chain of T-kininogen is structurally similar to HK and LK, it does not inhibit calpain. The heavy chain of HK shows a greater inhibition profile against papain than the heavy chain of the LK. The inhibitory potency against the different proteases shows species differences.

Although rat HK is similar in chemical structure to the human, guinea pig, and bovine molecules and subserves the known functions of being a substrate for the formation of bradykinin, a cofactor in the surface-mediated activation of HF, and an inhibitor of cysteine proteases (Hayashi et al., 1985a), a minor difference in amino acid sequence has been observed at the cleavage site for the enzymic action of kininogenases. In the rat HK, at the NH_2 terminus of the bradykinin sequence, an Arg-Arg sequence replaces the human Met-Lys-Arg.

Consequently, tissue kallikrein only releases bradykinin from rat HK (Kato et al., 1985).

iii. Kinin moiety. The only difference between the three proteins appears to reside at the amino terminus of the kinin segment. T-kininogen contains the sequence Met-Met-Ile-Ser-bradykinin instead of the Met-Lys-bradykinin of HK and LK (Kato et al., 1985; Furuto-Kato et al., 1985). The effector peptide, T-kinin (Ile-Ser-bradykinin), is formed by T-kininogenase (a thiol protease), cathepsin D, or trypsin, but not by tissue or plasma kallikrein (Okamoto and Greenbaum, 1983).

iv. Light chains. In each of the three kininogens, the light chain distal to the kinin moiety differs in length (Kitamura et al., 1983; Nawa et al., 1983; Takagaki et al., 1985; Lottspeich et al., 1985; Kellermann et al., 1986). The first 12 amino acids of the light chains are identical, but the molecules diverge in the subsequent COOH-terminal sequences. The light chain of HK (45 to 58 kDa) contains two domains, 5 and 6. Domain 5, rich in histidine, proline, and lysine (F-1.2), binds HK to the negative charge on damaged endothelial surfaces, crystals, and degraded cartilage products. Divalent metals (but not Ca^{2+} or Mg^{2+}) that accelerate contact activation of clotting bind to F-1.2. When plasma kallikrein releases from HK the kinin moiety embedded in the core of the molecule (domain 4), the downstream light chain enhances clotting through the sequential activation of the serine zymogen HF, prekallikrein, and factor XI. After active kallikrein is formed, the HK light chain continues to participate as an essential cofactor, promoting amplification of the activation cascade (Kaplan, 1978).

Domain 6 at the far end of the COOH terminus provides a unique site (Sugo et al., 1980) that specifically binds plasma prekallikrein or clotting factor XI with high affinity. A sequence of 31 residues (565 to 595) at the COOH terminus of domain 6 possesses a full binding capacity for prekallikrein (Tait and Fijikawa, 1987). A monoclonal antibody directed at the 31-amino acid peptide, which recognises the prekallikrein-binding site, inhibits HK-dependent activation of clotting in normal plasma (Reddigari and Kaplan, 1988, 1989a). The essential structural requirement for the attachment of prekallikrein is contained in 27 residues from amino acid 569 to 595; this finding was confirmed in experiments in which anti-idiotypic antibodies formed against a monoclonal antibody directed toward the 27 residues fingerprinted this binding site on HK (Vogel et al., 1990). Although, the core segment of 27 amino acids contains the minimum features for binding, the 31-residue peptide shows a fourfold greater affinity for prekallikrein and has a structural sequence (Scarsdale and Harris, 1991) that may be functionally important. In contrast, the binding site for factor XI stretches over a segment of 58 residues (positions 556 to 613) and overlaps the entire prekallikrein-binding site (Tait and Fugikawa, 1987).

In contrast to the light chain of HK which is about 45 to 58 kDa, the light chain of LK is only 4 to 5 kDa and lacks the contact activation and prekallikrein-binding sites. The light chains of LK and T-kininogen are formed by a single domain of unknown function that remains to be elucidated.

v. Cleavage sites in the kininogen molecule. One proposal has been that there are hyperfrangible regions in the kininogen molecule that could be attacked by proteinases. Using limited proteolysis, Müller-Esterl et al. (1985c) and Vogel et al. (1988) identified cleavage fragments of LK: domain 4 (kinin moiety), domain 2, and domain 3 (either still fused or detached) and domains 1 to 5 linked by a single disulphide bridge (Vogel et al., 1988). Therefore, the question still to be answered is which endogenous proteinase specifically cleaves the disulphide loops believed to be exposed on the surface of the molecule and positioned in the form of β turns that form the interdomain junctions.

d. CELL RECEPTORS AND BINDING. After synthesis, HK in platelets is transported to the plasma membrane (Schmaier et al., 1983, 1986b). On exteriorisation it attaches itself to the cell membrane by two sites, one of which is on the heavy chain and the other on the light chain (Schmaier et al., 1987). Binding experiments indicate the presence of specific receptors for HK on the cell membrane of both activated (Greengard and Griffin, 1984) and unstimulated (Gustafson et al., 1986a) platelets, human endothelial cells (van Iwaarden et al., 1988a,b; Schmaier et al., 1988b), and neutrophils (Gustafson et al., 1989). HK and LK bind specifically and reversibly to inhibit the binding of thrombin to platelets. Such binding to platelets and endothelial cells of the human umbilical vein is effected through domain 3 (Jiang and Schmaier, 1991). Another site for binding involves the histidine-rich domain (domain 5) that attaches HK to its receptor on the cell membrane through a Zn^{2+} bridge, thereby providing a mechanism for internalisation of circulating kininogen into endothelial cells. Surface-attached HK molecules interfere with the specific binding of fibrinogen to the C3R receptor located on the cell membrane of activated platelets and neutrophils (Gustafson et al., 1989). The nature of the kininogen receptor on cells and the mechanism involved in the internalisation of the kinin-free moiety of this protein are not known. Binding experiments using the cystatin domains, histidine-rich segment, or the plasma prekallikrein-binding site may help in elucidating the nature of the receptor involved in the binding of HK.

Although binding data indicated the presence of high-affinity sites for HK, positive evidence for HK attachment to the cell membrane of neutrophils has been provided using immunofluorescent labeling and confocal and electron microscopy (fig. 5) (Figueroa et al., 1990a, 1991b). Therefore, domain 6 of HK may serve as an anchor for the docking of plasma prekallikrein on the external surface of the neutrophil. This finding extends

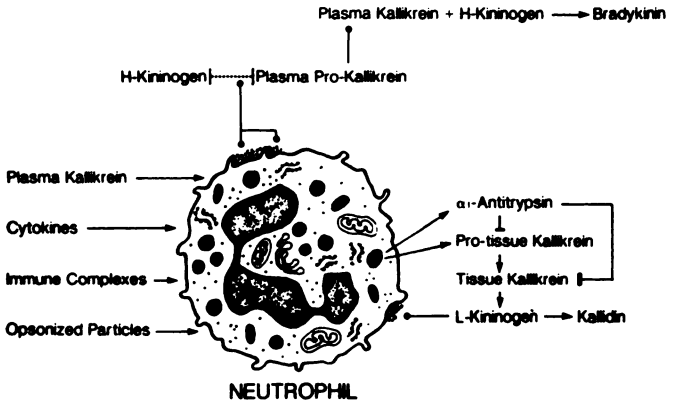

FIG. 5. Diagrammatic representation of the location and attachment sites of kallikreins and kininogens on the external surface of the neutrophil.

considerable physiological importance to the circulating neutrophil. Both cellular functions of kininogen, the formation of kinins, and the ability to initiate clotting may therefore occur on the external surface of its cell membrane.

e. CELL SYNTHESIS, STORAGE, AND SECRETION. Kininogens are synthesised by hepatocytes and as typical secretory proteins undergo posttranslational modifications such as glycosylation prior to secretion into the circulation (Kitamura et al., 1983; Takagaki et al., 1985). With the more recent availability of appropriate antibodies, kininogens have been localised in platelets (Schmaier et al., 1986b) and acinar cells of the rat SMG (Chao et al., 1988b). The localisation of immunoreactive kininogen in CD cells of the human kidney (Figueroa et al., 1988) was confirmed by the identification of LK mRNA in the human kidney cortex and medulla using Northern blot analysis (Iwai et al., 1988). The presence of kininogen in endothelial cells (van Iwaarden et al., 1988a) has been supported by the demonstration of HK synthesis and mRNA expression in human endothelial cells using [^{35}S]methionine (Schmaier et al., 1988b). Recently, immunoreactive LK and HK were identified in human neutrophils (Figueroa et al., 1990a, 1991b).

Liver cells in culture have been used to examine the factors that regulate the synthesis of these proteins. FAO cells, derived from a rat hepatoma cell line, synthesise and secrete kininogens into the culture medium; the basal rate of secretion of the HK and LK measured was similar to that produced normally by hepatocytes. In these cells, as in cultured hepatocytes, HK is stored after synthesis, whereas T-kininogen is secreted immediately upon synthesis. Dexamethasone enhances the synthesis of T-kininogen and HK by rat hepatocytes in primary culture and by FAO cells, in contrast to its action in vivo, where it suppresses only circulating levels of T-kininogen (Baussant et al., 1988).

f. CIRCULATING LEVELS. Plasma levels of total HK, determined in a sensitive immunoblotting assay in which the antibody was specific for the light chain of HK, amounted to 55 μg/ml in three healthy subjects. Cleavage of HK was detected when only 2% of plasma had been activated by dextran sulphate; the method has a detection limit of 2 ng of HK (Reddigari and Kaplan, 1989b).

2. T-kininogens. As stated, T-kininogens are unique to the rat (Okamoto and Greenbaum, 1983; Greenbaum, 1986; Bedi et al., 1983a; Hayashi et al., 1985b). They are a mixture of two homologous proteins. Of these, T-Iα was identical with T-kininogen I (Enjyoji et al., 1988) and T-Iβ was similar to the circulating α_1 major acute phase protein (α_1-cysteine proteinase inhibitor) that was induced by treating rats with turpentine (Urban et al., 1979; Anderson and Heath, 1985). An examination of the functional properties of T-kininogen indicates a loss of the calpain inhibition profile and coagulant function, a resistance to the proteolytic formation of T-kinin, but retention of the ability to inhibit lysosomal cysteine proteases (Moreau et al., 1986, 1989; Gauthier et al., 1988). It is therefore possible that the evolutionary segregation of the T-kininogen gene has occurred for the specific purpose of inhibiting cysteine proteinases.

Oestrogen influences the circulating levels of T-kininogen in the newborn rat; it is very high at birth because of the exposure to maternal oestrogen. Although T-kininogen levels decrease in both sexes and remain low in the male, ontogenic studies indicate a threefold increase in the female at puberty. The higher circulating levels of T-kininogen in the mature female can be dramatically reduced by ovariectomy (Bouhnik et al., 1989). The synthesis of this acute phase protein clearly appears to be modulated by oestradiol (Adam et al., 1989).

In normal rat plasma, the concentration of T-kininogen I (T-I) is much greater than that of T-kininogen II (T-II). It has been suggested that T-II is synthesised but not glycosylated or secreted by the hepatocytes into the circulation. However, after adjuvant treatment, levels of glycosylated T-II increase dramatically. Clearly, both T-I and T-II increase markedly in response to inflammation. The time course of change is interesting. After carrageenin is injected, the paw swelling peaks at 3 to 5 h, but the massive increase in T-kininogen does not occur until the second day (Utsunomiya et al., 1988). A similar temporal dissociation was noticed in carrageenin pleurisy: first, the pleural fluid increased; then, there was migration of leucocytes into the exudate; next, there was an increase in liver microsomal T-kininogen; and finally, almost simultaneously, there was an increase in this acute phase protein in plasma and the pleural exudate. It was concluded that T-kininogen had no proinflammatory properties (Oh-Ishi et al., 1989b). However, the precise cytokine that induces synthesis of this acute phase protein is not known, even though interferon has been shown to stimulate hepatic synthesis of T-kininogen (Ito et al., 1988). Treatment of experimentally created inflammation with dexamethasone either inhibits the formation of cytokines, possibly IL-8 (Oh-Ishi et al.,

1989b), or affects the induction of the T-kininogen gene, thereby affecting both synthesis and circulating levels (Howard et al., 1990). The thyroid status also affects acute phase responses; a reduction in circulating thyroid hormones causes an increase in T-kininogen during inflammation and vice versa (Bouhnik et al., 1988). Furthermore, whereas the normal hepatic synthesis of T-kininogens is enhanced by inflammation, that of the other two kininogenase substrates (HK and LK) is unaffected.

Unlike T-kininogen (65 to 77%), only small amounts of the HK (12 to 21%) and LK (9 to 14%) have been isolated from the plasma of WKY rats (Suzuki et al., 1987a). In contrast, although in Brown-Norway rats (from the BN/May Pfd f strain, later called Katholiek) the gene codes normally for T-kininogen (Damas et al., 1986; Oh-Ishi et al., 1989a), they show a complete congenital deficiency of HK and LK and a moderate absence of plasma prekallikrein (Fitzgerald trait) (Damas and Adam, 1979, 1980; Reis et al., 1985). The cellular response produced with urate, uric acid crystals, kaolin, or carrageenan in the paw of these animals was small in comparison with that observed in normal rats (Damas and Remacle-Volon, 1982; Damas et al., 1984; Oh-Ishi et al., 1986). This observation confirms the importance of circulating HK-prekallikrein complexes in the contact activation cascade that occurs during inflammation. Experiments designed to compare the rate of activation of plasma prekallikrein and HF by kaolin-cephalin, kaolin alone, and ellagic acid suggested that HK was a less important cofactor in rat than in human plasma for activation of the clotting cascade (Damas and Bourdon, 1990). Because abundant and functionally active HK can be detected in rat hepatic microsomes, but little or none in the cytosol, the abnormality may be a posttranscriptional one (Hayashi et al., 1989) with the hepatocytes unable to secrete the protein. The defect seems to reside in the terminal domain that complexes plasma prekallikrein; the uncomplexed form may be unstable and could account for the reduced concentration of plasma prekallikrein in the circulation of the Brown/Norway rats.

3. Avian (ornitho) kininogens. Unlike in mammals, only a single kininogen consisting of a heavy chain, a kinin, and a light chain has been identified in avian plasma. Because of the absence of factor XII in avian plasma, birds do not appear to have an intrinsic blood clotting pathway that could be initiated by contact activation. It is therefore curious that chicken kininogen has similar functional components to those delineated for mammalian HK. One clear difference is in the amino acid sequence of the kinin molecule. Ornitho-(avian)-kinin has Thr^6 and Leu^8 instead of Ser^6 and Phe^8 in mammalian bradykinin (Kimura et al., 1987). Further studies may provide interesting data concerning the evolutionary ancestry of nonmammalian kininogen molecules.

C. Kininases

The turnover of kinins depends on both the rate of formation and the rate of destruction. After kinins are formed, they are rapidly destroyed by the enzymic action of peptidases (fig. 6). This family of enzymes is generically called kininases (Erdös, 1989, 1990). So far, the KI family comprises KI-CPN and KI-CPM and the KII-ACE and KII-NEP. Additional kinin-hydrolysing enzymes are prolidase (aminopeptidase P) and two endopeptidases (kininase A and B). Sequences for the regulatory and catalytic subunits for most of these enzymes have been ascertained from cDNA clones. A series of selective enzyme inhibitors (D-L-mercaptoethanol-3-guanidino-ethylthiopropanoic acid, ethylenediaminetetraacetic acid, *o*-phenanthroline for KI-CP, captopril for KII-ACE, phosphoramidon for KII-NEP, and amastatin, bestatin, and puromycin for aminopeptidases) are available for regulating kininase activity, particularly for in vitro studies.

The cellular actions of kinins could be regulated acutely. The relative importance of each of the peptidases in controlling kinin levels varies with species, type of biological fluid, and tissue site of formation of the peptide. In humans, circulating levels are primarily regulated by KI-CPN but on endothelial surfaces, particularly in the pulmonary vascular bed, by KII-ACE. In contrast, the most potent kinin-degrading enzyme in rat plasma is KII-ACE with little contribution from other peptidases (Ishida et al., 1989).

1. Kininase I carboxypeptidases. KI is a family of two carboxypeptidases, KI-CPN and KI-CPM.

a. KININASE I-CARBOXYPEPTIDASE N. KI-CPN (EC 3.4.17.3) was originally discovered in Cohn fraction IV of human plasma (Erdös and Sloane, 1962) and named CPN to distinguish it from pancreatic carboxypeptidases, A and B (Erdös, 1979). It is an arginine carboxypeptidase, optimally active at pH 7.4 and activated by $CoCl_2$. The K_m values for bradykinin and the synthetic substrate, L-hippuryl-L-lysine are 0.43 and 1.25 mM, respectively. KI-CPN acts on the COOH-terminal end of the bradykinin molecule and by removing the Arg^9 residue produces a BK1 receptor agonist. This carboxypeptidase, synthesised by the liver, is secreted into the circulation where

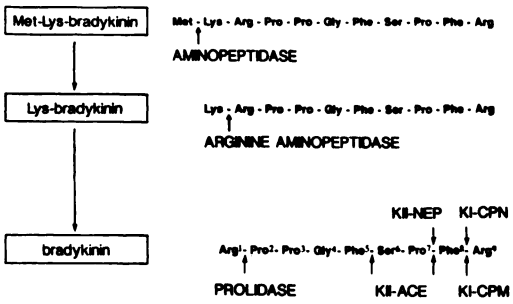

FIG. 6. Cleavage sites for the conversion of Met-Lys-bradykinin and Lys-bradykinin (kallidin) to bradykinin. Hydrolysis of the bradykinin molecule by kininases.

it probably accounts for about 90% of the bradykinin-destroying activity in human plasma (Zacest et al., 1974) and becomes associated with the vascular endothelium (Palmieri et al., 1986). The primary importance of this enzyme in cleaving kinins in the circulation varies with species. In addition to kinins, CPN hydrolyses the COOH-terminal basic amino acid of several biologically active peptides, namely, enkephalins, anaphylotoxins (C3a, C5a), and fibrinopeptides.

The plasma enzyme is a tetrameric complex of 280 to 320 kDa that can be dissociated into two large, identical subunits of 83 to 98 kDa and two small, identical subunits of 42 to 55 kDa (Plummer and Hurwitz, 1978; Skidgel et al., 1984; Gebhard et al., 1989). Catalytic activity resides only in the small, unglycosylated subunits. The large glycosylated subunits are supposed to prevent proteolytic degradation of the enzymatically active small subunits and/or their rapid removal from blood by glomerular filtration (Oshima et al., 1974; Levin et al., 1982). About a quarter of the molecular weight of the larger subunit is attributable to N- and O-linked carbohydrates (Skidgel and Weerasinghe, 1987; Tan et al., 1990). The large subunit, when cloned using a human liver cDNA library, revealed the presence of 12 leucine-rich tandem repeats of 24 residues each (Tan et al., 1990). The amino acid sequence of the active subunit has been determined from mirror-image cDNA clones derived from a human liver library (Gebhard et al., 1989). The COOH-terminal section of the active subunit has paired basic amino acid residues that could be cleaved by kallikrein or plasmin. In fact, the small subunit undergoes rapid hydrolysis from 55 to 48 kDa (the fragments still retaining catalytic activity), and subsequently to 27 and 21 kDa polypeptides, which when separated become inactive (Skidgel and Weerasinghe, 1987). The primary stucture of the active subunit of human KI-CPN is clearly related to bovine carboxypeptidase E with 49% of the amino acid residues identical and >40% of the divergent residues regarded as conservative changes. A more distant relationship exists with pancreatic digestive enzymes, carboxypeptidase A and B (Skidgel et al., 1988; Gebhard et al., 1989).

Occasionally, protamine, administered to reverse the anticoagulant effect of heparin, causes systemic hypotension and bronchoconstriction. The mechanism involved is considered to be by inhibition of KI-CPN, resulting in an increase in circulating kinins (Tan et al., 1989). Patients with familial deficiency of KI-CPN show a diminished activity in plasma that is accompanied by high circulating levels of kinins and a clinical syndrome characterised by erythema, dizziness, and hypotension (Streeten et al., 1972). During pregnancy, KI-CPN increases significantly from 14 weeks to reach the highest values at term but when complicated by severe pre-eclampsia attains even greater circulating levels (Ito et al., 1989, 1990). This effect appears to be specific for KI-CPN because values for KII-ACE are unaffected.

b. KININASE I-CARBOXYPEPTIDASE M. KI-CPM cleaves the COOH-terminal arginine of bradykinin and Lys-bradykinin, resulting also in the formation of des[Arg^9]-bradykinin and des[Arg^9]Lys-bradykinin. The optimum pH for degrading the two kinins is in the neutral range. KI-CPM has been purified from human urine and human placental microvilli and cloned from a human placental cDNA library (Skidgel et al., 1984, 1989; Tan et al., 1989). KI-CPM is a 62-kDa metallopeptidase activated by cobalt (more pronounced at pH 5.5) which replaces zinc as cofactor and is inhibited by guanidino-ethylmercaptosuccinic acid (Deddish et al., 1989). Structurally, KI-CPM is a single-chain glycoprotein with 426 amino acids and six asparagine-linked glycosylation sites. The molecule shows only 41% identity with the active subunit of KI-CPN.

KI-CPM is a cell membrane enzyme that has been located in human kidney, lung tissue and fibroblasts, pulmonary artery endothelial cells, and the placenta (Johnson et al., 1984). Because of its location on cell surfaces and its specificity restricted to arginine or lysine bonds, KI-CPM may play a role in processing prohormones. More important, because both CPN and CPM are not capable of degrading the kinin molecule further, increased levels of the desArg^9 metabolite may be observed in situations when kinin levels are increased. Fortunately, the metabolite des[Arg^9]-bradykinin is inert on BK2 receptors but, of course, active on BK1 receptors. A change in balance between the two agonists may shift the emphasis and make prominent a particular cellular action of kinins.

The release of arginine by KI-CPN or KI-CPM either close to or within endothelial cells or in synaptic clefts could provide the primary substrate for the formation of nitric oxide (EDRF) by nitric oxide synthase (Moncada et al., 1988; Bredt et al., 1990; Erdös, 1990). A functional link of this type would be of considerable physiological significance, particularly because several studies have suggested that nitric oxide may be a transmitter or neuromodulator at synaptic junctions (Bult et al., 1990; Schachter et al., 1991).

2. Kininase II-peptidases. The two peptidylpeptidases considered to belong to this family are KII-ACE and KII-NEP, both of which hydrolyse the Pro-Phe bond at the COOH terminus of the kinin molecule. The immunogold localisation of KII-ACE and KII-NEP on the brush border membranes of the proximal tubular epithelium (Schulz et al., 1988) suggests that by cleaving hormones and bioactive peptides, including kinins and ANF, these enzymes play a primary role in regulating renal function.

a. KININASE II-ANGIOTENSIN I-CONVERTING ENZYME (EC 3.4.15.1). Circulating bradykinin is inactivated mainly during its passage through the lung (Kroneberg and

Stoepel, 1963; Ferreira and Vane, 1967; Vane, 1969). At the same time, the enzyme converts angiotensin I (a decapeptide) to angiotensin II (an octapeptide) by the removal of the COOH-terminal His-Leu in the pulmonary circulation (Helmer, 1957; Biron and Huggins, 1968; Yang et al., 1970a). Although ACE was first described in the mid-1950s (Skeggs et al., 1956), it was not until the 1970s that the two enzymes, ACE and KII, were considered to be identical (Yang et al., 1970a,b; Igic et al., 1972).

KII-ACE is a single-chain, transmembrane enzyme bound to cell membranes with a COOH-terminal anchor sequence. It has a molecular mass that ranges from 195 to 200 kDa. The cDNA of the human enzyme has been cloned and the amino acid pattern determined. It hydrolyses two separate bonds on the COOH-terminal end of the bradykinin molecule. First, it removes the dipeptide Phe^{8}-Arg^{9} and next splits the Ser^{6}-Pro^{7} bond (Zacest et al., 1974; Erdös, 1979). Several other peptide substrates are hydrolysed by KII-ACE, namely, enkephalins, neurotensin, substance P, and luteinising hormone-releasing hormone (Skidgel and Erdös, 1985, 1987; Skidgel et al., 1987).

KII-ACE occurs as a soluble enzyme in biological fluids. The cellular location of KII-ACE in tissues where sodium exchange occurs could be of physiological relevance. By regulating levels of angiotensin, ANF, and possibly kinins, the enzyme could modulate epithelial cell transport of sodium. KII-ACE is primarily found in the vascular endothelium (Caldwell et al., 1976; Ryan et al., 1976), neuroepithelial cells (subfornical organ, pallido-nigral dendrites), brush border of the choroid plexus (epithelial cells that face the CSF), and the microvilli of the placenta, small intestine, and renal tubules (Hall et al., 1976; Erdös and Skidgel, 1986; Schulz et al., 1988). The enzyme occurs in abundance at the brush border of the inner cortical proximal renal tubules (Ward et al., 1976; Ikemoto et al., 1987; Schulz et al., 1988). High concentrations of KII-ACE have been demonstrated immunocytochemically on the luminal surface of the pulmonary blood vessels (Ryan et al., 1975, 1976). In humans, on a comparative basis, there is a sixfold greater amount of the enzyme in the kidney than in the lung. Although in the lung, kidney, and brain, KII-ACE is membrane bound, in the testis it is probably secreted by the luminal epithelium and may serve an exocrine function in the seminal plasma (Erdös et al., 1985).

Inhibitors of KII-ACE were first developed when the mode of action of bradykinin-potentiating peptides, derived from snake venoms, fibrinopeptides, and tryptic digests of plasma proteins, was elucidated. The rat heart seems to contain a sulphydryl protein that competitively inhibits KII-ACE (Ikemoto at el., 1989). Drug design based on the structure of the bradykinin-potentiating peptide molecules provided the first generation of KII-ACE inhibitors for clinical use in the treatment of hypertension. Recent reports indicate that the efficacy of KII-ACE inhibitors, as judged by lowering the blood pressure, does not always correlate with the level of inhibition of the circulating enzyme but rather with reduced activity in the brain, kidney, and vascular smooth muscle (Unger et al., 1987). A contradictory finding in the rat is the increase in the activity of inner cortical KII-ACE following the administration of captopril for 1 week (Song et al., 1988; Ikemoto et al., 1990). In experiments designed to elucidate their clinical importance, using isolated ischaemic guinea pig and rat hearts, beneficial effects were observed with ACE inhibitors on reperfusion arrhythmias, cardiac function, and metabolism. These functional improvements were believed to be due to a reduction in local angiotensin II levels and to prevention of kinin degradation (Schölkens et al., 1988; Linz et al., 1990). The hypotensive effect of KII-ACE inhibitors (captopril, ramipril) is believed to be partially mediated by increases in circulating levels of kinins, particularly in those patients with a low renin output; the increase in plasma kinin levels is associated with increases in the excretion of sodium and volume of urine following chronic usage (Iimura et al., 1986; Shimamoto et al., 1990). However, pretreatment with indomethacin inhibits the natriuretic effect and partially attenuates the increase in renal blood flow (Miura et al., 1985). Clearly, if there is any significant regulation of sodium excretion by kinins in patients, control is probably exercised indirectly through the release of prostanoids.

b. KININASE II-NEUTRAL ENDOPEPTIDASE 24.11 (EC 3.4.24.11; ENKEPHALINASE). The biosynthesis, glycosylation, and membrane localisation of the KII-NEP in cultured human fibroblasts indicated that the enzyme was first transcribed in a pro form. The enzyme is synthesised as an 88-kDa protein that increases in size to 94 kDa when four to five N-linked oligosaccharides are added. After synthesis, the enzyme migrates to the plasma membrane where it becomes translocated to be anchored on the outer surface of the fibroblast from which it may be secreted into body fluids (Lorkowski et al., 1987). KII-NEP is a 742-amino acid metallopeptidase that was cloned from a human placental cDNA library (Malfroy et al., 1988). The amino acid sequence of the enzyme shows homology between the rat, rabbit, and human forms (Erdös and Skidgel, 1989). It preferentially cleaves bonds on the amino side of hydrophobic amino acid residues. Like KII-ACE, KII-NEP inactivates kinins by removing the COOH-terminal Phe^{8}-Arg^{9} dipeptide (Gafford et al., 1983). Several of the biologically important peptides cleaved by KII-NEP, described in sequence of affinity for the enzyme, are substance P, kinins, enkephalins, ANF, and neurotensin (Gafford et al., 1983; Pozsgay et al., 1985; Skidgel and Erdös, 1986; Ura et al., 1987; Erdös and Skidgel, 1989).

KII-NEP is present in high concentrations in the

brush border of proximal tubules of the kidney, microvilli of the intestine, and fibroblasts of lung and skin (Kerr and Kenny, 1974; Danielsen et al., 1980; Johnson et al., 1985; Schulz et al., 1988). Further sites of location of KII-NEP are in the brain, where it is considered to be identical with enkephalinase A (Schwartz et al., 1981), placenta (Johnson et al., 1984), and pituitary (Almenoff et al., 1981). High concentrations of KII-NEP have been reported in the human male genital tract (Erdös et al., 1985). In the epididymis and prostate (luminal epithelial cells), KII-NEP activity is mainly associated with the high-speed centrifugation membrane fractions.

A finding of considerable significance is the citing of KII-NEP on the cell membrane of human neutrophils (Connelly et al., 1985). KII-NEP is identical with the common acute lymphoblastic leukaemia antigen on the plasma membrane of leukaemic cells (Shipp et al., 1989). One important action of KII-NEP is the hydrolysis of N-fMLP, a peptide that stimulates chemotaxis. Activation of neutrophil or antigen-antibody reactions triggers the internalisation of KII-NEP, with a rapid loss of its enzymic activity. Such an endocytotic effect can be induced by phorbol esters and diacylglycerol (Erdös et al., 1989). The reduction in KII-NEP activity could have a marked effect on the migration of neutrophils to sites of inflammation. Another very important regulatory action would be to control the biological activity of kinins formed on the external surface of the neutrophil membrane on which LK, HK (Figueroa et al., 1991b), and plasma prekallikrein (Henderson et al., 1992) have been localised.

Although KII-NEP is generally embedded in cell membranes, it seems to occur in biological fluids also. Circulatory levels of the enzyme increase in sarcoidosis and respiratory distress syndrome. When the kinin-degrading activity of rat urine was tested against KI-CPN, KII-ACE, and KII-NEP, the major kinin-destroying enzyme turned out to be KII-NEP. Infusion of the KII-NEP inhibitor, phosphoramidon, in the rat increases the urinary excretion of kinins (Ura et al., 1987). In normal subjects, plasma levels of KII-NEP are low, whereas circulating levels of KII-NEP increase 50- to 60-fold in adult patients with respiratory distress syndrome (Johnson et al., 1985). Elevated levels also have been observed in the serum of patients with pulmonary oedema and chronic obstructive lung disease (Johnson et al., 1985). The source of the KII-NEP is not known but is believed to come from subendothelial tissues or damaged neutrophils. Of the various biological fluids, only in seminal plasma does the enzyme appear to be membrane bound (Erdös et al., 1985).

3. Additional kinin peptidases. When studying the half-life and metabolised fragments of bradykinin in the pulmonary circulation, Ryan et al. (1968) identified a prolidase that cleaved the Arg^1-Pro^2 bond. So far, the prolidase (aminopeptidase P) has been described in erythrocytes, kidney (Erdös et al., 1963; Erdös and Yang, 1966), and lung tissue (Orawski et al., 1987). The activity and importance of this enzyme may need to be reassessed following the discovery of Hyp^3-kinins. The question of whether aminopeptidase P will split the Arg^1-Pro^2 bond of the NH_2-terminal Arg^1-Pro^2-Hyp^3 sequence of kinins remains unanswered.

A proline-specific endopeptidase, called postproline-cleaving enzyme (EC 3.4.21.26), cleaves peptides on the COOH side of internal proline residues and has been identified in the vasculature, kidney, and brain (kininase B, described by Camargo and colleagues, 1973). Postproline-cleaving enzyme has been purified from porcine kidney, and the kinetics for hydrolysis of its preferred synthetic substrate (Z-Gly-Pro-methylcoumarin) and the K_m (7.5 μM) for bradykinin were such to be consistent with an in vivo role for the enzyme (Ward et al., 1987). It is a cytosolic enzyme, and therefore, its in vivo function may reside in degrading emiocytosed kinins.

Two additional endopeptidases designated as kininase A and B have been purified from rabbit brain (Camargo et al., 1973; Oliveira et al., 1976). Kininase A, which has a molecular mass of 71 kDa and has recently been characterised from rat brain synaptic membranes as metalloendopeptidase 24.15 (Orawski and Simmons, 1989), cleaves the Phe^5-Ser^6 bond of bradykinin. A similar endopeptidase which splits bradykinin at the Phe^5-Ser^6 has been purified from the rat liver (Kouyoumdjian et al., 1984). On the other hand, kininase B, a peptidyl dipeptidase also identified in rat brain synaptic membranes, has a molecular mass of 68 kDa and hydrolyses the Pro^7-Phe^8 bond but, unlike ACE, does not split the peptide further, nor does it convert angiotensin I to angiotensin II. Synaptic membranes prepared from the rat brain contain a dipeptidase that splits the COOH-terminal Phe^8-Arg^9 bond (Orawski and Simmons, 1989). Should kinins perform a neuromodulatory role in the nervous system, then these synaptic peptidases may exert a regulatory function.

D. Kinins

1. Kallidin and bradykinin. a. CHEMICAL STRUCTURE. Kinins are potent bioactive peptides formed by the enzymic action of kininogenases on kininogen (Werle and Berek, 1948; Rocha e Silva et al., 1949; Schachter, 1956; Bhoola and Schachter, 1960; Bhoola, 1961; Bhoola et al., 1962). The first amino acid sequence proposed for bradykinin missed a single proline residue in position 7 (Elliot et al., 1960). The correct sequence was established by chemical synthesis (Boissonnas et al., 1960). It is generally accepted that kallidin (Werle et al., 1961) is released from LK by tissue kallikrein and bradykinin from HK by the action of plasma kallikrein (Fiedler, 1979). Some conversion of kallidin (Lys-bradykinin) to bradykinin may occur through removal of the NH_2-terminal lysine by aminopeptidases (fig. 6). Another

endogenous analogue, Met-Lys-bradykinin, is formed by pepsin and by uropepsin (Guimaraes et al., 1976; Hial et al., 1976).

Other mammalian kinins with the structure sequence of Ile-Ser-bradykinin (T-kinin) and Met-Ile-Ser-bradykinin (Met-T-kinin) have been described but so far only in the plasma and pouch fluid of rats with carrageenan-induced inflammation (Okamoto and Greenbaum, 1983; Sakamoto et al., 1987). More recently, Hyp^3-kinin has been reported by several groups (Kato et al., 1988; Maier et al., 1988). The fact that Hyp^3-kinins are formed by kallikrein from intact human kininogen suggests that the precursor contains the same hydroxylated residue in the kinin moiety of kininogen, probably a posttranslational modification (Maier et al., 1988; Sasaguri et al., 1988a,b). Indeed, in vitro hydroxylation of bradykinin and kallidin by a hydroxyl-4-prolinase was reported about 20 years ago (Rhoads and Udenfriend, 1969). The hydroxyl-4-prolinase selectively hydroxylated the Pro^3 residue of both the kinin molecules and any other protein containing the sequence Pro-Pro-Gly or Ala-Pro-Gly (McGee et al., 1971). So far, no particular function can be attributed to the hydroxylated proline, but one may reside in the reason why hydroxyproline is a constituent of connective and cell supportive proteins.

Jaques and Schachter (1954) were the first to describe, and Schachter and Thain (1954) characterised further, a kinin in the venom sacs of wasps. Subsequently, a kinin was discovered in the venom sacs of hornets (Bhoola et al., 1960). The pharmacological activity and the amino acid sequences of kinins, purified from the venom sacs of hymenotera of many genera, have been determined in the last decade (Nakajima et al., 1985; Piek and Spanjer, 1986). The *Vespinae* and *Polistinae* venoms contain (*a*) kinins that are hypotensive, produce pain and local swelling, contract smooth muscle preparations, and block synaptic transmission (Piek et al., 1987), (*b*) mastoparans that degranulate mast cells and release histamine, and (*c*) chemotactic peptides that attract human leucocytes (Toki et al., 1988). Structure-activity determinations on the venom kinins may be particularly valuable in the selective development of kinin agonists. Although Thr^6-bradykinin is less active than bradykinin on most pharmacological preparations, it is 10 times more active in relaxing the rat duodenum (Yasuhara et al., 1987). Of interest is the presence of Hyp^3-bradykinin in the venom of *Vespa mandarini* (Kishimura et al., 1976). Tracing the biological ancestry of hydroxylated kinins, should they exist in insect venoms, could form a fascinating study. Mammalian kinins possess a highly conserved structure that shows a remarkable degree of homology with insect, amphibian skin, reptile, and avian (ornitho) kinins (table 2).

b. HALF-LIFE AND LEVELS IN BIOLOGICAL FLUIDS. The effects of kinins in biological fluids are often very short-lived because they are rapidly destroyed by kininases. In blood, the half-life of bradykinin and kallidin is estimated to be <30 s (McCarthy et al., 1965; Ferreira and Vane, 1967). Similarly, the biological half-life of intracerebrally administered bradykinin in the conscious rat is about 27 s (Kariya et al., 1982b).

The concentration of bradykinin in tissue homogenates and biological fluids has been determined mainly by RIA. Early measurements of the peptide in the circulation revealed a large disparity with more recently reported values (Talamo et al., 1969; Mashford and Zacest, 1967). Subsequent studies give levels of kinins in human plasma of <50 pg/ml (Shimamoto et al., 1982; Scicli et al., 1982). The main reason for a lack of conformity in circulating values has been inadequate inhibition of kininogenases and or kininases and the type of anti-bradykinin antiserum used. More recently, when syringes containing 0.8 N HCl were used to withdraw blood samples, a mean value of 3.8 pg/ml was recorded (Shimamoto et al., 1988). Transferring the blood to a tube containing 0.8 N HCl increased the kinin levels sixfold to 24.4 pg/ml.

Urinary excretion of kinins amounts to 24.6 ± 1.0 μg/day, in samples collected in flasks containing HCl and pepstatin (Shimamoto et al., 1988). Kinin levels have also been reported in other body fluids such as nasal fluids (Shimamoto et al., 1988), bronchoalveolar lavage fluid (Christiansen et al., 1987), human (Scicli et al., 1984) and rat CSF (Hermann et al., 1986), and synovial fluid from patients with rheumatoid and osteoarthritis (E. Fink, personal communication).

So far, antisera to bradykinin have lacked precise specificity, because they will cross-react with larger and smaller analogues of the peptide. One solution may be to raise antibodies to epitopes in the peptide with different recognition sites for the parent molecule. One such attempt was described recently. Polyclonal antibodies were raised to Cys^6-bradykinin linked to bovine serum albumin through N-maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitro-4-benzsulphonic acid. The antibody reacted with bradykinin but not to kallidin; however, cross-reactivity with $desArg^9$-bradykinin was not excluded (Hilgenfeldt et al., 1990). Monoclonals could provide further selectivity. This view was confirmed by the development of two monoclonals, one specific for the COOH terminus that recognised bradykinin with a K_d of 0.67 ± 0.17 nM and a second which, in addition, recognised 1–7 bradykinin (Phillips and Webb, 1989).

c. CELLULAR ACTIONS. The two mammalian kinins, kallidin (Lys-bradykinin) and bradykinin, influence the cardinal features of inflammation as well as a number of cellular functions, including blood pressure and local blood flow, electrolyte and glucose transport, and cell proliferation. The cellular actions of kinins are modified by their ability to stimulate the release of many second generation mediators, e.g., platelet-activating factor, leukotrienes, prostaglandins, substance P (neurogenic in-

TABLE 2
Amino acid sequences of the kinin family of peptides

	1	2	3	4	5	6	7	8	9
Bradykinin	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg
Lys-bradykinin	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe-Arg
Met-Lys-bradykinin	Met	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro-Phe-Arg
T-kinin	Ile	Ser	Arg	Pro	Pro	Gly	Phe	Ser	Pro-Phe-Arg
Met-T-kinin	Met	Ile	Ser	Arg	Pro	Pro	Gly	Phe	Ser-Pro-Phe-Arg
T-kinin-Leu	Ile	Ser	Arg	Pro	Pro	Gly	Phe	Ser	Pro-Phe-Arg-Leu
Birds (ornitho) kinin	Arg	Pro	Pro	Gly	Phe	Thr	Pro	Leu	Arg
Insects									
Vespa-kinin X	Ala	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe-Arg-Ile-Val
Vespa-kinin M	Gly	Arg	Pro	Hyp	Gly	Phe	Ser	Pro	Phe-Arg-Ile-Asp

flammation), acetylcholine, and noradrenaline (sympathetic nerves). Kinins also stimulate the secretion of renin from the kidney (Beierwaltes et al., 1985a), release vasopressin from the neurohypophysis (Baertschi et al., 1981), and secretion of catecholamines from the adrenal medulla (Staszewska-Barczak and Vane, 1967). Therefore, the homeostatic regulation of regional blood flow by kinins could be modulated in a manner that would be difficult to evaluate, except with selective antagonists. In addition, it would be necessary to establish the precise role of the secondary autacoids in the control of systemic or local blood flow.

i. Smooth muscle of the intestine and uterus. Kinins contract extravascular, isolated smooth muscle preparations but relax the rat duodenum (Werle and Berek, 1948; Rocha e Silva et al., 1949; Schachter, 1956, 1960; Elliot et al., 1960; Bhoola, 1961). Apparently, several smooth muscle preparations have been reported to respond directly to tissue kallikrein, in the absence of added kininogen (Frey et al., 1950). Beraldo et al. (1966) made a similar observation on the isolated rat uterus and suggested that the contractions were due to a direct combination of tissue kallikrein with receptors on the muscle. The mechanism of this action is now clarified and the contraction is due to the release of kinin from kininogen molecules present within the uterine muscle (Figueiredo et al., 1990).

ii. Smooth muscle of the airways. Isolated airway smooth muscle of most species, including humans and guinea pig, shows a biphasic response to kinin peptides. At concentrations of 10^{-8} M, the relaxations are more prominent (Bhoola et al., 1989), whereas at higher concentrations only contractions are observed (Collier et al., 1960; Bhoola et al., 1962). Removal of the epithelium abolishes the relaxation phase, believed to be mediated by PGE_2. However, kinins also appear to release PGE_2 and the prostacyclin metabolite 6-keto-PGF_1 in a dose-dependent manner from cultured guinea pig smooth muscle cells. At high concentrations both prostaglandin-dependent and -independent contractions are observed. Intravenous and inhaled bradykinin causes bronchoconstriction in asthmatics but not in normal subjects. Bradykinin is a potent stimulant of bronchial C-fibres, and some of its actions are mediated through the release of sensory neuropeptides from capsaicin-sensitive nerves (Kaufman et al., 1980). Multiple mechanisms are probably involved in the in vivo bronchoconstrictor action of bradykinin, namely (*a*) release of prostanoids, thromboxanes, substance P, and neuropeptides; (*b*) C-fibre-evoked axon reflexes, and (*c*) direct stimulation.

iii. Vascular endothelium and smooth muscle. Kinin-induced actions in these cells depend on a number of factors: (*a*) presence of endothelium-relaxing factors, EDRF and PGE_2 (Furchgott, 1981; Toda and Okamura, 1989); (*b*) type and potency of kininases present on endothelial cells; and (*c*) type of kinin receptor. The relaxant effect of bradykinin and des[Arg^9]-bradykinin on the isolated human basilar artery and the rabbit mesenteric artery is dependent on the formation of a relaxing prostanoid (Whalley et al., 1987a; Nwator et al., 1989). In contrast, the dog renal artery is relaxed by both endothelium (EDRF, nitric oxide) and nonendothelium-dependent (prostaglandins) mediators (Rhaleb et al., 1989). The vasodilator properties of kinins are being increasingly investigated because of their clinical relevance in cardiac, cerebral, or renal ischaemia. The occurrence of increased amounts of bradykinin in cardiac muscle after acute myocardial infarction is considered to produce beneficial effects by reducing or limiting the size of the myocardial infarct. Coronary infusion of bradykinin causes improved cardiac function and metabolism, reduces reperfusion injury, and prevents arrhythmias in isolated ischaemic hearts (Schölkens et al., 1987, 1988; Linz et al., 1986, 1990).

iv. Modulation of glucose metabolism. A hypoglycaemic effect of kallikrein was first reported by Frey et al. (1932). Administration of kallikrein to diabetic patients or dogs resulted in a decrease in the concentration of circulating glucose. A similar finding in diabetics was reported by Wicklmayr and Dietze (1977). The notion that kinins may represent a protective mechanism in ischaemia by improving perfusion, oxygenation, and transport of glucose to the affected tissue was proposed by Needleman and colleagues (1975). Evidence for a metabolic role has been provided by the studies of Dietze and colleagues (Dietze et al., 1978; Dietze, 1982). Infusion

of bradykinin in an experimental model (in which blood flow is kept constant) improved impaired insulin function in patients with the postoperative stress syndrome, in which insulin resistance is due to a postreceptor defect in skeletal muscle. The infused kinin almost doubled muscle glucose uptake, without a change in the formation of lactate (Jauch et al., 1986, 1988). In non-insulin-dependent diabetics, unlike control subjects, captopril caused a significant increase in forearm glucose uptake and total body glucose utilisation. In hypertensive patients, monotherapy with enalapril produces a clear decrease in the fasting blood sugar concentration (Helgeland et al., 1986). Therefore, kinins may modulate glucose utilisation in patients with the postoperative stress syndrome in several ways: enhancing insulin-stimulated peripheral glucose uptake, increasing peripheral glucose utilisation, decreasing the elevated hepatic glucose formation, and reducing the increased gluconeogenesis (Jauch et al., 1989). Some doubt was cast on whether kinins directly increase glucose uptake and utilisation by the study of Shimojo et al. (1987), who failed to demonstrate the ability of bradykinin to stimulate glucose metabolism in the isolated soleus muscle of the rat. An opposite conclusion, confirming the in vivo experiments of Dietz and colleagues, was evident from the study by Symington et al. (1988) of adipocytes. Bradykinin increased glucose oxidation in rat adipocytes only in the presence of insulin. In adipocytes from streptozotocin diabetic rats, bradykinin increased glucose oxidation in a dose-dependent manner even in the absence of insulin. Characterisation of the receptor type and molecular mechanism involved in this action of bradykinin on glucose uptake and oxidation should establish whether the kinins have an important regulatory function in cell metabolism.

v. Role in inflammation. Of all the known cell mediators, kinins are considered to play a primary role in inflammation. All of the cardinal signs and symptoms of inflammation are observed when kinins are injected into the human skin. At first, there is pain due to direct stimulation of sensory C-fibre terminals and then the release of substance P which adds to the neurogenic inflammation. Next, there is an axon reflex-mediated flare caused by local vasodilation and oedema created by the increase in vascular permeability and extravasation of proteins and fluid. Subsequent kinin-stimulated release of cytokines from monocytes attracts leucocytes to the injection site. Of particular importance is the ability of kinins to release cytokines (IL-1, TNF)(Tiffany and Burch, 1989) and many second generation mediators of which prostaglandins and leukotrienes formed through activation of phospholipase A_2 are formed most frequently.

Both bradykinin and kallidin have similar pharmacological potency in causing vasodilation and increasing vascular permeability that results in local oedema (Bhoola and Schachter, 1960; Bhoola et al., 1960; Majno et al., 1969; Reis et al., 1971; Carter et al., 1974). At first, there is separation of endothelial cells, resulting in extravasation of fluid from the capillaries. This effect is enhanced by the simultaneous contraction of veins by the kinin peptides. More recently, the early experiments of Bhoola et al. (1960) on the capillary permeability effect of plasma kallikrein in the guinea pig skin have been shown to be due to the generation of kinins (Imamura et al., 1984). Locally injected kinins increase vascular permeability and paw volume in the guinea pig and rabbit pretreated with KII inhibitor (captopril), with a decreasing order of potency ranging from cyclical kinins, bradykinin, kallidin, Met-Lys-bradykinin, and des[Arg^9]-bradykinin (Whalley et al., 1987b). Enhanced vascular permeability arising from formation of kinins in cancerous tissue may have profound consequences for the metastatic spread of tumours. Kinin (bradykinin and Hyp^3-bradykinin) levels in ascitic tumour fluid from patients with ovarian, gastric, and hepatic carcinomas are sufficiently increased to cause enhanced vascular permeability (Matsumara et al., 1988, 1990).

Kinins are the most potent pain-producing substances when applied to a blister base (Bhoola, 1961; Keele and Armstrong, 1964; Whalley et al., 1987b) or when injected intradermally (Ferreira, 1972). When bradykinin is applied to a cantharidin blister base created on human skin, it causes a burning sensation (Armstrong et al., 1957). The painful sensation has a latency of 30 to 120 s and persists for at least 75 to 150 s, depending on the concentration used (Bhoola, 1961). When compared to 5-HT, bradykinin is at least 10 times more potent in causing pain on the blister base (Whalley et al., 1987b). The algesic action of bradykinin is potentiated by thromboxanes, protaglandins (Vane, 1978), and 5-HT. Assessment of the algesic action of kinins has been difficult in animals. One test described recently is the abdominal constriction assay (stretch test) in mice, which is considered to provide quantitative assessment of the algesic potency of bradykinin. Intraperitoneal injection of kaolin in mice causes a clear and reproducible stretching reaction (Fujiyoshi et al., 1989) that can be used to study the antialgesic potency of kinin antagonists.

Kinins evoke pain by stimulating nociceptive afferent nerves (Juan and Lembeck 1974; Steranka et al., 1988). Formalin injected subcutaneously into the rat paw causes prolonged activation of neurones receiving sensory information from that area. Formalin injected into the paw fails to fire the dorsal horn neurones when the bradykinin receptors are desensitised or if added in the presence of a kinin antagonist (Haley et al., 1989). Kinins generated in injured or inflamed tissues activate sensory receptors that relay through C and Aδ afferent fibres nociceptive information to the substantia gelatinosa of the spinal cord. Low concentrations of bradykinin excite rat trigeminal and dorsal root neurones grown in culture (Baccag-

lini and Hogan, 1983). Bradykinin depolarises postganglionic fibres of the superior cervical ganglion and the primary afferent terminals attached to the hemisected immature rat spinal cord. It is likely that this action of bradykinin leads to the release of excitatory amino acids in dorsal horn (Bhoola et al., 1987). Visceral chemosensory nerve terminals are also stimulated by kinins (Lew and Longhurst, 1986; Ammons, 1988). In fact, writhing movements are produced in mice when bradykinin is injected intraperitoneally (Emele and Shanaman, 1963). Retrograde intraarterial injection of bradykinin is particularly painful and causes reflexly mediated flexion of the limbs and vocalisation in animals (Riccioppo Neto et al., 1974). A functionally important role may exist for kinins in activating visceral and peripheral C and sensory mechanoreceptors that results in the perception of pain.

vi. Importance in clinical disorders. The resurgence of interest in kinin receptors as potential drug targets and the development of potent antagonists has led to further evaluation of some of the kinin-associated cellular actions. Their role in chronic inflammation was reviewed recently by Burch et al. 1989. Kinins act as mitogens, stimulating DNA synthesis and thereby promoting cell proliferation (Whitfield et al., 1970; Owen and Villereal, 1983; Goldstein et al., 1984; Marceau and Trembley 1986). The ability of kinins to induce cell division could enhance the spread of cancerous cells and increase the proliferation of epidermal cells in disorders such as psoriasis. This is an area in need of much new research effort. Involvement of kinins in inflammatory joint disease, in addition to arthritic pain and effusion, is further supported by the ability of kallidin and bradykinin to stimulate bone resorption in cultured mouse calvaria. Both mineral mobilisation and matrix degradation are stimulated by kinins, as measured by release of ^{45}Ca and [^{3}H]proline from the cultured calvaria (Gustafson et al., 1986b; Lerner et al., 1987).

Kinins also appear to play an important role in a number of pathological states, namely, reduced sperm motility, allergic and viral rhinitis and asthma, postgastrectomy dumping syndrome, inflammatory bowel diseases, carcinoid, anaphylactic shock, HAE, and septic shock. Some of these effects are discussed in greater detail in section V. With the recent advent of potent antagonists, it is only a matter of time before the participation of kinins in these pathological processes is elucidated.

d. MEMBRANE BINDING. Saturable binding of monoiodo-Tyr-kallidin to bovine and rat uterus membranes was first reported by Odya et al.(1980). Subsequently, [^{3}H]bradykinin-binding sites were demonstrated on membranes prepared from the guinea pig intestine (Innis et al., 1981; Manning et al., 1982) and cultured human fibroblasts (Roscher et al., 1983). Next, bradykinin attachment sites were localised in the spinal cord and sensory ganglia by autoradiographic techniques (Manning and Snyder, 1983). More recent binding studies have identified bradykinin receptors on sensory fibres and neurones in the dorsal root, substantia gelatinosa, and trigeminal ganglia (Steranka et al., 1988). Vascular smooth muscle cells derived from rat aorta bind ^{125}I-[Tyr^{8}]-bradykinin and then either rapidly degrade or internalise the peptide (Hirata et al. 1989).

Specific high-affinity-binding sites have been characterised in murine neuroblastoma clones, N1E-115 and in cultured rat neonatal brain cells (Snider and Richelson, 1984; Lewis et al., 1985). Additional studies revealed the presence of bradykinin-binding sites on neuroblastoma × glioma hybrid NG 108–15 cells (Reiser et al., 1984). Digitonin-solubilised BK2-binding sites from the rat uterus and NG 108–15 tumours showed the same affinity for a range of bradykinin analogues as did the receptor-binding sites in the membranes derived from these cells. The authors concluded that the solubilised binding site mirrored the physiological BK2 receptor (Snell et al., 1990). Another approach to identifying the binding ligand was to use an iodinated photoaffinity probe, N-4-azidosalicyclyl-kallidin. The probe labeled a bradykinin blockable glycoprotein of 166 kDa on NG 108–15 cells or tumour membranes that seemed to be precipitable with a polyclonal antiserum to ACE (de Vries et al., 1989). Although this strategy was unsuccessful, photoaffinity labeling of the new kinin antagonist Hoe 140 may prove to be more fruitful, because it is not a substrate for ACE.

e. RECEPTORS. The concept of receptor heterogeneity arises from a diverse pharmacological profile in which different analogues are responsible for different actions of the parent molecule. For receptor characterisation, there is an initial requirement of structure-activity studies to establish an order of potency of agonists, identification of differences in activity profile, measurement of the affinity of competitive antagonists, and mechanisms involved in signal transduction. So far, two types of kinin receptors have been characterised, BK1 and BK2 (Regoli and Barabe, 1980; Vavrek and Stewart, 1985; Roberts, 1989; Regoli et al., 1990).

Kinin receptors were first classified according to relative potencies of kinin agonists on isolated smooth muscle preparations (table 3). The des[Arg^{9}]-bradykinin metabolite showed a much greater affinity for contracting the rabbit aorta, mesenteric vein, and basilar artery than did either of the two standard kinins (Regoli et al., 1977, 1978; Whalley et al., 1983). A thousand-fold excess of the parent kinins was needed to achieve potency equivalent to that of desArg^{9}-bradykinin on the rabbit aorta (BK1 receptor). desArg^{9}-bradykinin produced relaxation and contraction of the rabbit mesenteric artery precontracted with phenylephrine; the relaxation was mediated by prostaglandins, and the contraction was a direct effect (Churchill and Ward, 1986, 1987). Stimulation of BK1 receptors on macrophages released IL-1 and TNF (Tiffany and Burch, 1989). Whereas the increase in protein

TABLE 3
Kinin receptors in isolated tissue preparations and cultured cells

	Receptor type	Effect
Isolated tissues		
Rat		
Duodenum	BK2	Relaxation
Vas deferens	BK2	Contraction
Uterus	BK2	Contraction
Rabbit		
Aorta	BK1	Contraction
Jugular vein	BK2	Contraction
Guinea pig		
Trachea		
+ epithelium	BK2	Relaxation
− epithelium	BK2	Contraction
Ileum	BK2	Contraction
Dog		
Renal artery	BK2	Relaxation
Carotid artery	BK2	Relaxation
Bladder	BK2	Contraction
Cell type		
Human fibroblasts	BK2	Ca^{2+} increase, cell division
Murine neuroblastoma (N1E-115)	BK2	cGMP increase, phospholipase A_2 activation
Murine neuroblastoma × glioma (NG108-15)	BK2	Phosphotidylinositol turnover, Ca^{2+} and cGMP increase
Bovine aortic endothelial cell	BK1/BK2	cGMP increase
Bovine pulmonary artery endothelial cell	BK2	EDRF release and Ca^{2+} increase
Murine macrophages (P388-D1 RAW 264.7)	BK1	IL-1, TNF release
Murine 3T3 fibroblasts	BK2	PGE_2 synthesis
Madin-Darby canine kidney cells	BK2	Phospholipase A_2 activation, arachidonic acid release

synthesis, collagen building and cell division in human foetal lung fibroblasts by desArg^9-bradykinin was inhibited by desArg^9[Leu^8]-bradykinin (BK1 antagonist), the formation of prostaglandins by bradykinin was unaffected. Therefore, both BK1 and 2 receptors may coexist on the same cell. An alternative explanation would be that the differences in potency are due to alterations in affinity for these molecules or to the presence or absence of secondary mediators such as prostaglandins or nitric oxide.

BK1 receptors seemed to be absent normally, but expression in smooth muscle cells and fibroblasts becomes evident in pathological states, particularly in inflammation or after exposure of tissue to noxious stimuli. When rabbits were injected with lipopolysaccharide, a hypotensive effect was observed with desArg^9-bradykinin (Regoli et al., 1981). Induction of BK1 receptors was observed in the cardiovascular responses of rabbits when KII-ACE inhibitors were injected (Nwator and Whalley, 1989) and in the isolated rabbit aorta as a selective time-dependent response to in vitro incubation (deBlois et al., 1991). The enhanced sensitivity of isolated tissues containing BK1 receptors increased with time in parallel with the specific binding of [^{3}H]desArg^9-bradykinin. Inhibition of BK1 receptor induction by cycloheximide or actinomycin-D and indomethacin has led to the proposal of de novo synthesis of BK1 receptors in some tissues as a result of inflammation or tissue trauma (Regoli and Barabe, 1980; Regoli et al., 1981). In fact, following an intravenous injection of *Escherichia coli* endotoxin in the rabbit, desArg^9-bradykinin produced pronounced hypotension, an effect not observed in control animals. IL-1 has been proposed as the endogenous trigger for the induction of BK1 receptors and the mediator that enhances the effects of desArg^9-bradykinin in inflammation (de Blois et al., 1991).

Most of the actions of bradykinin and kallidin are mediated through the BK2 receptor, which essentially does not respond to desArg^9-bradykinin or -kallidin (table 3). On most membranes, the K_d for the BK2 receptor is in the range 0.7 to 5 nM. In contrast to the rabbit aorta (a primary site for the BK1 receptors), the muscle relaxant actions of kinins on the dog carotid artery and bladder (Regoli et al., 1986)and isolated rabbit mesentery and human basilar arteries are effected through BK2 receptors (Whalley et al., 1987a; Nwator and Whalley, 1989), as is the venocontractile action of bradykinin on the rabbit jugular vein. Analysis of the potency of cyclical kinins, Met-Lys-bradykinin, kallidin, and bradykinin, in increasing paw volume and skin vascular permeability suggested species differences in BK2 receptors mediating these actions in the rat when compared to the guinea pig

and rabbit, even though the involvement of the BK1 receptor was excluded for all three species (Whalley, 1987).

Because bradykinin-induced bronchoconstriction and contraction of guinea pig tracheal and lung parenchymal strips is only partially inhibited by the Stewart and Vavrek antagonists, Farmer and colleagues (1989b) proposed the existence of a BK3 receptor in the airways. The claim for a third BK3 receptor remains controversial. However, some evidence exists for the presence of subtypes in the BK2 class. There are supposed to be two BK2 subtypes on intestinal epithelia, fibroblasts, and primary brain cultures (Lewis et al., 1985; Braas et al., 1988; Roberts and Gullick, 1989) and three on a neuroblastoma cell line (Snider and Richelson, 1984). The linking of each bradykinin receptor and the subtypes to a specific G protein-coupled second messenger system may explain why bradykinin produces different cellular actions on the same cell. Differences may also occur because of different affinities, arising from changes in sensitivity of bradykinin receptors to bradykinin produced by enzymes, hormones, and compounds that alter cell membrane characteristics. Until cellular actions of kinins are selectively and competitively inhibited by a specific molecular species of antagonist, the question of whether there are indeed more than two kinin receptors remains unanswered.

Several approaches have been directed to the identify the molecular features of the kinin receptor. A photoaffinity probe, although functionally active, failed to label the receptor. Active binding sites from bovine myometrium (Fredrick and Odya, 1987) and NG 108–15 cells (Snell et al., 1990) were recovered in too low yield for characterisation of the bradykinin receptor. In 1990, Mahan and Burch reported the expression of functional bradykinin receptors following the injection of BALB/c (SV-T2 3T3) mouse fibroblast mRNA; response to bradykinin was measured by the evoked release of oocytes preloaded with $^{45}Ca^{2+}$. More recently, the bradykinin receptor solubilised from the rat uterus was assessed using electrophoretic and chromatograhic techniques (Snell et al., 1991). The receptor bound strongly to phenyl-Sepharose from which it could be eluted with a gradient of increasing concentration of detergent. The solubilised receptor had a pI of 4.6 and bound strongly to diethylaminoethyl-Sepharose and hydroxyapatite. The bradykinin receptor is a glycosylated protein that binds to wheat germ agglutin but not to lentil lectin. On cross-linking with a radiolabeled ligand, the receptor revealed a molecular mass of 81 kDa (Snell et al., 1991).

A successful approach has been to clone the receptor gene. After a cDNA rat uterus library was constructed and when clonal selection was used as a strategy, the cDNA encoding of a functional bradykinin receptor was achieved in *Xenopus laevis* oocytes (McEachern et al., 1991); these cells can translate functional receptors from injected mRNA, including receptors coupled to specific G proteins (McIntosh and Catt, 1987). The clone expressing the bradykinin receptor in *X. laevis* oocytes was mapped by assaying the ability of bradykinin to stimulate chloride current. The receptor has a predicted protein sequence of 366 amino acids, a molecular mass of 41 kDa, three potential N-linked glycosylation sites, a Ser-318 as a protein kinase A phoshorylation site, and homology with the the seven transmembrane G protein-coupled family of receptors. Strong homology (23%) was observed with neurotensin and tachykinin receptors and to the COOH half of luteinising hormone/human chorionic gonadotrophin receptor. In pharmacological studies, bradykinin, but not desArg^9-bradykinin, stimulated chloride current (see section III.D.1.f) in oocytes expressing the BK2 receptor. Bradykinin stimulation of the chloride current was inhibited by [Thi^{5,8},D-Phe^7]-bradykinin; the concentration producing 50% inhibition was 400 nm which was close to its pA_2 value of 6.3 (500 nm) determined on the rat uterus. Using Northern analysis, McEachern et al. (1991) observed bradykinin receptor messages in the uterus, vas deferens, kidney, ileum, heart, lung, testis, and brain. Different clone lengths of the message in the various tissues may indicate subtypes.

Phillips et al. (1992) also successfully expressed the bradykinin receptor in *Xenopus* oocytes, injected with mRNA prepared from the NG 108–15 hybrid neuroblastoma × glioma cell line, rat dorsal root ganglia, rat uterus, and human fibroblast cell line W138. Expression of the receptor was followed by recording membrane currents generated by the application of bradykinin on voltage-clamped oocytes. The current responses of oocytes injected with NG 108–15 mRNA obtained with bradykinin were blocked by the BK2 antagonist, Hoe 140 (2 μM); in the case of the oocytes injected with human fibroblast, the electrophysiological response was stimulated by desArg^9-bradykinin (2 μM) and antagonised by desArg^{9}Leu^8-bradykinin (5 μM). Therefore, only BK2 receptor was expressed from the mRNA derived from NG 108–15 cells and rat uterus, but W128 human fibroblast cell mRNA expressed both BK1 and BK2 receptors (Phillips et al., 1992).

f. RECEPTOR COUPLING AND CELL SIGNALING. Unlike any other peptide, kinins promote every signal transduction event at the cellular level: (*a*) calcium mobilisation; (*b*) chloride transport; (*c*) activation of phosphotidylinositol-specific phospholipase C that results in the formation of inositol phosphate and diacylglycerol; (*d*) formation of nitric oxide, resulting in stimulation of guanylate cyclase with a transient increase in cGMP; (*e*) activation of phospholipase A_2, which forms prostaglandins and leukotrienes; and (*f*) stimulation of adenylate cyclase with an increase in cAMP (fig. 7, table 3).

At the receptor level, new advances will be achieved in elucidating the precise molecular events that link kinin formation of nitric oxide to second messengers. Although

bradykinin appears to stimulate the formation of nitric oxide in vascular endothelium, it is likely that a similar effect will be described in many other cell types, including tracheobronchial epithelial cells, neurones, neutrophils, and macrophages. The receptor-coupling steps are unclear, but it is possible for the terminal arginine of the kinin molecule to act as the precursor for the nitric oxide synthase, which by rearrangement of the guanidino nitrogen produces nitric oxide. After the nitric oxide is formed, it may modulate cellular events by one of three pathways: activation of guanylate cyclase and formation of cGMP (fig. 7), recruitment of calcium, or enhancement of phosphotidylinositol turnover.

The first demonstration of a kinin-mediated increase in electrogenic chloride secretion in rat colonic epithelia was reported by Cuthbert and Margolius (1982). Recent experiments confirm that the kinin-evoked chloride secretion by the mucosa of the rat colon and guinea pig ileum involves BK2 receptors (Gaginella and Kachur, 1989). The effect of kallidin (Lys-bradykinin) on chloride secretion by the HCA-7 series cells cultured from human colonic carcinoma was unaffected by inhibition of cycloogygenase, whereas the chloride secretion by Colony-29 series cells was partially dependent on eicosanoid formation (Cuthbert and McVinish, 1991).

Both isolated cell membrane fragments and intact cultured cells have been used to demonstrate the bradykinin receptor coupling to second messenger systems. Bradykinin increases cytosolic free calcium in many tissues. However, in vascular smooth muscle, the intracellular calcium is mobilised by receptor-coupled activation of phospholipase C, hydrolysis of inositol bisphosphate, and formation of inositol triphosphate (Takeuchi et al., 1988). Specific stimulation of the inositol phosphate pathway in human fibroblasts and the prostaglandin pathway in mouse fibroblasts is produced by anti-idiotypic antibodies bearing the internal image of the bradykinin molecule (Haasemann et al., 1991a). The effect on the prostaglandin pathway was inhibited by the BK2 receptor antagonist, NPC 567 (see section III.D.1.g). Binding of the anti-idiotypic antibodies to the BK2 receptors expressed by human foreskin fibroblasts and guinea pig ileum indicated that these receptor antibodies show cross-species reactivity (Haasemann et al., 1991a). The signal transduction mechanisms involved in the mitogenic action of bradykinin on cultured epithelial cells and epidermal cells in psoriasis are not known. Recent evidence suggests that the *ras* oncogene influences the expression of the bradykinin receptor gene in such a manner as to promote cell division by kinins (Roberts and Gullick, 1989). The sequence of membrane events involved in this action requires elucidation.

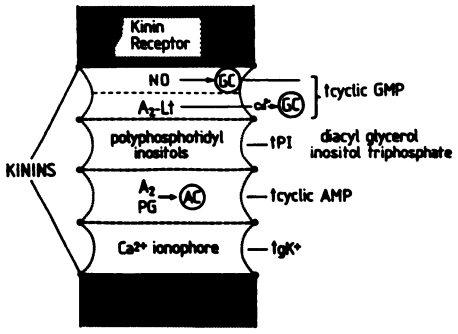

FIG. 7. Kinin-receptor coupling and signal transduction. NO, nitric oxide; A_2, phospholipase A_2;, Lt, leukotriens; PI, phosphotidylinositol second messengers (diacyl glycerol and inositol triphosphate); PG, prostaglandin; gK^+, potassium conductance; GC, guanylate cyclase (particulate and soluble); AC, adenylate cyclase.

In NG 108–15 neuroblastoma × glioma hybrid cells, bradykinin produces a transient hyperpolarisation followed by prolonged depolarisation (Higashida and Brown, 1987) . Bradykinin receptor coupling in these cells increases phosphatidylinositol turnover, with the resultant formation of the second messengers, diacylglycerol and inositol triphosphate, and a simultaneous, calcium-dependent increase in cGMP (Reiser and Hamprecht, 1982; Reiser et al., 1984; Snider and Richelson, 1984; Yano et al., 1984; Higashida and Brown, 1986; Higashida et al., 1986). Bradykinin also evokes an increase in cytosolic calcium in the NG 108–15 cells (Reiser and Hamprecht, 1985). In lung membranes, bradykinin produces a dose-dependent increase in cAMP (Gadd and Bhoola, 1988). Bradykinin and thrombin stimulate PGE_2 synthesis in murine 3T3 fibroblasts by means of a guanosine triphosphate protein linked to phospholipase A_2 (Burch and Axelrod, 1987). Each agonist causes desensitisation in the short term, but prolonged application of one agonist sensitises the response to the other. This amplification of the bradykinin activation of phospholipase A_2 was mediated by diacylglyerol independently of protein kinase C (Burch et al., 1987; Burch, 1989). Similarly, human recombinant ILs, IL-1α and IL-1β, promote PGE_2 synthesis but with a much slower time course. However, in cells pretreated with IL-1, bradykinin induces a 10-fold increase in PGE_2 synthesis. The mechanism involved is a combination of the activation of phospholipase A_2 and cyclooxygenase together with an enhanced stimulation of the bradykinin receptor coupled to guanosine triphosphatase activity. These actions of IL-1 were not associated with an increase in receptor number or affinity for bradykinin. The IL-1 modulation was not specific because a similar effect was observed with bombesin and thrombin (Burch et al., 1988).

The coupling of kinin receptors to second messenger systems is through a family of G proteins, but if multiple pathways are recruited, there could be modulation of the transduction signals. Changes in calcium and turnover of phosphotidylinositol produced by bradykinin are linked to pertussis toxin-sensitive and -insensitive G proteins. Furthermore, bradykinin activation of phospholipase C and A_2 in fibroblasts and Madin-Darby canine kidney cells involves independent and nonindependent sequential transduction of the receptor signal,

supporting the thesis that a family of G proteins is linked to the kinin receptor. The particular second messenger system initiated, therefore, may be a function of the receptor-coupled G protein in each cell type, indicating the occurrence of BK2 receptor subtypes. Further experimental evidence is required to elucidate whether any distinctive differences exist in the transduction of BK1 and BK2 receptors.

Receptor-coupled activation of second messengers frequently results in the phosphorylation of key membrane or cytoplasmic proteins and enzymes. Such a regulatory mechanism is believed to be involved in the forward transduction of the bradykinin signal in aortic endothelial cells (Demolle et al., 1988). In our experience, a single application of bradykinin to the perfused hemisected spinal cord of the immature rat caused a marked desensitisation of primary afferent terminals that lasted for up to 1 h (Bhoola et al., 1987). Such a desensitisation process may involve serine and threonine phosphorylation of the cytoplasmic segment of the bradykinin receptor with recovery accompanied by dephosphorylation (Roberts and Gullick, 1989).

g. ANTAGONISTS. Specific antagonists are essential to understanding the physiological role and cellular actions of endogenously active molecules. An important property of an antagonist is high affinity for its receptor without possessing any intrinsic activity. History shows that such compounds are obtained by careful structure-activity analysis of analogues. Ever since the description of the correct structure of bradykinin in 1960, many analogues have been synthesised. In the early 1980s, Regoli and colleagues (Marceau et al., 1983) reported that des-Arg^9[Leu^8]-bradykinin antagonised the potent action of desArg^9-bradykinin on the isolated rabbit aorta but showed no effect on many known biological actions of bradykinin. Initial constructs by Stewart and Vavrek resulted in the synthesis of superagonists. The increase in potency of extended NH_2-terminal kinin agonists was due to resistance to hydrolysis by kininases, and the replacement of proline residues in positions 2 and 3 led to increased selectivity for contraction of the uterus. The one single change that converted the molecule from an agonist to an antagonist was the replacement of Pro^7 by an aromatic D amino acid. The first bradykinin analogues to inhibit the standard pharmacological actions of bradykinin were published by Vavrek and Stewart in 1985.

Time-consuming and patient synthesis of hundreds of compounds by Stewart and Vavrek established the following structure-activity relationships: (*a*) replacement of Pro^7 usually destroyed agonist activity, but replacement by D-Phe gave the analogue an antagonist profile; (*b*) proline in positions 2 and 3 and the Ser^6 served as spacers for the correct conformational turns necessary for combination with the receptor, whereas Pro^2 was essential for agonist activity but not Pro^3; in contrast, introduction of hydroxyproline at positions 2 and 3 produced good antagonists; (*c*) the two phenylalanine residues at positions 5 and 8 were essential for agonist action of the molecule; and (*d*) finally, basic amino acid residues at positions 1 and 9 were essential if the biological actions of bradykinin were to be retained. Resistance to degradation by kininases was enhanced by extensions at the NH_2 terminus (Stewart and Vavrek, 1991). The most effective of the Stewart and Vavrek family of BK2 antagonists on standard assay tissues (D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, called NPC 567) was derived by the substitution of D-phenylalanine for proline (increased resistance to the action of peptidases) and by the inclusion of β-2-thienylalanine (increased potency).

All of the numerous sequence-related, synthetic kinin antagonists (Stewart et al., 1985; Stewart and Vavrek, 1986, 1988) have been tested against the classical biological activities of bradykinin that are mediated by BK2 receptors. Testing of such a vast number of analogues has revealed some interesting anomalies. Evidence from structure-activity experiments on the rat vas deferens indicated heterogeneity of BK2 receptors. These studies revealed differences in the degree of antagonism of pre- and postjunctional bradykinin receptors of the rat vas deferens. Also, [Dβ2-naphthyl-Ala^1-Thi^{5,8}-D-Phe^7]-bradykinin inhibits the contractile effect of bradykinin on the rat uterus but not the guinea pig ileum (Farmer et al., 1989a). The reason for such selectivity of action could imply the presence of BK2 receptor subtypes.

Regional blood flow in many organs is increased by both kinins. In rats with renovascular hypertension, a reduction in the decrease of blood pressure produced by KII-ACE inhibitors was antagonised by a BK2 receptor antagonist. This finding suggested a primary role for kinins in the hypotensive action of KII-ACE inhibitors (Benetos et al., 1986). Increased coronary blood flow, determined on the isolated perfused heart, was reduced in a dose-dependent manner by a BK2 antagonist (Barton et al., 1989). The effect of antagonists has also been examined on peripheral circulation (Barton et al., 1988). Whenever bradykinin was injected into the human skin, vascular permeability was increased, and the resultant extravasation of plasma proteins was produced by venoconstriction and prostaglandin release. Equimolar concentrations of a BK2 antagonist significantly reduced these vascular permeability and inflammatory actions of bradykinin. Whereas in the rabbit following the intradermal injection of bradykinin inhibition of protein leakage was a BK2 receptor event (Schachter et al., 1987; Lembeck and Griesbacher, 1990), in rat paw and skin there was a suggestion that vascular permeability was not mediated by either BK1 or BK2 receptor types (Carey et al., 1988).

Several studies now show that painful stimuli evoked by bradykinin, which is probably the most painful endogenous autacoid that we know, were suppressed by

BK2 antagonists (Stewart et al., 1985; Steranka et al., 1988). Antagonism of the algesic action of bradykinin on the human blister base was demonstrated with a BK2 but not a BK1 receptor antagonist (Whalley et al., 1989). The role of endogenous bradykinin in producing pain was examined by injecting urate crystals into the hind-paw of the rat, so that activation of HF and the ensuing contact cascade resulted in the appearance of kinins in the inflammatory exudate (Melmon et al., 1967). When mechanical pain was applied to the urate-injected paw, the preformed kinins induced a hyperalgesic response to the painful stimulus which was inhibited by potent BK2 antagonists (Steranka et al., 1988). The potency of algesic compounds injected into the arterial circulation of the rabbit ear (an isolated preparation in which neuronal contact with the animal is through the auricular nerve) can be assessed quantitatively by measuring the reflex changes that occur in systemic blood pressure. The stimulation of pain-conducting receptors by bradykinin in this preparation was specifically blocked by BK2 antagonists (Lembeck and Griesbacher, 1990). Bradykinin contracted the isolated iris sphincter muscle of the rabbit by releasing substance P from primary afferent neurones of the trigeminal nerve. Whereas these neuronal receptors were blocked by BK2 antogonists, the capsaicin-induced contractions of the muscle were unaffected.

Recently, a new class of kinin antagonist (Hoe 140, D-Arg-Arg-Pro-Hyp-Gly-(β-2-thienyl)-Ala-Ser-DTic-Oic-Arg) was developed by introducing several unnatural amino acids into the molecule (fig. 8) (Hock et al., 1991). It has a prolonged half-life in serum and synovial fluid (Bond et al., 1992). Up to 0.1 mg/Kg of Hoe 140 administered intravenously was well tolerated by conscious dogs (Wirth et al., 1991). Hoe 140 showed saturable, high-affinity binding to guinea pig ileum membranes (IC_{50} of 1.07×10^{-9} M and a K_I of 7.98×10^{-10} M), and efficacy was 40 times greater than with the NPC 567 antagonist. Hoe 140 inhibited bradykinin-evoked contractions of the isolated rat uterus, guinea pig ileum, pulmonary artery, and rabbit iris sphincter muscle; it antagonised the relaxation of the rat duodenum, vasoconstriction, and the release of PGE_2 in the isolated perfused ear of the rabbit (Hock et al., 1991). Hoe 140 also antagonised bradykinin-produced hypotension in the rat and dog, bronchoconstriction in the guinea pig, and carrageenan-induced oedema in the rat paw (Wirth et al., 1991). The kinin-mediated release of EDRF (nitric oxide), increases in cytosolic calcium in cultured endothelial cells from the porcine aorta, and release of prostacyclin from similar cells cultured from bovine aorta were all inhibited by Hoe 140 (Hock et al., 1991). Therefore, it specifically inhibited the actions of bradykinin on at least nine bioassay tests with no effects on responses produced by acetylcholine, noradrenaline, and histamine (Lembeck et al., 1991). The infarct-limiting and cardioprotective effect of bradykinin and KII-ACE inhibitors was reversed by Hoe 140. It was virtually inactive on the isolated rabbit aorta and, therefore, was considered selective for BK2 receptors.

Another class of bradykinin antagonist has been developed by the synthesis of N^1,N^6,bis-succinimidoalkane peptide dimers of the bradykinin antagonist, D-Arg^0-[Hyp^3,D-Phe^7,Leu^8]-bradykinin, in which each of the amino acids within the bradykinin molecule was systematically replaced by cysteine and the latter used as the dimeric bridge (Cheronis et al., 1991). The most potent compound to eventuate was one dimerised at Cys^6 (CP0127). This compound was about 50 to 100 times more potent than the parent antagonist molecule on the rat uterus, guinea pig ileum, and the rabbit jugular vein (pA_2 value of 10.5 on the rabbit jugular vein). Furthermore, CP0127 was a potent long-acting antagonist of bradykinin-induced hypotension and the hypotensive response to *E. coli* lipopolysaccharides in the rat and rabbit. In addition, CP0127 significantly increased survival in rat and rabbit models of endotoxic shock (Whalley et al., 1991). Because lipopolysaccharide induces BK1 receptors, the Cys^6 dimerised compound may antagonise both BK1 and BK2 receptors and turn out to be a very useful adjunct to the treatment of septic shock in humans (Whalley et al., 1991).

The hypothesis that a β turn in the four COOH-

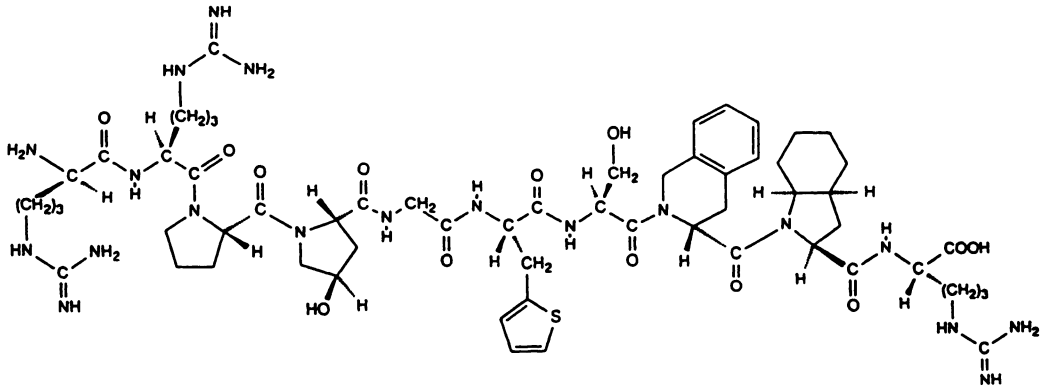

FIG. 8. Chemical structure of the kinin antagonist, Hoe 140.

terminal amino acids of bradykinin analogues may be an important requisite for high affinity of the receptor has led to the design of new bradykinin antagonists by molecular modeling (Kyle et al., 1990, 1991a,b). Of the five peptides prepared, D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-DTic^7-Tic^8-Arg^9 and D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-DTic^7-Aoc^8-Arg^9 proved to be highly potent bradykinin receptor antagonists when assayed against bradykinin-stimulated release of prostaglandin by SV-T2 mouse fibroblasts that express BK2 receptors only. Analysis of the environment surrounding the amphiphilic membrane-embedded bradykinin receptor should provide an insight into the precise side chains and atoms of the parent bradykinin molecule involved in its binding to the reactive groups on the receptor. Kyle and colleagues (1991a,b) have taken such an approach to probe the bradykinin receptor by preparing conformationally constrained peptide analogues of the natural peptide that would provide data relating to the formation of peptide-receptor complexes and delineate the topographical features of the bradykinin receptor. Therefore, the exciting challenge ahead is the development of new agonists and antagonists, either by synthesis or by molecular modeling, that would selectively affect specific actions of kinins. Little of what is known about nonpeptide antagonists of bradykinin was reviewed by Burch et al. (1990). The most promising of these was a terpene glycoside extracted from the Brazilian plant, *Mandevilla velutina*, which is used in folk medicine for treatment of inflammation and snake bites, including those caused by *B. jararaca* (Calixto et al., 1985; Calixto and Yunes, 1986).

IV. Cellular Biology and Functional Importance in Cells

A. Renal Cells

1. Kallikrein: tissue kallikrein. The initial observations of Abelous and Bardier (1909) and, subsequently, the experiments of Frey (1926) indicated the presence of a vasodepressor substance in urine. This molecule was isolated from the urine of several mammals and shown to possess kallikrein-like activity (Kraut et al., 1934). The first suggestion that the kidney may be an important source of the enzyme was made by Werle and Vogel in 1960 after the destruction of rat renal tubules by uranyl acetate and the discovery of tissue kallikrein-like activity in kidney homogenates (Werle and Vogel, 1961). Since then, many studies have been performed to determine both the synthesis (Nustad et al., 1975) and functional importance of the enzyme in the kidney.

a. PROPERTIES. Tissue kallikrein in renal extracts and urine is present in both active and inactive forms (Corthorn et al., 1977; Silver et al., 1980; Oza et al., 1981; Yamada and Erdös, 1982; Takaoka et al., 1982; Nustad et al., 1978b). As an example, human urinary kallikrein consists of about equal portions of active and inactive enzyme (Silver et al., 1980; Oza et al., 1981). On the other hand, about 70% of rat urinary kallikrein is in an active form (Noda et al., 1983). Renal prokallikrein has been partially purified by immunoaffinity and diethylaminoethyl-Sephadex A-50 column chromatography. When the purification was performed in the presence of SBTI, a greater recovery was achieved, suggesting that the pro form is activated by a trypsin-like enzyme (Nishimura et al., 1983). At acid pH, renal cortical extracts reduced the molecular mass of the urinary prokallikrein from 44 to 38 kDa (Takaoka et al., 1982, 1985). In fact, rat kidney cortex contains at least three prokallikrein-activating proteases, one of which (activator I), with a molecular mass of 57 kDa, has been purified (Nishii et al., 1989). Activator I is a thiol proteinase with a pH optimum of about 4.5. Although this is the first evidence of endogenous tissue prokallikrein processing proteases, further studies are necessary to determine the cellular relationship to tissue kallikrein and the mechanisms involved in the secretion of the activating enzymes.

i. Inhibitors of renal tissue kallikrein. In the course of experiments concerned with the isolation of tissue kallikrein from the rat kidney, Geiger and Mann (1976) found a 4.7-kDa polypeptide that selectively inhibited tissue and plasma kallikreins from different species but did not inhibit trypsin. Mono- and divalent cations inhibit the esterase or amidase activity of porcine, rat, and human urinary kallikreins (Fiedler and Werle, 1968; Lieberthal et al., 1982; Madeddu et al., 1985). Preincubation of purified rat urinary kallikrein with high concentrations of monovalent cations also decreased both the kininogenase and immunoreactivity of the enzyme (Chao et al., 1983).

b. CELLULAR LOCALISATION. Studies in the dog and rat kidney indicated that >90% of the enzyme was in the cortex, with the medulla and papilla containing 4.5 and 4.1%, respectively (Nustad, 1970; Scicli et al., 1974). In addition, isolated glomeruli were found to contain only 15% of the activity in the cortex, indicating that the primary localisation of tissue kallikrein was in the tubules. (Scicli et al., 1976; Ward et al., 1977). This view was supported by immunofluorescence experiments in the rat that localised tissue kallikrein in the distal tubules between the macula densa and the CDs (Orstavik et al., 1976a). Simultaneously, Ward et al. (1976) reported the absence of tissue kallikrein in a rat kidney tumour derived from cells of the proximal tubule. However, four different types of cells are known to occur in the distal nephron: DCT cells, CNT cells, CD cells, and intercalated cells (Le Furgey and Tisher, 1979). Three of these (DCT, CNT, and CD cells) are characterised by an infolding of the basal cell membrane, which is greatest in the DCT cells, intermediate in CNT cells, and least folded in CD cells (Kaissling, 1982). The decrease in the basal membrane infoldings from the DCT to the CCDs is paralleled by a similar decrease in Na^+,K^+-ATPase activity (Katz et al., 1979). The fourth cell type, inter-

calated cell, is characterised by a high carbonic anhydrase activity with little or no Na^+K^+-ATPase activity (Rosen, 1972; Ernst, 1975).

The aim in the early 1980s was to determine the precise cell type in which kallikrein was synthesised and stored. Further progress was made when measurement of tissue kallikrein in microdissected segments of rabbit and rat nephron localised the enzyme in CNTs (Tomita et al., 1981; Omata et al., 1982, 1983; Proud et al., 1982). Subsequent immunocytochemical studies, at the ultrastructural level, localised tissue kallikrein exclusively in the CNT cells of the rat nephron (Figueroa et al., 1984a,b; Vio and Figueroa, 1985). The intracellular distribution of the immunoreactivity confirmed the finding of previous subcellular studies that kallikrein activity resided in fractions rich in endoplasmic reticulum, luminal and basal cell membranes (Ward et al., 1975; Nishimura et al., 1980; Yamada and Erdös, 1982), and Golgi membranes (Brandan et al., 1982). In addition, immunoreactive tissue kallikrein was visualised in secretory-like vesicles present at both the luminal and basal poles of the CNT cells (Vio and Figueroa, 1985). Studies with hybridisation histochemistry have confirmed the expression of tissue kallikrein mRNA in the CNT cells of rat and mouse kidney (Coghlan et al., 1984; Fuller et al., 1985; Xiong et al., 1989). These observations on storage and synthesis, and the finding of a close anatomical relationship of the CNT cells with capillary vessels, may explain the appearance of tissue kallikrein in the venous effluent of the isolated perfused kidney (Roblero et al., 1976; Vio et al., 1983; Lauar and Bhoola, 1986).

The cellular localisation of tissue kallikrein in the human nephron proved to be difficult. A number of contradictory observations were reported. Orstavik et al. (1976b) failed to detect immunoreactivity to tissue kallikrein, whereas Pinkus et al. (1981) localised the enzyme in the reabsorption droplets of proximal tubules and in focal segments of the DCT. In contrast, Kimura and Moriya (1984) observed immunoreactivity to the enzyme only in the renal interstitium and postulated that this represented tissue kallikrein captured from the circulation. However, tissue kallikrein was localised unequivocally in CNT cells of the human nephron (Figueroa et al., 1988).

c. MECHANISMS POSTULATED FOR THE RELEASE OF RENAL TISSUE KALLIKREIN. A number of studies have been directed toward determining the factors involved in the release of renal tissue kallikrein. Severe restriction of sodium intake results in an increase in both the renal levels and the urinary excretion of tissue kallikrein (Geller et al., 1972; Margolius et al., 1974; Lieberthal et al., 1983; Omata et al., 1983; Bascands et al., 1987). These changes are accompanied by a twofold increase in the rate of synthesis of the enzyme, measured by the incorporation of [^{35}S]methionine (Miller et al., 1984). Although the regulatory control exercised by a low sodium diet on renal tissue kallikrein is generally accepted, the effects produced by a high sodium diet are controversial. Several authors have reported that high dietary sodium decreases urinary excretion (Marin-Grez et al., 1984; Shimamoto et al., 1980; Weinberg et al., 1987), whereas others have shown a significant increase but only when the enzyme was measured using a kininogenase assay (Yasujima et al., 1986; Bascands et al., 1987) rather than an RIA (Bascands et al., 1987).

i. Potassium. More recently, interest has shifted to the effect of potassium on the urinary excretion of renal kallikrein. An early study performed in humans by Horwitz et al. (1978) indicated that potassium increases kallikrein excretion in the urine. Experiments with renal cortical slices and isolated perfused kidney confirmed the stimulatory effect of potassium on the secretion of tissue kallikrein (Lauar et al., 1982; Lauar and Bhoola, 1986). Although these results were obtained with high concentrations of the ion (70 and 100 mM potassium chloride), values close to these have been reported (107 mM) in the distal tubular fluid of rats given a high potassium diet (Malnic et al., 1964). Furthermore, such a diet in rats produces hypertrophy and hyperplasia of the tissue kallikrein-containing CNT cells together with both an increase in the number of immunoreactive secretory vesicles and excretion of urinary kallikrein (Vio and Figueroa, 1987). Similar effects of potassium have been reported in mice (Guder et al., 1987), rats (Obika, 1987), and humans (Barden et al., 1987).

The influence shown by potassium on the secretion of tissue kallikrein by CNT cells may be influenced by prostaglandins (Vio et al., 1982; Kasai et al., 1984), particularly since Nasjletti et al. (1985) reported that high potassium intake selectively stimulates the formation of renal $PGF_{2\alpha}$, which is associated with an increase in the excretion of urinary kallikrein. Alternatively, the increased levels of tissue kallikrein generated following potassium loading may also enhance the formation of kinins, which stimulate the formation of renal prostaglandins. Potassium also stimulates the secretion of aldosterone (Frazer et al., 1979) which has been reported to have a stimulatory effect on renal kallikrein (Margolius et al., 1976). Early studies, using rat renal cortical cells, suggested that the synthesis of tissue kallikrein is switched on by aldosterone and inhibited by spironolactone (Margolius et al., 1976). However, recent experiments in which the tissue kallikrein content and its rate of synthesis were measured in the kidney after a single physiological dose of aldosterone have failed to confirm this effect (Miller et al., 1985). Adrenalectomy decreases the tissue kallikrein content of microdissected CNTs as well as that of Na^+,K^+-ATPase in cortical and medullary CDs (Marchetti et al., 1984). Following a single injection of aldosterone, the concentration of Na^+,K^+-ATPase returned to a normal value, whereas the tissue kallikrein content remained depressed. Such an acute effect seems

to differ from the changes induced by chronic administration of mineralocorticoids. Marchetti et al. (1984) provided convincing evidence that chronic treatment with deoxycorticosterone acetate increases the amount of tissue kallikrein in CNTs and urine. However, it is not established if this increase is due to a direct effect. The question whether the stimulatory effect exerted by potassium on renal tissue kallikrein is mediated directly and/or through secondary mechanisms involving the release of either mineralocorticoids or prostaglandins is not certain. The concept that renal tissue kallikrein is regulated by potassium needs serious consideration.

The potassium studies may provide new insight into the earlier observations on the beneficial effects of potassium on blood pressure (Iimura et al., 1981; MacGregor et al., 1982; Treasure and Ploth, 1983; Kaplan et al., 1985). In fact, recent studies of spontaneously hypertensive rats have shown that potassium supplementation (1% KCl in the drinking water) produced an increase in the urinary excretion of tissue kallikrein, which coincided with a simultaneous attenuation of the increase in blood pressure that occurs normally with age (Barden et al., 1988). In addition, plasma renin was reduced significantly after 5 weeks in those animals receiving potassium. A similar observation has been reported in humans (Kaplan et al., 1985).

ii. Antidiuretic hormone (arginine vasopressin). The effect of AVP on the release of renal tissue kallikrein has also been investigated. Experiments in which AVP was infused into dogs and rats showed a dose-dependent increase in the urinary excretion of renal kallikrein (Fejes-Toth et al., 1980). AVP stimulated the release of tissue kallikrein in isolated, renal cortical slices, an effect not mimicked by lysine vasopressin (Lauar et al., 1982). No such effect was observed when the cortical slices were prepared from the kidneys of spontaneously hypertensive animals, suggesting a mutational defect secondary to the development of hypertension (Chapman et al., 1986). It is not known whether the action of AVP on the release of tissue kallikrein is mediated directly through V1 or V2 receptors or indirectly by the formation of prostaglandins. A role for prostaglandins is supported by the finding that indomethacin blocks the action of AVP on the release of tissue kallikrein by isolated renal cortical slices (Chapman et al., 1986). Of the two AVP receptors, V2 is coupled to adenylate cyclase and is believed to be involved in water resorption by the CDs (Abramow et al., 1987). Human studies indicate that the urinary excretion of tissue kallikrein and kinins is lower in patients with diabetes insipidus when compared with normal subjects; however, after administration of AVP, both urinary kallikrein and kinins increase but with a greater response in normal subjects than in patients with diabetes insipidus. Identification of AVP receptors on CNT cells should provide supportive evidence for the view that AVP exercises regulatory control over the secretion of kallikrein (Luar et al., 1982).

iii. Neural control. The extent of neural control of kallikrein secretion remains to be established. Conflicting evidence exists concerning the role of sympathetic nerves. Early studies in conscious rats showed that the urinary excretion of tissue kallikrein was unaffected either by a sustained increase in plasma noradrenaline or by renal artery denervation (Diz et al., 1982). In contrast, evidence obtained after central β-adrenoceptor blockade and peripheral sympathectomy led Albertini et al. (1987) to postulate that the renal sympathetic nerves inhibit the urinary excretion of tissue kallikrein. In agreement with these results were the studies of Girolami et al. (1990) who found that noradrenaline acting through β-adrenoceptors inhibited kallikrein release from rat cortical slices in a dose-dependent manner.

2. Kininogen. Whereas renal tissue kallikrein has been extensively studied, there has been little experimental work on renal kininogen. The presence of kininogen was first detected in human urine more than a decade ago by Hial et al. (1976) and Pisano et al. (1978b). Recent studies have defined the biochemical characteristics of renal kininogen isolated from human urine by using an immobilised antibody reactive to the heavy chains of both kininogens (Proud et al., 1981). The additional finding that the electrophoretic pattern of the kininogen molecule isolated from urine resembled that of LK led Pisano and his colleagues to conclude that LK occurs in the tubule cells, thereby increasing the possibility of kinins being formed intrarenally (Proud et al., 1981). Subsequently, a protein with characteristics similar to those of LK was isolated from human urine by Müller-Esterl and Vohle-Timmermann (1984). This molecule consisted mainly of a single chain, but minor amounts were found in a two-chain, kinin-free form. Furthermore, Müller-Esterl and Vohle-Timmermann (1984) showed, by using an enzyme-linked immunosorbent assay, that the kininogen was present in concentrations from 1.6 to 6.2 mg/liter, which corresponded to about 10% of the total urinary protein. A recent report indicates that human urinary kallikrein releases kinins from renal medullary extracts that contain about 2.4 mg kininogen/g protein (Hallbach et al., 1987).

Proud and colleagues (1981) used their kininogen antibody to localise by immunofluorescence the kallikrein substrate in the kidney, where it was diffusely distributed within the distal tubule cells as well as in the CCD and medullary CD cells. No fluorescence was observed with an antibody raised against the light chain of HK. In the human kidney, kininogen has been localised in the CD cells (principal cells), whereas tissue kallikrein resides in the CNT cells. However, at the junction of the CNTs and CDs, the cells that contain the enzyme and the substrate are in close proximity to one another (fig. 9) (Figueroa et al., 1988). Confirmation of the view that LK

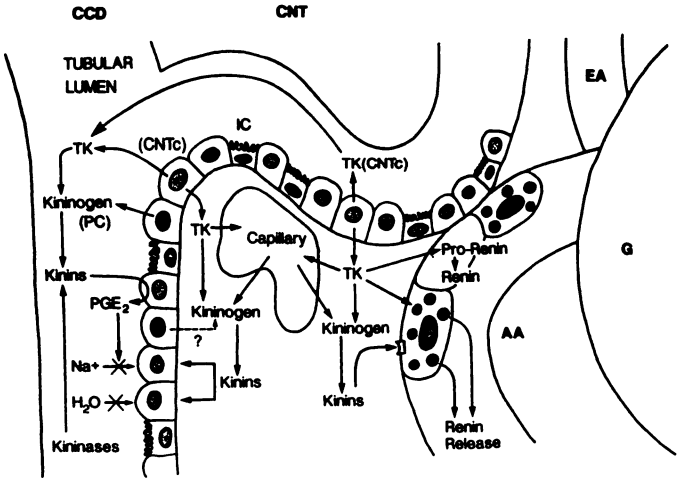

FIG. 9. Cellular localisation and humoral interactions among the kallikrein-kininogen-kinin, prostaglandin, and the renin-angiotensin systems. TK, renal tissue kallikrein; EA, efferent arteriole; AA, afferent arteriole; G, glomerulus; IC, intercalated cells; PC, principal cells.

was the kininogen species in the kidney was provided by Iwai et al. (1988) who showed expression of LK mRNA but not that of HK.

3. Kininases. The kidney is rich in kininases that are distributed all along the nephron. KI-CPM, KII-ACE, and KII-NEP have been isolated from human urine (Kokubu et al., 1978; Porcelli et al., 1987; Skidgel et al., 1987). In rat urine, NEP accounts for 68% of the total kininase activity, and ACE and CPM account for 23 and 9%, respectively, of the kinin-degrading activity (Ura et al., 1987).

The most studied of these, KII-ACE, was discovered originally in the kidney by Erdös and Yang (1966). Early experiments using fluorescein-conjugated antibodies showed that in the porcine and rabbit kidney ACE was concentrated in the proximal tubule (Hall et al., 1976; Caldwell et al., 1976) along the brush order membrane of these cells (Ward et al., 1975, 1976, 1977; Bruneval et al., 1986). These results were in agreement with those of Sudo (1981) who noted a higher ACE activity in the S3 proximal tubule segments of the rat inner cortex. A similar result was reported by Marchetti et al. (1987) in the microdissected rabbit nephron. More recently, two new ligands, radiolabeled ACE inhibitor (Chai et al., 1986) or antibodies directed against rat lung ACE (Ikemoto et al., 1987), have specifically localised the enzyme in cells of the inner cortex with the active site exposed on the luminal surface of the cell (Ikemoto et al., 1990).

Another enzyme with a kininase capacity in the kidney is KII-NEP. Like KII-ACE, KII-NEP occurs in the brush border of proximal tubules of several mammals (Kerr and Kenny, 1974; Gee et al., 1983; Gafford et al., 1983; Ura et al., 1987). A recent immunocytochemical study at the electron microscopic level localised human KII-NEP on the outer surface of the brush border membrane of proximal tubules. Immunoreactive KII-NEP also was visualised in vesicular organelles in the apical cytoplasm and the basal infoldings of proximal tubule cells (Schulz et al., 1988). Although stop-flow experiments suggested distal tubule site for KII-NEP (Skidgel et al., 1987; Sakakibara et al., 1989), no immunolabeling of the enzyme was observed in the distal portions of the nephron (Schulz et al., 1988).

The precise site of the nephron in which KI-CPM might be produced has not been fully identified, even though KI-CPM activity was found in collections from proximal and distal segments using stop-flow techniques (Sakakibara et al., 1989). Previously, a new KI type enzyme purified from human urine and kidney had been shown to differ in size, inhibitory profile, and immunogenic specificity from the enzyme that circulates in plasma (Marinkovic et al., 1980). Although it is believed to originate in the kidney, its precise localisation is unknown. More recently, a kinin-hydrolysing enzyme was localised along the cortical and medullary CDs of the rabbit (Marchetti et al., 1987); the enzyme did not respond to inhibitors of the KI and KII family of enzymes. A similar enzyme sensitive to $HgCl_2$ (Hallbach et al., 1987) and bacitracin was found in extracts of human kidney. Kininases in the renal vasculature (KI-CPN) and parenchyma (KI-CPM, KII-ACE, KII-NEP) provide an explanation for the complete loss of bradykinin when it has been injected into the renal artery or proximal tubules (Nasjletti et al., 1975; Carone et al., 1976). Kinins in the urine must, therefore, be formed either in or beyond the distal nephron.

4. Kinins. Three different kinins have been found in the human urine: kallidin (Lys-bradykinin), bradykinin [probably formed from kallidin by the action of a urinary aminopeptidase (Brandi et al., 1976)], and Met-Lys-bradykinin [formed by uropepsin, another serine protease that is active only at acid pH (Miwa et al., 1968, 1969)]. Twenty-four-hour urine collections contain microgram quantities of these kinins (Hial et al., 1976). Early experiments showed that when labeled bradykinin was injected into the renal artery or proximal tubules it did not appear in the urine, but the urine still contained unlabeled kinins (Yoshinaga et al., 1964; Nasjletti et al., 1975; Carone et al., 1976). However, when the peptide was injected into the distal tubule, it appeared almost intact in the urine (Carone et al., 1976). Using the stop-flow technique, Scicli et al. (1978) reported the presence of kinins in the luminal fluid of the distal nephron of the dog, with the highest concentrations located in the distal region and in the renal papilla and pelvis. No evidence of kinin formation was observed in the fluid obtained from the proximal tubule. Exocrine release of tissue kallikrein by the CNT cells and kininogen by the CD cells (principal cells) would support the view that urinary kinins are formed in the terminal segments of the distal nephron and beyond.

a. KININ RECEPTORS. Binding experiments with labeled bradykinin have been used to elucidate kinin re-

ceptors on microdissected nephron segments of the rabbit kidney. The highest binding was observed in the cortical and outer medullary collecting tubules. Low, but significant binding, was seen in the glomerulus and the proximal collecting tubules and DCTs. Kallidin, Met-Lys-bradykinin, and [Tyr^8]-bradykinin, but not desArg^9-bradykinin, competitively displaced bradykinin from its binding sites (Tomita and Pisano, 1984). A similar binding of bradykinin to medullary interstitial cells of the rat was reported by Fredrick et al. (1985). These findings suggest that both collecting tubule and interstitial cells express the classical BK2 receptor. bradykinin-binding sites have been localised in the outer and inner medulla and pelvic capsule of the rat kidney in autoradiographic studies in which [^{3}H]bradykinin was used. In addition, a relative absence of binding sites over the cortex was observed (Manning and Snyder, 1989). On the other hand, BK2 receptor-like binding has also been found in crude membrane preparations obtained from isolated rat glomeruli (Bascands et al., 1989). A more precise delineation of the BK2 receptor sites may provide further insights into the renal actions of kinins. Expression of BK1 receptors in the renal vascular bed seemed to occur only during trauma, inflammation, or exposure to des-Arg^9-bradykinin (Guimaraes et al., 1986).

b. FORMATION OF KININS. **i. In the tubular fluid (exocrine).** The close proximity of the cells containing immunoreactive tissue kallikrein and kininogen, together with a concomitant release of enzyme and substrate, makes the formation of kinins in the lumen of CDs inevitable. Kinins occur in the distal nephron, with the highest concentration in the final portion of this segment (Scicli et al., 1978) .

ii. In the extracellular tissue space (paracrine). The functionally controlled release of tissue kallikrein from the basolateral membranes of CNT cells (Yamada and Erdös, 1982; Figueroa et al., 1984b) into the interstitial tissue space could lead to the paracrine formation of kinins.

c. FUNCTIONAL IMPORTANCE OF RENAL KININS. Infusion of kinins into the renal artery increases renal blood flow, diuresis, and natriuresis without changes in glomerular filtration rate (Webster and Gilmore, 1964; Stein et el., 1972; Granger and Hall, 1985). The increase in renal blood flow, urine volume, and sodium excretion produced by intrarenal infusion of bradykinin was completely abolished by the administration of L-monomethyl-arginine, an inhibitor of nitric oxide synthesis (Romero et al., 1991).

i. Control of renal blood flow. The precise role of intrarenally formed kinins is not known. Faarup (1965) demonstrated that the "intercalated part of the distal tubule," which in the current nomenclature corresponds to the CNT, passed outward to the renal capsule, from whence it returned to the glomerular vascular pole and then ran alongside the afferent arteriole for a variable distance before reaching the collecting tubule. Such a morphological relationship has been described in the rat (Barajas et al., 1986) and human (Vio et al., 1988), although earlier studies failed to demonstrate it. Because the afferent arteriole is a well-documented site for the localisation of human renin (Celio and Inagani, 1981a; Orstavik and Inagami, 1982) and the CNT is the site for tissue kallikrein, this anatomical proximity points to a physiological relationship between the two humoral regulators of renal blood flow (fig. 10). A functional link between the two enzymes was first proposed by Sealey et al. (1978), who reported that tissue kallikrein activates prorenin in vitro; the suggested role in vivo is probably doubtful, because the K_m for the conversion of prorenin to renin is much higher than the K_m for the release of kinins. Apparently, both kinins and tissue kallikrein stimulate renin release from isolated rat glomeruli with the afferent arteriole attached (Beierwaltes et al., 1985a,b). Whereas angiotensin II primarily vasoconstricts the efferent arterioles of juxtamedullary and cortical nephrons (Navar et al., 1986), bradykinin decreases both afferent and efferent arteriolar resistance, with the decrease generally being proportionately greater in the afferent than in the efferent arteriole (Bayliss et al., 1976; Thomas et al., 1982). Therefore, paracrine-formed kinins may participate in a complex manner in the homeostatic control of renal blood flow and glomerular filtration, both by a direct vasodilatory action on the renal vessels and indirectly by increasing the resistance of efferent arterioles through the formation of angiotensin II as a result of the release of renin from the smooth muscle cells of the afferent arteriole (figs. 9 and 10).

Following an intravenous injection of enalapril or phosphoramidon, a 50% increase in renal papillary blood flow is observed. This vasodilatory participation of kinins in the presence of the inhibitors became more evident when the kinin antagonist, D-Arg-Hyp^3-Thi^{5,8}-D-Phe-

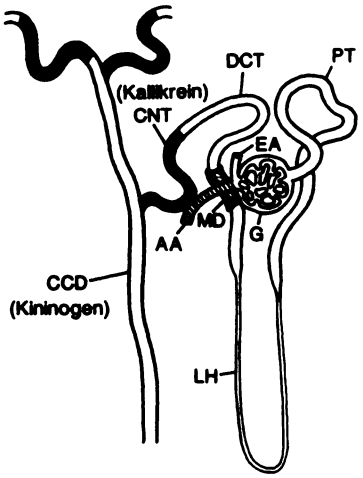

FIG. 10. Relationship between the CNT (kallikrein) and components of the juxtaglomerular apparatus (renin) in the human kidney. G, glomerulus; AA, afferent arteriole; EA, efferent arteriole; MD, macula densa; PT, proximal tubule; LH, loop of Henle.

bradykinin, returned papillary blood flow to control values. On the other hand, administration of the antagonist alone lowered papillary blood flow by 20%, without affecting outer cortical blood flow or glomerular filtration (Roman et al., 1988; Carretero and Scicli, 1990). Evidence so far confirms a humoral role for kinins in regulating renal papillary blood flow. The exact contribution of each of the two vasoactive peptides, kallidin and angiotensin II, in the homeostatic control of segmental blood flow in the kidney remains to be determined.

ii. Control of electrolyte and water transport. An extensive literature exists concerning the question whether kinins regulate electrolyte and water excretion (see review by Coyne and Morrison, 1991). Natriuresis occurred when bradykinin and ^{22}Na were injected simultaneously into the late proximal nephron (Kauker, 1980). However, even though the dose used was rather high (approximately 100 μM), urinary recovery of ^{22}Na only doubled. The lower than anticipated recovery may be because of the occurrence of kininases on the brush border of the proximal tubule and in the CDs. Intravenous administration of a kinin antibody in saline-infused rats decreased sodium and water excretion, whereas control nonimmune serum had no such effect (Marin-Grez, 1974). Captopril, an inhibitor of KII-ACE, produces diuresis and natriuresis which is accompanied by an increase in urinary kinins (McCaa et al., 1978).

Kinins affect electrogenic ion transport in primary cultures of porcine renal papillary collecting tubule cells. Activation of kinin receptors produces short current changes on both luminal and basolateral membranes, whereas AVP receptors appear to be confined to the basolateral side. The electrogenic response to kinins seems to be mediated partially by eicosanoid formation, because inhibition of cyclooxygenase activity by indomethacin or piroxicam almost abolished the short current effect (Cuthbert et al., 1985).

CDs isolated from the rabbit renal papillae are stimulated by kinins to form PGE_2. The three kinins (bradykinin, kallidin, and Met-Lys-bradykinin) caused, within 3 min, a four- to sixfold increase in the formation of PGE_2 (Grenier et al., 1981). Of particular importance is the finding that bradykinin releases PGE_2 only when added to the luminal surface of canine CCD cells cultured on Millipore filters (Garcia-Perez and Smith, 1984). This finding suggests that luminally, but not interstitially, produced kinins elicit PGE_2 formation in these cells. The formation of PGE_2 in the distal tubular or CD fluid could enhance the natriuretic action of kinins, because PGE_2 has been reported to inhibit Na^+ resorption in isolated, perfused rabbit CDs (Stokes and Kokko, 1977) (fig. 9).

Recent reports give further prominence to a role for kinins in regulating actions of AVP on water transport by CDs. It has been shown that kallidin has a marked inhibitory effect on vasopressin-stimulated water permeability and that bradykinin reversibly inhibits sodium resorption by the CDs (Schuster et al., 1984; Tomita et al., 1985). In both studies, the response was seen after the application of kinins to the serosal, but not the luminal, surface of the tubule. If the same effects occur in vivo that would imply a role for kinins on the serosal plasma membrane of CD cells to modulate water permeability and sodium reabsorption.

The physiological role of kinins in renal sodium transport may be secondary to that of renally produced ANF (Figueroa et al., 1990b) and circulating antidiuretic hormone, respectively. The humoral relationships among kinins, prostaglandins, and renin-angiotensin are shown in fig. 9. Answers to these questions will soon be realised with the use of specific and potent kinin antagonists.

5. Functional importance. a. HYPERTENSION. **i. Surgically created (Goldblatt) hypertension.** Tissue kallikrein is reduced in the urine of rats with experimentally induced renovascular hypertension (Croxatto and San Martin, 1970; Margolius et al., 1972; Jenner and Croxatto, 1973; Pisano et al., 1974; Croxatto et al., 1976; Keiser et al., 1976). In Goldblatt hypertensive rat models, tissue kallikrein activity in urine was decreased irrespective of whether plasma renin activity was normal (arterial stenosis with contralateral nephrectomy, Goldblatt one kidney-one clip model) or increased (arterial stenosis with contralateral kidney intact, Goldblatt two kidneys-one clip model) (Vio et al., 1980). Subsequent studies showed that, whereas tissue kallikrein in the urine from the stenotic kidney was decreased, enzyme levels in the urine from the normal kidney were unaffected (Girolami et al., 1983). In addition, these rats showed an increased propensity to inactivate circulating kinins.

In Goldblatt model rats, the decline in tissue kallikrein levels in urine is mirrored by a reduction of the enzyme in the kidney (Croxatto et al., 1974; Carretero et al., 1974; Nolly and Lama, 1981). Furthermore, Nolly and Lama (1981) reported that the in vitro release of active tissue kallikrein from kidney slices of Goldblatt two kidneys-one clip hypertensive rats was significantly lower than in normotensive sham-operated controls; no difference was observed in the levels of total tissue kallikrein. A recent study of the Goldblatt one kidney-one clip model indicates that tissue kallikrein gene expression in the kidney increases in parallel with the transient increase in plasma renin during the first week of renal artery constriction. After 3 weeks, the return of plasma renin to control values was followed by a decrease in renal tissue kallikrein mRNA (Schedlich et al., 1988).

ii. Genetic and salt-sensitive models of hypertension. The Okamoto (Geller et al., 1975), New Zealand (Carretero et al., 1976), Milan (Porcelli et al., 1975), and Fawn-hooded (Gilboa et al., 1984) rats are genetically derived spontaneously hypertensive animals. Only the Dahl strain (Carretero et al., 1978) is susceptible to becoming hypertensive when fed a high salt diet. The

excretion of urinary kallikrein is reduced in each of these hypertensive models.

There is consensus regarding the reduction of tissue kallikrein in the urine of SHO rats (Keiser et al., 1976; Ader et al., 1985, 1987; Chapman et al., 1986). The SHO strain shows decreased levels (active and total) of kallikrein in urine at 12 weeks, the reduction in values correlating directly with the increase in arterial blood pressure (Ader et al., 1985). A comparison was undertaken in SHO animals aged 52 and 78 weeks. The daily excretion of tissue kallikrein in milliunits per 24 h was reduced in the 52-week-old SHO rats, but the reduction appeared to occur before any obvious histological changes could be detected in the kidney. In contrast, at 78 weeks, these pronounced pathological changes in the kidney developed that were accompanied by a severe proteinuria (Figueroa et al., 1991a). Consequently, it is more than likely that in advanced hypertension a proportion of the tissue kallikrein measured in the urine of rats with severe nephropathy may be derived from the circulation. Of interest also is that at 78 weeks the normotensive animals excrete less active and proenzyme forms of tissue kallikrein when compared with 52-week-old rats. Furthermore, the reduction of the enzyme in urine was accompanied by a small increase in the systemic blood pressure. A similar ageing change was recently described by Girolami and Corman (1990) who found a progressive decrease in the excretion of urinary kallikrein in 10- to 30-month-old female rats; however, no information was provided concerning the systemic blood pressure in these animals. On the other hand, if the potassium in the diet of SHO rats is substantially increased, they excrete higher amounts of urinary kallikrein, which is paralleled by a reduction in blood pressure (Barden et al., 1988).

Salt-sensitive Dahl rats show reduced levels of kallikrein in urine when compared with the salt-resistant animals (Arbeit and Serra, 1985; Yamaji et al., 1987). Differences in the isoelectric focusing pattern with forms that show higher pI values (4.0 to 4.1) emerged in partially purified tissue kallikrein isolated from the urine of salt-sensitive rats (Rapp et al., 1984). The change in pI value was unique to the enzyme in urine but not to tissue kallikrein extracted from salivary glands. Rapp et al. (1984) also observed that the isoelectric focusing pattern for Dahl salt-resistant (pI 3.72 to 3.88) rats could be converted to a salt-sensitive (pI 4.0 to 4.1) pattern by treatment with neuraminidase, suggesting that the differing isoelectric focusing pattern was attributable to differences in the sialic acid content of tissue kallikrein.

The finding of reduced tissue kallikrein levels in the urine has led many investigators to postulate a role for renal tissue kallikrein in the pathogenesis of hypertension (Croxatto and San Martin, 1970; Margolius et al., 1971; Porcelli et al., 1975). Levels of tissue kallikrein in the kidney of spontaneously hypertensive rats have received some attention, but the findings are controversial. Reduced amounts of the active enzyme have been detected in Milan (newborn and 5 weeks) (Favaro et al., 1975; Baggio et al., 1979), Fawn-hooded (12 and 40 weeks) (Gilboa et al., 1984), and Dahl salt-sensitive rats (Yamaji et al., 1987). Although the rate of synthesis of the enzyme in the CNT cells of the Dahl salt-sensitive animals appears to be reduced (Yamaji et al., 1987), the level of expression of tissue kallikrein mRNA in the renal cells of genetically hypertensive rats was indistinguishable from the expression in appropriate controls (Fuller et al., 1986).

In a study using 8-week-old SHO rats in which both active and total renal tissue kallikrein was measured, Yasujima et al. (1987) reported values significantly lower than in WKY normotensive rats. At 10 weeks, Andersen et al. (1986) found no changes in the enzyme when measured by RIA. Opposite results were reported in an age-restricted study in which SHO rats showed an increase in renal tissue kallikrein (Ader et al., 1987). It was apparent that no previous studies had examined changes in the amount of the enzyme within the kidney at the time of onset and during progression to advanced hypertension. Consequently, an ontogenic study was planned in animals that ranged from 4 to 78 weeks of age with special emphasis on the renal levels of tissue kallikrein and the number of CNT cells that were immunoreactive to the enzyme in the spontaneously hypertensive animals. In addition, renal biopsies from patients with essential hypertension were used to visualise and measure tissue kallikrein in the human renal parenchyma. In fact, after the first 4 weeks of life, renal levels of both active and total tissue kallikrein increased and remained consistently higher in the SHO rats when compared with the WKY normotensive animals. In contrast, in the 78-week-old SHO rats, morphological changes indicative of renal parenchymal damage and tubular atrophy were accompanied by significantly reduced levels of the enzyme. In severe hypertensive rats, as illustrated by the 78-week-old SHO rats and by patients with established hypertensive nephropathy, the low values for tissue kallikrein in the kidney were probably due to a reduction in renal tubular mass (Figueroa et al., 1991a).

Clearly, the studies have so far established that, prior to the occurrence of advanced renal morphological changes, the SHO kidney contains higher levels of renal tissue kallikrein than normotensive WKY animals of comparable age. The increased renal values were associated with reduced excretion of the enzyme in urine. Several postulates have been offered to explain this finding. One reason could be that a molecular abnormality results in a defective release and increased storage of the enzyme in the CNT cells in early and moderate hypertension (Ader et al., 1987; Figueroa et al., 1991a). The molecular defect may prevent the release of the enzyme into the tubular fluid, resulting in reduced formation of kinins in the urine. However, apart from the

localisation of tissue kallikrein in luminally oriented granules, the enzyme is closely associated with the basolateral membranes of CNT cells. Therefore, the question that requires investigating is whether a similar defect affects the paracrine secretion of tissue kallikrein and the subsequent formation of kinins in the interstitial tissue fluid. These interpretations were further supported by the lack of response of SHO rat renal cortical slices to AVP, a stimulus able to release kallikrein from normal renal tissue (Chapman et al., 1986). Because no difference was observed in renal tissue kallikrein mRNA between SHO and WKY rats 40 to 50 days of age (Fuller et al., 1986), and if synthesis was indeed normal, then the increased kallikrein levels in the kidney could reflect increased storage associated with a molecular defect in the physiological release of the enzyme as indicated by the in vitro AVP experiments. An alternative explanation may reside with the concept of a tissue kallikrein inhibitor, called kallikrein-binding protein or kallistatin, that has been identified in many tissues, including the kidney, of WKY normotensive rats. This inhibitor, which forms complexes only with the active site of tissue kallikrein, is absent in the tissues of the SHO rat (Chao and Chao, 1988a). Even though enzymic measurements (amidase, kininogenase) could be influenced by the absence or presence of this inhibitor, it is difficult to conceive how the inhibitor can be responsible for the simultaneous increase in the tissue and reduction in urinary values of kallikrein in SHO rats.

Clearly, there is a need for comprehensive studies in which there is simultaneous measurement of the arteriovenous difference in tissue kallikrein and kinins in the renal circulation, together with urinary values for the enzyme, substrate, and the peptide. The completion of these experiments should be followed by a determination of the renal tissue levels of these components, by direct measurement and by immunocytochemistry. However, more immediate experiments are required to ascertain the levels of renal kininogen and urinary kinins during the period in which hypertension develops in the SHO animals. In particular, it is important to examine whether any change observed is a primary event or has occurred in response to an increase in systemic blood pressure.

It seems probable also that the higher tissue kallikrein levels arise in response to an elevated blood pressure. The question of whether this is a primary or secondary change requires further experimentation. However, it is tempting to suggest that, whereas the paracrine secretion into the tubular fluid is reduced, the increased storage and release of the enzyme may enhance the formation of kinins in the renal vasculature to counterbalance the vasoconstrictor effects of angiotensin II. However, if the in vitro action of kinins on renin release (Beierwaltes et al., 1985b) become functionally dominant in vivo, then the beneficial effects of kinins in hypertension could be negated.

With the advent of kinin antagonists it is now possible to critically examine the putative role of kinins in hypertension. The competitive kinin antagonist, [Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, caused an increase in blood pressure and a decrease in renal blood flow in anaesthetised male WKY and spontaneously hypertensive rats (Benetos et al., 1986; Seino et al., 1988). However, the magnitude of these changes was significantly lower in hypertensive than in normotensive rats (Seino et al., 1988). These results seem to indicate a failure in the kinin system in the hypertensive rats that may contribute to the development or the maintenance of hypertension. Either a mutation in the important coding regions of the gene or a defect in the promotor segment in spontaneously hypertensive rats may contribute to the pathogenesis of hypertension in these animal models (Woodley-Miller et al., 1989). Analysis of the cDNA sequences of the renal gene of spontaneously hypertensive animals with that of true tissue kallikrein will be valuable in examining this concept.

iii. Human hypertension. The earliest reports that urinary excretion of tissue kallikrein was significantly reduced in hypertensive when compared to normotensive individuals was published in 1934 by Elliot and Nuzum and in 1938 by Werle and Korsten. However, it took 37 years before supporting evidence for this observation began to appear in the literature (Margolius et al., 1971, 1974; Seino et al., 1975; Lechi et al., 1978; Weber et al., 1979; Kovatz et al., 1985; Nakahashi et al., 1986; Ura et al., 1986; Tanaka et al., 1986; Favre et al., 1986). Further evidence was the finding that when hog pancreatic kallikrein is administered orally to hypertensive patients, it lowers the blood pressure and at the same time corrects the lowered urinary excretion of tissue kallikrein and PGE (Overlack et al., 1979, 1980; Ogawa et al., 1985). In contrast, some authors have reported no difference in the excretion of tissue kallikrein between normotensive and hypertensive individuals (Lawton and Fritz, 1977; Zschiedrich et al., 1980; Holland et al., 1980). Uchiyama et al. (1984) found no relationship between blood pressure and tissue kallikrein excretion in a study involving 77 healthy Japanese children. The failure to demonstrate an overall difference in the excretion of urinary tissue kallikrein between 20- to 60-year-old normotensive and hypertensive patients, but a clear reduction in age-matched older hypertensive (40 years) patients led Koolen et al. (1984) to suggest that the discrepancies between the various studies could be accounted for by the heterogeneity of the populations analysed, degree of hypertensive nephropathy, and methods of measurement. A clearer relationship may become evident if subgroups of hypertensives are studied. In persons with essential hypertension with low renin levels, both active and immunoreactive urinary kallikrein are reduced (Ura et al.,

1983). In addition, these patients, together with patients with renoparenchymal hypertension, have higher proportions of a tissue kallikrein inhibitor in their kidneys or urine (Shimamoto et al., 1989).

In a survey of a large population of normal children and their mothers, there was a significant familial clustering of the excretion of urinary tissue kallikrein (Zinner et al., 1978). More recently, analysis of genetic contributions to hypertension revealed that a dominant allele that promotes high excretion of urinary tissue kallikrein may be associated with a decreased risk of essential hypertension (Berry et al., 1989). Therefore, families with a genetic predisposition to hypertension may exhibit abnormalities in the secretion of both kallikrein and kininogen and in the turnover of kinins. In fact, reduced levels of urinary kininogen and kinins have been reported in hypertensives (Shimamoto et al., 1978; Weinberg et al., 1985; Stahr et al., 1988). The decrease in kinins may be associated with increased levels of urinary kininases that are known to occur in hypertensive patients (Ura et al., 1985; Nakahashi et al., 1986).

b. GLUCOSE HOMEOSTASIS AND DIABETES MELLITUS. Renal haemodynamic differences have been reported in two models of streptozotocin-induced diabetic rats. Severely hyperglycaemic diabetic animals show increased glomerular arteriolar resistance and reduced glomerular plasma flow, filtration pressure, and single-nephron glomerular filtration rate (Hostetter et al., 1981). In contrast, moderately hyperglycaemic diabetic rats (treated with insulin) show entirely opposite effects for each of the above parameters determined in the severely hyperglycaemic animals (Hostetter et al., 1981; Harvey et al., 1990). Recent data indicate that the diabetic state suppresses tissue kallikrein expression in the kidney, whereas insulin treatment induces acutely an increase in tissue kallikrein mRNA levels (Jaffa et al., 1991). All of these findings suggest that changes in renal tissue kallikrein or renal kinin levels contribute to the haemodynamic differences observed in the two models of diabetic rats (Harvey et al., 1990; Jaffa et al., 1987; Mayfield et al., 1991). The spatial anatomical relationship that has been observed between CNT cells and the afferent arteriole in rat and human kidneys could, by implicating the kallikrein-kinin system, support such a hypothesis (Barajas et al., 1986; Vio et al., 1988). The possibility of the formation of kinins in this region is additionally supported by the finding that tissue kallikrein-containing (CNT) and kininogen-containing (CD) cells occur in close proximity to one another and the afferent arteriole (Figueroa et al., 1988).

The renal excretion of tissue kallikrein has been examined in patients with type I diabetes (Mayfield et al., 1984). The urinary excretion of the enzyme was measured in 20 type I diabetic patients and 10 normal subjects. Of these, 12, who were poorly controlled but receiving a normal 120-mEq sodium diet, showed a significantly greater excretion of kallikrein when compared either with the eight who were in good or moderately good control or with the 10 normal subjects. When the circulating glucose level was normalised in nine poorly controlled insulin-dependent diabetic patients, the urinary excretion of tissue kallikrein decreased toward the values observed in normal subjects. Furthermore, levels in the urine increased to a lesser extent in the diabetic patients than in normal subjects in response to daily low sodium in the diet (10 mEq) (Mayfield et al., 1984).

B. Sweat Gland Cells

1. Kininogenases and kininogen. a. TISSUE KALLIKREIN AND KININOGEN. The presence of a kinin-forming enzyme in normal human eccrine sweat was first reported by Fox and Hilton in 1958. A decade later, this kininogenase enzyme in sweat was characterised as a kallikrein (Fräki et al., 1970). However, these findings were questioned (Frewin et al., 1973), because of the inability to detect kinins or kininogenase activity in normal human sweat. Further differences emerged when a kininogenase, sensitive to inhibition by SBTI but immunologically distinct from plasma or tissue kallikrein, was purified from extracts of human skin (Toki and Yamura, 1979). The enzyme was a neutral, arginine esterase inhibited by α_1-antitrypsin but not by SBTI or by α_2-macroglobulin. If the enzyme was a tissue kallikrein, then the molecular mass of 104 kDa ascribed to it suggested a tetrameric structure. Using a specific RIA, Mann et al. (1980b) reported the presence of a tissue kallikrein in human sweat which was later partially purified by Hibino et al. (1988a). More recently, variations in the amount of immunoreactive tissue kallikrein have been reported in sweat collected from different regions of the body, the highest levels being found in samples obtained from the trunk and forehead (Mayfield et al., 1989).

i. Cellular localisation. Even though the presence of a bradykinin-forming enzyme was described in human sweat three decades ago by Fox and Hilton (1958), and a role had been proposed for kinins in sweat gland function, the precise segment of the gland and the cells that contained the enzyme(s) and the substrate (kininogen) necessary for the formation of kinins had not been delineated. Recently, the cellular localisation of immunoreactive tissue kallikrein and kininogen was determined in human sweat glands (Poblete et al., 1991). Tissue kallikrein gave a granular pattern of immunostaining in the acinar dark cells arranged around the lumen of the secretory unit. Other components of the secretory unit, such as the clear cells and myoepithelial cells, showed no immunostaining. Staining was also seen as an intense line along the luminal microvilli of the duct cells up to its opening on the surface of the skin. There was no evidence of specific immunostaining in the sebaceous glands, hair follicles, keratinocytes, dermal vessels, or the interstitial tissue space. Like tissue kallikrein,

an intense immunoreactivity to kininogen was observed only in the dark cells of the acinus and in the capillaries; no staining was noted either in other cells of the acinus or in the interstitial tissue space. Deep staining for kininogen was observed on the luminal surface of the microvilli of duct cells. Serial sections showed that there was a segmental distribution of the two antigens. Double immunostaining on serial sections allowed resolution of the two antigens to distinct regions of the fundus. In the junctional zone, the enzyme and substrate were each seen in separate dark cells of the same cross-sectional area of the acinus (fig. 11). A similar spatial relationship between tissue kallikrein and kininogen has been found in the human kidney (Figueroa et al., 1988). The observation that the secretory granules containing tissue kallikrein or kininogen are oriented toward the apical end of the cell suggests a primary secretion of the enzyme and substrate into the acinar lumen; the granules probably discharge by exocytosis. The structural organisation of the dark cells and intercellular tight junctions (McEwan et al., 1983) is such that, during active sweat formation, backward transport of macromolecules (Sato, 1977) into the interstitial tissue space is unlikely; indeed, no immunoreactivity to tissue kallikrein or kininogen was seen beyond the outer surface of the basal lamina of the normal secretory epithelium. During prolonged stimulation of sweating, the dark cells that cannot sustain secretion are discarded by the secretory epithelium (Montgomery et al., 1984). Disintegration of dark cells causes large intercellular gaps to appear, thereby exposing the myoepithelial cells of the secretory coil to the contractile action of kinins, in addition to the neuronally released acetylcholine. In the recovery phase following intense sweating, the dead cells are replaced by direct division of the remaining epithelial cells. The known cell proliferative property of kinins (Whitfield et al., 1970; Rixon and Whitfield, 1973) may be relevant to recovery of the secretory epithelium after a period of intense sweating. Of some relevance may be the localisation of IL-1α and -1β in the clear cells but not the dark cells of the secretory coil or the luminal cells of the coiled and straight ducts. After stressful exercise, intense staining is seen in the luminal cells of the dermal ducts (Reitamo et al., 1990). Following excessive sweating, the ILs may be involved with kinins in promoting cell differentiation. Whether such a relationship exists requires experimentation.

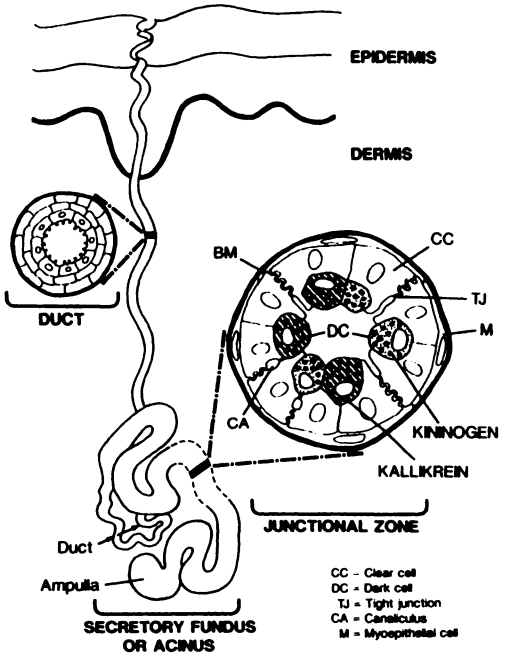

FIG. 11. Human sweat gland morphology and the spatial relationship between the cellular localisation of tissue kallikrein and kininogen. BM, basement membrane.

2. Kinins. a. ROLE OF KININS ON SWEAT GLAND CELLS. The iontophoretic application of bradykinin produces an abrupt decrease of sweat osmolality (Gordon and Schwartz, 1971). Moreover, kallidin (Lys-bradykinin) increases sodium absorption across cultured sweat epithelium in a way that seems to be independent of the generation of prostaglandins (Brayden et al., 1988). However, these effects were only observed when the peptide was added to the basolateral and not to the apical face of the monolayers (Brayden et al., 1988). During thermal stimulation of sweat glands, small clear vesicles appear on the luminal surface and constitute a resorptive mechanism for the movement of molecules to the serosal side (Montgomery et al., 1985). In addition, the intercellular spaces between the luminal cells of the duct widen during sweating. Therefore, kinins formed in the duct lumen could, after permeating to the serosal side of the luminal cells, modulate the transport of sodium and water by the gland, assuming that they survive the action of kininases. A kininase with properties similar to those of KII-ACE has been recently partially purified from human sweat by Hibino et al. (1988a).

Preference for basolateral versus apical side for bradykinin and kallidin on ion transport has also been described using isolated CCDs from rabbit kidneys. At this level, kinins inhibit sodium absorption and AVP-mediated water resorption (Schuster et al., 1984; Tomita et al., 1985). Furthermore, the formed kinins could enhance the contractile movements of the luminal microvilli of the duct cells to increase propulsion of sweat toward the epidermis.

C. Brain Tissue and Neuronal Cells

A current interest in the nervous system is the modulation of nociception and the release of excitatory amino acids by kinins. Furthermore, small amounts of bradykinin, when injected into the brain, produce a number of important physiological effects. All of the components of the tissue kallikrein-kinin system have been identified in the brain. The generation and destruction of kinins can occur in the CSF and in circumscribed regions of the

brain. The precise functional role for this system in the brain is unknown. One intrinsic function of tissue kallikrein may reside in its ability to process precursors of neuronal cell growth factors that maintain the integrity of transmitters and synaptic function. A reduction in the gene expression of the enzyme may result in the absence of a kallikrein-like moiety and contribute to Alzheimer's dementia (Aoyagi et al., 1990). Another importance may stem from the ability of kinins to release excitatory amino acids (Bhoola et al., 1987) and, should that occur in the region of a stroke, then nerve cells could be permanently damaged. The involvement of kinins in the cause of vasogenic brain oedema and neuronal cell damage merits study. So far, the clearest evidence for a primary neuronal role for kinins exists in the activation of peripherally located nociceptive receptors on C-fibre terminals that transmit and modulate pain perception.

1. Kininogenases and tissue kallikrein. Kinin-forming activity in neuronal tissue was first reported by Hori (1968) in the microsomal fractions of rabbit brain homogenates. Subsequently, regional distribution studies in the rat located the highest activity in the cerebral cortex and the lowest in the brainstem and cerebellum (Shikimi et al., 1973; Shisheva et al., 1985). In addition, a kininogenase, probably belonging to the family of acid cathepsins, has been reported (Kariya et al., 1984) to be distributed exclusively in the mitochondrial fraction and can be detected only in the acidic pH ranges, optimally at pH 3.0. The enzyme showed an inhibition profile characteristic of cathepsin D, and the kinin released from partially purified rat kininogen was found to be different from bradykinin, kallidin (Lys-bradykinin), or Met-Lys-bradykinin. More conclusive evidence confirming the presence of tissue kallikrein in the brain has been obtained following the development of specific RIAs. A higher concentration of tissue kallikrein was reported in the hypothalamus, anterior and neurointermediate lobes of pituitary, and pineal gland of the rat (Powers and Nasjletti 1983, 1984; Scicli et al., 1984; Chao et al., 1987). In the rat pineal gland, immunoreactive tissue kallikrein appears to be localised in perivascular and vascular walls rather than in the pinealocytes (Kitagawa et al., 1991). Lower concentrations have been detected in other sites such as cerebral cortex, brainstem, and cerebellum (Scicli et al., 1984; Chao et al., 1987). Immunocytochemical studies of the rat hypothalamus have shown immunostaining in the ependymal cells lining the third ventricle as well as in cell bodies of arcuate, supraoptic, paraventricular, and ventromedial nuclei (Simson et al., 1985). Specific cDNA probes have been used to detect tissue kallikrein mRNA in the hypothalamus, cerebral cortex, cerebellum, brain stem, and pineal gland; no sex differences were observed in any of the brain regions studied (Chao et al., 1987).

Kininogenase activity also has been reported in human CSF (Scicli et al., 1984). The kininogenase activity was augmented two- to threefold by preincubation with immobilised trypsin and was in part resistant to inhibition by SBTI. In addition, the kininogenase activity detected in untreated CSF was completely inhibited by SBTI (Scicli et al., 1984). The inability of SBTI to inhibit the kininogenase activity after trypsin preincubation suggests that a tissue kallikrein-like enzyme is present in normal human CSF.

2. Kininogen. There have been only a few reports concerning the identification of endogenous kininogen in the CSF and brain. The presence of kininogen has been reported in human CSF; RIA was used to measure kinins generated after treatment of the CSF with an excess of trypsin (Scicli et al., 1984). The presence of the kinin precursor was first reported in homogenates of perfused brain depleted of circulating kininogens (Shikimi et al., 1973). The authors measured the kininogen content of the brain in terms of kinins formed from it after treatment with trypsin and reported that the kininogen content of the cerebral cortex was one-third of that in the cerebellum or brainstem. In contrast, kininogen measured by bioassay methods was almost exclusively found in the hypothalamus and lower brainstem (Pela et al., 1975). Also, it was found in the culture medium of mouse neuroblastoma × glioma hybrid cells that secrete a tissue kallikrein-like enzyme (Chao et al., 1985). More recently, immunoreactive rat HK has been observed in neurones of the hypothalamic periventricular area, projecting to the external zone of the median eminence, whereas T-kininogen immunostaining was restricted to the magnocellular neurones of the paraventricular and supraoptic nuclei and to parvocellular neurones of the suprachiasmatic nucleus (Richoux et al., 1991). T-kininogen was also identified in hypothalamic (10 ng/mg protein) and cerebellar (5.3 ng/mg protein) homogenates (Richoux et al., 1991). Comparative studies are clearly necessary to identify and to delineate the cellular localisation of the precise kininogen moieties in the mammalian brain.

3. Kininases. Kininase activity has been identified in the brain of mammals (see section III.C.3). Several authors have reported that the kininase activity is higher in the supernatant or soluble fractions of brain homogenates (Hori, 1968; Camargo and Graeff, 1969; Iwata et al., 1969). Two neutral endopeptidases that selectively hydrolyse kinins were partially purified by Camargo et al. (1972) from the supernatant fraction of rabbit brain homogenates. One enzyme (kinin-converting aminopeptidase) converted Lys-bradykinin and Met-Lys-bradykinin into bradykinin. This conversion was inhibited by puromycin, and the properties of this enzyme were similar to those of brain arylamidases. A second enzyme, KII-NEP (enkephalinase), which inactivates kinins by removing the COOH-terminal Phe^{8}-Arg^{9} dipeptide (Gafford et al., 1983), is present in high concentrations in the brain (Schwartz et al., 1981). However, KII-NEP and

KII-ACE inhibitors did not seem to potentiate the effects of kinins on cerebral vessels (Whalley and Wahl, 1983), suggesting that probably other kininases are involved. Of relevance is the description by Bausback and Ward (1988) of a membrane-bound carboxypeptidase in purified cerebral microvessels. The enzyme hydrolysed the COOH-terminal Phe^8-Arg^9 bond of both bradykinin and Lys-bradykinin and showed characteristics similar to those of KI-CPN. The activity of this enzyme could result in the formation of desArg^9-bradykinin.

Two additional endopeptidases, designated kininase A and B, have been purified from brain tissue (Camargo et al., 1973). Kininase A (metalloendopeptidase 24.15) showed a molecular mass of approximately 71 kDa and hydrolysed the Phe^5-Ser^6 peptide bond of the peptide. The other enzyme, kininase B, a dipeptidyl peptidase with a molecular mass of approximately 68 kDa, hydrolysed the Pro^7-Phe^8 peptide bond. Both kininases were activated by dithiothreitol and inhibited by *p*-chloromercuribenzoate (Camargo et al., 1973; Oliveira et al., 1976). Even though brain kininase A and B individually hydrolyse Phe^5-Ser^6 and Pro^7-Phe^8 they are distinct from KII-ACE, which has the capacity to split both of these bonds sequentially from the COOH terminus but, unlike KII-ACE, are unable to further hydrolyse the remaining peptide or to convert angiotensin I to angiotensin II. Wilk and Orlowski (1979) isolated a prolyl endopeptidase from rabbit brain that cleaved bradykinin at the Pro^7-Phe^8 and Pro^3-Gly4 bonds. [^{3}H]Bradykinin was degraded by an enzyme similar to kininase A that splits the Phe^5-Ser^6 bond when incubated with rat hypothalamic slices (McDermott et al., 1987) .

Determination of the regional distribution of kininase activity shows higher activity in the cerebellum than in either the cerebral cortex or the brainstem of rats. The specific activities in the cerebellum were about two- to threefold that of the cerebral cortex, which showed the lowest activity in the brain. Similar activity levels were found in the midbrain, hippocampus, medulla oblongata, and hypothalamus, but less activity was observed in the spinal cord (Roth et al., 1969; Cushman and Cheung 1971; Yang and Neff, 1972; Kariya et al., 1981a,b; Elrod et al., 1986). In contrast, in the human brain, the lowest activity was found in the cerebellum (Poth et al., 1975). The time course and nature of the action of kinins on cerebral vessels may be modified by the presence of KII-ACE in cerebral microvessels (Churchill et al., 1987).

4. Kinins. Attempts to identify endogenous kinins in the brain have been frustrated by both the presence of kininases and the lack of specificity in the assays. For some time, bioassays of brain extracts on smooth muscle preparations suggested the presence of kinins (Inouye et al., 1961; Hori, 1968; Werle and Zach, 1970; Pela et al., 1975). More recently, immunoreactive bradykinin was visualised in neuronal bodies and fibres of the hypothalamus, with especially dense clusters overlying the periventricular and dorsomedial nuclei (Correa et al., 1979). Staining of fibres and varicose processes was observed in the periaqueductal grey matter, hypothalamus, perirhinal and cingulate cortices, ventral portion of the caudate-putamen, and lateral septal area. No bradykinin-like immunoreactivity was seen in the spinal cord at middle cervical and upper cervical regions (Correa et al., 1979). Unfortunately, these studies provide no data concerning the cross-reactivity of the antibodies with kininogen which contains antigenic determinants for bradykinin.

Perry and Snyder (1984) purified bradykinin from acetic acid extracts of saline-perfused rat brains by gel filtration chromatography and reverse phase high-performance liquid chromatography. Levels of 0.6 pmol/g whole rat brain were detected, with eight times higher levels in the hypothalamus. Detectable levels of immunoreactive bradykinin were also found in cerebral cortex, cerebellum, pons, corpus striatum, hippocampus, and spinal cord. A similar study was carried out by Kariya et al. (1985) who found the highest content in the pituitary gland, followed by the medulla oblongata, cerebellum, and cortex. In the pituitary, the kinins were much more concentrated in the posterior lobe (neural) than in the anterior lobe. Furthermore, pituitary kinins were separated into bradykinin (87%), Lys-bradykinin (10%), and Met-Lys-bradykinin (3%).

Recently, Elrod et al. (1986), using focused microwave irradiation to inactivate brain kininases and kallikrein, reported the presence of T-kinin (Ile-Ser-bradykinin; Greenbaum, 1986) in rat brain homogenates. The authors suggested that the cerebral blood supply was the most likely source of T-kinin, because removal of cerebral blood prior to extraction resulted in samples containing only bradykinin.

a. KININ RECEPTORS. A saturable binding of [^{3}H]bradykinin to membranes prepared from whole rat and guinea pig brain and bovine cerebellum was first reported by Innis et al. (1981). Bradykinin receptors were later localised in guinea pig and canine spinal cord and sensory ganglia by autoradiographic techniques (Manning and Snyder, 1983). More recently, binding sites have been identified on sensory C-fibres, neurones of the dorsal root and trigeminal ganglia, and sites in the substantia gelatinosa (Steranka et al., 1988).

Tissue culture systems have been used successfully to demonstrate and characterise kinin receptors and to obtain information regarding the molecular events that follow the kinin-receptor binding. Specific high-affinity-binding sites have been characterised in murine neuroblastoma clone N1E-115 and in cultured brain cells from neonatal rats (Snider and Richelson, 1984; Lewis et al., 1985). Additional studies performed on neuroblastoma × glioma hybrid NG 108–15 cells have shown that these cells have bradykinin receptors (Reiser et al., 1984) and respond to bradykinin by a transient cell hyperpolarisa-

tion followed by a long-lived depolarisation (Reiser and Hamprecht, 1982). In these cells, bradykinin also stimulates phosphatidylinositol turnover (Yano et al., 1984) and produces a large, rapid and calcium-dependent increase in cGMP (Snider and Richelson, 1984). Furthermore, bradykinin elicits a transient increase of the cytosolic Ca^{2+} activity in a dose-dependent manner in mouse neuroblastoma × glioma hybrid cells and polyploid rat glioma cells (Reiser and Hamprecht, 1985).

b. PHARMACOLOGICAL RESPONSES TO KININS INJECTED INTO THE CEREBRAL VENTRICLES. Even though the biological half-life of bradykinin administered intracerebrally in the conscious rat is about 27 s (Kariya et al., 1982b), small doses of bradykinin injected intraventricularly in the brain produce a variety of central actions in different experimental animals (Clark, 1979). Usually one observes an increase in systemic blood pressure and a biphasic behavioural change consisting of short-lasting excitation followed by sedation (Graeff et al., 1969; Okada et al., 1977; Kariya et al., 1982a), electroencephalogram alterations (Pearson et al., 1969; Kariya and Yamauchi, 1981), hyperthermia (Almeida e Silva and Pela, 1978), analgesia (Ribeiro et al., 1971), and vasopressin release (Rocha e Silva and Malnic, 1964; Hoffman and Schmid, 1978).

i. Behavioural responses. Injection of bradykinin into a lateral cerebral ventricle depresses neuronal activity prior to the development of catatonia (Graeff et al., 1969; Moniuszko-Jakoniuk and Wisniewski, 1974). The sedatory effect is often preceded by a short period (2 to 5 min) of excitation (Graeff et al., 1969; Lambert and Lang, 1970; Okada et al., 1977; Kariya et al., 1982a). Several studies have reported changes in electroencephalogram patterns associated with the behavioural responses (Graeff et al., 1969; Pearson et al., 1969; Kariya and Yamauchi, 1981). A similar effect was produced by intracarotid injections of bradykinin in anaesthetised cats (Pearson et al., 1969).

ii. Effects on brain amines. Injections of bradykinin into the ventricles causes a significant decrease in mouse brain levels of noradrenaline (Capek et al., 1969) which is probably due to an enhanced release of the amine into the circulation (Benetato et al., 1967). The work of Pirola et al. (1987) suggests that both 5-HT and adrenaline are involved in the mediation of the pressor effect of central bradykinin. Evidence exists for the alteration of brain dopamine and 5-HT levels in response to bradykinin injection, but conflicting data from different species mean that the overall significance of these observations is not known (Graeff et al., 1969; Przesmyckia and Kleinrok, 1976).

iii. Vascular responses. Substantial evidence indicates that intraventricular injection of bradykinin is followed by a dose-dependent increase in blood pressure accompanied by tachycardia (Diz and Jacobowitz, 1984; Pearson et al., 1969). This centrally mediated effect appears to be primarily due to cardiac stimulation, although an increased sympathetic tone to blood vessels has also been reported. Correa and Graeff (1974) demonstrated an analogous, although transient, response to kallidin administration. Bradykinin injection into several brain sites showed that the lateral septal region is a relatively specific site for the pressor action of bradykinin (Correa and Graeff, 1975). It is thought that the lateral septal area is responsible for the integration of certain behavioural patterns and for the adjustment of arterial blood pressure connected with these behavioural responses (Pirola et al., 1987). Several workers have correlated the changes in behaviour or electroencephalograph seen after bradykinin administration with alterations in blood pressure (Kariya et al., 1982a).

Recent work by Kariya and Yamauchi (1987) demonstrated that the hypertensive response to kinin could be induced by an intraventricular injection of tissue kallikrein and that brain kinin levels increased during hypertension. No effect on blood pressure was seen in the presence of the kallikrein inhibitor aprotinin, suggesting that the hypertensive action is mediated via bradykinin liberated from a kininogen in the brain. In addition, Ile-Ser-bradykinin (T-kinin) produces a similar increase in the mean arterial blood pressure when injected intravenously (Lindsey et al., 1989).

The mechanism underlying the pressor effect of centrally administered bradykinin appeared to involve both prostaglandins and adrenoceptors (Lambert and Lang, 1970; Correa and Graeff, 1974; Thomas et al., 1987). The use of indomethacin and phentolamine to inhibit one or the other of these systems led to the suggestion that immediate blood pressure responses to bradykinin were mediated by noradrenaline, with a secondary, more potent, effect dependent upon the production of prostaglandins (Kariya et al., 1982a; Takahashi and Buñag, 1981). In fact, the pressor response to bradykinin was blocked by the BK2 receptor antagonist, D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe]-bradykinin but not by the BK1 receptor antagonist desArg^9-[Leu^8]-bradykinin (Lindsey et al., 1989). This finding would be difficult to reconcile with the action of bradykinin being secondary to the release of noradrenaline and prostaglandins.

iv. Antidiuretic effects. In concert with the pressor response to bradykinin, a reduction in urine flow has been reported in the rat (Rocha e Silva and Malnic, 1964; Harris, 1971; Hoffman and Schmid, 1978). These were abolished by median eminence lesions, suggesting the involvement of AVP. Recent work has shown that intracerebroventricular administration of bradykinin (1 to 20 μg) into conscious rats results in significant dose-dependent increases in the plasma AVP concentration together with an increase in mean arterial blood pressure and heart rate (Brooks et al., 1986).

c. PHARMACOLOGICAL RESPONSES OF BRAIN BLOOD VESSELS. In addition to centrally mediated constrictor

effects on the peripheral vasculature, bradykinin causes direct dilation of cerebral arterioles in a wide variety of species including humans, rabbit, and cat (Toda, 1977; Whalley et al., 1983; Wahl et al., 1983). Analogous effects can also be produced by tissue kallikrein, indicating that endogenous production of kinins from brain kininogen can occur (Kamitani et al., 1985). Furthermore, a kinin antagonist reduced the dose-dependent vascular dilation produced by bradykinin (Ellis et al., 1987). A role for kinins during the development or enhancement of vasogenic brain oedema has been postulated (Maier-Hauff et al., 1984; Unterberg and Baethmann, 1984). Decreased formation of posttraumatic swelling, following a standardised cold lesion to the brain, has been demonstrated by the selective use of inhibitors of kallikrein (aprotinin) (Unterberg et al., 1986) and kinins (Arg-Pro-Pro-Gly-Thi-Ser-D-Phe-Thi-Arg) (Ellis et al., 1987).

d. NOCICEPTIVE ACTIONS OF KININS. Of the known autacoids, kinins are the most potent in producing pain. When bradykinin is applied to a blister base evoked on human skin, it causes a burning sensation (Armstrong et al., 1957). The painful sensation has a latency of 30 to 120 s and persists for at least 75 to 150 s (Bhoola, 1961). Dose dependency is usually difficult to assess because of tachyphylaxis. The subcutaneous injection of formalin in the rat produces a prolonged activation of nociceptive neurones receiving sensory information from the appropriate receptive field. Following desensitisation of the bradykinin receptors or the prior injection of a bradykinin receptor antagonist (D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin) into the cutaneous field, formalin fails to fire the dorsal horn neurones (Haley et al., 1989). In addition to activation of cutaneous receptors, kinins are known to stimulate visceral chemosensory nerve endings (Lew and Longhurst, 1986; Ammons, 1988). Mice respond rapidly after intraperitoneally injection of bradykinin and display writhing movements (Emele and Shanaman, 1963, 1967) that are inhibited by either narcotic or anti-inflammatory analgesics (Collier et al., 1968). Retrograde intraarterial injection of bradykinin, depending on the depth of anaesthesia, activates vascular sensory receptors that produce reflexly mediated flexion of extremities and vocalisation in animals (Riccioppo Neto et al., 1974). Prostaglandins appear to potentiate the algesic effects of bradykinin in humans (Ferreira, 1972) and cardiovascular reflexes in dogs (Ferreira et al., 1973) and in the isolated perfused rabbit ear (Juan and Lembeck, 1974). Evidence suggests that prostaglandins and the thromboxanes may be even more potent than the classical endoperoxides in potentiating the algesic actions of bradykinin (Vane, 1978).

It is conceivable, therefore, that kinins generated in injured or inflamed tissue activate receptors that transmit, via C and A afferent fibres, nociceptive information to lamina V of the spinal cord. After integrative summation, nociception is relayed through bulbospinal tracts to the brainstem and higher centres. The presence of bradykinin receptors in the central nervous system poses the essential problem of the manner in which such receptors can be activated by kinins which, so far, have not been identified in nerve endings or synaptic junctions. In the superior cervical ganglion, kinins depolarise the postganglionic but not the preganglionic sympathetic trunk (Bhoola et al., 1987). Application of bradykinin to the hemisected spinal cord of the immature rat depolarises the primary afferent terminals to release excitatory amino acids (Bhoola et al., 1987). Excitatory amino acid transmitters seem to play an important role in the spinal processing of primary afferent nociceptive input, because central integrative processes involve their release from primary C-fibres (Evans, 1989). In contrast, intrathecal administration of bradykinin exerts an antinociceptive action by the presynaptic release of noradrenaline from bulbospinal terminals (Laneuville et al., 1989). Although neuronal kinin receptors have been reported and the application of bradykinin produces neuronal effects, there is as yet no evidence for the formation of kinins at synaptic junctions.

At the molecular level, stimulation of kinin receptors on sensory neurones (dorsal root ganglion) activates phospholipase C which results in the formation of inositol triphosphate and diacylglycerol; furthermore, stimulation of phospholipase A_2 causes the release of arachidonic acid and formation of prostaglandins. The overall neuronal excitatory effects of bradykinin on dorsal root ganglion cells are probably due to an increase in intracellular calcium and, through a release of eicosanoids and further production of cAMP, an increase in potassium conductance. Kinins appear to be the only peptides that seem to recruit all the known second messengers in the mediation of their cellular actions.

D. Prolactin-secreting Cells

1. Kininogenases. a. TISSUE KALLIKREIN-LIKE PROTEASE. An enzyme similar to tissue kallikrein was first identified in porcine anterior pituitary and rat neurointermediate lobe by Powers and Nasjletti (1982, 1983). In more detailed studies in the rat, the enzyme was found in the anterior and intermediate lobes of the pituitary, with females showing levels that were 20 greater than those in the male (Powers and Nasjletti, 1984; Powers, 1986; Hatala and Powers, 1988). No change in enzyme levels was observed during the various phases of the oestrous cycle. However, a rapid increase was measured on the day 15 of pregnancy, with levels only returning to normal values by day 5 of lactation (Powers and Westlin, 1987). The tissue kallikrein, induced by oestrogen and inhibited by dopamine, showed, concomitantly with an increase in prolactin, a dramatic (up to 20-fold) increase in females achieving puberty; no appreciable change was noticed in the male (Hatala and Powers, 1988). Although tissue kallikrein in the anterior and intermediate lobes

appeared to be identical with urinary kallikrein, only the enzyme in the anterior lobe was regulated by oestrogens (Chao et al., 1987). The oestrogen induction was highly sensitive to the action of tamoxifen (a triphenylethylene antioestrogen) (Powers et al., 1989).

i. Enzymic properties. Subcellular experiments indicated that tissue kallikrein in the pituitary was mainly sequestered in organelles derived from the transcisterna of the Golgi apparatus, with >90% in the pro form (Hatala and Powers, 1989). The latent enzyme was activated fivefold when incubated with trypsin. Extracts of the rat neurointermediate lobe also contain thrombin-like proteases in a proenzymic form (Powers, 1986). Another serine protease, reactive toward synthetic fluorogenic tripeptide substrates containing basic amino acids (bz-Ala-Lys-Arg-methylcoumarin amide), was isolated from the pituitary. It resembled the tryptases from the lung, skin, and pulmonary mast cells (Cromlish et al., 1987). The identity, or otherwise, of this protease to tissue kallikrein remains to be determined.

ii. Oestrogen dependence. The control of kallikrein gene expression in the rat anterior pituitary was studied with a rat pancreatic tissue kallikrein cDNA probe. Very much higher levels of mRNA were coded for in the female than the male, with levels declining markedly after ovariectomy. Oestradiol injections restored expression in the ovariectomised female and increased values in the male. Clearly, the tissue kallikrein gene in the anterior pituitary shows a marked oestrogen dependence (Fuller et al., 1985; Clements et al., 1986, 1989; Chao et al., 1987). High levels were recorded in oestrogen-induced prolactin tumours (Powers, 1987). The question whether kallikrein is under dopaminergic control was examined in male rats in which haloperidol (a dopamine receptor antagonist) was injected for about 6 days. Tissue kallikrein levels doubled in these animals, indicating that synthesis of the enzyme was restrained by dopaminergic nerves (Powers, 1986). A marked attenuation of the oestrogen-induced increase in tissue kallikrein in the gland was observed following treatment with the dopamine agonist, bromocriptine (Powers and Hatala, 1986).

Administration of diethylstilboestrol to F344 rats induces lactotroph hyperplasia, increases in tissue kallikrein, and the formation of prolactin-secreting pituitary tumours (Powers, 1987; Hatala and Powers, 1988). In these tumours the increase in prolactin gene expression was paralleled by an increase in tissue kallikrein (Fuller et al., 1985; Clements et al., 1986). Although the 240-fold increase in tissue kallikrein plateaued after 5 weeks, synthesis of prolactin continued to increase beyond that point (Hatala and Powers, 1988). The reason for terminating the synthesis of tissue kallikrein is not known. The enhanced levels of tissue kallikrein and of the prolactin mRNA are partially reversed when bromocriptine is given in association with oestrogen withdrawal (Fuller et al., 1988). The finding that oestrogens enhance and bromocriptine inhibits tissue kallikrein gene expression in the anterior pituitary certainly merits further investigation, and this phenomenon may account for the ability of bromocriptine to shrink human prolactinomas.

iii. Cellular localisation. The rat pituitary GH3 cell line synthesises and secretes tissue kallikrein concomitantly with prolactin and growth hormone (Chao and Chao, 1988b). Subsequently, prolactin and tissue kallikrein were colocalised in the rat anterior pituitary (Vio et al., 1989, 1990; Kizuki et al., 1990). A simultaneous study identified immunoreactive tissue kallikrein in human prolactin-secreting adenomas in which the enzyme was colocalised with prolactin (Bhoola et al., 1990; Jones et al., 1990). All of the human tumours revealed a high percentage of prolactin-immunoreactive cells that corresponded with high levels of circulating prolactin. Strong immunoreactivity to tissue kallikrein was visualised as a cap over the nucleus which, at higher magnification, gave a cisternal appearance (Jones et al., 1990). An identical localisation in the Golgi complex was observed in subcellular experiments by Hatala and Powers (1989). Both the hormone and the enzyme colocalised in the same cells of normal anterior pituitary tissue and the adenomas. No immunostaining to tissue kallikrein was observed in null cell adenomas that did not contain immunoreactive prolactin. So far, in nine of 11 purely growth hormone-staining human pituitary adenomas, no immunoreactivity to tissue kallikrein has been observed; in the remaining two, only very patchy staining was observed. No immunostaining of any other anterior pituitary hormone was observed in the human prolactin-secreting adenomas (Jones et al., 1990). Clearly, the only association of tissue kallikrein in the human anterior pituitary is with lactrotophs (Jones et al., 1991).

iv. Functional importance. The function of tissue kallikrein in the normal lactotroph, as well as in prolactin-secreting adenomas, is not fully understood. Three possible actions come to mind: (*a*) the enzyme may act as a growth hormone-processing molecule by producing an enzymically active form of the epidermal growth factor from its precursor (Isackson et al., 1987a); epidermal and other growth factors known to occur in the pituitary could modulate cell proliferation; (*b*) the morphological appearance of the immunostaining strongly suggests that tissue kallikrein is located mainly in the Golgi cisternae of prolactin-secreting cells. Such an intracellular localisation may relate to the function of converting the precursor forms of prolactin to the active molecule or, alternatively, (*c*) kallikrein may degrade stores of prolactin produced in excess. At least three possible cleavage sites with paired basic amino acids have been identified in the prolactin molecule. The conversion of the parent prolactin molecule to novel forms by the tissue kallikrein was reported recently by Powers and Hatala (1990).

However, it is conceivable that the kininogenase func-

tion of tissue kallikrein is of primary importance in this tissue. The release of tissue kallikrein from prolactinoma cells, resulting in the formation of kallidin, may induce the paracrine secretion of prolactin. In fact, both kallidin and bradykinin (Jones et al., 1989) stimulate the release of prolactin from normal rat pituitary cells in primary culture, with kallidin approximately 10 times more potent than bradykinin (T. H. Jones, B. L. Brown, and P. R. M. Dobson, unpublished results). Furthermore, both kinins stimulate phosphotidylinositol turnover in rat anterior pituitary cells (Jones et al., 1989). There may be an important link between phosphoinositide turnover and cell growth; kinins stimulate DNA synthesis and cell division (Whitfield et al., 1970; Rixon and Whitfield, 1973; Owen and Villereal, 1983; Tilley et al., 1987), thereby promoting the proliferation of lactotrophs.

Angiotensin II also stimulates prolactin release (Schramme and Denef, 1983), and acts as a pituitary paracrine agent mediating gonadotrophin-releasing hormone-stimulated prolactin secretion (Jones et al., 1988). In addition, angiotensin II evokes prolactin release from human prolactinomas (T. H. Jones, unpublished observations). Interactions may, therefore, occur between angiotensin II and kallidin in the modulation of prolactin secretion.

E. Cardiac Myocytes

Many hormone-synthesising cells seem to contain a serine protease with enzymic properties very similar to those of tissue kallikrein. The importance of the heart as an endocrine organ was realised only about a decade ago. Although several morphological studies pointed to the presence of secretory granules in mammalian atrial myocytes (Kisch, 1956; Jamieson and Palade, 1964; Dorey et al., 1974), it was de Bold et al. (1978) who saw the similarity between atrial myocyte and endocrine cell granules and had the vision to suggest that the atrial granules contained a hormone. Soon afterward, the function of the atrial hormone was described (de Bold, 1979; de Bold et al., 1981; Garcia et al., 1982) and the chemical structure of the atrial natriuretic peptides determined (Kangawa et al., 1985).

1. Kininogenases. The first indication of a kininogenase with an acid pH optimum came from enzymic studies of extracts of human, rat, and dog hearts (Britos and Nolly, 1981; Nolly et al., 1981). At least three kininogenases have been identified: a pepstatin-sensitive, aspartic protease enzyme, an arginine esterase A, and a tissue kallikrein (Zeitlin et al., 1989; Simson et al., 1989; Xiong et al., 1990b). A tissue kallikrein with esterolytic and kinin-forming activities has been isolated from the rat heart. The cardiac enzyme was identified as a serine protease with a molecular mass of 38 kDa and was immunologically identical with tissue kallikrein. The enzyme was localised in the sarcoplasmic reticulum and granules of the atrial myocytes by immunoelectron microscopy. Expression of the tissue kallikrein mRNA in rat heart myocytes was confirmed with a specific cDNA probe (Xiong et al., 1990b). The functional importance of the enzyme in the heart is a matter for debate. The in vitro conversion of pro-ANF to ANF by tissue kallikrein (Currie et al., 1984) lends some credence to the view that it may be one of a family of enzymes that process pro-ANF (Seidah et al., 1986; Wypij and Harris, 1988). The conversion in vivo is believed to occur during or just after secretion of the peptide hormone (Thibault et al., 1985; Schwartz et al., 1985; Sugawara et al., 1985).

2. Kinins. a. EXCITATION OF EPICARDIAL AFFERENT TERMINALS. The application of bradykinin to the epicardium of several mammal species excites some of the unmyelinated and myelinated afferent terminals of the spinal sympathetic nerves. Impulses generated in the terminals cause reflex tachycardia and an increase in blood pressure (Uchida and Murao, 1974; Staszewska-Barczak et al., 1976; Baker et al., 1980). Activation of vagal terminals produces a reflex bradycardia and a decrease in blood pressure (Neto et al., 1974; Lombardi et al., 1982). When porcine pancreatic kallikrein was applied to the surface of the heart, like bradykinin, it elicited reflex cardiovascular effects, and these effects were blocked by aprotinin (Staszewska-Woolley and Woolley, 1989). This action of kallikrein was considered to be due to the release of kinin from kininogen in the heart muscle. The reflexogenic responses of bradykinin, kallidin, and kallikrein applied to the epicardium were either abolished or attenuated by the BK2 receptor antagonist, D-Arg[Hyp^3,Thi^{5,8},D-Phe^8]-bradykinin. The inhibition by the BK2 antagonist was specific and did not affect the nerve terminal activation by capsaicin or potassium chloride (Staszewska-Woolley and Woolley, 1989).

b. EFFECT OF KININS ON CORONARY VASCULATURE AND CIRCULATION. Kinins dilate coronary arteries in isolated hearts and in vivo (Maxwell et al., 1962; Antonio and Rocha e Silva, 1962; Montague et al., 1963; Parratt, 1964). Increase in coronary circulation produced by bradykinin in isolated, perfused guinea pig hearts was inhibited by D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]bradykinin in a dose-dependent manner, whereas the BK1 receptor antagonist, desArg^9[Leu^8]-bradykinin, was ineffective. Of interest was the failure, in these experiments, of acetylcholine, vasoactive intestinal peptide, and calcitonin gene-related peptide to significantly influence, even in large doses, coronary blood flow (Barton et al., 1989). Isolated human coronary arteries relaxed to bradykinin, histamine, and substance P through the release of EDRF and formation of cGMP but showed virtually no response to acetylcholine or 5-HT.

At the cellular level, the dilation of arterial smooth muscle by bradykinin is mediated by the release of prostaglandins and/or EDRF; the primary mediator involved depends on the species (Terragno et al., 1975; Crutchley

et al., 1983; de Nucci et al., 1988). The cyclooxygenase inhibitor, indomethacin, inhibits the vasodilatory effect of bradykinin on the coronary circulation of the isolated perfused rabbit heart (Needleman et al., 1975). Endothelium removal from the dog coronary artery affects the kinin relaxation response (Cherry et al., 1982). Experimentally induced phasic contraction of the coronary artery indicated a mechanism different from that involved in vascular relaxation. Phasic coronary vasospasm created by blocking K^+ conductances (3,4-diaminopyridine) was inhibited by bradykinin and by calcium channel blockers. This effect of bradykinin seemed to be mediated through an increase in K^+ conductance.

c. CARDIOPROTECTIVE ACTIONS OF KININS. Kinins increase heart rate, cardiac output, and oxygenation of the heart (Maxwell et al., 1962; Parratt, 1964; Lochner and Parratt, 1966). Coronary artery perfusion, of either the isolated rat heart or the exposed dog heart (in situ) in which the vessels had first been subjected to localised ischaemia by the application of a temporary clip on one of the major coronary vessels, with bradykinin (1 fM to 10 nM) showed a significant reduction in the incidence and duration of reperfusion arrhythmias. Cardiac function and metabolic parameters (ATP, creatine phosphate, and glycogen levels) improved during reperfusion, together with a reduction in lactate dehydrogenase, creatine kinase, and lactate in the coronary venous perfusate. A similar effect was observed with KII-ACE inhibitors, administered to the animal before removal of the heart or perfused either prior to, during, or after the ischaemic injury. The beneficial effects of bradykinin and KII-ACE inhibitors were reversed by the kinin antagonist, D-Arg[Hyp^2,Thi^{5,8},D-Phe^7]-bradykinin. Following coronary perfusion of the heart with angiotensin I or II, the duration of the postischaemic reperfusion arrhythmias increased and cardiac function and metabolic parameters deteriorated (Linz et al., 1986; Schölkens et al., 1987; Linz et al., 1990).

2. Functional importance in myocardial ischaemia and infarction. Arrhythmias and shock are major causes of death following myocardial infarction. Several studies appear to indicate a primary role for kinins in poorly perfused or damaged cardiac muscle. There is consensus in the literature with regard to a decrease in circulating levels of kininogen (Sicuteri et al., 1967; Wiegershausen et al., 1970; Kedra et al., 1973; Hashimoto et al., 1978) and prekallikrein (Sicuteri et al., 1972; Kolber-Postepska, 1975; Gomazkov, 1974) following myocardial infarction. Some evidence suggested that patients with cardiogenic shock deteriorated more quickly when shock was accompanied by reduced kininase levels. Although no clear correlation was observed, this latter aspect should be reinvestigated with the new, specific methods available for the measurement of kallikrein, prekallikrein, and the kininogens.

Plasma levels of kinins increase in some patients with acute myocardial infarction simultaneously with a decrease in blood pressure (Hashimoto et al., 1978); this finding was confirmed by Ando and Shimamoto (1983). A considerable increase in kinin levels appeared to occur in the coronary sinus after acute myocardial ischaemia (Kimura et al., 1973; Matsuki et al., 1986). Although a likely source of the kinin-forming enzymes may be from ischaemic or infarcted muscle or even neutrophils that have migrated to the damaged site, their origin and mechanism of formation in cardiac pathology remains uncertain.

F. Spermatozoa, Sertoli Cells, and Epithelial Cells of the Prostate, Epididymus, and Coagulating Glands

1. Kininogenases. a. TISSUE KALLIKREIN-LIKE PROTEASES. The impetus for kallikrein research in the reproductive tract came from the finding of a potent kininogenase in the coagulating gland, seminal vesicles, and prostate of the guinea pig but not in the testis or ovary. The enzyme released a peptide with a pharmacological profile identical with that of bradykinin from crude kininogen preparations and the plasma of several mammalian species (Bhoola et al., 1962). The kininogenase was purified and characterised by Moriwaki and colleagues (1974, 1975b). Comparison of the coagulating gland enzyme, when purified to homogeneity, with that from the SMG revealed differences in their amino acid composition and in their ability to hydrolyse synthetic substrates. The SMG enzyme was named tissue kallikrein I and the coagulating gland/prostate protease was called tissue kallikrein II (Mayer et al., 1989).

The importance of kallikreins and kinins in male reproductive function began to be realised when Schill and Haberland (1974) reported that porcine pancreatic kallikrein stimulates the motility of human ejaculated spermatozoa. The first identification of a tissue kallikrein in porcine and human seminal plasma was by RIA (Fink et al., 1979a,b; Mann et al., 1980b). The purified enzyme resembled tissue kallikrein in its properties (Geiger and Clausnitzer, 1981). Measurement of the enzyme by RIA presented problems including nonlinearity of dose-response curves when compared to standards. The reason for the difficulty was attributed to the formation of complexes having a molecular mass of about 72 to 80 kDa between tissue kallikrein and α_1-proteinase inhibitor (Fink et al., 1985). Levels of α_1-proteinase inhibitor, which is considered to be the main inhibitor affecting the activity of the enzyme in a time-dependent manner, seem to be about 20 times lower in seminal plasma than in serum (Schill, 1976). The prostate was considered to be the most probable source of the enzyme (Fink et al., 1985). Kallikrein enzyme in semen is considered to be derived from two sources, namely, the testis and the prostate. Because of local synthesis of kallikrein by Sertoli cells, a 10-fold greater concentration of the enzyme was detected in the testicular semen than in the

blood of normal volunteers. Levels in testicular semen of patients with azoospermia showed a twofold increase compared with normal men (Saitoh et al., 1987). The reason for the enhanced synthesis is unclear but could be a secondary response to improve the functional viability of the reduced number produced by the spermatogonia.

Another kininogenase contained within the acrosome, located on the head of spermatozoa, has been identified in human seminal plasma (Schill et al., 1976; Palm et al., 1976). This sperm-specific proteinase is called acrosin (EC 3.4.21.10). The enzyme probably exists in the acrosome of the sperm as a complex with its natural inhibitor, acrostatin. Enzyme-inhibitor complexes occur in the seminal plasma.

i. Cellular localisation. Luminal epithelial cells show immunoreactivity to the coagulating gland kininogenase (tissue kallikrein II) (Schachter et al., 1978). Tissue kallikrein II is mainly concentrated in the Golgi area of these cells (C. D. Figueroa and K. D. Bhoola, unpublished); a similar appearance was observed in the prolactin-secreting cells of the anterior pituitary (Jones et al., 1990). In human male genital organs, the enzyme was localised with antibodies raised to human urinary kallikrein. Specific staining for the tissue kallikrein was recorded in the Sertoli cells of the testis, epithelial cells of the epididymis, and the adenocytes of the prostate (Saitoh et al., 1987; Kumamoto et al., 1989). The presence of the enzyme in the Sertoli cells of the seminiferous tubules suggests a functional role in spermatogenesis. Localisation in the epididymis may relate to the maturation that sperm undergo in this organ. Although at least two genes appear to regulate the synthesis of kininogenases in the prostate (PSA and hGK-1 in humans and PI and S3 in the rat), so far, expression of true tissue kallikrein has not been observed in the human or rat prostate. The question of the occurrence of true tissue kallikrein in the human prostate remains an enigma. However, prostatic proteases are believed to increase testicular blood flow, accelerate spermatogenesis, promote maturation of nerve and epidermal growth factors, and cause liquefaction of seminal fluid clots; all are functions potentially important in reproductive physiology (Saitoh and Kumamoto, 1988; Clements et al., 1988).

2. Kininases. In the seminal plasma, the main kinin-inactivating enzyme is KII-ACE. The source of the highest activity of KII-ACE in the rat is the testis and epididymis (Cushman and Cheung, 1971), whereas in humans, although significant amounts occur in these organs, about 70% of the enzyme is found in membrane vesicles of the prostatic cells (Krassnigg et al., 1986, 1989). The presence of kininases in human ejaculates was first reported by Palm et al. (1976). Of these, KII-ACE, purified from seminal plasma, is considered to be a protein with four subunits, two having molecular masses of 55 and 46 kDa and the remaining two being of higher molecular mass. Like KII-ACE, KII-NEP has been identified in the human prostate and the epididymis and is inhibited by ethylenediaminetetraacetic acid, 1,10 phenanthroline, and phosphoramidon (Almenoff and Orlowski, 1983; Erdös et al., 1985). Of interest is the apparent absence of KI-CPN in human genital fluids.

3. Kininogens and kinins. Although the occurrence of kininogen in human seminal plasma has been reported (Palm and Fritz, 1975; Fink et al., 1989b), the type of kininogen molecule from which these peptides are derived is not known. The cellular origin and localisation of kininogen merits study. Should the spatial relationship between the localisation of kininogen and tissue kallikrein be similar to that observed in the CNT cells of the renal nephron and the dark cells of the sweat gland, then such a fact could be of functional significance.

Evidence appears to suggest the presence of low concentrations of free kinins in human seminal plasma. Unlike desArg^9-bradykinin and inhibitors of KII-ACE, bradykinin displaced the specific binding of [^{3}H]bradykinin from human spermatozoal membranes, with 50% inhibition achieved at 3 to 5 nM (Schill et al., 1989). Sperm motility showed a dose-dependent increase when bradykinin was added to washed human spermatozoa. Kinins increase the mean velocity and motility of the sperm (Schill, 1974; Steiner et al., 1977); they rejuvenate nonmotile (sleepy) sperm and viability, increase fructose and oxygen consumption, and improve the production of lactic acid and carbon dioxide in them (Schill and Haberland, 1975; Leidl et al., 1975; Schill, 1977). Kinin receptor activation results in a small increase in cAMP. Of particular importance is the enhancement of the capacitance reaction (Saito et al., 1984; Shinohara et al., 1985). Similar effects of tissue kallikrein on isolated spermatozoa probably indicate the presence of kininogen on the plasma membrane of the sperm.

Spermatogenesis in the rat has been studied after administration of porcine pancreatic kallikrein. Sertoli cell function was improved, with an increase in the number of spermatocytes. There was incorporation of [^{3}H]thymidine into the DNA of testicular tissue (Matthiessen and Rohen, 1975), enhancement of glucose intake, and an increase in testicular blood flow (Saitoh and Kumamoto, 1988). The extent to which kallikrein and kinins may modulate the pituitary-gonadal hormonal loop requires investigating. Another use of kinins could be in subfertile women to improve sperm migration and conception during ovulation. A challenge for the future is to transform the sperm capacitance-related action of kinins into a therapeutic possibility.

G. Leucocytes

1. Kininogenases. Early studies indicated that human PMN leucocytes contain two families of kinin-forming enzymes. One consists of leucokinin-forming enzymes (leucokininogenases) that release leucokinins from a sub-

strate (different from HK or LK) called leucokininogen (Greenbaum, 1979). The second group is a family of neutral proteases that act on plasma kininogens to produce kinins (Movat et al., 1973; Lupke et al., 1982).

a. LEUCOKININOGENASES. Human and rabbit PMN leucocytes contain an enzyme able to release a kinin-like peptide from plasma previously heated to 57°C (Greenbaum et al., 1969). The enzyme was active at neutrality (pH 7.0), but the activity was doubled at acid pH (pH optimum, 4.0). The leucokinin-forming enzyme was later considered to be very similar to, or the same as, cathepsin D in view of its acid optimum pH and inhibition by pepstatin. Cellular fractionation studies indicate that the leucokininogenase was present in the nuclear and lysosomal fractions. Detergent treatment of the fraction with sodium dodecyl sulphate solubilised some of the enzyme activity, suggesting binding to membranes (Greenbaum, 1972). The same leucokininogenase was reported to occur in other white blood cells such as monocytes and lymphocytes (Engleman and Greenbaum, 1971).

The substrate for leucokininogenase proved to be different from HK and LK and was therefore called leucokininogen (Greenbaum et al., 1972). Rabbit plasma contained adequate amounts of this substrate, but initially only small amounts were found in human plasma. However, warmed human plasma (45°C for 15 min) contained high concentrations of the substrate. This finding and the fact that temperature activation was inhibited by SBTI or trasylol led to the view that leucokininogen was formed from a precursor protein (proleucokininogen) by a plasma protease. The substrate was later purified from ascites fluid obtained from a patient with ovarian carcinoma (Roffman and Greenbaum, 1979). Leucokininogen is hydrolysed by leucokininogenase and trypsin but not by plasma kallikrein. It has a molecular mass of about 41 kDa, does not cross-react with antisera against LK, and has a different amino acid composition from both that of human and bovine LK.

The enzymic activity of leucokininogenase on leucokininogen results in the formation of leucokinins, bioactive peptides comprising 21 to 25 amino acids (Greenbaum, 1979). In this regard, they are different from bradykinin, kallidin (Lys-bradykinin), and Met-Lys-bradykinin. Furthermore, amino acid analysis showed that the leucokinin peptides (A, H, M, and PMN) did not contain the typical bradykinin sequence in their primary structure (Freer et al., 1972; Chang et al., 1972; Greenbaum, 1979). The biological properties of leucokinins, namely, an increase in vascular permeability, arterial vasodilation, contraction of the isolated rat uterus and guinea pig ileum, and relaxation of the isolated rat duodenum, were similar to those typical of kinins (Greenbaum, 1979).

b. NEUTRAL KININOGENASES. The presence of a neutral protease with kininogenase activity was first described in isolated human neutrophils by Movat et al. (1973). Neutrophils incubated with immune complexes released an enzyme capable of liberating a kinin-like substance from kininogen at neutral pH. The same kinin-forming activity was found in granules and lysosomes isolated using cell fractionation techniques. Of the several inhibitors tested on this enzyme, di-isopropyl-fluorophosphate, produces 100% loss of activity, whereas SBTI gave 65% and trasylol 41% inhibition. Of the circulating inhibitors, only α_1-antitrypsin was effective. The kininogenase was later purified by Movat et al. (1976). The molecular mass of the protease was estimated to be between 20 and 28 kDa, depending on the separation method used (gel filtration or sodium dodecyl sulfate gel electrophoresis). The kinin-like peptide contracted the rat uterus and seemed to resemble bradykinin and Met-Lys-bradykinin in its properties. Intradermal injections of the protease in the guinea pig induced hyperaemia and moderate enhancement of vascular permeability. The latter response was markedly enhanced when the protease was mixed with kininogen prior to injection.

A kininogenase that liberates kinin-like activity (contraction of the isolated guinea pig ileum) at neutral pH was identified in neutrophil extracts by Lupke et al. (1982). Later, the same authors reported the purification of two kininogenases from neutrophils that had molecular weights similar to that of tissue kallikrein. However, the kininogenase activity was inhibited by SBTI, and the enzyme did not cross-react with anti-tissue kallikrein antibodies in an enzyme immunoassay (Lupke et al., 1983). Furthermore, an enzyme similar to, but not identical with, elastase, which releases Met-Lys-bradykinin from kininogen, was reported in azurophilic granules (Wendt and Blümel, 1979).

Another kallikrein-like enzyme has been described in human leucocytes isolated from patients allergic to ragweed or grass pollen (Newball et al., 1975). The leucocytes released in parallel both a TAMe esterase activity and histamine when challenged with anti-IgG or purified proteins from ragweed or grass pollen. Because basophils are believed to be the only type of white blood cell that fixes IgE and contains histamine, Newball and his colleagues (1975) concluded that the TAMe esterase activity was derived from basophils. Purification by gel filtration resolved the enzyme into three components with molecular masses of 1200, 400, and 100 kDa (Newball et al., 1979). The TAMe esterase had the capacity to release kinins from citrated human plasma.

2. Identification of tissue kallikrein and kininogen in human neutrophils. During the immunocytochemical localisation of tissue kallikrein in the human kidney, PMN leucocytes (neutrophils) infiltrating a region of infection within the renal parenchyma showed an intense granular pattern of immunostaining. Although previous reports suggested the presence of a number of kininogenases, none was identified as a tissue kallikrein. This important chance finding merited detailed investigation, in partic-

ular, in diseases in which there is an accumulation of neutrophils. Large numbers of neutrophils were observed in the synovial fluid and membranes of patients with rheumatoid arthritis. The demonstration of immunoreactive HK in human neutrophils (Gustafson et al., 1989), and the availability of specific antibodies to HK and LK, provided the opportunity to determine whether the neutrophil, in addition to tissue kallikrein, contained the two kinin-releasing substrates.

a. IMMUNOCYTOCHEMICAL VISUALISATION. **i. Tissue kallikrein in human neutrophils.** A range of fixatives, except Bouin's fluid, revealed, in paraffin wax sections or smears, the presence of immunoreactive tissue kallikrein in human leucocytes. In addition, each of five antibodies to human urinary and salivary kallikrein reacted positively for tissue kallikrein. Counterstaining with haematoxylin and eosin or Giemsa showed that the immunoreactivity was present only in neutrophils, whereas other blood cells such as eosinophils, lymphocytes, monocytes, platelets, and erythrocytes did not stain for the enzyme. Neutrophils consistently displayed a granular pattern of cytoplasmic immunoreactivity that was particularly evident in blood smears (Figueroa and Bhoola, 1989).

The enzyme also was seen in cytoplasmic granules of precursor cells of the neutrophilic cell line in bone marrow. Strong immunostaining was observed in cells that could be identified as myelocytes and metamyelocytes, whereas no such staining was seen in precursor cells containing eosinophilic granules. Cells visualised as monocytes, lymphocytes, plasma cells, megakariocytes, and erythroblasts showed no immunostaining (Figueroa et al., 1989). Smears prepared from the blood of patients with chronic myeloid leukaemia revealed intense immunostaining to tissue kallikrein in both mature and immature forms of neutrophil precursor cells. As with the bone marrow samples, the immunostaining was confined entirely to neutrophilic myelocytes and metamyelocytes (Figueroa and Bhoola, 1989).

Ultrastructural studies revealed localisation of the enzyme in the larger sized granules. Although subcellular fractionation studies seem to suggest sequestration in organelles, as yet it has not been possible to precisely identify whether the enzyme is contained in azurophilic or specific granules. One possible source of tissue kallikrein in these cells could be through the endocytosis of circulating tissue kallikrein-α_1-antitrypsin complexes. However, the presence of immunoreactive tissue kallikrein in precursor cells of the neutrophil cell line in both the bone marrow and in the blood of patients suffering from myeloid leukaemia suggests that the enzyme is probably synthesised in myeloid stem cells. Nevertheless, definitive evidence for synthesis must await in situ hybridisation with the appropriate cDNA probes to detect the specific mRNA for tissue kallikrein.

The question of whether human basophils contain tissue kallikrein requires investigation, particularly because Newball et al. (1975) reported that, when preparations of sensitised human leucocytes were challenged with anti-IgE, they coreleased a TAMe esterase activity and histamine. On this indirect evidence, they based their conclusion that basophils contain a kallikrein. Because basophils constitute about 0.5% of the total white blood cell count, one could not be certain of the apparent absence of tissue kallikrein in these cells.

Extracts prepared from purified PMN leucocytes possessed both amidase and kininogenase activities (Figueroa and Bhoola, 1989; Figueroa et al., 1989). The enzymic activity of the PMN leucocyte extracts was characterised further using of a range of inhibitors. The SBTI-ethylenediaminetetraacetic acid-resistant amidolytic activity was inhibited by anti-tissue kallikrein antibodies and by aprotinin. Of particular importance is the finding of two kininogenases in the neutrophil extracts: (*a*) an SBTI-sensitive enzyme similar to that described by Movat et al. (1973) and by Lupke et al. (1982) and (*b*) an SBTI-resistant but, aprotinin-sensitive, enzyme; the vasoactive molecule formed by the enzymic action of the extract on human kininogen gave a pharmacological profile characteristic of that of the kinins, kallidin and bradykinin (Figueroa and Bhoola, 1989).

The mechanisms involved in the release of tissue kallikrein from human neutrophils was studied recently by Cohen et al. (1991) and K. Worthy, K. D. Bhoola, C. Elson, and P. A. Dieppe (unpublished). Both groups failed to effect a release of tissue kallikrein from human neutrophils with the chemotactic peptide, fMLP. The unpublished results of K. Worthy et al. indicate that, although fMLP causes degranulation, but myeloperoxidase is released into the incubation medium, tissue kallikrein becomes endocytosed into the plasma membrane of the degranulating neutrophil. However, Cohen et al. (1991) demonstrated the release of tissue kallikrein by thrombin using the chromogenic substrate, H-D-Val-Leu-Arg-paranitroaniline, and Western blot analysis in which four immunoreactive bands that ranged from 32 to 45 kDa were observed (Cohen et al., 1991). Additional autoradiographic studies of the proteins secreted by the thrombin-challenged neutrophils revealed the incorporation of [^{35}S]methionine in the 35-kDa immunoreactive band (Cohen et al., 1991). Clearly, further experiments need to be designed to determine the precise mechanism involved in the release of tissue kallikrein during both physiological and pathological events. The observations of Cohen et al. (1991) suggest a new functional link between clotting and inflammation, in which the neutrophil may play a dominant role.

ii. HK, LK, and plasma prekallikrein on human neutrophils. HK was identified in lysates of washed neutrophils by a competitive enzyme-linked immunosorbent assay (Gustafson et al., 1989). In addition, these cells were found to possess surface-binding sites for this

molecule. HK and LK were identified on the neutrophil by immunocytochemistry (Figueroa et al., 1990a, 1991b). The localisation of the kininogens on the external surface of the neutrophil plasma membrane was confirmed using confocal microscopy and by electron microscopy immunogold studies. This suggested the presence of surface receptors that capture circulating complexes of HK bound to plasma prekallikrein. In very recent experiments, we showed, by confocal microscopy, that plasma prekallikrein localises as a circular ring along the cell membrane of nonfixed neutrophils (Henderson et al., 1992). The question of whether a plasma prekallikrein-like enzyme also exists inside the neutrophil remains to be determined. The release of active tissue kallikrein or the activation of plasma prekallikrein-HK complexes could result in the formation of kinins on or in the vicinity of the neutrophil membrane. Future experiments should determine which of the two primary functions of the neutrophil kininogens is of biological relevance, serving as substrates for the formation of kinins and/or acting as inhibitors of cysteine proteases.

3. Neutrophil function in inflammation. Circulating neutrophils respond to chemotactic factors (Seligman et al., 1981; Harvath and Leonard, 1982) by expressing Fc receptors (Klempner and Gallin, 1978) and by binding to monoclonal antibodies (Ball et al., 1982; Clement et al., 1983). These features seem to indicate the presence of various subpopulations of neutrophils.

a. MATURATION. Neutrophils, like the other granulocytes, originate from stem cells located in the bone marrow. It is believed that stem cells originate from morphologically undefined pluripotential stem cells, referred to as the colony-forming unit. This cell possesses a high capacity for self-replication into several different types of progenitor stem cells, one of which becomes committed to the formation of granulocytes. Immunoreactive tissue kallikrein has been identified in precursor myelocytes. The transit time for complete maturation of the neutrophil from the stem cell takes about 10 days. Thereafter, the neutrophil remains in the bone marrow for about 5 days before migration into the circulation, extracellular tissue space, and biological fluids (bronchoalveolar secretions, saliva, urine).

b. GRANULE CONTENT. At least three types of granules occur in the cytoplasm of PMN leucocytes. Two types, azurophilic and specific granules, are known to contain a large number of enzymes (Baggiolini et al., 1969). The azurophilic or primary granules are rich in myeloperoxidase, elastase, cathepsins B, D, and G, and probably tissue kallikrein. The specific or secondary granules are characterised by their content of lysozyme, lactoferrin, and collagenase. A third type of granule, the secretory or tertiary granule, has recently been identified and seems to contain alkaline phosphatase and other proteins that are translocated to the plasma membrane during exocytosis (Borregaard et al., 1987; Borregaard, 1988). The existence of yet another type of granule, a gelatinase-containing granule, has been suggested (Dewald et al., 1982), but recent evidence based on immunocytochemistry indicates that gelatinase is a constituent of specific granules. Some functionally important proteins are also associated with the granules, namely, receptor proteins for fMLP (Jesaitis et al., 1982; Fletcher and Gallin, 1983), cytochrome *b* (Borregaard et al., 1983), and the adherence protein Mac-1 (a macrophage differentiation antigen; Sanchez-Madrid et al., 1983).

c. CHEMOTAXIS. Neutrophils are attracted to an inflammatory site by chemotactic factors formed in the very early phase of inflammation. They initiate migration by causing neutrophils to marginate and adhere to the endothelium of capillaries (Ryan and Majno, 1977; Issekutz, 1984) prior to diapedesis through the endothelial cell junction gaps. The orientation and migration of neutrophils are regulated by concentration gradients of chemotactic factors in the interstitial fluid between the inflammatory site and the lumen of the capillary (Gallin et al., 1980).

Some of the most important chemotactic substances are IL-1 (Luger et al., 1983), the complement component C5a (Johnston and Stroud, 1977), and some proteins of the clotting system, e.g., plasma kallikrein (Kay and Kaplan, 1975). Neutrophils express specific receptors for chemotactic factors such as fMLP, leucotriene B4, and the complement product C5a. In addition, an intracellular reservoir of fMLP receptors has been identified in the specific granules of neutrophils (Jesaitis et al., 1982). These receptors and the Mac-1 adherence protein are translocated to the plasma membrane during stimulation of neutrophils.

d. ADHERENCE. Neutrophils are the most mobile inflammatory cells. Calcium ions are essential for leucocyte-endothelial cell adhesion, possibly by acting as a bridge to overcome the net negative membrane charge of both cell types involved (Atherton and Born, 1972; Hoover et al., 1978). In vivo studies suggest that it is the endothelial cell that primarily undergoes certain specific changes following injury, becoming "sticky" for circulating leucocytes (Allison et al., 1955). It has been shown that IL-1 induces endothelial cells to become hyperadhesive for neutrophils and monocytes (Dunn and Flemming, 1984; Bevilacqua et al., 1985). The increased adhesiveness was protein synthesis dependent and specific for endothelial cells. Similar results have been obtained with TNFα (Pohlman et al., 1986). These observations are in agreement with some of the effects produced by IL-1 in vivo that are characterised by extensive leucocyte migration through the endothelial cells (Dunn et al., 1987). Kinins release IL-1 from monocytes (Tiffany and Burch, 1989) and thereby may indirectly affect adherence.

e. DIAPEDESIS OF NEUTROPHILS. An acute inflammatory response consists of several features, including

changes in vascular calibre and tone, as well as an increase in vascular permeability that results in the formation of a protein-rich exudate (Lewis, 1986). As soon as neutrophils receive chemotactic signals, they marginate and adhere to the vascular endothelial cells. Two mechanisms have been proposed for the adhesion of neutrophils to the endothelial cell wall: one depends on the neutrophil and the other on the endothelial cell. The interaction between these two cells may occur simultaneously and may involve similar processes. Pretreatment of human umbilical vein endothelial cells with IL-1 (Bevilacqua et al., 1985) or TNFα (Gamble et al., 1985; Pohlman et al., 1986) promotes subsequent neutrophil adherence by a mechanism that requires de novo synthesis of adhering surface proteins on endothelial cells (endothelial-dependent mechanism).

After neutrophils are attached to endothelial cells, the next phase is the opening of endothelial gaps that permit the migration of neutrophils into the interstitial tissue space. The mechanism(s) by which neutrophils form endothelial gaps and migrate through them remains undetermined. It seems likely that neutrophils secrete substances that affect blood vessels. Such a secretion seems to be independent of phagocytosis, because soluble chemotactic agents (leucotriene B4, fMLP, IL-1) produce the same response as particulate substances such as zymosan (Lewis, 1986). The very recent identification of tissue kallikrein in, and HK, LK, and plasma prekallikrein on, the neutrophil provides a new and novel mechanism for the diapedesis of these cells between capillary cells. Neutrophils have receptors for HK (Gustafson et al., 1989), and because plasma prekallikrein colocalises with HK on the neutrophil membrane (Henderson et al., 1992), activation of this enzyme could result in the discrete and circumscribed formation of bradykinin. The peptide has a potent effect on endothelial cells, causing them to retract and permit both the migration of neutrophils and transudation of plasma constituents (fig. 12) (Oyvin et al., 1970). The simultaneous secretion of progelatinase and procollagenase (Schettler et al., 1991) and the subsequent activation of these enzymes by tissue kallikrein (Tschesche et al., 1991) may be another important step in the movement of the neutrophil through the basement membrane in its passage from the gap junction to the interstitial tissue space (fig. 12).

The function of forming a kinin close to the endothelial junction may not be restricted to plasma kallikrein. Neutrophils contain a number of enzymes that have the capacity to form kinins (Movat et al., 1973; Greenbaum, 1979; Lupke et al., 1982). Like tissue kallikrein, the other kininogenases are stored intracellularly in cytoplasmic granules. However, the mechanisms that mobilise the granules that contain these enzymes to the plasma membrane and the factors that control its fusion with the granular membrane are not known. Nevertheless, exocytosis of specific granules has been demonstrated during

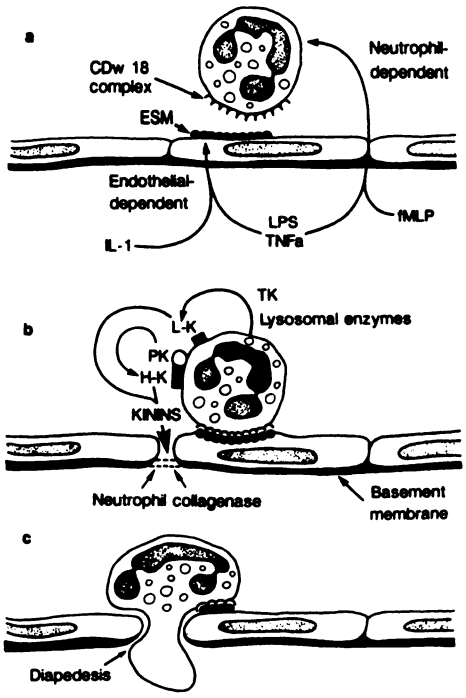

FIG. 12. A novel concept for the diapedesis of neutrophils: involvement of kinins. ESM, endothelial surface molecules; CDw 18, adhesion complex; LPS, lipopolysaccharide; TK, tissue kallikrein; L-K, LK; H-K, HK; PK, plasma prekallikrein.

adherence in vitro and during diapedesis in vivo (Wright and Gallin, 1979). The half-life of kinins in this environment may be regulated by the KII-NEP located on the surface of the neutrophil (Connelly et al., 1985).

f. MIGRATION TO AN INFLAMMATORY NIDUS. Psoriasis is an immune-driven inflammatory skin disease in which neutrophils orient and then migrate into the epidermis either individually or in clusters to accumulate in psoriatic scales (Pinkus and Mehregan, 1966). An additional characteristic, histopathological feature of psoriasis is microabscess formation within the stratum corneum (Pinkus and Mehregan, 1966) into which neutrophils infiltrate in large numbers. Psoriatic scales contain leucotactic substances that correspond to complement-associated peptides, possibly a C5 cleavage product, generated by the action of proteolytic enzymes (Tagami and Ofuji, 1976; Lazarus et al., 1977). Circulating neutrophils that infiltrate into the epidermis of psoriatic patients contain immunoreactive tissue kallikrein (Poblete et al., 1991). In addition, the neutrophils contain immunoreactive LK and HK on the external surface (Figueroa et al., 1990a, 1991b). Kininogen is increased in several inflammatory diseases including rheumatoid arthritis (Zeitlin et al., 1976a) and psoriasis (Winkelmann, 1984). Because kininogens are synthesised by hepatocytes in the liver, cytokines or similar mediators produced locally in inflamed tissue may be responsible for switching on the hepatic synthesis and secretion of the kininogen into the circulation.

In recent receptor and autoradiographic studies, bradykinin-binding sites were demonstrated on human ker-

atinocytes (Schremmer-Danninger et al., 1991; Heinz-Erian et al., 1991). Kinins are believed to act as mitogens, stimulating DNA synthesis and cell division (Rixon and Whitfield, 1973; Owen and Villereal, 1983: Marceau and Trembly, 1986). In psoriasis, intradermal occlusions of the sweat gland ducts together with the migration of neutrophils into pustules and microabscesses may result in an increased formation of kinins. The higher kinin levels could, in part, account for the increased mitotic activity seen in the psoriatic epidermis. If kinins contribute to the pathogenesis of psoriasis, then it is conceivable that locally applied kinin antagonists may prove of therapeutic value.

g. DEGRANULATION AND SECRETION. When neutrophils reach the source from which the chemotactic factors are emanating, contact with noxious agents initiates the next phase: phagocytosis. If phagocytosis is to be effective, particles usually need to be coated with opsonins, although some bacteria or noxious molecules are readily engulfed without opsonisation (Wade and Mandell, 1983). The most important opsonins are IgG and C3b. Contact with surface receptors for IgG and complement triggers cellular events that lead to the internalisation and destruction of the noxious agents (Stossel, 1978). After contact is initiated, the particle is surrounded by pseudopods that meet, fuse, and engulf the particle into a phagocytic vacuole (Stossel, 1974, 1978). At the same time, the intracellular granules fuse their membranes with the vacuole's membrane, sequentially liberating enzymes to destroy the offending particle. This process is usually referred to as degranulation.

Secretion of granule contents may occur as an independent event or as a sequential step with degranulation. Secretion expresses the active release of the granule contents to the external environment by exocytosis. The factors that regulate the exocytosis of PMN leucocyte granules are not known. Of the factors that trigger exocytosis, fMLP, when applied in concentrations that normally cause chemotaxis, produces the release of secretory granule contents by exocytosis (Borregaard et al., 1987). Because neutrophils migrate in response to the chemotactic signal produced by fMLP, this peptide may cause a similar effect during margination, diapedesis, and movement of the neutrophil to the site of inflammation. In fact, exocytosis of specific granules has been demonstrated during adherence in vitro and during exudation in vivo (Wright and Gallin, 1979).

The identification of a tissue kallikrein in the neutrophil merits isolation in a pure form to determine its chemical structure and properties. The functional importance of our new and exciting discovery of the coexistence in the neutrophil of tissue kallikrein and the kininogens (Figueroa et al., 1991b) together with plasma prekallikrein (Henderson et al., 1992) clearly requires further research. The precise conditions necessary for the formation of kinins by neutrophils and the physiological role of released kinins may provide new insights into the migration of neutrophils through capillary gap junctions to travel to sites to which they are attracted by chemotactic agents.

V. Selected Topics concerning the Clinical Pharmacology of the Kallikrein-Kinin System

A. Allergic Rhinitis

A number of time-dependent phases are involved in the allergic responses of epithelial and mucosal cells. The allergic disorder may be immune driven, involving antigen-presenting cells, or may be dependent on the formation of complexes with IgE. The development of a controlled, reproducible model of nasal inflammation permitted the study of immediate and late responses to allergen challenge (Proud et al., 1983; Naclerio et al., 1985). A particular advantage of this model was the ability to collect nasal lavage fluid and to undertake quantitative measurement of mediator release following challenge with allergens or with cold, dry air. Nasal secretions of patients with allergic rhinitis subjected to antigen challenge or symptomatic rhinoviral infections contain HK, LK, bradykinin, and kallidin in the nasal secretions (Proud et al., 1983; Togias et al., 1985; Baumgarten et al., 1986). During the immediate phase of the challenge response, transudation of HF and the plasma prekallikrein-kininogen complex occurs. The precise mechanism of contact activation involved is not known, but participation by negatively charged mucous macromolecules or mast cell heparin has been suggested. Another kininogenase that could contribute to the formation of bradykinin may be mast cell tryptase, but of the two, plasma kallikrein is the preponderant enzyme (Proud and Kaplan, 1988). The finding of kallidin in these nasal secretions indicated the additional presence of tissue kallikrein. Values for tissue kallikrein increased following antigen challenge, and this enzyme was considered to be the major kinin-forming enzyme in the postchallenge lavages (Baumgarten et al., 1986). The source of the tissue kallikrein was probably from neutrophils and/or from local secretory glands because human nasopharyngeal and tracheal submucous glands contain immunoreactive tissue kallikrein (Figueroa and Bhoola, unpublished). Kinin degradation in nasal secretions during an allergic response was due primarily to an arginine aminopeptidase and to KI-CPN. The duration of action and potency of the formed kinins will depend on the type of kinin receptors in the nasal mucosa.

B. Asthma

Airway hyperreactivity is a feature of asthma and inflammatory lung diseases such as bronchitis. Irritant stimuli (allergens, cold air, air pollutants), sensitising mediators (platelet-activating factor), and spasmogens (histamine, acetylcholine, eicosanoids, kinins) all contribute to the pharmacology of airway hyperreactivity.

Application of bradykinin causes mucosal oedema and chloride secretion by airway epithelial cells. Activation of capsaicin-sensitive airway C-fibre terminals by kinins initiates axon reflexes that lead to the local release of sensory neuropeptides near blood vessels, secretory glands, and smooth muscle (Barnes, 1987). The question to answer is the relevance of kinins in the symptomatology of airway hyperreactivity. Kinins produce mucosal oedema and in the absence of mucosal epithelium marked and persistent contraction of bronchial smooth muscle. Corroboration for this in vitro finding (Bhoola et al., 1989) relies on inhalation studies in which bradykinin caused severe bronchospasm in asthmatics but not in normal subjects (Herxheimer and Stesemann, 1961; Varonier and Panzani, 1968). Further firm evidence came from a seminal study in which free kinin levels and kininogenase activity were detected in bronchial lavage fluids from asthmatic patients but not from control subjects (Christiansen et al., 1987). Sources for tissue kallikrein are the pulmonary submucous gland (Bhoola et al., 1989) and the neutrophil (Figueroa et al., 1989). Kininogens are probably derived from plasma transudate and the neutrophil (Figueroa et al., 1991b). Hence, both enzyme and substrate can be available in the bronchopulmonary tree for the formation of kinins. Some doubt exists as to the nature of the receptor involved in kinin-induced bronchoconstriction. Bradykinin-evoked bronchoconstriction was clearly inhibited by a BK2 receptor antagonist (Jin et al., 1989), whereas Farmer et al. (1989b) concluded that pulmonary tissues contain a novel BK3 receptor, particularly in the large airways. Hoe 140, the new BK2 receptor antagonist, inhibits all pulmonary effects of kinin. Clinical use of Hoe 140 will certainly establish the precise spasmogenic role played by kinin in patients with airway hyperreactivity, especially asthmatics.

C. Arthritis

Cellular mediators in the synovium and synovial fluid of patients with inflammatory joint disease include cytokines, components of the complement cascade, products of arachidonic acid metabolism, platelet-activating factor, and the vasoactive kinins. The initiators of the synovitis in each inflammatory joint disease seem to be different; rheumatoid arthritis is immune driven (probably initiated by immune complexes), and gout is linked to urate crystals. Recent evidence suggests a primary role for kinins in the causation of synovitis and the appearance of symptoms of inflammatory joint disease. Both kallikreins and kininogens may enter the synovial joint space either with migrating neutrophils or by transudation from the plasma (Worthy et al., 1990b; Bhoola and Dieppe, 1991).

1. Kallikreins. a. PLASMA KALLIKREIN. This enzyme was first implicated in gout (Kellermeyer and Breckenridge, 1965). Later, significantly higher plasma kallikrein activity was recorded, using the synthetic substrate Pro-Phe-Arg-methylcoumarin, in synovial fluid aspirated from the knee joint of patients with rheumatoid arthritis when compared to fluid taken from patients with osteoarthritis. Most of the plasma kallikrein in synovial fluid exists as a proenzyme complexed to HK. Furthermore, at least 50% of the active form of the enzyme is trapped within the protease inhibitor, α_2-macroglobulin (Suzuki et al., 1987b). Conversion of plasma prekallikrein to its active form may be triggered through activation of HF by tissue matrix components such as proteoglycans, urate crystals, or pyrophosphates (Kaplan et al., 1989a). Plasma kallikrein has a chemotactic potency similar to that of fMLP and apparently primes neutrophils for superoxide production in the joint. Furthermore, plasma kallikrein is believed to cause significant conversion of latent collagenase to its active form in vitro (Nagase et al., 1982); this could be a significant property of plasma kallikrein should it occur within the joint space.

b. TISSUE KALLIKREIN. Tissue kallikrein has been identified in synovial fluid aspirated from swollen knee joints of patients with rheumatoid arthritis or osteoarthritis by enzymic (amidase assay) and immunological (RIA) criteria. Because α_2-macroglobulin traps, but does not inactivate, some proteases that could interfere in the amidase or the kininogenase assays, prior removal of this inhibitor is essential before precise values for both the active and proenzyme forms of tissue kallikrein can be determined in synovial fluid (Worthy et al., 1990a). An amidase resistant to SBTI with kinin-forming activity was first described more than a decade ago in inflammatory exudates and in human and animal synovial biopsy specimens (Thomas and Zeitlin, 1981; Al-Haboubi et al., 1986). Part of the amidase activity in the synovial

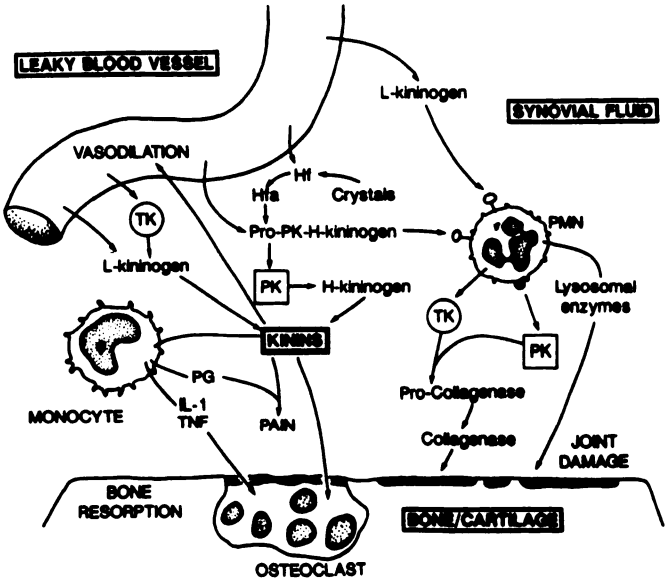

FIG. 13. An overview of the pivotal role of kinins and cytokines in inflammatory joint disease. PK, plasma kallikrein; TK, tissue kallikrein; PG, prostaglandins. From Bhoola and Dieppe (1991) and used with permission.

fluid of patients with rheumatoid arthritis and osteoarthritis can be ascribed to tissue kallikrein because about 15% of this activity is inhibited by anti-tissue kallikrein antibodies (Worthy et al., 1990a) and immunoreactivity to tissue kallikrein can be shown by RIA (Selwyn et al., 1989).

Many neutrophils invade rheumatoid synovial vessels, migrate to the luminal surface of the synovial membrane, and accumulate in large numbers in the synovial fluid (Dularey et al., 1990). Therefore, tissue kallikrein probably reaches the synovial fluid from both neutrophils and/or from plasma transudates. Secretion from even a small number of neutrophils in rheumatoid arthritis synovial fluid could result in the activation of the enzyme and the subsequent formation of kinins during episodes of acute inflammation within the joints. Recent experiments indicate that mast cells also migrate into synovial membranes of rheumatoid arthritis joints and accumulate at sites of bone erosion close to or within the junctions of cartilage and pannus (Crisp et al., 1984). Degranulating mast cells release a kininogenase that forms bradykinin at acid pH (5.5) but is chemically different from plasma and tissue kallikreins. Synovial fluid contains considerable amounts of both α_1-antitrypsin and α_2-macroglobulin. The clinical relevance of these observations will become evident only when appropriate inhibitors become available for use in patients.

Human granulocyte procollagenase is activated much more rapidly by tissue kallikrein than by bovine trypsin, without further degradation of the enzyme (Eeckhout and Vaes, 1977; Tschesche et al., 1989). This action appears to be specific to both tissue and plasma kallikreins because it is not shared by human leucocyte elastase. The activated collagenase could, at least in part, be responsible for the extensive cartilage and bone destruction seen in the joints of patients with rheumatoid arthritis, in which neutrophils invade the synovium and migrate in large numbers to the synovial fluid. The importance of these latter observations needs to be determined.

2. Kininogens. Both HK and LK are present in synovial fluid (Jasani et al., 1969). Until such time as specific assay methods are developed for measuring each of the two kininogens, precise values cannot be determined for synovial fluid. Tentative evidence suggests that HK, but not LK, is reduced in rheumatoid arthritis but not in osteoarthritis synovial fluid when compared to plasma (Sawai et al., 1978). A fascinating aspect could be the finding of a pulsatile formation of kinins in the synovial fluid. Furthermore, it is conceivable that kininogens not only serve as substrates for the kallikreins but also perform an important function as cysteine proteinase inhibitors in synovial fluid.

3. Kininases. The rate of conversion of bradykinin to desArg^9-bradykinin and, thereafter, to smaller peptides by the serum of normal persons and patients with rheumatoid arthritis and osteoarthritis was studied by Sheikh and Kaplan (1987). They found that the first step, i.e., conversion of bradykinin to desArg^9-bradykinin, was much faster in patients with rheumatoid arthritis than in those with osteoarthritis and normal individuals. Although the concentration of kininase was always lower in synovial fluid when compared to blood, higher levels of KI were seen in patients with rheumatoid arthritis, gout, and psoriatic arthritis than in those with osteoarthritis (Chercuiffe et al., 1987). Recently, aminopeptidase M (EC 3.4.11.2), KII-NEP, and dipeptidyl peptidase IV (EC 3.4.14.5) were identified on the cell surface of human synovial cells (Bathon et al., 1991). In contrast, no significant aminopeptidase A (EC 3.4.11.7), KII-ACE, or KI-CPN activities were found. The degradation of bradykinin by the synovial cells was attributed entirely to KII-NEP and the conversion of Lys-bradykinin into bradykinin by aminopeptidase M (Bathon et al., 1991). It is known that bradykinin and desArg^9-bradykinin interact with different receptors so that formation of specific kinin metabolites could result in a change in the inflammatory response.

4. Kinins. For many years kinins have been implicated as mediators of pain and swelling associated with inflammatory joint disease. In 1957, Armstrong and her colleagues described a pain-producing substance in the synovial fluid of patients with rheumatoid arthritis. This compound resembled bradykinin and was produced only after contact with glass (activation of plasma prekallikrein). Subsequently, elevated levels of kinins, determined by bioassay, were reported in inflamed and gouty joints (Eisen, 1966). Kinin levels increase markedly during an acute attack of gout or following intraarticular injection of urate crystals (Melmon et al., 1967). So far, it is considered that levels of kinins in inflammatory exudates are similar in magnitude to those known to increase vascular permeability and activate pain receptors.

Both bradykinin and kallidin appear to enhance bone resorption in mouse calvaria. They mobilise bone minerals and cause degradation of proteoglycan matrix, as indicated by the release of ^{45}Ca and ^{3}H from [^{3}H]proline (Gustafson et al., 1986b; Lerner et al., 1987). Because these effects on bone resorption were inhibited by indomethacin and corticosteriods, it has been suggested that kinins act through the formation of prostaglandins by activating phospholipase A_2 in osteoclasts, the bone-resorbing cells. Very recent experiments indicate that desArg^9-bradykinin stimulates the secretion of IL-1 and TNF by murine macrophages (Burch et al., 1989). Of particular interest is the ability of both bradykinin and kallidin to induce the secretion of IL-1β from human monocytes (B. Dularay, C. J. Elson, and K. D. Bhoola, unpublished observations). The answers to questions of whether kinin receptors are expressed on the synovial

cell membrane and of the precise second messengers coupled to these receptors are not known.

Leucocyte infiltration, capillary transudation, and oedema are consistent features of inflammation. Both the kallikreins and the kininogens enter the local inflammatory milieu from the capillary circulation and degranulating leucocytes. After the prokallikreins are converted to an active form, interaction of the enzyme with its selective kininogen substrate results in the formation of kinins (fig. 13). A primary mediator role for kallidin and bradykinin still has to be established. There is a clear need for the development of new kinin antagonists of greater potency for the treatment of inflammatory joint disease. It is anticipated that the new generation of kinin antagonists, Hoe 140 or the dimeric CP0127, will soon be available for therapeutic treatment and confirmation of the primary importance of kinins in inflammatory joint disease.

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