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BRADYKININ ANTAGONISTS

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INTRODUCTION

The history of the kallikrein-kinin system can be traced back to the observation of Abelous & Bardier in 1909 that intravenous injection into the dog of an alcohol-insoluble fraction of human urine caused a pronounced, but reversible, fall in systolic blood pressure (1). It was not until almost 20 years later, however, that this observation was pursued by Frey (2), who established that the substance responsible for this hypotensive effect was nondialysable and thermolabile. Further studies, in collaboration with Werle & Kraut, showed that a similar activity was present in blood and in the pancreas (3, 4). On the erroneous assumption that the active substance, in each case, was identical and was probably derived from the pancreas, it was named *kallikrein* (from the Greek "kallikreas", meaning pancreas). In 1937, Werle demonstrated that kallikrein could release, apparently enzymatically, a substance from plasma that was capable of contracting smooth muscle (5). This generated material was later named "kallidin". It is now over 40 years since Rocha e Silva and colleagues, working independently, coined the name "bradykinin" for a similar smooth muscle spasmogen liberated from plasma by the action of either trypsin or the venom of the snake, *Bothrops Jararaca* (6).

In the decades since these pioneering studies, our knowledge of the biochemistry of the kallikrein-kinin system has increased dramatically. Moreover, our appreciation of the pharmacologic actions of exogenously administered kinins has expanded to encompass considerably more than their abilities to contract smooth muscle and to lower blood pressure. Despite significant

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advances in these areas, however, our understanding of the role of endogenously generated kinins in pathophysiology has developed painfully slowly. This has been due, in part, to the technical difficulties that had to be overcome to permit meaningful measurements of the components of the system in biological fluids. Even more important, however, in terms of being unable to establish the importance of kinins in pathophysiological conditions, has been our inability to selectively antagonize their actions. Only in the last few years have we seen the successful development of the first competitive kinin antagonists and have attempts been made to begin to characterize kinin receptors and to define receptor subtypes. In the present chapter, we review this rapidly developing field. We begin by providing the necessary background on the biochemistry and potential pathophysiologic roles of kinins and subsequently summarize our present knowledge of the distribution and properties of B_1 and B_2 kinin receptor subtypes. Finally, we review what is known about the currently available kinin antagonists, focusing on their structures, their application in various in vitro and in vivo systems, and on the evidence for the existence of new receptor subtypes.

BIOCHEMISTRY AND PATHOPHYSIOLOGY OF KININS

The substrates for kinin-generating enzymes are α_2-globulins called kininogens. Two such substrates exist in man. High molecular weight (HMW) and low molecular weight (LMW) kininogens are derived from a single gene as a consequence of alternative RNA processing events (7). The two kininogen molecules are single-chain glycoproteins, but release of the kinin moiety produces heavy and light chains that are linked by a single disulfide bond. Both kininogens display identical amino acid sequences for their amino terminal heavy chains, the kinin moiety and the first 12 residues of their carboxy terminal light chains (8). HMW kininogen, however, then displays a considerably extended light chain that is responsible for conferring on this molecule its unique properties in the intrinsic coagulation cascade (9). The principal site of kininogen synthesis is the liver. In human plasma, HMW kininogen is present at a concentration of approximately 90 μg/ml (10) and represents approximately one third of the total level of plasma kininogens, with LMW kininogen constituting the remainder. This means that there is the potential to release as much as 3 μg of kinin from the kininogens present in 1 ml of plasma. These plasma kininogens also gain access to the lymph and interstitial fluid (11).

Although, in general, enzymes that can liberate kinins are called kininogenases, the historical name kallikrein is still ascribed to the most important physiological kinin-generating enzymes from blood (plasma kallikrein) and from the major exocrine glands (tissue, or glandular, kallikrein). This com-

mon nomenclature is unfortunate, since the plasma and tissue enzymes are physicochemically and immunologically distinct from one another. Plasma kallikrein is synthesized in the liver and circulates in the blood stream as an inactive precursor, prekallikrein. Prekallikrein is converted to kallikrein via the Factor XII-dependent pathway of contact activation (9). When active plasma kallikrein is produced during contact activation, it generates bradykinin (BK) from HMW kininogen, its preferred substrate (12). Indeed, in vivo, plasma kallikrein does not produce kinin from LMW kininogen. In contrast, tissue kallikreins are single-chain, acidic glycoproteins that readily release kinin from both HMW and LMW kininogens (12). Originally believed to be derived only from the major exocrine glands, these enzymes are now known to enjoy a more widespread distribution that is not restricted to exocrine tissues (9). Within a given species, the kallikreins from all organs are immunologically identical (13). To date, tissue kallikreins are the only human enzymes known to hydrolyze two dissimilar bonds within the kininogen molecules to liberate lysylbradykinin (14). It is now known that lysylbradykinin (Lys-BK) is the same entity described by Werle as "kallidin" and the two names tend to be used interchangeably in the literature.

In humans, the nonapeptide BK (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and Lys-BK are the principal kinins of physiological relevance, and these two peptides display essentially identical pharmacologic properties (15). Although older reports describe the generation of methionyl-Lys-BK (16), this latter peptide is now known to be produced only under conditions of acidification when it is generated from already cleaved kininogen by the actions of pepsin (17, 18). In rats, the peptide Ile-Ser-BK (T-kinin) has been isolated (19) and shown to have similar properties to those of BK and Lys-BK (20), but this peptide is not detectable in humans and is apparently unique to the rat. More recently, variants of BK and Lys-BK, in which the proline at position 3 is hydroxylated, have been reported to exist (21–23). These variants are found in human and primates but not in other species examined, such as rodents and cattle (22). In humans, this variant represents between 25–40% of the kinin contained in kininogens (21, 22) and is presumed to arise as a result of posttranslational modification of the kininogen molecule. The pathophysiological significance of this variation is unknown, however, since the hydroxyproline analogs show essentially the same properties and potencies as their unmodified counterparts (24).

In addition to their abilities to contract many types of smooth muscle and to induce hypotension by acting as peripheral vasodilators, BK and Lys-BK are approximately 100-fold more potent than histamine in increasing vascular permeability and, as a direct consequence of this, they are powerful inducers of edema (25). Kinins can also stimulate sensory Aδ and C-fibers to cause pain and hyperalgesia (26–30) and are capable of stimulating epithelial ion

transport in the renal tubule, intestine, and airway (31–36). In addition, these peptides have been reported to cause bone resorption (37, 38), to stimulate mitosis in some cells (39–41), and they are clearly capable of causing the release of biologically active lipids, such as platelet activating factor and prostaglandins, from a variety of cell types (42–44). These properties, together with a range of studies purporting to demonstrate activation of the kallikrein-kinin system during various pathophysiological states, have led to kinins being implicated in a plethora of clinical conditions (45, 46). Perhaps the strongest evidence to date, however, supports a role for kinins in the pathogenesis of various inflammatory disorders, including allergic reactions, asthma, viral rhinitis, hereditary angioedema and inflammatory arthritides (9, 47), and in the regulation of circulatory homeostasis and blood pressure, particularly during certain types of hypertension and during endotoxic shock (46, 48–50). It is hardly surprising, therefore, that many of the first studies performed using the recently developed kinin antagonists have focused on further defining the proinflammatory and vascular effects of kinins.

KININ RECEPTORS

The classification of receptor subtypes and the development of antagonists are, inevitably, intimately linked. As recently available kinin antagonists have been used in an increasing number of tissues and cells, several investigators have interpreted their data as supporting the existence of novel receptor subtypes. Although we will critically evaluate this evidence (see below), we begin by focusing on the properties of the two putative receptor subtypes that have formed the basis of the kinin receptor literature since 1980, when Regoli & Barabe (51) proposed the classification of B_1 and B_2 kinin receptors based upon the relative bioassay potencies of a series of bradykinin analogs and competitive antagonists in isolated vascular smooth muscle preparations.

B_1 Receptors

The principal characteristic attributed to the B_1 receptor is that the kinin metabolites, des-Arg^9-BK and des-Arg^{10}-Lys-BK, are more potent agonists than the parent peptides or the analog, [Tyr(Me^8)]BK. In addition, effects mediated via B_1 receptors can be competitively inhibited by the antagonists, [Leu^8]des-Arg^9-BK and [Leu^9]des-Arg^{10}-Lys-BK (discussed below). Conversely, the B_2 receptor exhibits much higher affinity for BK and Lys-BK, and their analog, [Tyr(Me^8)]BK, than for the carboxypeptidase metabolites, des-Arg^9-BK and des-Arg^{10}-Lys-BK. In addition, B_2-mediated effects cannot be antagonized by [Leu^8]des-Arg^9-BK. Correlation of these pharmacologic criteria for defining a B_1 receptor with binding properties has not, in general, been rigorously pursued.

PROPERTIES Determining the presence of a receptor in various tissues usually depends on the application of a selective antagonist in the tissue in question. As such, we focus on the topic of B_1 receptor distribution in more detail when considering the application of B_1 antagonists. In general, however, the B_1 receptor appears to be fairly limited in distribution, with the primary site being rabbit vascular (arterial and venous) smooth muscle (51–57), although nonvascular localizations also exist. Not surprisingly, given the location of B_1 receptors in multiple vascular preparations, the predominantly reported physiological effect associated with their presence is contraction or relaxation of smooth muscle. The type of response elicited—that is, contraction or relaxation—may depend upon the type of cell responding to the peptide and/or on the receptor coupling mechanism specific to that tissue. For example, while relaxation, in response to des-Arg^9-BK, of rabbit mesenteric artery was observed to be dependent upon the presence of endothelium (58), relaxation of rabbit celiac artery was not (57). Furthermore, while des-Arg^9-BK induced relaxation of rabbit mesenteric artery could be inhibited by indomethacin (56, 58), arachidonic acid metabolites did not appear to play a role in the contraction of several other vascular tissues (59).

An interesting body of evidence has now accumulated, indicating that B_1 responsiveness can be induced under certain conditions. In 1978, Regoli et al (60) described the spontaneous development, over a six hour period of in vitro incubation, of contractile responsiveness of isolated rabbit mesenteric vein to des-Arg^9-BK, but not to substance P or noradrenaline. A similar phenomenon has been confirmed in other isolated tissues, including rat duodenum (61), human colon (62), rabbit aorta and mesenteric artery (52, 58, 63), although the response in the last was less specific, as comparable time-dependent responses to angiotensin II and 5-hydroxytryptamine were also observed. The mechanism by which this upregulation of response to des-Arg^9-BK occurs has not been entirely clarified but may be due, at least in part, to the de novo synthesis of B_1 receptors during in vitro incubation. This hypothesis is supported both by the prolonged time of incubation necessary to observe, and the ability of protein synthesis inhibitors to ablate, the spontaneous generation of peptide responsiveness (60). Furthermore, Barabe et al (64) were able to correlate an increase in specific binding of [^{3}H]-des-Arg^9-BK with its time-dependent ability to induce contractility in rabbit mesenteric vein. Like the biological response, specific ligand binding could be ablated by pretreatment with cycloheximide.

The development of responsiveness to des-Arg^9-BK during in vitro incubations prompted investigators to propose that noxious stimuli from tissue damage or inflammation may be responsible for the induction of B_1 receptor synthesis and that the same process may occur in vivo (47). In support of this hypothesis, Deblois et al (65, 66) have shown that, when applied to isolated

rabbit aortic strips in vitro, a number of soluble products of bacteria, and of monocytes and macrophages, are capable of amplifying and hastening the development of the contractile response to des-Arg^9-BK. These factors include lipopolysaccharide (LPS), interleukin-1 and -2, epidermal growth factor, phorbol esters and muramyl dipeptide (66). In contrast, dexamethasone downregulates this response (65). While a series of other factors, such as platelet-derived growth factor, interferon gamma, and fibroblast growth factor, have been reported to have no effect on B_1 responsiveness (66), it should be noted that many of the factors studied were human recombinant proteins whose effects may be species-specific.

In vivo studies, in both vascular and nonvascular smooth muscle, have lent further credence to the concept that responsiveness to des-Arg^9-BK is induced during tissue damage. Marceau et al (67) showed that, following the induction, using Triton X-100, of chemical cystitis in rats, des-Arg^9-BK was capable of inducing bladder smooth muscle contraction in treated, but not in control, rats. In addition, in rabbits that were pretreated with intravenous LPS, but not in control animals, des-Arg^9-BK induced a hypotensive response (68). In both situations, the in vivo response to des-Arg^9-BK, similar to that in vitro, required five or more hours of pretreatment with the noxious agent to develop and was blocked by [Leu^8]-des-Arg^9-BK. Furthermore, mesenteric veins and aortae removed from LPS treated rabbits and incubated in vitro demonstrated an earlier and more pronounced contractile response to des-Arg^9-BK than did vessels from saline-injected animals (64, 66).

Unfortunately, data relating physiological activities of des-Arg^9-bradykinin to receptor occupancy are limited, to our knowledge, to only two reports. In the study cited previously, Barabe et al (64) confirmed the presence of specific binding of [^{3}H]des-Arg^9-BK to intact rabbit mesenteric vein. This study was limited, however, in that the apparent binding affinity was low (104 nM) and full saturation curves were not presented. In addition, the authors were unable to demonstrate similar specific binding for this peptide in rabbit aortae (64), where its physiological potency is identical to that in rabbit mesenteric vein. Sung et al (69) have demonstrated, in bovine endothelial cell membranes, the existence of both a high-affinity and a low-affinity kinin binding site. The latter site is present on untreated cells and appears to be of the B_1 subtype but high concentrations of des-Arg^9-BK are necessary to saturate this receptor or to induce a physiological response.

B_2 Receptors

In light of the relatively limited distribution of the B_1 receptor, it must be assumed that the majority of actions of kinins are mediated, according to the classification of Regoli & Barabe, via occupancy of the B_2 receptor. Only with the availability of B_2 antagonists has it proven possible to begin testing

this assumption. Accordingly, we will address which functional responses to kinins have been attributed to B_2 receptor occupancy when examining the applications of currently available antagonists. In the present section, we delineate our current knowledge regarding the properties of this putative receptor subtype. We focus, in particular, on its binding characteristics, including a consideration of the evidence for multiple types of binding sites, on the regulation of B_2 receptor expression, and on how the functional responses to this receptor may be expressed through different signal transduction pathways.

PROPERTIES In contrast to the B_1 receptor, a sizeable body of knowledge has been accumulated regarding the properties and regulation of the B_2 receptor. Specific binding of radiolabeled BK to a variety of tissues and homogenous cell populations has now been demonstrated (70–84). While precise comparisons of data among these studies are difficult because of variations in methodologic approaches, the affinity constant (K_d) for bradykinin binding has generally been reported to range from 1 to 12 nM, while maximum binding (B_{max}) has varied from 10 to 230 fmol per mg protein or per 10^6 cells. More importantly, several groups of investigators have successfully correlated ligand binding with physiological function in the tissues employed. For example, the rank order potency of kinin analogs in displacing radiolabeled BK from binding sites on homogenized membranes correlated well with their potencies in stimulating contraction of guinea pig ileum and uterine myometrium (71, 85), intestinal chloride secretion (78), release of endothelium-derived relaxing factor from (and elevation of cytosolic calcium in) endothelial cells (60), and of inositol phosphates from murine neuroblastoma cells (84). In the limited number of studies using intact, rather than homogenized, cells, good correlation of potency of radioligand displacement with parameters such as cyclic GMP formation (71), stimulation of prostacyclin release (73), and DNA synthesis (80) have been demonstrated.

Several of these studies have demonstrated the presence of more than one class of binding site, raising the intriguing possibility that the so-called B_2 receptor may, in fact, represent more than one subtype of kinin receptor. Odya et al (85), utilizing [^{125}I]Tyr-BK in radioligand-binding studies of particulate bovine uterine membranes, identified both a high- (100 pM) and low-affinity (20 nM) binding site. In a similar manner, but utilizing [^{3}H]BK as the radioligand, Manning et al (70) hypothesized the presence of two high-affinity kinin binding sites (K_ds of 13 and 910 pM) in guinea pig ileum, based upon the biphasic nature of both the saturation curve and the association/dissociation curves. Although the two binding sites could not be distinguished by specific physiological functions, they did differ slightly in their selectivities for kinin analogs. In a cloned neuroblastoma cell line,

N1E-115, Snider & Richelson (71) proposed the existence of three kinin binding sites (K_ds of 0.83 pM, 1.0 nM and 4.9 nM), based upon computer analysis of saturation data with intact cells; however, true saturation of binding was not adequately demonstrated. Cyclic GMP production appeared to correlate with the 1.0 nM binding site. Roberts & Gullick (80), also using intact cells, demonstrated both a high (K_d 4.9 nM, B_{max} 52,000 per cell) and a very high (K_d 2.7 pM, B_{max} 1100 per cell) affinity binding site in *ras*-transformed Rat 13 cells. The high-affinity site appeared to mediate BK's mitogenic effect but, because of the small numbers of the very high-affinity site, no physiological function could be ascribed to it. In all of the above cited reports, des-Arg^9-BK was ineffective both in eliciting physiologic effects and in displacing labeled BK from binding sites, thereby supporting the concept that these receptors were not of the B_1 subtype. Whether the multiple binding sites described in these various tissues represent different receptor subtypes remains to be determined. While the identification of physiological functions, and specific antagonists, for each binding site, may help to clarify this issue, it is likely that a definitive answer will await purification and cloning of each putative receptor subtype.

Preliminary evidence, both from binding and from functional studies, suggests that the B_2, like the B_1, receptor is regulated by a number of physiological factors. There is good agreement among various tissues (70–72, 85) that cations, particularly manganese, zinc, and cobalt, significantly reduce BK binding. This effect may be due, however, to activation of peptidases rather than to any direct effect on the receptor itself. BK itself is also a potent regulator of kinin binding. In human dermal fibroblasts (86, 87), exposure to 1 μM BK for 5 minutes at 37°C results in a marked reduction in total number, but not affinity, of [^{3}H]BK binding sites as well as in bradykinin-induced prostacyclin production. In contrast, prolonged exposure of Rat 13 fibroblasts to BK at 37°C results in reduction of receptor-ligand affinity but not in total number of binding sites (81). Several cytokines and growth factors have also recently been shown to influence putative B_2 receptors. Pretreatment of human synovial cells with interleukin-1 (88) was shown to greatly enhance subsequent release of PGE_2 in response to BK, but not to des-Arg^9-BK or other unrelated peptides. Preliminary data suggests that interleukin-1 upregulates the number of kinin binding sites on these cells (J. Bathon, D. Proud, unpublished observations), although postreceptor induction of cyclooxygenase (89) may also be contributory. Simultaneous addition of BK (but not des-Arg^9-BK) and any one of several growth factors to fibroblasts or PC12 cells results in synergistic enhancement of the resulting cytosolic calcium response (90, 91); a similar synergistic response to BK and interleukin-1 is also observed for proliferation of BALB/3T3 cells (92). In studies of binding, nerve growth factor was demonstrated to alter the shape of

the BK-binding isotherm (79), and to induce a fourfold increase in kinin binding sites (82), on PC12 cells, while also enhancing BK-induced phenotypic changes (79) and uptake of inositol (82). In addition, transformation of cells by *ras* (80, 93, 94) or *dbl* (95) oncogenes was associated with increased BK receptor number and, in the case of the *ras* oncogene, as noted previously, the appearance of a very high-affinity kinin binding site (80). These alterations in binding correlated with BK's ability to induce DNA synthesis (80) and to stimulate phosphatidylinositol turnover (95).

Our understanding of the coupling of the B_2 receptor to signal transduction events is still evolving. BK has been reported to stimulate a variety of intracellular events, including accumulation of cyclic AMP (96, 97) and GMP (71, 98); activation of phospholipase C (99, 100), leading to enhanced levels of inositol phosphates (101, 102), cytosolic calcium (69, 103) and diacylglycerol (103); and activation of phospholipase A_2 (104–107). BK-induced phospholipase C activation appears to be coupled via a guanine nucleotide-binding (G) protein in a variety of cells, but the sensitivity of this protein(s) to pertussis toxin is variable (102, 103, 107–110). Phospholipase A_2 activation by BK has also recently been linked to a G protein (107) in murine 3T3 cells. While BK may activate a calmodulin-dependent protein kinase in dermal and lung fibroblasts (111), its effect on protein kinase C (PKC) is less clear and appears to depend upon the effector molecule being measured. For example, in studies using pharmacological agents rather than direct enzyme measurements, PKC activation appears to enhance kinin-induced prostaglandin E_2 production (105, 106) while inhibiting its ability to stimulate inositol phosphate production (102, 107). In a direct enzyme assay, Dixon et al (103) found a fivefold increase in PKC activity in cortical collecting tubular cells, in response to BK, that correlated with the ability of BK to inhibit vasopressin-stimulated cAMP production. Whether BK routinely activates PKC or, alternatively, PKC serves as a negative regulator of BK's actions remains unclear and additional, direct enzyme measurements are needed to clarify this issue.

The confusing array of postreceptor events that have been reported to be activated in various cells in response to kinin receptor occupancy could potentially be explained on the basis of the same receptor being coupled to different signal transduction pathways in each cell type examined (particularly if species variations in coupling exist). Alternatively, one could hypothesize, for example, that different receptor subtypes are each coupled to a specific signal transduction pathway (e.g. phospholipase C vs phospholipase A_2-dependent prostaglandin formation). Several recent studies, showing that activation, within the same cell, of phospholipase C and phospholipase A_2 are totally independent of each other (104–107), would argue against this latter possibility unless it is assumed that two receptor subtypes coexist on each cell

type in question. Certainly, the potential for interaction among intracellular mediators, as well as for overlap between receptor subtypes and second messenger molecules (such as that seen with muscarinic receptors), necessitate caution in the classification of receptor subtypes on the basis of second messenger production (112). Further clues regarding possible heterogeneity of the B_2 receptor and its coupling to second messenger systems may be obtained with the development of specific antagonists capable of discriminating between putative subtypes of the B_2 receptor or as we gain more information on the structure of kinin receptors.

KININ ANTAGONISTS

B_1 Antagonists

STRUCTURE The derivation of competitive peptide antagonists for the B_1 receptor arose from the observation that the agonist effect of des-Arg^9-BK was dependent upon the presence of the phenylalanine residue at position 8. The N-terminal heptapeptide of BK was completely ineffective, whereas replacing Phe^8 with a tyrosine led to a 2 log reduction in potency. Replacement of Phe^8 with aliphatic amino acids, however, was found to confer selective competitive antagonist activity (52). Of the antagonists originally produced, [Leu^8]des-Arg^9-BK was among the most potent and this compound, and its related analog [Leu^9]des-Arg^{10}-Lys-BK, have been by far the most widely employed B_1 antagonists. They are selective antagonists in that they do not antagonize the actions of BK on tissues, such as rat uterus (53), dog carotid artery, or rabbit jugular vein (54), that contain only B_2 receptors, nor do they antagonize the actions of structurally unrelated agonists, such as angiotensin, noradrenaline, or histamine (52). Since the proline at position 7 of the kinin sequence renders these antagonists resistant to the actions of carboxypeptidases, it is perhaps not surprising that the introduction of structural changes designed to render [Leu^8]des-Arg^9-BK even less susceptible to metabolism by carboxypeptidases, such as amidification or esterification of the C-terminal carboxyl group or the use of a DLeu, led to no significant increase in the duration of antagonist action and generally resulted in antagonists with a lower affinity (51). A modest increase in potency and a marked increase in duration of action on the rabbit aorta was seen, however, upon replacement of Phe^5 with a carboranylalanine residue, an amino acid with a rigid ring containing 10 atoms of boron that occupies a volume similar to that of phenylalanine (51). This increased duration of action presumably reflects an increased resistance to metabolism by endothelial angiotensin converting enzyme, which normally hydrolyzes analogs of des-Arg^9-BK at the Phe^5-Ser^6 bond to release the C-terminal tripeptide (113, 114). The importance of peptidase degradation must always be remembered when con-

sidering the actions of kinins. Not only do tissue peptidases produce a functional antagonism of kinin responses via their degradative action, but carboxypeptidase metabolism of peptide B_2 agonists and antagonists can convert them into peptides that are active on B_1 receptors (115). Thus, blockade of a kinin response by a B_2 antagonist in intact tissues cannot be assumed to imply the presence of a B_2 receptor unless it is demonstrated that metabolism of the compound by carboxypeptidase does not occur, or that specific B_1 agonists and antagonists have no effect. Alternatively, this problem could be overcome by using recently developed pseudopeptide analogs of BK, in which the Phe^8-Arg^9 peptide bond is replaced by the isosteric CH_2-NH bond, that are resistant to hydrolysis by both carboxypeptidase N and angiotensin converting enzyme and have no effect on B_1 receptor-dependent systems (116).

APPLICATIONS The application of B_1 receptor antagonists in vitro and in vivo has revealed that this receptor subtype has a much more limited distribution than the B_2 receptor. The predominant use of [Leu^8]des-Arg^9-BK has been in examining the actions of des-Arg^9-BK on vascular smooth muscle of certain animal species, particularly the rabbit. The presence of B_1 receptors has been confirmed in isolated rabbit aorta, mesenteric vein, and in mesenteric, celiac, and basilar arteries (51–57). These studies have revealed intriguing differences, however, between different vascular beds within the same species. Thus, while rabbit mesenteric vein contains exclusively B_1 receptors, the jugular vein of this animal contains entirely B_2 receptors. [Leu^8]des-Arg^9-BK has also been used to determine the specificity of kinin effects in nonvascular tissues. The effects of des-Arg^9-BK on thymidine incorporation and mitosis in both human embryonic lung fibroblasts (40) and rabbit dermal fibroblasts (41) were clearly inhibited by [Leu^8]des-Arg^9-BK, suggesting a B_1 receptor-mediated event. Moreover, while bovine endothelial cells possess a high-affinity B_2 receptor, they are also responsive to high concentrations of des-Arg^9-BK (69, 117). The ability of des-Arg^9-BK to stimulate the release of prostanoids (117) and endothelium-derived relaxing factor (69) was inhibited by [Leu^8]des-Arg^9-BK, while this B_1 antagonist had no effect on responses to BK mediated via the high-affinity B_2 receptor. The existence of B_1 receptors on these cells was further confirmed by demonstrating that this antagonist was effective in displacing low-affinity BK binding from these cells (69). In contrast, while des-Arg^9-BK displayed a similar potency to BK in causing bone resorption, this property cannot be attributed to B_1 receptor occupancy, since [Leu^8]des-Arg^9-BK was ineffective in blocking this effect (38). This latter observation highlights the need for caution in using a response to des-Arg^9-BK as the sole criterion for defining the existence of B_1 receptors in a tissue. Thus, while the contractile action of BK on the rat duodenum has

been attributed by some (118) to effects via B_1 receptors because it can be mimicked by des-Arg^9-BK, others have suggested that the rank order of potency of BK and des-Arg^9-BK in causing duodenal contraction argues against a role of B_1 receptors (61). The failure to use [Leu^8]des-Arg^9-BK to antagonize these effects leaves the role of B_1 receptors in this tissue unclear. Antagonists have been used, however, to confirm the presence of B_1 receptors in isolated rat kidneys (119) and in some nonvascular preparations, including rat bladder (67) and human circular colonic smooth muscle (62), although data suggest the coexistence of both B_1 and B_2 receptors in these tissues.

In vivo studies using B_1 antagonists have been extremely limited. It has been shown that [Leu^9]des-Arg^{10}-Lys-BK can inhibit the hypotensive response to exogenously administered des-Arg^9-BK in rabbits pretreated with lipopolysaccharide (120). A role for B_1 receptors has also been inferred in capsaicin-induced ear inflammation in mice, based upon the observation that [Leu^8]des-Arg^9-BK was more potent than a B_2 antagonist in inhibiting this response (121). To our knowledge, no study in humans has demonstrated blockade of a physiologic response by administration of a B_1 antagonist.

In summary, therefore, the B_1 receptor subtype has been described principally in animal tissues, where it exists primarily under pathological and/or inflammatory conditions. Even when present, however, its putative physiological effects often require high concentrations of peptide (100 nM or greater). These features, together with its limited distribution in human tissues, suggest that this receptor subtype is unlikely to play a major role in the actions of kinins in human disease states.

B_2 Antagonists

Although the ability to inhibit actions of BK has been attributed to many compounds over the years, most have failed to show selectivity and have often proven to be inhibitors of signal transduction pathways that are common to many agonists. Such compounds are not considered here. Similarly, although it has recently been demonstrated that a series of compounds, believed to be terpene glycosides (122–124), derived from the Brazilian plant *Mandevilla velutina,* show competitive functional antagonism of the actions of BK on some smooth muscle preparations, they are of low potency, have not been shown to cause displacement of BK in binding studies, and their structures have yet to be fully elucidated. In the present chapter, therefore, we will not consider these compounds further but focus on our knowledge of synthetic peptide antagonists of BK.

STRUCTURE It is now almost six years since the first effective B_2 antagonists were discovered (125). After over two decades of frustrating attempts to modify the structure of BK to yield an antagonist, Vavrek & Stewart

found that the essential structural change for conferring antagonist properties was the replacement of the proline residue at position 7 in the BK sequence with a D-aromatic amino acid. The most widely used replacement, and the one that has yielded the most successful antagonists, has been D-phenylalanine. Although [DPhe^7]BK itself was a weak antagonist of BK on the guinea pig ileum, it was a weak agonist on rat uterus. Clearly, additional modifications of the kinin molecule were necessary to generate useful antagonists. Replacement of the phenylalanine residues at positions 5 and 8 of BK with isosteric β-(2-thienyl)alanine (Thi) residues had previously been shown to increase the specific binding affinity of agonist analogs to bovine uterine myometrium (85), and incorporation of such residues into [DPhe^7]BK led to the first effective kinin antagonist (125). Since that time, literally hundreds of analogs with additional or alternative structural modifications have been synthesized (126–129). It is not possible to make many comprehensive statements about the effects of specific types of sequence changes, but two additional types of structural modifications have proven useful in producing some of the more potent peptide antagonists. First, replacement of one, or both, of the proline residues at positions 2 and 3 with trans-4-hydroxyproline has led to enhanced potency, while at the same time producing compounds with differing degrees of selectivity in antagonizing the effects of BK on rat uterus and guinea pig ileum (127–129). The second change that has been incorporated into several of the most potent antagonists is an extension of the amino terminus of the kinin moiety, usually with Lys-Lys or with DArg. Although all [DPhe^7]BK analogs so far tested are resistant to hydrolysis by angiotensin converting enzyme (130), the amino terminal extension of the kinin sequence may confer better resistance to other peptidases as well as perhaps causing an increase in affinity (128, 129). The issue of peptidase degradation remains an important one in considering the utility of these compounds and the antagonists synthesized to date have shown half-lives of only a few minutes in rats in vivo. As mentioned above, the presence of a carboxyterminal arginine residue renders these molecules susceptible to metabolism by carboxypeptidase N, with the resultant des-Arg^9 metabolites displaying antagonist properties on B_1 receptors (115). Interestingly, however, although replacement of Arg^9 with phenylalanine would be expected to confer resistance to carboxypeptidase N, Phe^9-substituted antagonists have not shown significantly prolonged half-lives in rats in vivo (129), suggesting that other peptidases play important roles in degradation.

The most potent, broad-spectrum antagonists used to date have incorporated most or all of the structural modifications described above. Additional modifications have been incorporated in an attempt to confer specificity of antagonists for selected smooth muscle preparations. One such change was to replace Arg^1, usually with a β-2-naphthyl-alanine (Nal) residue. This

modification converted several analogs that were antagonists on both rat uterus and guinea pig ileum into antagonists that were selective, albeit with low potency, for rat uterus (127). Studies by Farmer et al (131) demonstrated that one such antagonist, [DNal[1]-Thi[5,8]-DPhe[7]]BK, also inhibited vasopressin-induced contraction of uterine smooth muscle, a property not observed for the antagonist without this modification at Arg[1]. [DNal[1]-Thi[5,8]-DPhe[7]]BK did not compete, however, with radiolabeled vasopressin in uterine binding studies, nor did a whole series of Arg[1] modified analogs, including [DNal[1]-Thi[5,8]-DPhe[7]]BK, show significant competition with BK for binding on either uterus or ileum, suggesting that these modified analogs may not be BK receptor antagonists (131). These observations underline the need for combining binding studies with functional antagonism data before defining a compound as a receptor antagonist and indicate that results obtained with other analogs that are "tissue selective" must be viewed with caution until their ability to compete in specific binding assays is evaluated.

APPLICATIONS Although several hundred kinin analogs have already been synthesized in the search for the ideal antagonist, by far the majority of the studies performed to date have employed one or more of the analogs listed in Table 1. Because many authors have chosen to use abbreviations for these analogs that are based either on batch synthesis numbers of Stewart & Vavrek ("B" numbers) or on numbers assigned by Nova Pharmaceutical Corporation ("NPC" numbers), these have been included in Table 1 for reference purposes. In general, the compounds listed show pA_2 values of approximately 6 for BK-induced contraction of guinea pig ileum and pA_2s of 6 to 6.9 for rat uterus (126–129). They show relative selectivity in that they do not antagonize the ability of angiotensin II or substance P to contract guinea pig ileum (125), nor do they inhibit the actions of vasopressin or $PGF_{2\alpha}$ on isolated rat uterus (131). It must be emphasized, however, that these first generation antagonists have not been subjected to exhaustive specificity testing.

Cardiovascular system It is well established that BK can alter the tone of isolated vascular smooth muscle preparations, causing either contraction or relaxation depending upon the species and anatomic location from which the vessel is derived (132). Although the responses of vessels such as the rabbit aorta (52) and rabbit mesenteric artery (56) have been attributed to B_1 receptor-mediated effects, the actions of BK in many other vessels are clearly mediated via B_2 receptors. For example, BK-induced contraction of rat jugular vein and relaxation of dog carotid artery can be blocked by [Thi[5,8],DPhe[7]]BK (133). This same B_2 antagonist also inhibits the dilation of rabbit cerebral arterioles induced by BK and kallikrein but not by other

Table 1 The most commonly used B_2 kinin receptor antagonists

Structure	Pseudonyms
[Thi^{5,8}, DPhe^7]BK[a]	NPC 431; B3592, B3880, B3926
[Hyp^3, Thi^{5,8}, DPhe^7]BK	NPC 394; B3820, B4146
[DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK	NPC 349; B3824, B4162, B4881, B6572
[DArg^0, Hyp^3, DPhe^7]BK	NPC 567
Lys-Lys-[Thi^{5,8}, DPhe^7]BK	NPC 420; B3878
Lys-Lys-[Hyp^3, Thi^{5,8}, DPhe^7]BK	NPC 415; B3814, B4310

[a]BK = bradykinin

Numbering system for residues is based on the bradykinin sequence (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Scr^6-Pro^7-Phe^8-Arg^9)

agonists, such as adenosine, acetylcholine, or vasoactive intestinal polypeptide (134). Finally, in the only study, to our knowledge, performed to date on isolated human vessels, [Thi^{5,8}, DPhe^7]BK inhibited BK-induced relaxation of the human basilar artery. This observation, together with the lack of effect of des-Arg^9-BK as an agonist, led Whalley and colleagues to conclude that the effects of BK in this tissue are mediated via B_2 receptors (135). The ability of BK to relax several types of isolated vessels is dependent on the presence of endothelium (133, 135). Although there is some evidence for the presence of a low-affinity B_1 receptor on bovine endothelial cells (69), increases in cytosolic calcium, and release of endothelium-derived relaxing factor, in response to BK are inhibited by [Thi^{5,8},DPhe^7]BK and [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK but not by the B_1 antagonist, [Leu^8]des-Arg^9-BK (69). Similarly, BK-induced increases in cyclic GMP in porcine endothelial cells are mediated via B_2 receptors and can be antagonized by [DArg^0, Hyp^3, DPhe^7]BK (98).

Despite the variable effects of BK on vascular smooth muscle preparations in vitro, the overall effect of this peptide when administered in vivo is to cause peripheral vasodilatation and hypotension. These properties have led many investigators to suggest that BK may play a role in the maintenance of blood pressure during normal and pathologic states (48–50). Several studies have employed peptide B_2 antagonists to probe the role of kinins in regulating blood pressure in normotensive rats. Because of the problems associated with the short half-life of these compounds in vivo, they have been administered as either high-dose bolus injections or as continuous infusions. Different studies have produced conflicting results and it is difficult to determine how much this reflects variations in dosage and method of administration. Intra-aortic injection of 1 mg of [Hyp^3, Thi^{5,8}, DPhe^7]BK into normotensive, unanesthetized rats produced a variable response, causing a rise in blood pressure in some animals but a decrease in others (136). Another study using an infusion of high concentrations of [Hyp^3, DPhe^7]BK resulted in a significant

rise in blood pressure, but this was due to stimulation of the sympathoadrenal system and was prevented by adrenergic blockade (137). Again, the results of this latter study emphasize the need for caution in assuming that all effects seen with these antagonists relate specifically to kinin receptor antagonism. Administration of 1 mg of [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK into normal rats led to a transient biphasic response characterized by a pressor response followed by a depressor effect (138). The depressor response was not observed in nephrectomized rats or in animals pretreated with indomethacin, suggesting that it was due to a release of prostanoids from the kidney. In contrast, the pressor effect was not affected by either of these treatments, by adrenalectomy, or by the use of vasopressin antagonists or of ganglionic and adrenergic receptor blockers and it was suggested, therefore, that kinins may contribute to the regulation of blood pressure (138). However, if either [Hyp^3, Thi^{5,8}, DPhe^7]BK (139) or [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK (140) were infused at doses sufficient to block the effects of exogenous BK, but lower than those used for the above bolus injections, no effect on blood pressure was seen in normotensive rats. Similarly, whereas a 400 μg bolus of [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK was sufficient to block the effects of exogenously administered BK, it also had no effect on normal blood pressure (141). An interesting aspect of these latter two studies arose, however, when the same doses of antagonists were administered to animals receiving nonpressor doses of angiotensin II or methoxamine, or pressor doses of vasopressin or methoxamine. In each case, the antagonist significantly increased blood pressure, suggesting that endogenous kinins participate in the control of blood pressure by attenuating the effects of pressor hormones (140, 141).

The development of kinin antagonists has also prompted several investigators to reexamine the controversy regarding the role of kinins in the hypotensive and cardioprotective effects of angiotensin converting enzyme (ACE) inhibitors. Since ACE will both inactivate BK and convert angiotensin I to angiotensin II, inhibiting this enzyme could possibly lower blood pressure not only by reducing the formation of angiotensin II but also by reducing degradation of endogenously generated kinins. Attempts to confirm this theory based on direct measurements of plasma kinins during therapy have led to inconclusive results (142–145). In normotensive rats, acute administration of an ACE inhibitor did not modify the rise in blood pressure associated with administration of a 1 mg bolus of [Hyp^3, Thi^{5,8}, DPhe^7]BK (136), nor did converting enzyme inhibition modify the effects of [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK on blood pressure in normotensive rats pretreated with a nonpressor dose of angiotensin II (140). In contrast, infusion of [Hyp^3, Thi^{5,8}, DPhe^7]BK into renovascular hypertensive rats blocked by 30% the fall in blood pressure induced by administration of enalapril but had no effect on blood pressure changes induced by saralasin or by sodium nitroprusside

(139). The relative specificity of the antagonist effect suggests that kinins play a role in the hypotensive effects of converting enzyme inhibitors in experimental renovascular hypertension. Moreover, a similar effect of [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK was seen in blunting the antihypertensive effects of ACE inhibition in rats with severe hypertension induced by aortic ligation between both renal arteries (146). There is also good evidence that [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK inhibits, in a dose-dependent manner, vascular prostacyclin synthesis induced by three different converting enzyme inhibitors (147, 148). Finally, ACE inhibitors have been shown to display cardioprotective effects during congestive heart failure and cardiac ischemia (149, 150). Evidence for a role of kinins in these cardioprotective effects has been provided by recent studies showing that the ability of BK, or of ACE inhibition, to increase coronary flow and to protect against perfusion ventricular fibrillation in isolated, ischemic working rat hearts, can be completely blocked by perfusion with 10^{-5} M [DArg^0, Hyp^2, Thi^{5,8}, DPhe^7]BK. Increasing the doses of ACE inhibitor or BK used resulted in competitive reversal of the effects of the antagonist (151).

The ability of kinins to modulate blood pressure has also been related to their actions on the kidney. Various studies have suggested that kinins can cause natriuresis and diuresis, stimulate renin and prostaglandin release, and affect the renal vasculature, both directly and by modulating the effects of other hormones (152). The use of [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK, in an attempt to better understand the effects of endogenously produced kinins on renal function, has led to conflicting results. In one study, this antagonist had no appreciable effects when given to normal rats (153), whereas other investigators reported that the same compound selectively lowers papillary blood flow (154). In both studies, however, effects of the antagonist were seen when rats were pretreated with captopril and phosphoramidon to reduce the activity of renal kininases, implying that a regulatory role of kinins on renal function is more easily demonstrable when renal kinin levels are increased. In this situation, Nakagawa and colleagues (153) reported that the antagonist lowered renal plasma flow, glomerular filtration rate and sodium excretion, while Roman and coworkers (154) observed reduced papillary blood flow and sodium excretion. In salt-restricted dogs, [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK led to renal vasoconstriction and reduced renal blood flow without affecting sodium excretion or glomerular filtration rate, but a cautionary note regarding the specificity of the kinin antagonist was introduced when the effects of the compound were attributed to stimulation of renin secretion (155).

Hypotensive shock Several studies have reported systemic activation or consumption of kinin system components during shock associated with sep-

ticemia (156, 157), anaphylaxis (158), or acute pancreatitis (159), and it has been demonstrated that rapid activation of the kinin system associated with infusion of a plasma fraction containing Hageman factor fragments led to hypotensive crises (160). Several investigators have used kinin receptor antagonists in an attempt to clarify the role of kinins in shock reactions. Weipert and coworkers (161) used Lys-Lys-[Hyp^2, $Thi^{5,8}$, $DPhe^7$]BK at a dose that effectively blocked the hypotensive response to exogenously administered BK, but not that to acetylcholine, to examine the involvement of kinins in endotoxic shock. Infusion of drug or saline was carried out over a 25 minute period beginning 5 minutes prior to a bolus injection of lipopolysaccharide (LPS). A biphasic alteration in blood pressure was seen upon LPS administration. An acute fall in blood pressure immediately upon injection that lasted less than 2 minutes was not affected by the kinin antagonist, whereas a second, more chronic pressure drop, lasting approximately 20 minutes, was significantly attenuated by administration of the antagonist, leading these authors to conclude that kinins play a role in endotoxic shock. Another, more recent study (162) attempted to repeat this protocol and found no effect of Lys-Lys-[Hyp^2, $Thi^{5,8}$, $DPhe^7$]BK on the hypotensive response to LPS. Moreover, a variety of protocols using another antagonist, [$DArg^0$, Hyp^3, $Thi^{5,8}$, $DPhe^7$]BK, also failed to display any protective action in shock initiated in rats by endotoxin, anaphylaxis, or the development of acute pancreatitis (162). The issue is further confused by a study reporting that infusion of [$DArg^0$, Hyp^3, $DPhe^7$]BK, initiated 30 minutes before administration of LPS and continued throughout the course of a 10 minute LPS infusion and for an additional 90 minutes thereafter, not only blunted the acute hypotensive response to LPS but reduced by half the 100% mortality rate seen at 24 hours in the control group (163).

Gastrointestinal system Contraction of the guinea pig ileum has been used as a bioassay for the measurement of kinins since the original discovery of BK (6). Its use as a screen in the development of antagonists means that all peptide analogs produced to date have been tested in this system (126–129). The ileal smooth muscle is presumed to contain principally B_2 receptors and all of the antagonists used in in vivo models are capable of inhibiting the actions of BK in this regard. The more recent observations that some newer analogs are "tissue selective" and inhibit rat uterine contractions caused by BK without inhibiting its actions on guinea pig ileum has led to the suggestion that receptor subtypes exist. This subject is discussed in more detail below. BK-induced ion transport mechanisms in intestinal mucosal preparations are also susceptible to inhibition by B_2 antagonists. In the guinea pig ileum, [$DPhe^7$]BK had no effect on basal electrolyte transport but inhibited that induced by bradykinin, whereas the B_1 antagonist, [Leu^8]des-Arg^9-BK, was

without effect (164). BK-induced chloride secretion from rat colonic mucosa is inhibited by [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK (165) and this same antagonist also inhibits ion transport induced by BK, but not that induced by forskolin or PGE_2, in an epithelial cell line derived from a human colonic adenocarcinoma (166). Finally, in a recent report on the ability of BK to contract esophageal longitudinal smooth muscle in the opossum, all analogs tested, including [DPhe^7]BK, [Thi^{5,8}, DPhe^7]BK and [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK, were found to be agonists (167). The evidence suggesting that this reflects the presence of novel subtypes of BK receptors is discussed below.

Inflammation and pain Several studies have focused on the use of antagonists to further study the role of BK as a mediator of inflammation. This peptide is extremely potent in causing increased vascular permeability and edema. In the rabbit skin, several studies have demonstrated that vascular permeability induced by BK is not affected by [Leu^8]des-Arg^9-BK (168, 169), but can be effectively inhibited by numerous B_2 antagonist analogs (168–170). In contrast, Whalley and coworkers (168) have reported that such compounds are ineffective in inhibiting BK-induced vascular permeability in the rat skin. Indeed, both [DPhe^7]BK and [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK enhanced the effects of BK. Moreover, although the rank order of potency for kinin agonists is the same in rabbit and guinea pig skin, it is different in the rat skin (171). The effects of kinins in rat skin were assumed not to be due to mast cell histamine release, since mepyramine at a concentration sufficient to inhibit the effects of administered histamine had no effects on the response to BK (171). In a subsequent report, however, Steranka and coworkers (172) observed that use of cyproheptidine, a mixed serotonin and histamine antagonist, abolished the agonist effects of kinin analogs and permitted the demonstration that [DArg^0, Hyp^3, DPhe^7]BK could block bradykinin-induced vascular permeability.

The ability of BK to cause pain has been known since the pioneering studies of Armstrong and colleagues (173, 174) who used the cantharadin-induced blister base in human skin as a model system. It is fitting, therefore, that one of the earliest studies using bradykinin antagonists to examine BK receptors involved in pain responses should return to this original model. The subjective assessment of pain was not affected by the B_1 antagonist, [Leu^8]des-Arg^9-BK, but was significantly inhibited by doses of [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK that had no effect against pain caused by serotonin or potassium chloride (175). Several studies have also demonstrated that peptide analogs can inhibit BK-induced pain responses in animal models. In the isolated, perfused rabbit ear, the reflex hypotensive response to injected BK is used as an index of nociception. Lys-Lys-[Hyp^3, Thi^{5,8}, DPhe^7]BK antagonized the response to BK infusion and also inhibited BK-induced release

of PGE_2 into the perfusate (170). This compound had no effect on acetylcholine-induced responses. Several B_2 receptor antagonists have also been shown to block BK-induced vascular pain, and BK-induced paw hyperalgesia, in rats (83). These same compounds were shown to be capable of competing with BK for specific binding sites on sensory nerves and to display efficacy in inhibiting urate induced paw hyperalgesia, an effect presumed to reflect antagonism of endogenously generated kinins (83). Support for this assumption is provided by a report that [DArg^0, Hyp^3, DPhe^7]BK suppresses the hyperalgesia, hyperthermia, and edema associated with subcutaneous injection of carrageenan into the rat paw (176). Recent electrophysiologic studies also support a role of B_2 kinin receptors in chemical nociception [DArg^0,Hyp^3,Thi^{5,8},DPhe^7]BK, but not [Leu^8]des-Arg^9-BK, has been shown to inhibit BK-induced activation of neurones in the dorsal horn of the spinal chord of anesthetized rats. The B_2 antagonist, or prior desensitization of the receptive field with BK, also inhibited neuronal responses to subcutaneous formalin, whereas the B_1 antagonist was again ineffective (30). The ability of BK to stimulate polymodal nociceptors in the dog testis spermatic nerve was also inhibited by [Thi^{5,8}, DPhe^7]BK, but not by [Leu^8]des-Arg^9-BK (177).

Respiratory tract Recently interest has been revived in the role of kinins in inflammatory diseases of the airways (9). Kinin generation has been shown to occur in humans, and to correlate with symptoms, during allergic reactions in the upper and lower airways (178–180) and during experimental and natural rhinovirus colds (181, 182). Moreovcr, administration of BK to the lower airways causes profound bronchoconstriction in asthmatics (183–185), while, in the upper airways, BK induces symptoms of rhinitis and a sore throat regardless of atopic status (186). Definitive evidence that kinins are involved in the pathogenesis of airway inflammation can obviously only be obtained by specific blockade of their actions. It is not surprising, therefore, that several studies have been performed in animals and in humans to determine if existing B_2 peptide antagonists are suitable for this purpose. Attempts to block the actions of exogenously administered BK have produced mixed results. In guinea pigs, bronchoconstriction elicited by intravenous (i.v.) administration of BK was inhibited by i.v. [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK. No inhibition was seen using [Leu^8]des-Arg^9-BK, nor did the B_1 agonist, des-Arg^9-BK, elicit bronchoconstriction (187). Farmer and coworkers also demonstrated a partial inhibition of the effects of i.v. BK on pulmonary inflation pressure using i.v. [DArg^0, Hyp^3, DPhe^7]BK. In the same study, however, aerosolized administration of either [DArg^0, Hyp^3, DPhe^7]BK or [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK produced only weak inhibition of the effects of i.v. BK, whereas aerosolized [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK had no effect on the response induced by aerosolized BK (188). To better understand these disparate find-

ings, these investigators studied BK-induced contraction of isolated guinea pig tracheal preparations from which the epithelium had been removed to prevent the release of relaxant prostaglandins. Neither the B_1 antagonist, [Leu^8]des-Arg^9-BK, nor any of a series of B_2 antagonists inhibited BK-induced contractions. Moreover, none of these analogs was able to compete successfully with labeled BK for specific binding sites in homogenized tracheal smooth muscle, although competition was seen in preparations of lung parenchyma (188). These observations led these authors to postulate the existence of a novel B_3 receptor subtype on guinea pig trachea (see below).

In vivo studies of allergic sheep (189) have shown that inhalation of [DArg^0, Hyp^3, DPhe^7]BK, thirty minutes before challenge with aerosolized BK, significantly inhibited the BK-induced bronchoconstriction, a surprising observation in light of the prevailing view that these antagonists have short half-lives in vivo. In the same study, this antagonist was capable of inhibiting both the influx of neutrophils and the hyperreactivity to methacholine that are usually observed 2 hours after antigen challenge in this model. The antagonist had no effect, however, on the immediate allergic response. Using the same model, it has also been reported that this antagonist will inhibit the late response to allergen challenge (190).

Unfortunately, results in the upper airways in humans have been less encouraging. As a prelude to attempting to use [DArg^0, Hyp^3, DPhe^7]BK to delineate the role of kinins in various types of rhinitis, the ability of this compound to inhibit the effects of nasal provocation with BK were examined in the first placebo controlled, double-blind study of a kinin antagonist in humans. A 500 μg dose of [DArg^0, Hyp^3, DPhe^7]BK was administered 5 minutes prior to challenge with 20 μg of BK, having previously shown that doses of the antagonist up to 2 mg per nostril had no partial agonist activity. These conditions were selected to favor a drug effect, both by using a 25-fold excess of antagonist compared to BK and by preadministration of drug to favor receptor occupancy. Under these conditions, the antagonist had no effect on the symptomatic response to kinin challenge, nor did it inhibit BK-induced increases in vascular permeability, as assessed by the levels of albumin in recovered nasal lavages (191). To evaluate if the lack of antagonism was due to clearance or degradation of the compound in the 5 minutes prior to kinin challenge, a second double-blind study was performed in which the antagonist or placebo were coadministered with the BK. Again no effect of the antagonist was seen on any parameter. In additional limited, single-blind studies, increasing the dose of antagonist used to 2 mg, or varying the time period between drug administration and kinin challenge also failed to demonstrate any inhibitory effect (191). The failure of this antagonist to block the actions of BK in this system may reflect an inability of the antagonist to gain access to the appropriate receptor or it could be that BK is exerting its

effects via a receptor for which the analog used is not an antagonist. It is also possible that the antagonist is simply of insufficient potency to be effective. Prior to this study, experiments of the effects of B_2 antagonists on human tissues or cells had been extremely limited. In experiments using [DArg^0, Hyp^3, DPhe^7]BK on relevant target tissues, although 10^{-6} M antagonist blocked BK-induced prostanoid production from cultured human tracheal epithelial cells, it also completely inhibited basal prostanoid release. When used at concentrations that did not inhibit basal release it failed to inhibit the increased production to BK. Moreover, at a concentration of 10^{-6} M, [DArg^0, Hyp^3, DPhe^7]BK showed only a modest inhibition of prostanoid release induced by 10^{-8} M BK in interleukin 1-pretreated human synovial cells, a finding that correlated well with its relative potency in displacing specific [^{3}H]BK binding from these cells (J. M. Bathon, L. Churchill & D. Proud, unpublished observations). Thus, the efficacy of this analog seems limited in at least two human cell preparations.

Nonreceptor-mediated effects The need for caution in interpreting results obtained using high concentrations of these peptide analogs in vivo is emphasized by a number of studies, both in vitro and in vivo, in which these compounds clearly function via nonreceptor-mediated mechanisms. As mentioned above, [Hyp^3, DPhe^7]BK increased blood pressure in rats by stimulation of catecholamines from the adrenal gland, rather than by antagonizing endogenous kinins (137), while the effects of [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK on renal blood flow in salt-restricted dogs was attributed to stimulation of renin release (155). The antagonism of bradykinin-induced uterine contractions by [DNal^1, Thi^{5,8}, DPhe^7]BK was also shown to be unrelated to receptor antagonism (131). Although BK is not a secretagogue for human mast cells or basophils, a whole series of kinin analogs, including [DPhe^7]BK, induces histamine release from human skin mast cells via a mechanism that is unrelated to specific kinin receptors (192). A similar conclusion was drawn regarding the mechanism by which these compounds induce histamine release from rat peritoneal mast cells (193). Intracerebroventricular administration of DPhe^7-substituted kinin analogs has been reported to induce postural distortions and barrel rotation in rats, an effect not seen with BK administration, nor could it be prevented by preadministration with BK (194). Finally, some antagonists have been reported to inhibit human tissue kallikrein (195).

Evidence for New Receptor Subtypes

The existence of subclasses of receptors is generally suggested in pharmacological studies by observed differences in physiological response to a series of receptor agonists and antagonists (196). Ideally, the proposed subclassifica-

tion is then confirmed in radioligand binding studies, by correlating the binding affinities of ligands with their physiologic potencies (197). A strict definition of a new kinin receptor subtype, therefore, would require a target tissue or cell displaying specific binding properties for, and functional responses to, BK that are insensitive to selective B_1 and B_2 antagonists. In addition, the binding, and actions, of BK should be able to be antagonized by compounds selective for this receptor-mediated response. No study to date has rigorously satisfied these criteria, but several investigators have presented intriguing results that they have suggested may be indicative of the presence of new receptor subtypes.

The first type of evidence to be put forward has been based upon heterogeneity of responses of different tissues, as well as differing responses within an individual tissue, to kinin agonists and antagonists. The description of kinin analogs that selectively inhibit the actions of BK on guinea pig ileum but not rat uterus (or vice versa) was clearly provocative (128, 129). The specificity of such analogs with Arg^1 modifications has clearly been disproven, however, since these analogs also inhibit vasopressin-induced contractions of rat uterus and do not compete for specific kinin binding sites (131). In light of this, the assumption that other classes of analogs that display tissue-selective inhibition reflects the presence of novel receptor subtypes must be viewed with skepticism until supported by binding studies. Attempting to define the presence of new receptor subtypes based entirely on functional responses of intact tissues is always extremely difficult. While variability of responses among different tissues to a given kinin analog may indeed reflect multiple receptor subtypes, alternative explanations may include tissue differences in the rate or method of kinin degradation, in access of ligand to the receptor site, in binding to nonspecific membrane molecules, in transduction signaling mechanisms or in the capacity to generate a specific end product responsible for the biological response. The different rank order of potency for kinin agonists in inducing increased vascular permeability in rat, as compared to rabbit, skin, and the reported ineffectiveness of B_2 antagonists in the rat model (168, 171), could have many explanations. The suggestion that release of histamine and serotonin from mast cells may contribute to the actions of BK (172) in the rat may help to explain different rank order potencies for agonists without the need to invoke new receptor subtypes, since the ability of peptides to act as mast cell secretagogues is unrelated to activation of specific kinin receptors (192, 193). The effects of kinin antagonists on the dual response of the rat vas deferens to BK has also led to suggestions that multiple receptor subtypes exist (198, 199). The direct musculotropic effect of BK was antagonized by [$Thi^{5,8}$, $DPhe^7$]BK, while this analog acted as an agonist in potentiating the muscular response to transmural electrical stimulation. B_1 agonists and antagonists were inactive both pre- and

postjunctionally (198). The differential effects of the B_2 analog were suggested to reflect different receptor subtypes. No evidence was provided, however, that the agonist effect of [Thi^{5,8}, DPhe^7]BK was receptor mediated. Moreover, this analog potentiated the direct musculotropic effect of norepinephrine. Thus, prejunctional effects of the analog likely reflect a similar potentiation, since neurogenic effects in this tissue depend upon the presence of functional adrenergic nerve terminals. In a second study (199), two other antagonists, [Hyp^3, Thi^{5,8}, DPhe^7]BK and [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK, both functioned as pure antagonists both pre- and postjunctionally, albeit with somewhat differing potencies at the two sites. For the reasons documented above, however, it is difficult to interpret the meaning of such a differential potency effect when monitoring functional responses in intact tissues. Concerns regarding specificity must also be raised when considering a report suggesting that BK's effects on contraction of opossum esophageal smooth muscle are mediated via two novel subtypes of kinin receptor (167). In this tissue, the contraction to BK failed to achieve a true maximum, even at an administered dose of 5×10^{-5} M, and all of the bradykinin analogs examined showed agonist activity. A putative B_3 receptor subtype was characterized by its activation by very high concentrations (pD_2 values ranges from 5.2 to 5.4) of three putative "tissue-selective" antagonists, by rapid desensitization, and by its ability to cause prostaglandin-mediated contraction. In view of the problems mentioned above with other "tissue-selective" analogs, it is likely that these analogs stimulated prostaglandin release via a nonreceptor-mediated mechanism (particularly at the concentrations used) and that the desensitization of the response to these analogs by BK occurs at a post receptor level. The putative B_4 receptor in this tissue was activated by [Thi^{5,8}, DPhe^7]BK and [DArg^0, Hyp^3, Thi^{5,8}, DPhe^7]BK and did not utilize a prostaglandin-dependent pathway. In light of the unusual tissue-dose response to BK and of the lack of binding data to support the functional studies, the possibility of a nonreceptor-mediated mechanism must again be considered.

Although examining the responses of individual cell populations avoids some of the problems associated with intact tissues, the question of receptor specificity can again arise if functional responses are monitored in the absence of binding studies. The differential responsiveness of prostanoid production from 3T3 fibroblasts and an endothelial cell line to various kinin analogs led to the suggestion that the kinin receptors on these cells types were different (117). This is complicated, however, by the fact that prostanoid production by the two cell types involves different signal transduction pathways. Thus, the differential potency of [DArg^0, Hyp^3, DPhe^7]BK in inhibiting BK-induced prostanoid generation in the two cell types may relate to differential signal transduction coupling of the same receptor subtype on the two cells. The analog [p-chloro-DPhe^6, DPro^7]BK was an agonist in the two cell types, but an

unusual bell-shaped curve was seen in the endothelial cell line. Although this may reflect a variation in receptor subtype, the decline in agonist activity at high concentrations in the endothelial cell line could also be related to a nonreceptor-mediated inhibition of a post receptor event in this cell.

To our knowledge, only two studies suggesting the existence of novel receptor subtypes have employed specific binding studies in conjunction with the measurement of functional responses. Braas and coworkers (84) studied the ability of several analogs to compete with BK for specific binding sites on guinea-pig ileal membranes and neuroblastoma cells with their ability to inhibit BK-induced ileal contractions and inositol phosphate production in the neuroblastoma cells. It was stated that differences in binding affinities and agonist or antagonist properties were consistent with the presence of multiple receptor subtypes. Although K_i values for the analogs did differ quantitatively, always being lower in ileal membranes than in neuroblastoma cells, the rank order of potency was virtually identical. Thus, the variation in K_i values may reflect technical differences between the two preparations. It is intriguing that pA_2 values reported for effects of analogs in inhibiting BK-induced ileal contractions did not show the same rank order potency observed in membrane binding studies, again emphasizing the complex nature of functional responses in intact tissues. Those analogs that behaved as antagonists in neuroblastoma cells showed a good correlation between rank order potencies in functional and binding experiments. The fact that two analogs acted as agonists for inositol phosphate turnover may reflect receptor heterogeneity, but could also reflect an ability of these particular analogs to have additional, nonreceptor-mediated effects on these cells. The final study combining binding studies and functional measurements was that of Farmer and colleagues in guinea pig trachea (188). As mentioned above, a series of B_2 antagonists produced only weak inhibition of BK-induced bronchoconstriction in vivo and failed to antagonize the ability of BK to contract the epithelium-denuded guinea pig trachea in vitro. Binding studies confirmed that these antagonists could compete for specific bradykinin binding sites in lung parenchyma but were unable to displace specific binding in tracheal homogenates. These results seem consistent with the presence of a novel receptor subtype, but final proof must await the development of an antagonist selective for this binding site.

SUMMARY AND FUTURE DIRECTIONS

The provocative studies outlined above clearly indicate that the development of kinin antagonists and the characterization of kinin receptors are still in their early stages. The production, in particular, of the first B_2 kinin antagonists has led to substantial advances in our understanding of the mechanisms of

kinin action in several cells and tissues and has generated a resurgence of interest in the role of kinins in pathophysiologic functions relating to inflammation and hypertension. It must be remembered, however, that these are the first generation of such antagonists. As such, it is appropriate to adopt a cautious approach to interpretation of data obtained using these analogs, particularly if concomitant binding studies and selectivity controls cannot be incorporated into a study design. The application of these first generation compounds to clinical problems in which kinins may play a role is likely to be restricted by their susceptibility to rapid degradation and by their relatively low potencies. The results obtained with these analogs, however, have provided an impetus for several companies and individual laboratories to focus on developing compounds that overcome these deficiencies. Recently, for example, a compound (Hoe 140) has been produced with considerably increased potency and a greatly prolonged in vivo efficacy. Preliminary studies reveal that it displays comparable potency to BK for specific binding sites on cultured human synovial cells and that it is an effective antagonist of BK-induced prostanoid release from both synovial cells and respiratory epithelial cells (J. M. Bathon, L. Churchill & D. Proud, unpublished observations).

With the continued development of potent and selective kinin antagonists, the next few years seem certain to permit continued improvements in our understanding both of kinin receptor subtypes and of the role of kinins in pathophysiological events. In the longer term, such compounds may hold promise as novel therapies for combating the pain and inflammation associated with several disease conditions.

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