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# BIOCHEMICAL AND MOLECULAR PHARMACOLOGY OF KININ RECEPTORS

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## INTRODUCTION

Bradykinin (BK; Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is a potent inflammatory peptide whose generation in tissues and body fluids elicits numerous responses including vasodilation, edema, smooth muscle spasm, as well as pain and hyperalgesia via stimulation of C- and A-fibers. There is substantial evidence that BK and related kinins, such as kallidin (Lys-BK, or KD), contribute to the inflammatory response in acute and chronic diseases including allergic reactions, arthritis, asthma, sepsis, viral rhinitis, and inflammatory bowel diseases.

The past few years have witnessed an abundance of reviews on regulation of the biosynthesis, release, and degradation of kinins, in addition to their physiological and pathophysiological effects (1–13). Here we highlight recent advances in our understanding of biochemical and molecular aspects of kinin receptor pharmacology.



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## KININ RECEPTORS

Kinin receptors were originally classified according to the relative potencies of agonists in isolated vascular smooth muscle preparations (14), and subdivided into two classes, $B_1$ and $B_2$. Thus, $B_2$ receptors in rabbit jugular vein or dog carotid artery respond to kinin agonists with the following rank order of potency:

$$KD \geq [Tyr(Me)^8]\text{-}BK \geq BK > desArg^{10}\text{-}KD > desArg^9\text{-}BK$$

Most physiological effects of BK are mediated by $B_2$ receptors although there is increasing evidence for heterogeneity among $B_2$ receptors, and even for novel receptor subtypes (discussed later).

By comparison, $B_1$ receptors, in rabbit aorta or mesenteric vein, exhibit almost the opposite rank order of sensitivity to kinin agonists:

$$desArg^{10}\text{-}KD > desArg^9\text{-}BK > KD > BK > [Tyr(Me)^8]\text{-}BK$$

Conventionally, $B_2$ receptors exhibit much higher affinity for kallidin or BK than for the carboxypeptidase N metabolites, $desArg^{10}$-KD or $desArg^9$-BK, whereas $B_1$ receptors are more sensitive to the desArg metabolites. For this reason, the relative potencies of BK and $desArg^9$-BK are frequently used to classify a particular response as $B_1$ or $B_2$.

### *$B_1$ Receptors*

Although $B_1$ receptors have been studied largely in rabbit vascular smooth muscle (10, 15), they have also been demonstrated in various cells maintained in tissue culture. In human fibroblasts, for example, $desArg^9$-BK induced collagen formation, protein synthesis, and cell proliferation, which were inhibited by $desArg^9[Leu^8]$-BK, a $B_1$ receptor antagonist (16). BK also stimulated prostaglandin formation in these cells, an effect that was unaffected by the $B_1$ antagonist (16). As noted later, $B_2$ receptors have recently been solubilized from human foreskin fibroblasts (17). Faussner and colleagues demonstrated that BK and KD, but not $desArg^9$-BK, displaced specific $[^3H]$-BK binding in these cells with $IC_{50}$ values of approximately 8 nM. Data from these studies, therefore, indicate that human fibroblasts express $B_1$ and $B_2$ receptors.

Burch et al recently confirmed that $B_1$ receptors are expressed in P338-D1 murine macrophages (18). This study reported that BK-induced release of the cytokines, tumor necrosis factor (TNF), and interleukin 1 (IL-1) from these macrophages was mimicked by $desArg^9$-BK, and antagonized by $desArg^9[Leu^8]$-BK. In an earlier study, BK and $desArg^9$-BK were equipotent in

releasing TNF and IL-1 from the macrophage cell lines, RAW2647.7 and P338-D1 (19). The kinins' effects were inhibited by desArg$^9$[Leu$^8$]-BK and by DArg-[Hyp$^3$,DPhe$^7$]-BK (NPC 567), a mixed $B_1$ and $B_2$ receptor antagonist, leading the authors to conclude that murine macrophages express $B_1$ receptors.

Ljunggren & Lerner (20) described $B_1$ and $B_2$ receptor expression in osteoblast-like cells. BK and desArg$^9$-BK (10 nM-1 $\mu$M) stimulated $^{45}Ca^{2+}$ release (an index of bone resorption) from neonatal mouse calvarial bones. However, only the response to desArg$^9$-BK was blocked by a $B_1$ antagonist (20). Osteoblasts, maintained in tissue culture, synthesized $PGE_2$ and prostacyclin in response to BK and KD, and this response was unaffected by the $B_1$ receptor antagonist. In contrast, after prolonged incubation ($>$ 24 h), these cells developed the capacity to respond to desArg$^9$-BK by releasing $PGE_2$, an effect that was inhibited by desArg$^9$[Leu$^8$]-BK. Although the effects of a $B_2$ receptor antagonist were not examined, these data in mouse osteoblasts indicate the presence of both BK receptor subtypes (20).

Although $B_1$ receptor-mediated $^{45}Ca^{2+}$ release was evident acutely, it is curious that desArg$^9$-BK-induced prostaglandin synthesis appeared only after several hours. This may indicate that there are two subtypes of $B_1$ receptor, one (mediating $^{45}Ca^{2+}$ release) that is expressed either "normally" or induced very rapidly, and another (mediating prostaglandin production) that is induced over the several hours of the incubation period (see below). Alternatively, it is possible that the transduction mechanism for the $B_1$ receptor-mediated prostaglandin synthesis may be induced.

Bovine pulmonary artery endothelial cells (BPAEC) responded to both BK and desArg$^9$-BK by synthesizing prostacyclin (21–23), platelet activating factor (21), and endothelium-derived relaxant factor (23). These effects were inhibited by mixed $B_1$ and $B_2$ receptor antagonists such as [Thi$^{5,8}$,DPhe$^7$]-BK (NPC 431) (21) or NPC 567 (22). In contrast, desArg$^9$[Leu$^8$]-BK, inhibited only the effects of desArg$^9$-BK (22, 23).

Thus, like rat renal vasculature (24) and duodenum (25), mouse bone tissues, and human fetal lung fibroblasts (16), BPAEC appear to express $B_1$ and $B_2$ receptors (21–23). Other tissues reported to express both BK receptor subtypes include rabbit vena cava, renal vein and trachea, rat mesenteric vein, stomach, and urinary bladder, and canine jugular and mesenteric veins, vena cava, and subclavian artery (14).

INDUCTION OF SMOOTH MUSCLE $B_1$ RECEPTORS $B_1$ receptors are often not "normally" present in some tissues. Rather, a characteristic of $B_1$ receptors is their apparent induction during incubation in vitro, or following pathological or traumatic insults in vivo (reviewed in refs. 9, 10, 15). In rabbit isolated vascular smooth muscle, $B_1$ receptors are generated de novo in a time-

dependent manner. This phenomenon was first reported in anterior mesenteric vein (26), in which responsiveness to desArg$^9$-BK-induced contraction, initially nonexistent, increased with time of incubation in vitro, to a maximum level after 6 h. The increase in responsiveness was abolished by drugs that inhibit synthesis of protein or RNA (26). Further evidence that increased responsiveness to desArg$^9$-BK was due to de novo synthesis of $B_1$ receptors was provided by a report that incubation of chopped mesenteric artery for 24 h caused an increase in specific binding of desArg$^9$-BK, an effect that was abolished by cycloheximide (27). DesArg$^9$[Leu$^8$]-BK competed with [$^3$H]-desArg$^9$-BK binding with a $pK_i$ value of 6.86.

Induction of $B_1$ receptors has also been demonstrated in other rabbit blood vessels including aorta (26, 28–30), mesenteric artery (31–33), celiac artery (34), and basilar artery (35). Furthermore, $B_1$ receptors are evidently also induced by inflammation or prolonged incubation in visceral smooth muscles such as rat and human intestinal tissue, as well as rat bladder (25, 36–38).

It is noteworthy that certain vascular beds express $B_1$ receptors under apparently normal circumstances, and without pathological insult. In dogs, for example, infusion of BK into the saphenous vein caused venoconstriction secondary to thromboxane synthesis (39). This response was mediated by $B_1$ receptors, since desArg$^9$[Leu$^8$]-BK was a potent antagonist. Additionally, a mixed $B_1$ and $B_2$ receptor antagonist (NPC 431) blocked BK-induced venoconstriction (39). Similarly, kinin-induced relaxation of rabbit celiac artery is elicited by desArg$^{10}$-kallidin, and inhibited by $B_1$ antagonists (34).

MECHANISMS OF $B_1$ RECEPTOR INDUCTION It has been proposed (10, 15) that de novo synthesis of $B_1$ receptors results from trauma during tissue isolation and incubation, or from inflammation. The hypothesis was advanced that $B_1$ receptor induction is a manifestation of a homeostatic mechanism during inflammation whereby formation of $B_1$ receptors reveals local responses to normally inactive desArg$^9$ metabolites of kinins (10, 15).

The mechanisms underlying induction of $B_1$ receptors in rabbit aorta have been investigated. Incubation of aorta with endotoxin from *Escherichia coli* caused an approximately 100% increase in magnitude of contraction after 3 and 6 h exposure, as compared with controls (28). Similarly, aortic strips prepared from rabbits pretreated with endotoxin also exhibited enhanced $B_1$ responsiveness (40, 41). Intravenous desArg$^9$-BK was hypotensive in endotoxin-treated rabbits, but not in controls (28, 40-42).

Since endotoxin exerts many of its effects via macrophage protein synthesis (43–45), the effects of other macrophage stimulants on $B_1$ receptor induction were examined. Muramyldipeptide (MDP) and phorbol-12-myristate-13-acetate (PMA) enhanced $B_1$ receptor-mediated contraction after 3 and 6 h incubation (28). Moreover, the effects of these substances were selective for

kinins, as responses to neither norepinephrine nor histamine were affected.

Subsequent studies (29, 46) provided evidence that cytokines are involved in $B_1$ receptor induction. In particular, interleukin 1 (IL-1) was potent in its stimulation of responses to desArg$^9$-BK, and it may be significant that endotoxin and MDP both promote IL-1 synthesis and release from macrophages (44, 45), and fibroblasts (47), as well as from vascular endothelial (48) and smooth muscle cells (49). That IL-1 may be important in induction of aortic responses to desArg$^9$-BK was evidenced by the observation that glucocorticoid treatment of these tissues inhibited the development of $B_1$ responsiveness (29). Glucocorticoids, among their myriad pharmacological actions, inhibit IL-1 synthesis (50, 51).

Recent studies provide further, indirect evidence for a role for cytokines in $B_1$ receptor induction. Antigen arthritis in rabbits was associated with induction of $B_1$ receptors in isolated aorta smooth muscle 24 h following injection of antigen into the knee joint of sensitized animals (30). The arthritis was characterized by acute phase protein synthesis and joint swelling. Even at the beginning of experiments, aortae isolated from these animals, unlike those from controls, responded to desArg$^9$-BK. In addition, the time-dependent increase in $B_1$ responsiveness was greater in tissues from arthritic rabbits than from controls. This phenomenon was specific for $B_1$ receptors, as responses to an $\alpha$-adrenoceptor agonist were unaffected. This is the first demonstration of induction of smooth muscle $B_1$ receptors associated with an immune complex disease. The observation that fibrin challenge, administered locally, induced responsiveness to desArg$^9$-BK at a distant site implies that circulating cytokines are involved in inducing $B_1$ receptors during inflammation (30).

## *$B_2$ Receptors*

Traditionally, the lack of effect of desArg$^9$-BK in a tissue that nevertheless responded to BK, by default, resulted in the classification of the kinin receptor in that tissue as $B_2$ (14, 15). Similarly, the inactivity of $B_1$ antagonists to inhibit BK-induced responses suggests that the response is mediated by $B_2$ receptors. This is perhaps paradoxical since $B_2$ receptors are far more ubiquitous and, yet, until the discovery of $B_2$ receptor antagonists in 1985 (52), $B_1$ receptors were better characterized.

$B_2$ receptors are widespread, and mediate a multitude of physiologic responses (reviewed recently in refs. 2–4, 9, 53–55). Briefly, kinin-induced responses such as contraction of guinea-pig intestine (56–61), contraction and relaxation of rat duodenum (25, 62–64), and contraction of rat uterus (52, 61, 65–71) are mediated by $B_2$ receptors. In addition, endothelium-dependent relaxation of arterial smooth muscle from several species, including man, is mediated by $B_2$ receptors (23, 72–76). Intestinal and airway anion secretion,

induced by kinins, are mediated by $B_2$ receptors (53, 58, 76, 77), as is BK-induced stimulation of tracheal ciliary motility (78).

As noted earlier, BK is one of the most potent nociceptive substances known (79). BK has several effects on sensory and efferent autonomic nerve fibers, including membrane depolarization and modulation of neurotransmitter release, effects that are mediated by $B_2$ receptors (80–87).

## KININ RECEPTOR ANTAGONISTS

### *$B_1$ Receptor Antagonists*

Analogues of desArg$^9$-BK or desArg$^{10}$-KD are selective for the $B_1$ receptor, exhibiting $pK_b$ values in the range 6.00–8.40 (reviewed in ref. 10). The two most potent $B_1$ antagonists are desArg$^9$[Leu$^8$]-BK and desArg$^{10}$[Leu$^9$]-KD. These antagonists are $B_1$-selective, and do not antagonize the effects of BK mediated via $B_2$ receptors, e.g. in rabbit jugular vein, dog carotid artery, guinea-pig ileum (56, 61, 88) and trachea (88, 89), and rat (38) and human colon (37).

### *$B_2$ Receptor Antagonists*

ANALOGUES OF [DPHE$^7$]-BRADYKININ The report by Stewart & Vavrek (52) that substitution with DPhe for LPro in position 7 of the BK sequence conferred the ability to inhibit $B_2$ receptor-mediated effects, was the first description of selective $B_2$ receptor antagonists. Since 1985, hundreds of analogues, based on [DPhe$^7$]-BK (NPC 361), have been synthesized, and detailed analyses of the structure-activity relationships of these antagonists are reviewed elsewhere (3, 54, 90).

Although the [DPhe$^7$]-substituted analogues of BK have made a remarkable contribution to our understanding of kinin-receptor pharmacology, they have relatively weak affinity for $B_2$ receptors (3, 9, 54, 90, 91). Furthermore, these peptides often actually elicit responses such as smooth muscle contraction (61, 88, 92, 93), phosphatidylinositol (PI) metabolism (66) and mast cell degranulation (94–97). Several of these peptides suppress $B_2$ receptor-mediated effects of BK in some tissues, but not in others (61, 86, 98). Indeed, the supposed "partial agonist" and/or "tissue selectivity" of several $B_2$ receptor antagonists has resulted in reports of $B_2$ receptor subtypes (86, 98), and even novel receptors (93). Kinin receptor heterogeneity, real and fancied, is discussed later in this review.

NEW GENERATION $B_2$ RECEPTOR ANTAGONISTS Recently reported are novel peptide $B_2$ receptor antagonists that are not analogues of NPC 361, but rather are BK analogues containing modified amino acids in positions 7 and 8 of BK's primary sequence (74, 99–102). Peptides such as DArg[Hyp$^3$,

Thi[5],DTic[7],Oic[8]]-BK (HOE 140), DArg-[Hyp[3],Thi[5],DTic[7],Tic[8]]-BK (NPC 16731), DArg-[Hyp[3],Thi[5],HypE(*trans*-propyl)[7],Oic[8]]-BK (NPC 17684), and DArg-[Hyp[3],Thi[5],HypE(*trans*-propyl)[7],Tic[8]]-BK (NPC 17643) are several orders of magnitude more potent $B_2$ receptor antagonists than the [DPhe[7]]-substituted analogues in various assays in vitro (74, 99–101, 103) and in vivo (101, 102, 104, 105). Furthermore, these peptides, unlike some [DPhe[7]]-BK analogues, exhibit no spasmogenic activity in isolated smooth muscle (74, 101, 103). HOE 140 was reported to be selective for $B_2$ receptors, as it had no effect on desArg[9]-BK-mediated contraction of rabbit aorta (74). These potent, selective antagonists promise to advance our understanding of kinin receptor pharmacology.

## BRADYKININ RECEPTOR HETEROGENEITY AND SUBTYPES

Many studies have purported to demonstrate heterogeneity among $B_2$ receptors, and new terminology, including "$B_3$ receptors" (88) and "$B_4$ receptors" (93), has also been submitted. As addressed above, however, until recently the only available $B_2$ antagonists were relatively weak in their ability to inhibit responses to BK. In addition, these drugs commonly exhibit effects that probably cannot be explained by actions at BK receptors.

Considering our currently limited understanding of BK receptors, and given the lack of selective antagonists for any of the receptor subtypes reported in the literature, the results of certain investigations may require reinterpretation. Undoubtedly, the discovery of more selective and potent antagonists, such as those alluded to above, will contribute much to this aspect of kinin receptor pharmacology. On the other hand, other studies also provide persuasive evidence for heterogeneity among $B_2$ receptors, and even for novel kinin receptors.

### *$B_2$ Receptor Heterogeneity*

RAT DUODENUM Desensitization of rat duodenum to the contractile, but not relaxant effects of BK, coupled with the different potencies for BK-induced relaxation and contraction, were interpreted as evidence for different receptors mediating each response (25). The inactivity of desArg[9]-BK confirmed that this tissue normally lacks $B_1$ receptors. However, the "selective" desensitization to BK-induced contraction may be explained by the fact that much higher concentrations of BK were required to elicit contraction than relaxation. In addition, different signal transduction systems certainly mediate the two different effects, and it may be a second messenger rather than a receptor that was desensitized.

RAT VAS DEFERENS In this tissue, BK elicited contraction by a direct action on smooth muscle cells, and potentiated neurogenic contractions via a prejunctional modulation on intrinsic sympathetic nerve endings (83, 92, 106–108). DesArg$^9$-BK was inactive, and the effects of BK were unaltered by a $B_1$ receptor antagonist. Llona and coinvestigators (92) provided evidence that the smooth muscle effects of BK were mediated by a different receptor than the neuronal effects. The ability of several BK analogues to cause contraction presented a rank order of potency that differed somewhat from "classical" $B_2$ receptors:

$$BK = KD > Met\text{-}KD = [Tyr(Me)^8]\text{-}BK$$

In contrast, the ability of these analogues to potentiate neuronal norepinephrine release was:

$$Met\text{-}KD > BK > KD > [Tyr(Me)^8]\text{-}BK > NPC\ 431$$

In addition, NPC 431, normally considered to be a $B_2$ receptor antagonist (3), while inhibiting the neuromodulating effect of BK ($pK_b$ value, 5.60), caused smooth muscle contraction (92).

The different structure-activity relationships for smooth muscle stimulation and for increasing transmitter release, in addition to the observation that NPC 431 behaved as an "agonist" prejunctionally, were employed as evidence for subtypes of $B_2$ receptors (92). These observations should be interpreted with caution since it is unknown whether the "agonist" effect of NPC 431 in vas deferens was mediated by kinin receptor activation. Indeed, and as discussed later, this peptide possesses only *very* weak agonist activity ($EC_{50} >> 1$ mM) in mammalian $B_2$ receptors expressed in frog oocytes (109).

There is other evidence for differences between the smooth muscle and neuronal $B_2$ receptors in rat vasa deferentia. Thus, [Hyp$^3$,Thi$^{5,8}$,DPhe$^7$]-BK (NPC 394) was fivefold more potent as an antagonist of the postjunctional than the prejunctional effects of BK (107). Conversely, DArg-[Hyp$^3$, Thi$^{5,8}$,DPhe$^7$]-BK (NPC 349) was approximately fourfold more potent as an antagonist of prejunctional BK actions as compared with postjunctional effects. Unlike NPC 431, neither antagonist had excitatory activity (107). These studies, therefore, support the hypothesis that rat vas deferens contains pre- and postjunctional $B_2$ receptors of different subtypes.

OPOSSUM ESOPHAGUS It was recently shown that BK, NPC 361 and NPC 431 caused contraction of the longitudinal smooth muscle of opossum esophagus (93). In addition, the novel peptides [Phe$^2$,DPhe$^7$]-BK (B4772), [DPhe$^2$,DPhe$^7$]-BK (B4404) and [DPhe$^7$,Hyp$^8$]-BK (B5092), putative "tis-

sue-selective" $B_2$ antagonists (110; but, see below) elicited contractions. However, the antagonists were considerably less potent as spasmogens than BK, exhibiting $EC_{50}$ values of only 4–10 $\mu$M. Indeed, BK itself was a relatively weak and inefficacious agonist ($EC_{50}$ 0.26 $\mu$M, $E_{max}$ 44% of the maximum response to carbachol), with a very shallow concentration-response curve (93).

It was proposed that since contractions in response to B4404, B4772, and B5092 were subject to tachyphylaxis (unlike BK-induced contractions), they were mediated by "$B_3$ receptors". However, no cross-tachyphylaxis between BK and [DPhe$^2$,DPhe$^7$]-BK could be demonstrated (93). This observation suggests that contractions of opossum esophagus, in response to BK (presumably the endogenous agonist) and its analogues, may not be receptor-mediated. Moreover, although the authors did not examine the ability of these peptides to displace BK binding in esophagus, neither B4404, B4772, nor B5092 have significant effects in BK binding in guinea-pig ileum (90). Thus, the propositions that BK caused contraction via activation of "$B_4$ receptors" (93), in addition to the "$B_3$" receptor effects (93) of the analogues, have little basis. It would be interesting to learn whether HOE 140 or NPC 16731, nonspasmogenic antagonists, inhibit BK-induced contraction of opossum esophagus.

GUINEA-PIG TRACHEAL EPITHELIUM BK stimulates chloride transport in tracheal epithelial cells (111–114), and studies on the signal transduction processes involved suggested BK receptor heterogeneity. Thus, in dog tracheal epithelial cells, BK increased arachidonic acid release and stimulated PI turnover in a concentration-dependent fashion (115). In addition, these two effects of BK were independently activated, since PMA amplified BK-induced arachidonic acid release, but inhibited PI turnover (115). Furthermore, phosphoinositide metabolism was achieved at considerably lower concentrations ($10^{-12}$–$10^{-10}$M) of BK than was arachidonic acid release ($10^{-8}$–$10^{-6}$M). In polarized cultures of these cells, submucosal application of BK stimulated increases in both second messengers. In contrast, exposure of only the mucosal surface to BK resulted in arachidonic acid release, without any changes in PI turnover (115).

Differences in the ligand-binding characteristics were observed in basolateral and apical membranes prepared from tracheal epithelia, a high-affinity binding site in the latter, and a low-affinity site in the former (115). NPC 567 inhibited [$^3$H]-BK binding equally well at both sites (115). Previous studies showed that tracheal epithelial chloride secretion, induced by BK, was unaffected by $B_1$ antagonists (112, 114). Although a $B_2$ receptor antagonist inhibited BK binding in both sites, the observations of two different BK binding sites, apparently coupled to different signal transduction mechanisms,

raised the possibility of subtypes of $B_2$ receptors in canine tracheal epithelia (115).

"TISSUE-SELECTIVE ANTAGONISTS" Other studies reported the possibility of tissue differences in $B_2$ receptors. Whereas NPC 361 was a weak agonist in rat uterus, it antagonized BK-induced contraction in guinea-pig ileum ($pK_b$, 5.00) (52, 91). This contrasted with other analogues, Lys-Lys-[Thi$^{5,8}$,DPhe$^7$]-BK (NPC 420) and NPC 431, $B_2$ antagonists in both preparations. Furthermore, while NPC 361 and NPC 431 actually induced PI turnover in N1E-115 neuroblastoma cells, several other [DPhe$^7$]-substituted analogues inhibited BK-induced PI turnover (66). The differential effects of these analogues in separate bioassay procedures, along with the observation that desArg$^9$-BK was inactive, led to the proposal for multiple $B_2$ receptors (66).

However, while these BK analogues were shown to bind with nanomolar affinities to ileal or N1E-115 cell membranes, no direct evidence that the "agonist" effects of NPC 361 or NPC 431 were mediated via BK receptors. We consistently find that another of these antagonists, NPC 567, is spasmogenic in rat uterus and guinea-pig trachea (61, 88). Again, however, whether the stimulation of smooth muscle reflects a receptor mechanism is unknown. It will be useful to determine the effects of newly described, potent $B_2$ receptor antagonists such as HOE 140 upon the so-called "agonist" effects of kinin analogues.

In contrast to [DPhe$^7$]-BK analogues, [DArg$^1$]-substituted analogues of BK, such as [DNal$^1$,Thi$^{5,8}$,DPhe$^7$]-BK (NPC 573), inhibited BK-induced contractions of rat uterus, but were inactive against BK-induced contraction of guinea-pig ileum (86). Moreover, this analogue did not displace [$^3$H]-BK binding from ileal or N1E-115 membranes. The "uterus-selectivity" of NPC 573 resulted in the proposal that $B_2$ receptors in ileum are different from those in uterus (86).

Another study confirmed that, unlike the well-characterized $B_2$ receptor antagonist, NPC 567, NPC 573 inhibited BK-induced contraction in uterus but not ileum (61). Binding studies, nevertheless, revealed that several [DArg$^1$]-substituted analogues, including NPC 573, were either inactive or very weakly displaced [$^3$H]-BK binding in both tissues. Thus, the inhibitory action of NPC 573 on BK-induced smooth muscle contraction is not mediated by BK receptors. NPC 573 also inhibited vasopressin-induced uterine contractions, although it did not bind to vasopressin receptors. [DArg$^1$]-substituted analogues of BK, therefore, are not BK or vasopressin receptor antagonists (61). The proposal that ileal $B_2$ receptors are different from uterus $B_2$ receptors, based upon observations with these peptides (86), is without foundation.

## *Evidence For a $B_3$ Receptor In Guinea-Pig Trachea*

BK causes concentration-dependent relaxation of guinea-pig trachea in which the tone has been increased histamine (116, 117). DesArg$^9$-BK was inactive and BK-induced relaxation was not blocked by antagonists of $B_1$ or $B_2$ receptors (117). This was the first observation that kinin receptors, mediating guinea-pig tracheal relaxation, may not fall into the typical $B_1$ or $B_2$ classification.

Likewise, BK-induced contractions of guinea-pig trachea and lung parenchyma were resistant to inhibition by several [DPhe$^7$]-BK analogue $B_2$ receptor antagonists, including NPC 349, NPC 361, NPC 431, and NPC 567 (88). DesArg$^9$-BK was inactive, and BK-induced contraction was unaltered by desArg$^9$-[Leu$^8$]-BK. Binding studies in guinea-pig lungs and tracheal smooth muscle revealed saturable, specific binding sites for BK, but not desArg$^9$-BK. Neither desArg$^9$-[Leu$^8$]-BK nor NPC 567 displaced BK from tracheal binding sites and, in lung membranes, the $B_2$ receptor antagonist displaced only 60% of total specifically bound [$^3$H]-BK (88).

Binding data in guinea-pig lungs were verified by Mak & Barnes (118), in that a $B_1$ receptor antagonist had no effect, whereas NPC 349, a $B_2$ antagonist, displaced only 70% of specific BK binding. Thus, guinea-pig lungs may contain two kinin receptor subtypes, $B_2$ and $B_3$, whereas trachealis appears to contain one binding site, representing $B_3$ receptors, from which $B_1$ and $B_2$ receptor antagonists displaced little or no binding of [$^3$H]-BK (88).

A detailed examination of BK binding sites in guinea-pig lungs was recently published by Trifilieff et al (119). Scatchard and kinetic analyses of [$^3$H]-BK binding indicated the presence of two BK binding sites, with high and low affinity. Although $B_1$ receptor ligands had no effect on [$^3$H]-BK binding, NPC 361 and NPC 567 caused 100% displacement. On the other hand, NPC 431 inhibited high-affinity binding completely, but inhibited only 80% of BK binding under conditions of high and low affinity binding (119). These binding data are at variance with other data in guinea-pig lungs (88, 118). The reasons for this variance are unknown, but may reflect dissimilar experimental conditions, or differences in the health status of the animals.

Nevertheless, there are other indications of a third kinin receptor in airway tissues. Like guinea-pig airway tissues, sheep tracheal smooth muscle and lung contain BK binding sites from which NPC 567 displaced only 28% and 73% [$^3$H]-BK binding, respectively (88). DesArg$^9$-[Leu$^8$]-BK also had no effect in sheep-airway binding studies.

In biochemical studies in guinea-pig tracheal smooth muscle cells, BK stimulated both $PGE_2$ and prostacyclin biosynthesis, and the efflux of $^{45}Ca^{2+}$ (120). Indomethacin abolished BK-induced prostaglandin release, but the cyclooxygenase inhibitor did not affect $^{45}Ca^{2+}$ efflux, indicating that it was not mediated by prostaglandins. NPC 567 inhibited BK-induced pros-

taglandin synthesis, indicating the presence of $B_2$ receptors in the cultured cells. The efflux of $^{45}Ca^{2+}$, however, was completely resistant to the $B_2$ receptor antagonist. Moreover, desArg$^9$-BK had no activity in the tracheal smooth muscle cells (120). These functional studies, therefore, illustrated that $^{45}Ca^{2+}$ efflux in trachealis may be mediated by $B_3$ receptors.

We recently examined the effects of NPC 16731 (see above), a potent $B_2$ receptor antagonist in guinea-pig ileum (100). Confirming our original observations (88), NPC 567 (10 $\mu$M) displaced only 20% of [$^3$H]-BK (103). In contrast, NPC 16731 completely displaced [$^3$H]-BK trachealis binding. NPC 16731 also inhibited BK-induced tracheal contractions ($pA_2$ 7.63). Furthermore, NPC 16731, unlike NPC 567, completely inhibited BK-induced $^{45}Ca^{2+}$ efflux in cultured tracheal smooth muscle cells (-log $IC_{50}$ 8.48) (103). Perkins and colleagues (121) also recently confirmed that NPC 567 had no effect on BK-induced contractions of guinea-pig trachea. However, HOE 140 inhibited contractions, exhibiting an $IC_{50}$ value of less than 0.1 $\mu$M. Thus, data with these novel BK antagonists provide further evidence for the hypothesis that $B_3$ receptors are expressed in guinea-pig trachea.

NONRECEPTOR EFFECTS OF BK AND ITS ANALOGUES Several studies have demonstrated that cationic amphiphilic molecules, including peptides, can activate guanine nucleotide-binding proteins (G proteins) and hence G protein targets such as PLC (122). BK acts in this manner in mast cells, activating the endogenous G proteins in a pertussis toxin-sensitive manner, which leads ultimately to release of histamine (123). Thus, BK and certain analogues may be classified with other direct activators of G proteins, including mastoparan, substance P, and compound 48/80 (122).

The signal transduction mechanisms underlying the ability of NPC 567 to cause histamine release from human skin mast cells was recently examined (124). Whereas IgE-mediated histamine release was inhibited by agents that increase intracellular cyclic AMP levels, no effect on NPC 567-induced release was evident. In addition, the stimulatory effect of NPC 567, unlike that of IgE, did not appear to involve protein kinase C (PKC) (124). Interestingly, while IgE stimulates the release of several other mediators, such as leukotriene $C_4$ and prostaglandin $D_2$, NPC 567 released predominantly histamine, by a mechanism involving a rapid, transient increase in $[Ca^{2+}]_i$ (124). The ability of certain kinin analogues to stimulate mast cells may contribute to erroneous "receptor" subclassification.

## KININ RECEPTOR SIGNAL TRANSDUCTION

Activation of $B_2$ receptors, in virtually all tissues so far described, leads to stimulation of the activity of PI-specific phospholipase C (PLC), resulting in

the formation of inositol phosphates and diacylglycerol (3, 125–130), and subsequent elevations in $[Ca^{2+}]_i$. In addition, in human airway smooth muscle, BK-induced increases in $[Ca^{2+}]_i$ appear to be mediated both by activation of PLC/PI turnover and also $Ca^{2+}$ influx via receptor-operated $Ca^{2+}$ channels (131).

In most tissues, eicosanoid synthesis is also enhanced by $B_2$ receptor activation, potentially by release of arachidonic acid from the diacylglycerol produced by inositol lipid hydrolysis. In several tissues, however, inositol lipid hydrolysis is pharmacologically independent of arachidonate release, and has been proposed to result from activation of phospholipase $A_2$ ($PLA_2$) (132, 133). Both $B_2$ receptor-mediated inositol lipid hydrolysis and eicosanoid release are dependent on coupling of the receptors to G proteins (132, 134–136), suggesting that $B_2$ receptors belong to the class of receptors composed of seven hydrophobic transmembrane domains, at present known to include adrenoceptors, muscarinic receptors, and receptors for serotonin, neurokinins, and dopamine (137).

In addition, $B_2$ receptor activation often leads to enhanced cellular accumulation of cyclic AMP (114, 138, 139). Frequently, this is due, indirectly, to stimulation of the biosynthesis of eicosanoids, which subsequently bind to their respective receptors and activate adenylate cyclase (114, 139), but in certain tissues the BK receptor may be directly coupled to adenylate cyclase by $G_s$ (140).

The transduction pathways activated by $B_1$ receptor stimulation are less clear. In rabbit vascular smooth muscle, eicosanoid synthesis is apparently not stimulated as inhibitors of fatty acid cyclooxygenase were without effect on desArg$^9$-BK-induced contraction (14). Similarly, in murine macrophages (19) and human fibroblasts (16), desArg$^9$-BK did not stimulate eicosanoid synthesis. Conversely, eicosanoid production by $B_1$ agonists has been reported in endothelial cells and other tissues (21–23, 31).

The vast majority of signal transduction studies demonstrate that kinin signaling is initiated by activation of phospholipases. In particular, BK-induced activation of PI-specific PLC causes elevations in cellular inositol tris- and tetraphosphates, which initially release calcium from intracellular stores and secondarily increase calcium influx (for reviews see refs. 3, 9), as well as elevations in diacylglycerols that activate PKC.

Kinins often increase arachidonic acid release and metabolism. Over the years arachidonic acid release has been argued to result from direct activity of $PLA_2$, or from sequential activation of PLC to release diacylglycerol, from which arachidonic acid might be released by diacylglycerol lipase or monoacylglycerol lipase (3, 9). The calcium release elicited by the inositol phosphates, formed in response to PLC activation, has also been proposed to secondarily activate $PLA_2$. More recently, phosphatidylcholine-specific PLC

and phospholipase D/phosphatidic acid hydrolase pathways have been demonstrated to be activated by BK (141–144). These transduction pathways could also result in release of arachidonic acid in response to $B_2$ receptor activation. More work is clearly required to understand the very complex lipid metabolic pathways induced by kinins.

### *G Protein Coupling*

Many studies have indirectly suggested that $B_2$ BK receptors are coupled to G proteins (132, 135, 136, 145), and the recent cloning of a $B_2$ receptor (109) confirms this hypothesis (see below). Thus, BK-induced eicosanoid synthesis and inositol phosphate production were sensitive to guanine nucleotide analogues (132); similarly, BK-induced inositol phosphate production was pertussis toxin-sensitive (135,
endogenous G proteins had been inactivated using pertussis toxin, readdition of a combination of $G\alpha_0$ and $G\alpha_{i2}$, but neither by itself, reconstituted the ability of BK to initiate calcium currents (145). Similarly, Leeb-Lundberg & Mathis (69) found that guanine nucleotide analogues decrease affinity of BK for a high-affinity receptor in myometrial membranes.

In an extension of the studies by Burch & Axelrod (132), murine fibroblasts, permeabilized with streptolysin O, were used to further demonstrate $B_2$ receptor-coupled G protein activation as well as G protein activation of $PLA_2$ (146). In this study, when cells were permeabilized with streptolysin O, then $PLA_2$ activity assayed, BK did not stimulate the enzyme when free calcium was maintained at 100 nM. Addition of GTPγS to the media stimulated $PLA_2$ activity in a concentration-dependent manner, and, in the presence of GTPγS, BK further stimulated $PLA_2$ in a concentration-dependent manner.

Liebmann and colleagues (70) have studied the biochemistry of BK-receptor activation in myometrial membranes. BK, in picomolar concentrations, stimulated GTPase activity in membrane preparations, which was inhibited by BK antagonists and pertussis toxin. Similar to the findings of Miller et al (145), $G\alpha_{i2}$ was implicated as the BK-coupled G protein. In a complementary study (147), BK was demonstrated to increase GTPγS binding to the myometrial membranes. Based on $B_{max}$ for binding of BK and $B_{max}$ for binding of GTPγS, it was concluded that binding of BK leads to a large signal amplification, one BK receptor apparently activating up to 100 G proteins.

## REGULATION OF KININ RECEPTOR NUMBER

Several studies have examined regulation of BK receptor number. In murine fibroblasts (148) and human synovial cells (149, 150) IL-1 dramatically enhanced the ability of BK to induce eicosanoid synthesis. Binding studies

suggested that the mechanism of action of IL-1 was on signal transduction, since neither affinity nor number of BK receptors was affected, at least in murine fibroblasts (148). Similarly, other growth factors have been shown to enhance responsiveness to BK without altering receptor number (151, 152).

Other stimuli, however, may alter expression of BK receptors. In particular, several laboratories have demonstrated that expression by cultured cells of the *ras* oncogene led to increased expression of $B_2$ receptors (153–155). Another oncogene, *dbl,* also increased the expression of BK receptors (156). In our laboratory we have found that the Balb/c 3T3 fibroblast expresses only one tenth the number of BK receptors as its SV-40-transformed daughter line, SV-$T_2$ (157). While the mechanisms for oncogene-induced increases in BK receptor number are presently undetermined, transformation per se is not the cause, since *v-src, v-abl, v-mos, v-raf,* and *v-fos* transformed cells did not express increased numbers of BK receptors (156).

In rat kidney glomeruli, a $B_2$ receptor has been characterized. When animals were fed a low-sodium diet for 28 days, density of BK receptors was decreased from 32 to 12 fmol/mg protein (158). Similarly, water deprivation decreased receptor density to 13 fmol/mg protein. These decreases were not accompanied by altered receptor affinity. Interestingly, renal kallikrein and presumably renal kinin were increased by these maneuvers, suggesting that the alterations in receptor density represent down-regulation in response to kinin stimulation. In related studies, it was found that in the presence of two-kidney, one-clip Goldblatt hypertensive rats, renal kallikrein was decreased, while BK-receptor density was increased (159).

The suggestion that BK receptors undergo ligand-induced desensitization (158, 159) is supported by many functional studies and by several binding studies. Rat 13 fibroblasts expressed about 40,000 receptors per cell basally, with $K_d$ 2 nM (160). Exposure of these cells to BK-reduced receptor number to undetectable amounts with a half time of about 2 h. In concert with the loss of high-affinity receptors, low-affinity receptors ($K_d$ 40 nM) increased with the same half time, achieving a concentration of about 40,000/cell. NG108-15 cell BK receptors also experience ligand-induced desensitization (161). In these cells desensitization is very rapid, in that 5 min incubation with BK reduced $B_{max}$ from 240 fmol/mg protein to 71 fmol/mg protein. The mechanism for desensitization in these cells is unknown.

## ISOLATION AND PHYSICAL CHARACTERIZATION OF THE $B_2$ RECEPTOR

Several attempts have been made to solubilize BK receptors from uterus (162), NG-108-15 neuroblastoma-glioma cells (71), and human fibroblasts (17). In the most successful of these studies (17), CHAPS was used to

solubilize $B_2$ receptors from human fibroblasts in active form. The receptors were solubilized in 70% yield (compared to less than 20% yield in the other studies), based on $B_{max}$ estimates in binding assays, and bound BK with $K_d$ of about 2 nM, similar to its affinity in intact cells (17). The receptor presumably was a glycoprotein since it was retained on wheat germ lectin columns. Gel filtration revealed an apparent molecular mass of 250,000 Da, representing a complex of receptor with other membrane components, possibly including G protein and/or lipid-detergent micelles. Hopefully, protocols for successfully solubilizing BK receptors will lead to their purification and physical characterization.

## CLONING AND EXPRESSION OF $B_2$ RECEPTORS

Mahan & Burch (163) recently reported the successful expression of mammalian $B_2$ receptors in frog oocytes, which do not normally express kinin receptors. mRNA from murine 3T3 fibroblasts was injected into Xenopus oocytes and, 3–4 days later, it was shown that BK stimulated $^{45}Ca^{2+}$ efflux and increased cellular inositol-1,4,5-trisphosphate. These effects of BK were inhibited by NPC 567, but not desArg$^9$[Leu$^8$]-BK (163). These data suggested that the $B_2$ receptor expressed in these oocytes were coupled to PLC, activation of which resulted in increased $[Ca^{2+}]_i$.

A $B_2$ receptor was recently cloned from rat uterus (109), and its sequence predicts a protein of 366 amino acids with a molecular mass of 41,696 Da, belonging to the superfamily of G protein-coupled receptors, containing seven transmembrane domains. The receptor was expression-cloned using Xenopus oocytes. BK and KD activated the oocyte-expressed receptor equipotently, exhibiting $EC_{50}$ values of around 3 nM. The rank order of kinin agonist potency for activation of the cloned receptor was BK $\geq$ KD $>$ [Tyr$^8$]-BK $>$ [Phe$^6$]-BK, typical of $B_2$ receptors. DesArg$^9$-BK was inactive and NPC 431 was a weak partial agonist. Distribution of the message was to uterus, vas deferens, kidney, lung, heart, ileum, testis, and brain. Several of the tissues contained two or three message species (the predominant form being of 4 kb), suggesting the possibility of receptor subtypes. The sequence contains putative protein kinase A and PKC phosphorylation sites. The greatest sequence homology was with the canine histamine $H_2$ receptor (25%), with somewhat lesser homology for the neurotensin, substance P, neurokinin A, and neuromedin K receptors.

## STRUCTURE OF THE BRADYKININ BINDING DOMAIN OF THE $B_2$ RECEPTOR

Since the BK receptor, either by itself or in complex with a ligand, has never been obtained in crystalline form, deducing the geometric and electronic

characteristics of the solution conformation of BK may elucidate the receptor's BK binding site. A recent review describes early efforts in this regard (164).

Kyle and colleagues (164) were the first to study the peptide at 500 MHz in 90% dioxane as a simple mimic of the dielectric and hydrophobic character of a cell membrane. NPC 567 was also studied under the same conditions. The important structural feature observed in both peptides was described as a $\beta$-turn (a 1–4 hydrogen bond) spanning the C-terminal four residues of each peptide. The origin of the turn was the carbonyl oxygen of $Ser^6$ and the terminus, the amide proton of $Arg^9$. Since the only difference between the C-terminal four residues in BK and NPC 567 is the i+1 position (LPro in the former, DPhe in the latter), it was suggested that the difference between BK agonists and antagonists might be the type of turn adopted. By definition this is derived from the f, y dihedral angles of the i+1 and i+2 residues in the turn. In more complex NMR experiments with BK in the presence of micelles, Laidig and colleagues (165) subsequently confirmed the $\beta$-turn in BK.

Recently, Hock and colleagues have described a series of decapeptides containing the modified amino acids DTic (1,2,3,4-tetrahydroisoquinnoline-3-carboxylic acid) and LOic (octahydroindole carboxylic acid) as replacements for the 7 and 8 positions in the primary amino acid sequence of NPC 567-like peptides (74, 102). These decapeptides, highly constrained in their C-terminal portions, were the first examples of a new generation of potent BK receptor antagonists that were several hundred times more potent than [$DPhe^7$]-substituted BK analogues in guinea-pig ileum. In an effort to quantify the conformational impact of the C-terminal substituents within these peptides, a systematic 10·grid search was performed on model dipeptides derived from those of the Hoechst group (100). The global energy minima for dipeptides derived from active analogues corresponded to a $\beta$-turn structure, was proposed to dominate the overall population of unique conformations in solution and thus be similar to the bioactive conformation.

To further pursue the hypothesis that a $\beta$-turn in the four C-terminal amino acid residues of BK analogues might be a prerequisite for high receptor affinity, a simple chemical surrogate was reported (99) that would not only preferentially induce the turn into the C-terminus of a decapeptide, but could also be functionalized with a variety of groups that would serve as "probes" of the allowed steric binding environment. This system has proven valuable in mapping the geometric topography about the proposed $\beta$-turn-accepting portion of the BK receptor. Moreover, compounds contained in this series represent the first examples of highly potent peptides (at least as potent as the $DTic^7$-containing peptides) that do not contain an aromatic amino acid at position 7 as had been previously considered essential. The desired $\beta$-turn was confirmed when the residue in position 7 was an alkyl ether of D-4-

hydroxyproline in either the *cis* or *trans* geometric state and the eighth residue was Tic or Oic (99).

Analysis of the binding affinities for the series of decapeptides containing these amino acid pairs at position 7–8 reveals that the receptor has a zone of steric intolerance adjacent to the backbone at position 7 and *cis* to the carbonyl group. In contrast, there is a zone of steric tolerance on the opposite side of the backbone at position 7 (*trans* to the carbonyl group). This conclusion is based on the relative low affinity of *cis* ethers compared to the very high affinity of the *trans* ethers. Furthermore, the size of the zone of steric tolerance is better approximated by the propyl group than the smaller methyl group, based on their relative affinities.

From the wealth of information obtained using these new, highly constrained, highly potent BK antagonists, one is able to construct an "image" of the putative receptor binding site corresponding to the C-terminal portion of the ligands. A 1–4 hydrogen bond extending from the $Ser^6$ carbonyl oxygen to the $Arg^9$ amide proton characterizes the turn in the ligand backbone, the presumed purpose of which is to spatially orient the side chain groups in a fashion consistent with receptor topography. There is a zone of steric intolerance about position 7 *cis* to the carbonyl group of the amide bond linking residues 7 and 8. Furthermore, there is a zone of steric tolerance *trans* to that same carbonyl, also about position 7. The space about position 8 can accommodate steric bulk on either side of the turn.

## CONCLUDING REMARKS

Since the initial observation, over 50 years ago, that proteolytic degradation of a plasma globulin by a snake venom liberated a substance that caused slow contraction of isolated intestine (166), many hundreds of papers have been published on the biology of the kinins. Yet only in the past two or three years have scientists begun using sophisticated and also more modest experimental tools to classify kinin receptor subtypes, and their biochemical and molecular aspects. Although kinin receptors were originally categorized as $B_1$ and $B_2$ receptors (14), only in the mid-1980s were the first relatively selective antagonists of $B_2$ receptors acquired (52). These agents have led both to a greater understanding of kinin receptor pharmacology and, because of their low potency and "unusual" actions, they have also contributed to misinterpretations regarding receptor classification.

The recent cloning and expression of a rat smooth muscle $B_2$ receptor (109) advances the exciting probability that human kinin receptor subtypes will soon be isolated, cloned, expressed, and their gene sequences learned. This, coupled to current developments in the understanding of the conformational

structures of BK and its potent antagonists, while bound to the receptors (99, 100, 164), may lead to a potent, selective nonpeptide antagonist of BK receptor subtypes. Such agents will be invaluable instruments for dissecting the intricacies of kinin receptor pharmacology, and may further our knowledge of the role of kinins in disease.

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