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The B_1 Receptors for Kinins

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Foreword

A literature review on kinin pharmacology that appeared in this journal in 1980 was based on a systematic effort to define two receptor types for bradykinin- (BK [b]) related peptides, and proposed a receptor nomenclature (B_1, B_2) (Regoli and Barabé, 1980). Since then, that historical paper has been frequently cited and the nomenclature, widely adopted. The physiologically prominent B_2 receptor (B_2R) subtype has certainly been the subject of more intensive efforts in drug development, structure-function studies, and physiological investigations. However, the B_1 receptor (B_1R), activated by a class of kinin metabolites (des-Arg^9-BK and Lys-des-Arg^9-BK), is now a defined molecular entity (fig. 1) and its characterization, including its rapid up-regulation in tissue injury, has emerged as an important area of in-

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[b] Abbreviations: ACE, angiotensin I converting enzyme; AMP, adenosine monophosphate; BK, bradykinin; CGRP, calcitonin-gene related peptide; CHO, Chinese hamster ovary; EGF, epidermal growth factor; HMW, high molecular weight; i.c.v., intracerebroventricular; IL, interleukin; LMW, low molecular weight; LPS, lipopolysaccharide; MAP, mitogen-activated protein; PDGF, platelet-derived growth factor; PG, prostaglandin; PKC, protein kinase C; PLC, phospholipase C; RT-PCR, reverse transcriptase polymerase chain reaction; SMC, smooth muscle cell.

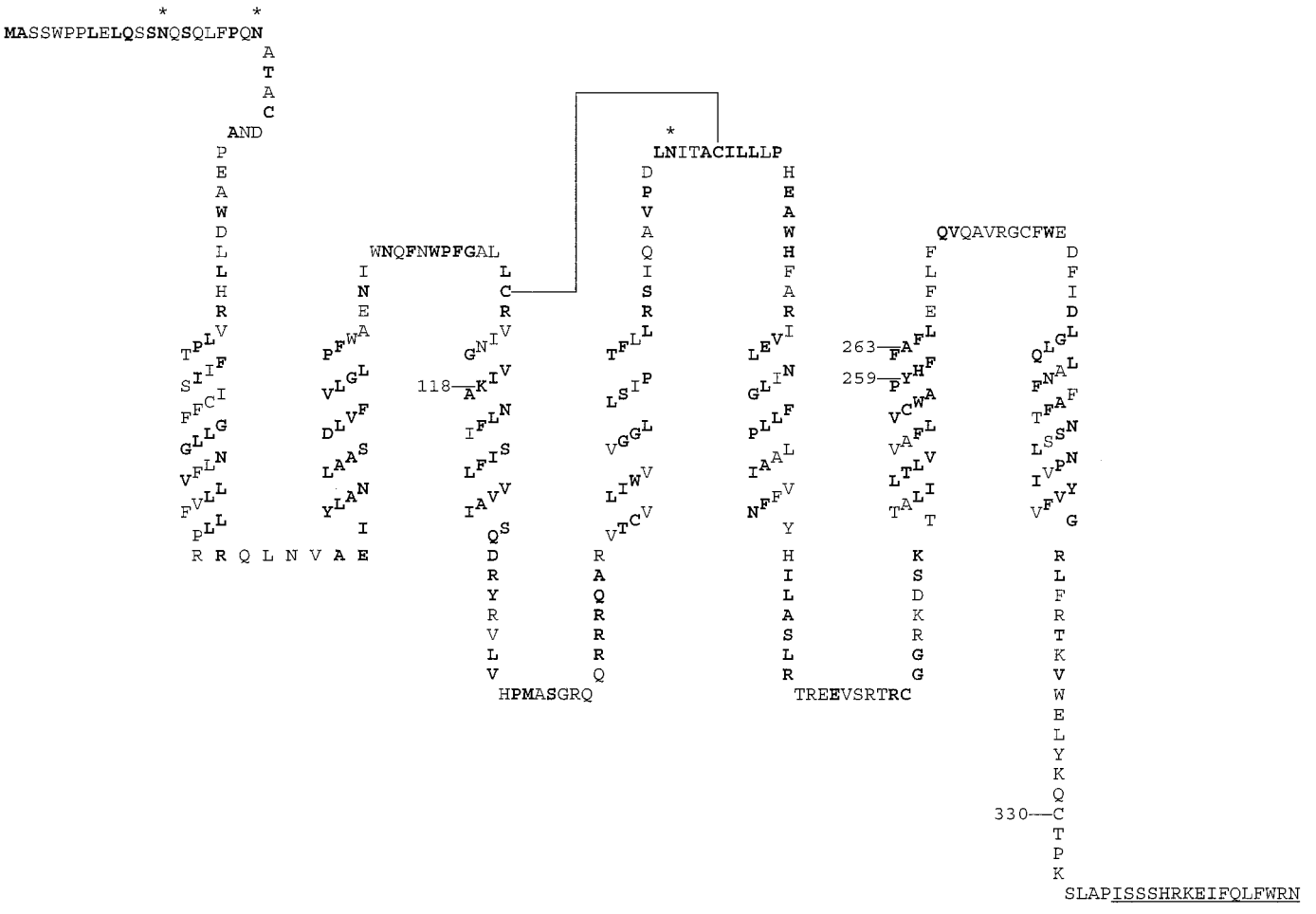

FIG. 1. Primary structure and putative domains of the human B_1R. The amino acid sequence (one letter code) starts at the upper left, an extracellular domain. Asterisks represent putative sites of N-glycosylation. A putative disulfide bond between Cys residues of the second and third extracellular domain is also represented. Residues in bold are identical in all four species for which the B_1R has been sequenced (human, rabbit, mouse, rat). The underlined C-terminal sequence, a part of a sequence extension found only in the human molecule, has been the immunogen for the production of anti-B_1R antibodies. Numbered residues are referred to in the description of mutant or chimeric constructions (see text). Data from Hess *et al.* (1996), GenBank file no. U66107, MacNeil *et al.* (1995), and Menke *et al.* (1994). Sequence variants for human B_1R are reported: R^{146} (Bastian *et al.*, 1997), R^{246}, and S^{259} (GenBank file no. U22346).

vestigation within the study of the kallikrein-kinin system. Indeed, a major interest in the B_1R is its inducible character, an unusual feature for a G-protein coupled receptor.

We will present the findings on the kinin B_1R with emphasis on contemporary molecular research and, when possible, on the human molecules, cells, tissues, and subjects. We do not aim at an exhaustive or historical coverage of early work on B_1Rs, which was essentially based on functional approaches and structure-activity investigations of peptide analogs; the reader is referred to *earlier reviews* for coverage of these aspects (Marceau, 1995; Marceau and Regoli, 1991; Regoli and Barabé, 1980). Finally, the field of B_1R research cannot be isolated from pharmacological and molecular studies of the BK B_2Rs or from other components of the kallikrein-kinin system. A comparative and parallel coverage will be used in several sections of the present text.

I. Introduction: The B_1 Receptors in the Kallikrein-Kinin System

A. Older and Novel Molecular Elements of the Kallikrein-Kinin System

Knowledge concerning the molecular elements of the kallikrein-kinin system is being constantly refined (table 1; reviewed by Kaplan *et al.*, 1997; Margolius, 1995). A single human kininogen gene codes for the hepatic production of both high (HMW) and low molecular weight (LMW) kininogen via alternative splicing. Kininogens are multi-domain proteins that include the BK sequence, but also domains capable of binding Ca^{2+}, binding to the cell surface or inhibiting the cysteine proteinases. Kallikreins are the enzymes that cleave kininogens and liberate kinins (defined as BK-related peptides). Plasma prekallikrein is cofactor of coagulation that is involved in a reciprocal activation reaction with the Hageman factor in the contact system. Plasma

TABLE 1
Molecular elements of the human kallikrein-kinin system

Class of molecules	Molecule	Molecular weight (kDa)	Cloning and sequencing[a]
Substrates	HMW-kininogen	88–120	A differentially spliced single gene product codes for both (Margolius *et al.*, 1995)
	LMW-kininogen	50–68	
Kininogenases	Plasma prekallikrein	82–83 (Chung *et al.*, 1986)	619 a.a. mature protein; 19 a.a. signal peptide; (Chung *et al.*, 1986)
	Tissue kallikrein hK1	40–45 (Chen *et al.*, 1995a)	238 a.a. mature protein; 24 a.a. profragment (Fukushima *et al.*, 1985)
	Tissue kallikrein hK2	40–45 (Schedlich *et al.*, 1987)	261 a.a. preproprotein; 237 a.a. mature protein; (Schedlich *et al.*, 1987)
Kallikrein inhibitors	C1q-inhibitor	21–750 range; 30 (core protein) (Ghebrehiwet and Galankis, 1993)	Not available
	Kallistatin, a serpin	58 (Zhou *et al.*, 1992)	401 a.a. mature protein; 26 a.a. signal peptide; (Chai *et al.*, 1993)
Kininases	Carboxypeptidase N	50 (catalytic subunit); 83 (carrier subunit) (Tan *et al.*, 1990)	438 a.a. mature protein + 20 a.a. signal peptide (catalytic subunit—Gebhard *et al.*, 1989) 536 a.a. carrier subunit (Tan *et al.*, 1990)
	Carboxypeptidase M	62 (Skidgel *et al.*, 1989)	439 a.a. protein (Tan *et al.*, 1989)
	Kininase II; angiotensin I converting enzyme	170 (Takeuchi *et al.*, 1989)	1306 a.a. total with 29 a.a. signal peptide (Soubrier *et al.*, 1988)
	Neutral endopeptidase	100 (Shipp *et al.*, 1988)	749 a.a. protein (Shipp *et al.*, 1989)
	Aminopeptidase M	150, 110 and 56/52 (Terashima and Bunnett, 1995)	966 a.a. (in the rat—Malfroy *et al.*, 1989)
	Aminopeptidase P	91 (Hyde *et al.*, 1996)	673 a.a. (in the pig—Hyde *et al.*, 1996)
G protein-coupled receptors	Receptor B_1	Predicted (without carbohydrates): 40	353 a.a. G protein-coupled receptor (Menke *et al.*, 1994)
	Receptor B_2	Predicted (without carbohydrates): 41; found 69	364 a.a. G protein-coupled receptor (Hess *et al.*, 1992)

[a] The human sequence is reported, unless otherwise indicated.

kallikrein releases BK (the nonapeptide H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) from HMW-kininogen (Margolius *et al.*, 1995). There is also evidence that HMW-kininogen adsorbed on extracellular (M) proteins of *Streptococcus pyogenes* can release BK in the presence of prekallikrein, thus extending the physiopathological applications of the contact system to sepsis (Ben Nasr *et al.*, 1997).

Tissue kallikreins are represented in humans by three related enzymes (hK1 to hK3). Tissue kallikrein (hK1) is widely expressed in glandular and duct cells, as well as in neutrophils (Wu *et al.*, 1993), cultured vascular smooth muscle cells (SMCs), colonic goblet cells, and renal distal tubules and collecting ducts (Chen *et al.*, 1995a, 1995b; Nolly *et al.*, 1993; Oza *et al.*, 1990). Tissue kallikreins are synthesized as zymogens and little is known of their production, storage, or release. Pure tissue kallikreins are not equally capable of generating biologically active kinins: hK1, the classical tissue kallikrein, is highly effective, whereas hK3 (also called prostate-specific antigen) is inactive, hK2 being of intermediate potency (Deperthes *et al.*, 1997). The exclusive prostatic localization of hK2 and hK3 is currently being challenged, as they are found in other cell types (e.g., a human breast cancer line expresses hK2; Hsieh *et al.*, 1997). Although hK1 is preformed in the human kidney, further synthesis of this protease occurs in this organ after trauma or immunopathological insult (Cumming *et al.*, 1994). Human tissue kallikrein preferentially releases the decapeptide Lys-BK (kallidin) from kininogens, as found in the nasal secretions of subjects with allergic or viral rhinitis (Nacleiro *et al.*, 1988; Proud *et al.*, 1983).

B. Current Issues in the Analytical Chemistry and Metabolism of Kinins

The metabolism of kinins will be briefly summarized here, because B_1Rs are selectively and exclusively stimulated by one class of kinin metabolites (fig. 2). BK exhibits a remarkably short half-life in the blood plasma in vitro (10 to 50 sec, depending on species; Décarie *et al.*, 1996b) or in vivo. The great susceptibility of these peptides to hydrolysis accounts for this rapid disposal. BK and Lys-BK are predominantly metabolized in the circulation by the angiotensin I converting enzyme (ACE; EC 4.4.15.1; kininase II; Erdös, 1990). ACE removes the C-terminal dipeptide from BK or Lys-BK, which leads to their complete inactivation (K_m of about 1 μM for this reaction). ACE is predominantly a surface

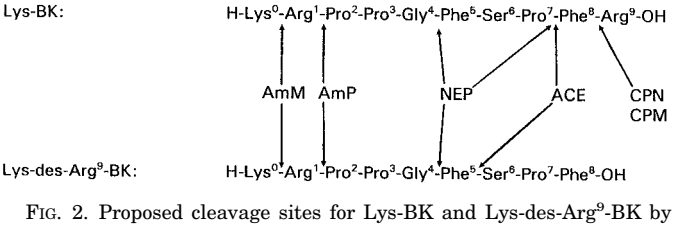

FIG. 2. Proposed cleavage sites for Lys-BK and Lys-des-Arg^9-BK by the kininases. Abbreviations: AmM, aminopeptidase M; AmP, aminopeptidase P; NEP, neutral endopeptidase 24.11; ACE, angiotensin I converting enzyme; CPN, carboxypeptidase N; CPM, carboxypeptidase M.

enzyme located on the luminal membrane of endothelial cells, an observation which explains the extensive pulmonary inactivation of kinins. The proximal nephron epithelium is also rich in ACE (Erdös, 1990). ACE eventually cleaves further its primary metabolite, BK_{1-7}, into shorter fragments (e.g., BK_{1-5}).

An aminopeptidase *P* (aminoacyl proline aminopeptidase; EC 3.4.11.9) which cleaves the Arg^1-Pro^2 bond, may also contribute to the pulmonary inactivation of BK (Ryan *et al.*, 1994; Ward, 1991). Another form of membrane-bound peptidase, the "neutral" endopeptidase (E.C. 3.4.24.11), cleaves two bonds in BK (Gly^4-Phe^5 and Pro^7-Phe^8; Zolfaghari *et al.*, 1989). Endopeptidase 24.11 is notably located at a high concentration in the proximal nephron epithelium and is also present on the membrane of some leukocytes, but barely detectable in the vasculature or plasma (Erdös, 1990; Ward, 1991).

Kininase I activity is of particular importance, because it is believed to generate the physiological agonists of B_1Rs, the des-Arg^9-kinins, from native kinins. This activity is composed of the arginine carboxypeptidases, carboxypeptidase N from plasma (EC 3.4.17.3), and carboxypeptidase M, predominantly membrane-bound and widely distributed, including in the microvasculature (table 1; Erdös, 1990; Ward, 1991). These are enzymes of rather low affinity (K_m 20 to 50 μM for BK and carboxypeptidase N) and broad specificity, digesting several other peptides generated by trypsin-like enzymes, such as the anaphylatoxins derived from complement. The relative role of arginine carboxypeptidases (kininase I) in the metabolism of kinins is discussed below.

The metabolism of des-Arg^9-kinins differs from that of native kinins in several important points. First, des-Arg^9-BK does not react with arginine carboxypeptidases, as it is devoid of the corresponding C-terminal residue (Drapeau *et al.*, 1991a). Second, ACE cleaves a C-terminal tripeptide from B_1R agonists, yielding BK_{1-5}, but at a slower rate, and with much less affinity (K_m 130 to 240 μM) than the removal of a C-terminal dipeptide from BK (Drapeau *et al.*, 1991a). The C-terminal position of Phe^8 in des-Arg^9-BK may protect the Pro^7-Phe^8 bond from the *endo*peptidase 24.11; this requires confirmation.

Aminopeptidase M (EC 3.4.11.2), present in plasma, hydrolyzes Lys-BK into BK, and Lys-des-Arg^9-BK into des-Arg^9-BK (Proud *et al.*, 1987; Sheik and Kaplan, 1989). This reaction is pharmacologically neutral for the B_2R agonists BK and Lys-BK, because these peptides exhibit similar potencies. However, it represents a relative inactivation for the B_1R agonists, as des-Arg^9-BK is an agonist of lesser affinity for the B_1Rs than Lys-des-Arg^9-BK in some species (see below).

The conversion effectiveness of native kinin sequences (BK, Lys-BK) into their respective des-Arg^9 metabolites is a question of considerable interest for the physiological role of B_1Rs, because the other competing metabolic pathways lead to fragments inactive on both B_1 and B_2Rs (Regoli and Barabé, 1980; see also below). Novel analytical techniques for BK- and des-Arg^9-BK-like immunoreactive peptides have shown that ACE accounts for more than 75% of the hydrolysis of exogenous synthetic BK (500 nM) in the plasma of humans, rabbits, or rats (Décarie *et al.*, 1996b), and more than 40% in cardiac membrane preparations derived from the same species (Blais *et al.*, 1997b). The formation of des-Arg^9-BK is minor in these species (0.9 to 3.4% of BK converted), much less than previous estimates based on high concentrations of exogenous BK as a substrate (Marceau *et al.*, 1981; Proud *et al.*, 1987). Blocking the competing ACE pathway with enalaprilat improved the conversion in all species (Blais *et al.*, 1997b; Décarie *et al.*, 1996b). Thus, when considering the relative contribution of several kininases, the mere existence of measurable des-Arg^9-kinins in vivo could be questioned. However, in the plasma or cardiac membrane preparations of the four cited species, the half-life of exogenous des-Arg^9-BK was 4- to 12-fold longer than that of BK under the same experimental conditions (Blais *et al.*, 1997b; Décarie *et al.*, 1996b). If this is an accurate representation of the situation in vivo, it may explain why the in vivo concentration of immunoreactive des-Arg^9-BK is consistently higher than that of immunoreactive BK. Simply put, des-Arg^9-BK, although not effectively produced, has a much greater capacity to accumulate than BK.

Kinins are very difficult to measure accurately, in part because the sampled blood contains all the necessary components to generate and destroy these peptides in vitro. For instance, the contact of glass in test tubes activates the plasma kallikrein. Current methods for measuring kinin concentrations rely on antibody-based techniques (radioimmunoassays, enzyme immunoassays) preceded by complex extraction procedures (Décarie *et al.*, 1994; Odya *et al.*, 1983; Raymond *et al.*, 1995). In venous blood sampled from normal humans, the average concentration of immunoreactive des-Arg^9-BK (204 pg/ml) is higher than that of BK (67 pg/ml) (Odya *et al.*, 1983). These values are not significantly changed inpatients with essential hypertension or under ACE blockade with enalapril (Odya *et al.*, 1983). The urine of these individuals also contains both BK and des-Arg^9-BK immunoreactivities that do not differ between groups. The authors point out that venous blood or urine measurements may not reflect the status of the kinins at the tissue level, because of metabolism by multiple pathways. In the arterial blood of anesthetized rabbits, captopril treatment increases the BK immunoreactive concentration, but does not influence the concentration of des-Arg^9-BK. The latter could be selectively and importantly increased by treatment with bacterial lipopolysaccharide (LPS), and even more so in animals pretreated with captopril (Raymond *et al.*, 1995). Species-dependent differences in the metabolism and the rate of activation of the kallikrein-kinin system may be important parameters which determine the relative concen-

trations of BK and des-Arg^9-BK. Local concentrations of kinins may generally be of greater interest, as the kinins are autacoids rather than hormones. Interestingly, both BK and des-Arg^9-BK immunoreactivities increase in inflammatory lesions caused by carrageenan in the rat (Burch and DeHaas, 1990; Décarie *et al.*, 1996a).

The discussion above is centered on the C-terminal structure of kinins, so important for receptor subtype selectivity (see below). However, various limitations are apparent in the analytical approach for des-Arg^9-kinins. The antibodies efficient to discriminate the C-terminal structure do not differentiate between kinin homologues that differ in their N-terminus (e.g., BK, Lys-BK, Ile-Ser-BK; Décarie *et al.*, 1994; Raymond *et al.*, 1995). This represents a major limitation of our analytical capabilities, as Lys-des-Arg^9-BK is the most potent B_1R agonist in humans, des-Arg^9-BK being relatively ineffective (see below, Section II.A.). Combining the antibody-based method with chromatographic separation of homolog peptides may be helpful to discriminate among them (Décarie *et al.*, 1994), although with a probable loss of sensitivity. An unambiguous analytical method for measuring Lys-des-Arg^9-BK still has to be invented. An additional problem is that the Pro (3) residue in either BK or des-Arg^9-BK may rather be hydroxyproline in a sizable proportion of the kinin molecules, caused by a partial posttranslational modification of high molecular weight kininogen in the human species (Schlüter *et al.*, 1997). Although this substitution does not adversely affect receptor affinity (Regoli *et al.*, 1996), its effect on metabolism or antigenicity is unknown. Finally, an inflammation-induced increase of immunoreactive des-Arg^9-BK, as in the case of acute edema produced by carrageenan in the rat paw, does not necessarily predict an important role for B_1Rs, as these receptors are apparently absent in this acute model that dissociates the agonist from the response (Décarie *et al.*, 1996a).

II. Seven Criteria to Classify Kinin Receptors into the B_1 and B_2 Subtypes

Kinins, positively charged peptides, influence tissues and cells by stimulating membrane receptors. Two types of receptors are currently completely defined in several species using a series of seven criteria, detailed in this chapter, although very significant species-specific pharmacological differences are documented. The first three criteria are pharmacological as defined by Schild (1973). The other ones are of more biochemical and molecular nature. Binding (table 2) and biochemical experiments based on cloned receptor subtypes have been valued in our coverage, because they offer the ultimate proofs regarding the behavior of definite molecular entities.

A. Potency Order of Agonists

In vitro smooth muscle preparations assume a particular importance in the history of kinin receptor classification. The first hint of the receptor heterogeneity was the atypical order of agonist potency found for BK and its fragment on the isolated rabbit aorta: the fragment without the C-terminal arginine residue, des-Arg^9-BK, was more potent than the intact nonapeptide (Regoli *et al.*, 1977). A few other preparations are worth mentioning at this point, as they are useful to compare potency orders obtained in binding assays with cloned receptors in each species: comprehensive data are available for the contractility mediated by B_1Rs in the human umbilical vein (Gobeil *et al.*, 1996b), the longitudinal muscle of the rat ileum (Meini *et al.*, 1996), and the mouse stomach (Allogho *et al.*, 1995). These preparations were further used to determine the potency and intrinsic activity of antagonists and the postisolation regulation of B_1Rs (see below). Of the preparations mentioned above, the rabbit aorta and the rat ileum respond to kinins only via B_1R, whereas the human umbilical vein and mouse stomach possess both receptor subtypes, a situation that complicates pharmacological analyses.

The potency order of agonists, as established in separate binding assays (table 2), confirms that the removal of the C-terminal arginine is essential for high affinity on the B_1R and detrimental for affinity toward the B_2R. This holds true for Lys-BK (kallidin) and Lys-des-Arg^9-BK (des-Arg^{10}-kallidin), which have higher affinities for the human and rabbit B_1Rs compared with BK and des-Arg^9-BK, respectively (table 2). Met-Lys-BK, resulting from a different cleavage of the kininogen sequence, does not significantly differ from Lys-BK, being a relatively selective B_2R agonist in the human and rabbit (table 2). The only natural kinin sequence with a subnanomolar affinity for human and rabbit B_1Rs is Lys-des-Arg^9-BK, a fact of particular significance that may suggest that the B_1R belongs to the "tissue" kallikrein-kinin system, jointly with tissue kallikrein that generates Lys-BK (kallidin) from low molecular weight kininogen. This does not invalidate the experimental use of des-Arg^9-BK in certain animal species as a tool of high specificity for B_1Rs; however, this peptide has a low absolute affinity for the human B_1R. This is reflected by the low relative potency of functional responses induced by des-Arg^9-BK as compared with those elicited by Lys-des-Arg^9-BK, in the human umbilical vein assay (Gobeil *et al.*, 1997), or in cells expressing recombinant human B_1Rs (MacNeil *et al.*, 1997).

Limited functional data based on porcine renal vein contractility show that this B_1R, not yet cloned, is also preferentially stimulated by Lys-des-Arg^9-BK (pD_2 of 8.4, versus 7.2 for des-Arg^9-BK; Rizzi *et al.*, 1997). By contrast, Lys-des-Arg^9-BK has no decisive advantage over des-Arg^9-BK in small rodents such as mouse (table 2) or rat. The sequenced kininogen genes in small rodents differ from the human ones, as the amino acids preceding the BK sequence are arginine, or Ile-Ser in T-kininogen, not lysine (Hess *et al.*, 1996; Takano *et al.*, 1997). In view of this recent discovery the precise sequence released by tissue kallikreins in rats and mice

TABLE 2

Nanomolar affinity estimates and structure-activity relationships for drugs binding to cloned B_1 and B_2 receptors in three species for which both receptors have been cloned and sequenced

Compound	Structure														Cloned human receptors		Cloned rabbit receptors		Cloned mouse receptors	
	−4	−3	−2	−1	0	1	2	3	4	5	6	7	8	9	B_1R[a]	B_2R[b]	B_1R[c]	B_2R[d]	B_1R[e]	B_2R[f]
Native kinins (B_2R agonists)																				
BK						Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	2000	0.54	>5000	4.5	200[g]	0.48
Lys-BK					Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	42	0.63	19	2	510	0.52
Met-Lys-BK				Met	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	70	2.1	16			3.6
B_1R agonists																				
des-Arg[9]-BK						Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe		720	8100	32	>1000	0.7	8.1
Lys-des-Arg[9]-BK					Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe		0.2	>30000	0.23	>1000	1.7	6400
Sar-[D-Phe[8]]des-Arg[9]-BK					Sar[h]	Arg	Pro	Pro	Gly	Phe	Ser	Pro	D-Phe				68[i]			
Tyr-Gly-Lys-Aca-Lys-des-Arg[9]-BK	Tyr	Gly	Lys	Aca	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe				0.2[j]			
Peptide B_2R antagonist																				
Hoe 140					D-Arg	Arg	Pro	Hyp	Gly	Thi	Ser	D-Tic	Oic	Arg	>10000	0.41	>5000	2	>10000	0.23
NPC 17731					D-Arg	Arg	Pro	Hyp	Gly	Phe	Ser	X[k]	Oic	Arg	126[l]	0.18[m]				
B_1R antagonists																				
[Leu[8]]des-Arg[9]-BK						Arg	Pro	Pro	Gly	Phe	Ser	Pro	Leu		440	>30000	80		4.1	>30000
Lys-[Leu[8]]des-Arg[9]-BK					Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Leu		1.3	>30000	0.43	>100	7.5	>30000
Ac-Lys-[MeAla[6], Leu[8]]des-Arg[9]-BK					Ac-Lys	Arg	Pro	Pro	Gly	Phe	Me-Ala	Pro	Leu		670[n]	>10000[n]			>5000[n]	>10000[n]
R-715					Ac-Lys	Arg	Pro	Pro	Gly	Phe	Ser	βD-Nal	Ile		0.66[n]	>10000[n]			41[n]	>10000[n]
Mixed B_1 and B_2R antagonists																				
Hoe 140-des-Arg					D-Arg	Arg	Pro	Hyp	Gly	Thi	Ser	D-Tic	Oic		60		27		24	
NPC 18565					D-Arg	Arg	Pro	Hyp	Gly	Phe	Ser	X[k]	Oic		0.07[l]	26.1[o]				
B9858				Lys	Lys	Arg	Pro	Hyp	Gly	Igl	Ser	D-Igl	Oic		0.04[n]	146[n]			5.4[n]	44[n]
B9430					D-Arg	Arg	Pro	Hyp	Gly	Igl	Ser	D-Igl	Oic	Arg	12.6[p]	0.25[p]			248[n]	0.33[n]
Nonpeptide B_2R antagonists																				
WIN 64338	[[4-[[2-[[bis(cyclohexylamino)methylene]amino]-3-(2-naphthyl)-1-oxopropyl]amino]phenyl]methyl]tributylphosphonium chloride monohydrochloride															64[q]		150		
FR 173657	8-[3-[N-[(E)-3-(6-acetamidopyridin-3-yl)acryloylglycyl]-N-methylamino]-2,6-dichlorobenzyloxy]-2-methylquinoline														>10000[r]	8.9[r]				

[a] Nanomolar affinity estimates are IC_{50} values from binding competition with 1 nM [^{3}H]Lys-des-Arg[9]-BK to transiently expressed human B_1R in COS-7 cells. From Menke *et al.* (1994), except if otherwise indicated. Other comparative estimates based on cloned human B_1Rs are found elsewhere (Bastian *et al.*, 1997; Aramori *et al.*, 1997; Austin *et al.*, 1997).

[b] Nanomolar affinity estimates are IC_{50} values from binding competition with 100 pM [^{3}H]BK to stably expressed human B_2R in CHO cells. From Hess *et al.* (1994), except if otherwise indicated. Other comparative estimates are found elsewhere (Aramori *et al.*, 1997).

[c] Nanomolar affinity estimates are IC_{50} values from binding competition with 0.5 nM [^{3}H]Lys-[Leu[8]]des-Arg[9]-BK to transiently expressed rabbit B_1R in COS-7 cells. From MacNeil *et al.* (1995), except if otherwise indicated.

[d] Nanomolar affinity estimates are IC_{50} values from binding competition with 1 nM [^{3}H]BK to transiently expressed rabbit B_2R in COS-1 cells (Bachvarov *et al.*, 1995).

[e] Nanomolar affinity estimates are K_i values from binding competition with [^{3}H]Lys-des-Arg[9]-BK to transiently expressed murine B_1R in COS cells. From Hess *et al.* (1996), except if otherwise indicated. Other comparative estimates are found elsewhere (Pesquero *et al.*, 1996).

[f] Nanomolar affinity estimates are IC_{50} values from binding competition with 100 pM [^{3}H]BK to transiently expressed murine B_2R in COS cells (Hess *et al.*, 1994).

[g] Pesquero *et al.* (1996) also report an IC_{50} of about 200 nM, but the value is much higher (>1 μM) if the assay is run in the presence of the kininase I inhibitor mergepta, suggesting that the formation of des-Arg[9]-BK may distort the estimate.

[h] Abbreviations: Ac, *N*-acetyl; Aca, ϵ-aminocaproic acid; Hyp, 4-hydroxy-L-proline; Igl, α-(2-indanyl)glycine; β-Nal: β-2-naphthylalanine; Oic, octahydroindole-2-carboxylic acid; Thi, β-2-thienylalanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Sar, sarcosine (*N*-methyl-glycine).

[i] Nanomolar IC_{50} value from the binding competition of 1 nM [^{125}I]Tyr-Gly-Lys-Aca-Lys-des-Arg[9]-BK to wild type rabbit B_1R in cultured aortic SMCs (Levesque *et al.*, 1995a).

[j] K_D value for the iodinated form of the peptide in a saturation binding assay to wild type rabbit B_1R in cultured aortic SMCs; (Levesque *et al.*, 1995a).

[k] X = D-HypE*trans*-propyl.

[l] Nanomolar K_i value from the binding competition of 1 nM [^{3}H]Lys-des-Arg[9]-BK to transiently expressed human B_1R in HEK 293 cells (Leeb *et al.*, 1997).

[m] Nanomolar K_D value for binding of [^{3}H]NPC 17731 to transiently expressed human B_2R in HEK 293 cells (Leeb *et al.*, 1997).

[n] Data from MacNeil *et al.* (1997). K_i values derived from human B_2R stably transfected in CHO cells, or human or murine B_1R or murine B_2R transiently transfected in COS cells.

[o] Nanomolar K_i value from the binding competition of 1 nM [^{3}H]NPC 17731 to transiently expressed human B_2R in HEK 293 cells (Leeb *et al.*, 1997).

[p] Nanomolar IC_{50} value from the binding competition of 0.5 nM [^{3}H]Lys-des-Arg[9]-BK to wild type human B_1R in IMR-90 cells or of 0.3 nM [^{3}H]BK to stably expressed human B_2R in CHO cells. (Gera *et al.*, 1996; Stewart *et al.*, 1996).

[q] K_i value from binding competition with 1–2 nM [^{3}H]BK to wild type human B_2R in IMR-90 cells (Sawutz *et al.*, 1994).

[r] Nanomolar affinity estimates are IC_{50} values from binding competition with 500 pM of either [^{3}H]Lys-des-Arg[9]-BK (B_1R) or [^{3}H]BK (B_2R) to stably expressed human receptors in CHO cells (Aramori *et al.*, 1997).

becomes an analytical issue of interest. Only BK has been isolated from rat urine and other experimental systems based on rat tissue kallikrein, but not Arg-BK, Ile-Ser-BK or Lys-BK (Hagiwara *et al.*, 1995). A form of coevolution of the B_1R with the kininogen genes may have occurred in rodents to maintain the B_1R functional by conferring a subnanomolar affinity to des-Arg^9-BK, which is reflected by the high potency of this peptide (superior or equal to Lys-des-Arg^9-BK in functional assays based on rat or mouse smooth muscle contractility; Allogho *et al.*, 1995; Meini *et al.*, 1996; or cultured rat SMCs, where des-Arg^9-BK exerts its effect with an EC_{50} of about 300 pM; Dixon and Dennis, 1997).

Thus, the B_1R is obviously specialized across species to respond to different kinin metabolites, either des-Arg^9-BK or Lys-des-Arg^9-BK, generated by arginine carboxypeptidases, such as carboxypeptidase N and M. No other kinin fragment seems to retain pharmacological activity. For instance, des-Arg^1-BK or des(Phe^8, Arg^9)-BK (the primary metabolite generated by ACE) do not retain significant activity on either known receptor type (Regoli and Barabé, 1980). The presence of arginine carboxypeptidases in functional systems frequently distorts the potency estimates for BK or Lys-BK on the B_1R, as these sequences are transformed into their respective des-Arg metabolites (discussed by Marceau and Regoli, 1991). Even a binding assay based on the cloned mouse B_1R seems to be prone to this error, as the apparent potency of BK in a competition with the ligand [^{3}H]Lys-des-Arg^9-BK is decreased by a factor of 5 in the presence of the arginine carboxypeptidase inhibitor MERGETPA (Pesquero *et al.*, 1996). This was not the case for des-Arg^9-BK. Thus, intact BK and Lys-BK may exhibit a very high selectivity toward B_2R.

Most published binding studies to human or animal B_1Rs have used the agonist ligand [^{3}H]Lys-des-Arg^9-BK (table 2). [^{125}I]Tyr-Gly-Lys-Aca-Lys-des-Arg^9-BK is an alternative high affinity B_1R ligand which can be also used (Levesque *et al.*, 1995a). This agonist incorporates a N-terminal extension which essentially acts as a spacer between iodotyrosine and the Lys-des-Arg^9-BK sequence.

A synthetic agonist modeled on Lys-des-Arg^9-BK incorporates modifications to improve resistance to metabolism: Sar-[D-Phe^8]des-Arg^9-BK is a high affinity B_1 receptor agonist of high selectivity that is completely resistant to blood aminopeptidase, kininases I and II (angiotensin I converting enzyme) and kidney neutral endopeptidase (Drapeau *et al.*, 1991b, 1993). In a binding assay to rabbit B_1 receptors, this analog was 7-fold more potent than des-Arg^9-BK, but 11-fold less potent than Lys-des-Arg^9-BK (Levesque *et al.*, 1995a). It is very potent in inducing contraction of isolated rabbit aorta (fig. 3D). Contractility data from the human umbilical veins also suggest an intermediate potency between des-Arg^9-BK and Lys-des-Arg^9-BK in the human B_1R (Gobeil *et al.*, 1997). However, Sar-[D-Phe^8]des-Arg^9-BK is equipotent to Lys-des-Arg^9-BK as an hypotensive agent in LPS-pretreated rabbits (Drapeau *et al.*, 1991b) and the hypotensive episodes are prolonged (fig. 3C). The analog is inert in rabbits not treated with LPS, caused by the lack of cardiovascular B_1Rs in these animals (see below). The hypotensive effect of Sar-[D-Phe^8]des-Arg^9-BK is antagonized by Lys-[Leu^8]des-Arg^9-BK, a B_1R antagonist, and its analogues, but not by a B_2R antagonist Hoe 140 (Drapeau *et al.*, 1993). A formal pharmacokinetic approach showed that Sar-[D-Phe^8]des-Arg^9-BK has a longer half-life than Lys-des-Arg^9-BK in the rabbit circulation (Audet *et al.*, 1997), probably accounting for the decisive advantage of the modified analog in vivo. Sar-[D-Phe^8]des-Arg^9-BK is also more potent than des-Arg^9-BK as an algesic agent in a rat model of chronic inflammation (Davis and Perkins, 1994a), and may be superior to any natural sequence for demonstrating the LPS induction of hypotensive responses mediated by B_1Rs in this species (Nicolau *et al.*, 1996). Sar-[D-Phe^8]des-Arg^9-BK appears to be a valid tool across different species to demonstrate the presence of a B_1R population. The replacement of a central tetrapeptide (Pro^2-Phe^5) in Lys-des-Arg^9-BK by alkyl spacers, optimally 12-aminododecanoic acid, is another reported approach to modify a natural B_1R agonist (Tancredi *et al.*, 1997). In this series of compounds, the best one retained 10% of Lys-des-Arg^9-BK potency in the rat ileum contractility assay.

B. Affinities of Antagonists

The development of kinin receptor antagonists has been pursued for more than two decades (Stewart, 1995). Selective antagonists have been crucial to define kinin receptor subtypes. The prototype of the kinin B_1R antagonists was [Leu^8]des-Arg^9-BK (Regoli *et al.*, 1977). The B_1 nomenclature was latter applied to the rabbit aortic preparation in which the receptors were initially defined by both a typical order of agonist potency and by these antagonists. However, it became clear that these antagonists were not active in the most common contractile bioassays for BK (e.g., rat uterus, guinea pig ileum) or in vivo (hypotension) (Regoli and Barabé, 1980). The B_2R was not well defined up to Schild's criteria until 1985, when the first generation of antagonists based on [D-Phe^7]BK were produced (Vavrek and Stewart, 1985). Since then, more efforts have been invested into the development of B_2R antagonists, relative to B_1R antagonists. Hoe 140 (icatibant; D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK; abbreviations for unusual residues defined in footnote h to table 2, previous page; Hock *et al.*, 1991) is a representative of the "second generation" B_2R antagonists, as it has several hours duration of action in animal models. Hoe 140 also exhibits a very high affinity (table 2) and is a competitive antagonist in most species with the noticeable exception of the rabbit (nonequilibrium insurmountable antagonism; Bachvarov *et al.*, 1995). The effect of Hoe 140 on the human B_1R has

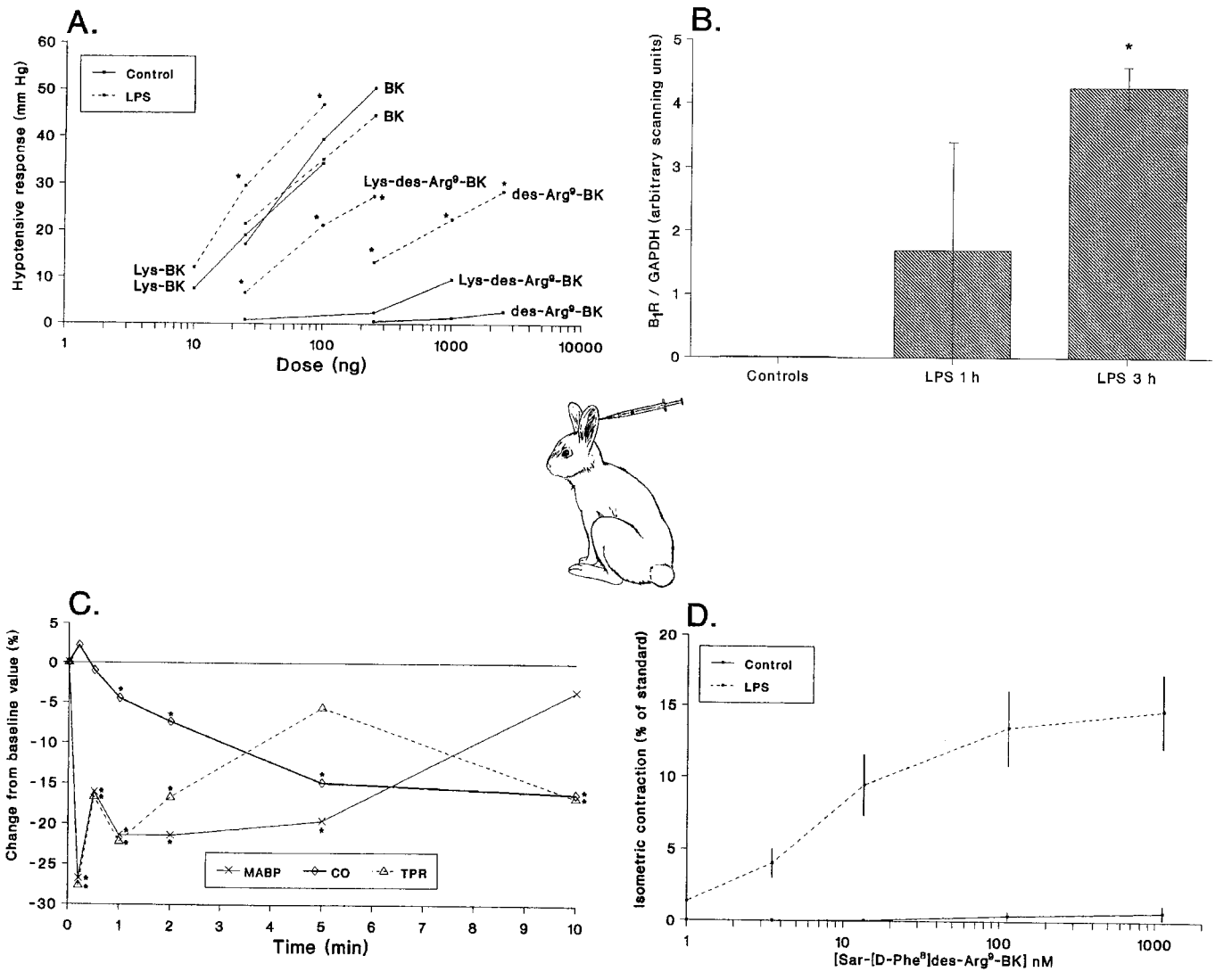

FIG. 3. The rabbit treated with lipopolysaccharide (LPS), an animal model for the study of the regulation of the B_1R. (A) Hypotensive effect of BK, Lys-BK and their des-Arg^9-BK fragments in anesthetized rabbits. The endotoxin-treated animals (dotted lines) were injected with 10 μg of *E. coli* LPS 5 h before anesthesia. Peptides were injected intra-arterially as boluses. Abcissa: dose of peptide (ng per 1.5 to 2 kg animal) ; ordinate: fall of the mean arterial pressure, mmHg. Points are means of 5 to 18 determinations; the asterisk indicates value significantly changed by the LPS treatment. Reprinted in a modified form from Marceau and Regoli (1991), p. 40, by permission from Marcel Dekker, Inc. (B) B_1R mRNA concentration in rabbit hearts relative to those of a housekeeping gene, GAPDH. Animals were untreated (controls) or injected with 25 μg/kg LPS 1 or 3 h before they were killed. Values are means ± SEM of triplicate determinations (with permission from Marceau *et al.*, 1997, and the NRC Research Press). (C) Hemodynamic changes induced by an intraarterial bolus injection of the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK (750 ng/kg) in anesthetized rabbits previously treated with LPS (30 μg/kg i.v. 5 h before anesthesia). CO, cardiac output reading derived from an electromagnetic flowmeter placed on the thoracic aorta; MABP, mean arterial blood pressure; TRP, calculated total peripheral resistance. The asterisk indicates value significantly different from baseline. Modified, from Audet *et al.* (1997), with permission from ASPET. (D) Initial responsiveness of aortic rings derived from control (saline vehicle i.v.) or LPS-treated rabbits (25 μg/kg, i.v. 8 h before they were killed). The cumulative concentration-effect curve of the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK was established after a short in vitro incubation (45 min) to minimize the influence of isolation and tissue incubation on the responses. Values (means ± SEM of eight determinations from four animals in each group) are expressed as a percent of an internal contractile standard, the maximal effect of phenylephrine, established in each tissue. The maximal effects of the kinin differed significantly between groups (J.-F. Larrivée, D. Bachvarov, and F. Marceau, unpublished).

probably been underestimated in the binding study quoted in table 2, because other functional or binding experiments rather indicate a K_i around 100 to 400 nM (Aramori *et al.*, 1997; Bastian *et al.*, 1997; Gobeil *et al.*, 1996b; Zuzack *et al.*, 1996). However, in practical terms, this peptide still exhibits a high selectivity toward B_2Rs. Hoe 140 has been used in many studies, particularly to assess the role of kinins in various models of experimental pathology. NPC 17731 is a B_2R antagonist that is chemically and pharmacologically similar to Hoe 140 (table 2). The first nonpeptide kinin antagonists, the phosphonium WIN 64338 and the quinoline FR 173657, are selective B_2R antagonists (Aramori *et al.*, 1997; Sawutz *et al.*, 1994; table 2). The latter compound is orally bioavailable, an important step toward the clinical evaluation of such antagonists.

Thus far, nonpeptide B_1R antagonists have not been yet reported. Structure-activity relationships for early

peptide antagonists of the B_1R are discussed elsewhere (Regoli and Barabé, 1980). In summary, the substitution of Phe^8 in des-Arg^9-BK by a residue with an aliphatic (Ala, Ile, Leu, D-Leu, norleucine) or saturated cyclic hydrocarbon chain (cyclohexylalanine) produced antagonists of variable potency. Early experiments have also shown that substituting Pro^7 (e.g., with Ala or Gly) can produce partial agonists or antagonists (Regoli and Barabé, 1980). Substitutions at this position were introduced in some of the more recent B_1R antagonists. The N-terminal extension of [Leu^8]-des-Arg^9-BK by a Lys residue confers a higher affinity in the species which have a similar relationships for agonists: the rabbit and human (table 2). Thus, Lys-[Leu^8]des-Arg^9-BK is the optimal B_1R antagonist based on natural amino acids. This family of peptides exhibits a competitive behavior and an almost ideal selectivity for the B_1R subtype (table 2). A tritiated form of Lys-[Leu^8]des-Arg^9-BK has been used as a ligand for the human B_1R in a recent study (Bastian *et al.*, 1997), and binding competition data were very similar to those obtained with the agonist ligand [^{3}H]Lys-des-Arg^9-BK. Thus, the antagonist activity of [Leu^8]des-Arg^9-BK and its homologues is a reasonable basis for the pharmacological definition of the kinin B_1R.

Improving the stability and selectivity of B_1R antagonists is critically important for physiopathological investigations and therapeutic applications. Peptides generally suffer from many disadvantages as potential therapeutic agents, such as metabolic instability, poor oral absorption, rapid elimination, short duration of action and, in some cases, partial agonist activity. A metabolically stable peptide B_1R antagonist, Ac-Lys-[$MeAla^6$, Leu^8]des-Arg^9-BK, has been elaborated by successive modifications of the high affinity prototype, Lys-[Leu^8]des-Arg^9-BK, with the main purpose of documenting the metabolic pathways of this class of agents (Drapeau *et al.*, 1993). The N-methyl-alanine residue in position 6 removes recognition sites for ACE and neutral endopeptidase (fig. 2). Thus, although the novel antagonist peptide was not very potent (pA_2 of 6.5 in the rabbit aorta; K_i of 670 nM on cloned human B_1R, table 2), it showed resistance to ACE, neutral endopeptidase and also aminopeptidases, and exhibited an increased duration of action (15–30 min) as compared with the reference compound, against cardiovascular responses induced by an exogenous B_1R receptor agonist in LPS-pretreated rabbits. Ac-Lys[βD-Nal^7, Ile^8]des-Arg^9-BK (also referred to as R-715) has been designed with a similar rationale, and proved to be partially resistant to ACE, although retaining a high affinity for the human and rabbit B_1R (pA_2 of 8.5 in the rabbit aorta; Gobeil *et al.*, 1996a; table 2).

Recently, metabolically stable B_1 and mixed B_1 + B_2 antagonists of high affinity and stability have been synthesized. B9858 [Lys-Lys-[Hyp^3, Igl^5, D-Igl^7, Oic^8]des-Arg^9-BK] belongs to this series and has some selectivity for B_1Rs (table 2; Gera *et al.*, 1996; Gobeil *et al.*, 1997). This peptide retains the Lys^0 residue, a favorable feature for binding to human B_1Rs (table 2). In binding assays, B9858 is more potent on the human B_1Rs than on its murine homologues: it is functionally highly active as a competitive antagonist against Lys-des-Arg^9-BK-induced contractility of the human umbilical vein (pA_2 9.2) and in the rabbit aorta (pA_2 8.4; Gobeil *et al.*, 1997). In the anesthetized dog, representing a hemodynamic system coexpressing B_1 and B_2Rs (see below, Section III.C.), B9858 is reported to be 20 times more potent than Lys-[Leu^8]des-Arg^9-BK in antagonizing des-Arg^9-BK-induced hypotension, and the antagonist effect lasts for more than 4 h (versus about 15 min for Lys-[Leu^8]des-Arg^9-BK; Stewart *et al.*, 1996).

Optimal peptide B_2R antagonists retain both Arg^1 and Arg^9 residues, and B_1R agonists or antagonists typically lack Arg^9. However, some novel antagonists with backbones constrained by nonnatural residues are somewhat more promiscuous in their selectivity. The des-Arg^9 fragment of Hoe 140 has been studied by some investigators (table 2). This peptide may not be a metabolite of Hoe 140. It exhibits increased affinity toward the B_1R in all species studied, but retains fairly high residual antagonistic effects on B_2Rs in functional in vitro (Rhaleb *et al.*, 1992) or in vivo studies (rat blood pressure, Lagneux and Ribuot, 1997). Similarly, NPC 18565 is the des-Arg^9 fragment of NPC 17731, but exhibits a better selectivity and affinity for the human B_1R than Hoe 140 des-Arg (table 2). However, recently produced antagonists retaining Arg^9, such as B9430 (D-Arg-[Hyp^3, Igl^5, D-Igl^7, Oic^8]BK) represent combined B_1 and B_2R antagonists, although the corresponding des-Arg^9 fragment has a large selectivity toward B_1Rs (Burkard *et al.*, 1996). B9430 has been deliberately exploited as a dual kinin receptor antagonist in the blood pressure assay of the dog where it can abolish, at certain doses, hypotensive responses to either BK or des-Arg^9-BK without affecting the effects of several other unrelated agonists (Stewart *et al.*, 1996). The development of this compound not only demonstrates that a "polypharmaceutic" approach covering both receptor types is possible, but also that the structures of the B_1 and B_2R molecules are sufficiently similar to be antagonized by a single drug, a fact not appreciated until recently. Conformational analyses of B-9430 and B-9858 have been performed (Sejbal *et al.*, 1997). The peptides exhibit no observable secondary structure in aqueous solution. However, some common and divergent elements emerge under certain experimental conditions, such as a type II β-turn involving residues 2 to 5 common to both B_1 and B_2 antagonists. Such efforts may lead to the discovery of a universal "pharmacophore" for antagonizing kinin receptors.

Partial agonists are often discovered upon development of antagonist drugs. For example [Leu^8]des-Arg^9-BK, the prototype B_1R antagonist, may exhibit fairly high partial agonist behavior in some species, especially

in the rats and mice. This has practical implications, because one of the most exciting emerging therapeutic applications of B_1R antagonists, analgesia, is commonly based on models involving these species (see below, Section IV.C.). The molecular structure of both the drug and the receptor are critically important for the partial agonist behavior of certain antagonists, as shown in a study involving the cloned human or murine B_1Rs transiently expressed in a cell line that constitutively produces the calcium sensitive photoprotein aequorin (MacNeil *et al.*, 1997). [Leu^8]des-Arg^9-BK or Lys-[Leu^8]des-Arg^9-BK increased intracellular calcium concentrations in cells expressing the murine receptor (about 40% of the maximal activity of the full agonist des-Arg^9-BK), whereas B9858 and R-715 were not active in this respect. None of these peptides acted as agonists on cloned human B_1Rs. Functional studies based on mouse or rat isolated tissues that express B_1Rs have confirmed that R-715 and des-Arg-Hoe 140 possess no or very low intrinsic stimulant activities in these species (Meini *et al.*, 1996; Teater and Cuthbert, 1997; Allogho *et al.*, 1995). However, partial agonist behavior of [Leu^8]des-Arg^9-BK or Lys-[Leu^8]des-Arg^9-BK was observed in some preparations (e.g., in the mouse isolated stomach, the rat isolated colon; Allogho *et al.*, 1995; Teater and Cuthbert, 1997). This does not necessarily indicate the existence of B_1R subtypes that could be differentiated using a partial agonist behavior of some antagonists. If a pharmacological effect requires the stimulation of a very large proportion of the receptors on each cell, ligands with reduced efficacy are more likely to behave as antagonists (discussed by Leslie, 1987). This model may be applied quite well to the interaction of [Leu^8]des-Arg^9-BK on rat B_1Rs, as the maximum contractile (agonist) effect of [Leu^8]des-Arg^9-BK, relative to that of des-Arg^9-BK, increases as a function of incubation time in the rat longitudinal ileum (i.e., the intrinsic activity is variable and increases; Meini *et al.*, 1996). This is one of the smooth muscle preparations that exhibit in vitro up-regulation of B_1Rs upon isolation (see below, Section III.A.).

A partial agonist behavior may explain why the analgesic effect of [Leu^8]des-Arg^9-BK in rats and mice may be limited to a relatively narrow window of dosage (around 30 nmol/kg), as higher doses may exacerbate pain perception (Rupniak *et al.*, 1997). No existing peptide antagonist seems ideal in term of affinity or selectivity to fully characterize the analgesic potential of B_1R blockade in small rodents (Rupniak *et al.*, 1997).

C. Absence of Cross-Desensitization

Cross-desensitization is a valid criterion to distinguish substances acting on the same receptors (Schild, 1973). This implicitly refers to adaptation mechanisms present in, or closely associated with the receptor molecules. Thus, a "fading" of a response caused by the depletion of intracellular stores of an ion or some other desensitizing mechanisms distal to the receptors cannot be linked to receptor selectivity and would not qualify in exploiting this approach for receptor classification. In rat mesangial cells, a line that coexpresses both B_1 and B_2Rs, it has been possible to down-regulate a functional response (acute increase of $[Ca^{2+}]_i$) to BK without depressing that to des-Arg^9-BK, and vice versa (Bascands *et al.*, 1993). An inositol trisphosphate formation or calcium response to des-Arg^9-BK has been demonstrated upon BK desensitization in bovine endothelial cells or rabbit mesenteric artery SMCs, but the inverse was not readily demonstrable, because B_1R-mediated responses were not as tachyphylactic (Mathis *et al.*, 1996; Smith *et al.*, 1995;).

The two types of kinin receptors differ significantly in regard to receptor-mediated ligand internalization: exposure of cloned human B_2R to BK results in a rapid receptor-mediated ligand internalization and the sequestration of both the receptor and the G protein α subunit in caveolae, accompanied by a profound loss of surface receptor binding (Austin *et al.*, 1997; de Weerd and Leeb-Lundberg, 1997). In contrast, the activation of the cloned B_1R is not associated with ligand-induced receptor internalization at 37°C (Austin *et al.*, 1997), and exhibits much less desensitization (further discussed below, Section III.D.). Experiments using truncated or chimeric human B_1 and B_2Rs in which the cytoplasmic carboxy terminus is either missing, or exchanged between the two receptor subtypes have indicated that the cytoplasmic carboxy terminus of the human B_2R contains sequences responsible for the receptor-mediated ligand internalization and receptor sequestration, which are not present in the C-terminal cytoplasmic domain of the B_1R subtype (Faussner *et al.*, 1996), a domain of low interspecies conservation (fig. 1). Replacement from Cys^{324} of the cytoplasmic carboxy terminus of the B_2R with that of the B_1R has greatly reduced ligand-receptor complex internalization, whereas replacement from Cys^{330} of the cytoplasmic carboxy terminus of the B_1R (see fig. 1) with the B_2R counterpart has lead to a striking increase (~80% within 10 min) of internalization. Another study has confirmed the important role of the carboxy tail in B_2R internalization, identifying also other structural determinants (Prado *et al.*, 1997). Thus, the consistent differences in signaling persistence between B_1 and B_2Rs appear to have a definite molecular basis and are of great potential interest to understand the relative importance of kinin receptor types in physiopathology. Whether other molecular mechanisms of desensitization are functionally important for kinin receptors (temporary loss of coupling of the G proteins, transcriptional suppression) is yet to be determined.

D. Second-Messengers

The structural features of the B_1R defined upon the expression cloning of the receptor cDNA confirmed its belonging to the superfamily of G protein-coupled recep-

tors, exhibiting a typical 7 transmembrane domain architecture (fig. 1). Recently, it has been shown that CHO cells stably transfected with human B_1Rs cause Gαq/11 and, to a lesser extent, G$\alpha i_{1,2}$ to bind GTP upon stimulation with Lys-des-Arg^9-BK (Austin *et al.*, 1997). Thus, the identity and relative importance of G protein subtypes linked to the B_1R are similar to the ones coupled to B_2R (e.g., see de Weerd and Leeb-Lundberg, 1997). Both B_1 and B_2 receptors are primarily linked to polyphosphoinositide phospholipase C (PLC) activation. Specifically, activation of the naturally regulated B_1R is linked to increased phosphatidylinositol turnover in all systems studied up to now (reviewed by Marceau, 1995; see also Smith *et al.*, 1995; Butt *et al.*, 1995; see application to rabbit aortic SMCs, fig. 4A). Human B_1Rs stably expressed in Chinese hamster ovary (CHO) or 293 cells increase the turnover of inositol-phosphates when stimulated with Lys-des-Arg^9-BK (Austin *et al.*, 1997; Bastian *et al.*, 1997). This effect was not prevented by pertussis toxin treatment in CHO cells, suggesting that the Gq/11 protein may be the more important than G$\alpha i_{1,2}$ for coupling the B_1R to PLC (β-isoform; Austin *et al.*, 1997).

Calcium signaling was instrumental in the expression cloning of the B_1R (Menke *et al.*, 1994). A transient increase in $[Ca^{2+}]_i$ (largely dependent on intracellular stores) is conceivably mediated by inositol 1,4,5-trisphosphate, one of the direct products of PLC, and this has been observed in response to stimulation of naturally expressed B_1Rs in animal or human cells (Bascands *et al.*, 1993; Bkaily *et al.*, 1997; Marsh and Hill,

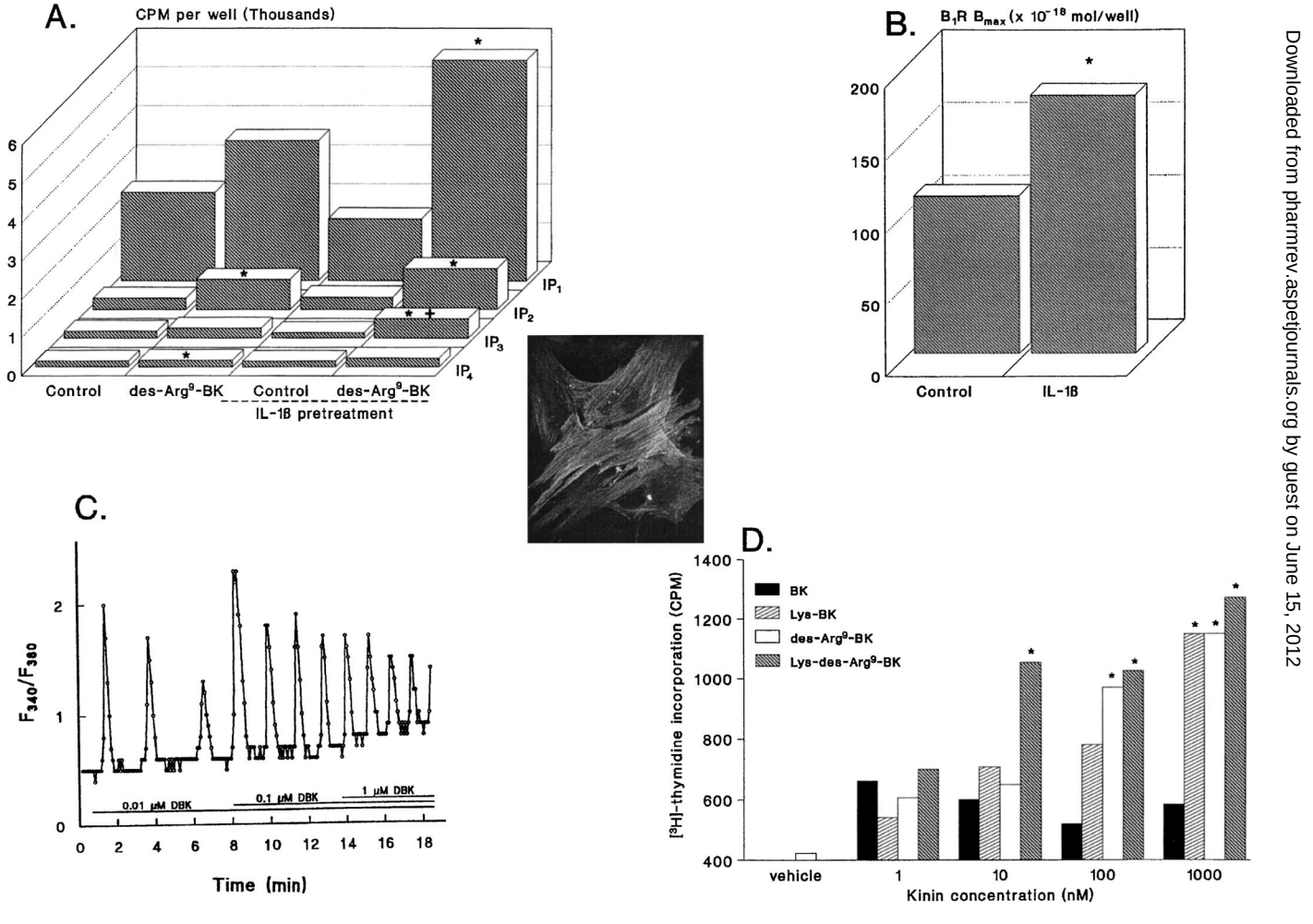

FIG. 4. The rabbit cultured arterial smooth muscle cell (SMC), a model for the regulated cellular effects of the B_1R. Middle: immunofluorescence pattern for α-actin in rabbit aortic SMCs, passage 1. (A) Inositol phosphates (IP) extracted from rabbit aortic SMCs 5 min after exposure to des-Arg^9-BK (1.7 μM) and pretreated or not with human recombinant IL-1β (5 ng/ml, 20 h before challenge). The asterisk indicates value significantly different from the corresponding control; the dagger indicates value significantly different from the corresponding one without IL-1β treatment. (Modified from Levesque *et al.*, 1993, with permission.) (B) The B_1R B_{max} is significantly (asterisk) increased in these cells by the 16- to 20-h IL-1β treatment in a proportion very similar to the effect of the cytokine on IP hydrolysis. Nevertheless, the baseline population of B_1Rs is important under the culture conditions employed (Levesque *et al.*, 1995a). (C) Persistent $[Ca^{2+}]_i$ signaling in a single representative SMC derived from the rabbit mesenteric artery and exposed to increasing concentrations of des-Arg^9-BK (DBK). The signal (Fura-2 fluorescence) is composed of oscillating peaks, of an increase of the baseline, or both, depending on the individual cell and the agonist concentration. From Mathis *et al.* (1996), with permission of the authors, editor, and ASPET. (D) Effects of kinins on [^{3}H]-thymidine incorporation into rabbit aortic SMCs in cells preteated with IL-1β (0.25 ng/ml, $-$24 h) and diclofenac (500 nM, $-$4 days). Both types of treatments are required to record a significant (asterisk) incorporation. Modified from Levesque *et al.* (1995b), with permission. Figures 4A and 4D were used with permission from the British Journal of Pharmacology; 109:1254–1262,1993; and 116:1673–1679,1995, respectively.

1994; Mathis *et al.*, 1996; Smith *et al.*, 1995; fig. 4C) or cloned human or murine B_1R expressed in heterologous systems (Austin *et al.*, 1997; Bastian *et al.*, 1997; MacNeil *et al.*, 1997). The sustained or oscillating phase of intracellular calcium increase is relatively more dependent on calcium influx by largely uncharacterized mechanisms (Bascands *et al.*, 1993; partial blockade by nickel: Mathis *et al.*, 1996; Smith *et al.*, 1995). There is preliminary evidence for the activation of multiple types of calcium channels by the B_1R agonists in vascular SMCs (L, T, and R types, the latter being relatively more important in the sustained phase, Bkaily *et al.*, 1997).

The moderate stimulatory effect of B_1R agonists on DNA synthesis in rabbit vascular SMCs (fig. 4D) is apparently dependent on another product of PLC, diacylglycerol, that activates a protein kinase C (PKC) (Levesque *et al.*, 1995b). The SMC contractility mediated by B_1Rs (in the rabbit aorta) probably involves the cooperation of PKC and Ca^{2+} of both intra- and extracellular sources (Levesque *et al.*, 1993). Filamin translocation from the membrane to the cytosol is another example of a Ca^{2+}-dependent response elicited by either B_1 or B_2R stimulation in cultured bovine ECs (Wang *et al.*, 1997). In this case, PKC action attenuates the response by limiting changes in calcium concentrations.

Thus, the identity of second-messengers is of limited value for the classification of the kinin receptors caused by common G protein subtypes and downstream elements. The most interesting differences observed so far may rather relate to the temporal patterns in systems that naturally coexpress both receptors. In bovine endothelial cells, rat mesangial cells, and rabbit mesenteric SMCs, the rise in $[Ca^{2+}]_i$ is more persistent and less tachyphylactic if elicited by B_1R stimulation, as opposed to B_2 activation, (Bascands *et al.*, 1993; Mathis *et al.*, 1996; Smith *et al.*, 1995). Similarly, the effect of the B_1R stimulation on PLC activation is similarly more persistent than that the B_2R, in separate transfected CHO cell lines expressing analogous densities of each receptor type (Austin *et al.*, 1997). In addition, these comparable cell lines were used to show that B_1Rs mediate much less receptor-ligand complex internalization than B_2Rs (Austin *et al.*, 1997), a fact that could explain the persistence of B_1R signaling. Structural determinants for B_2R internalization not shared by the B_1R, are discussed above (Section II.C.) and may be the molecular basis of these temporal differences.

The kinin receptors might be involved in other transduction mechanisms, different from those described above. For instance, both B_1 and B_2Rs are equally capable of suppressing platelet-derived growth factor-(PDGF) induced DNA synthesis in rat arterial SMCs (Dixon and Dennis, 1997). The mechanism of this suppression is still obscure, as the effects were not dependent on PLC, prostaglandins (PGs), or cyclic adenosine 3′,5′-monophosphate (AMP), or correlated with the extent of PLC activation (the B_1R being relatively ineffective in this respect; Dixon and Dennis, 1997).

Secondarily released mediators formed by Ca^{2+} dependent enzymes, such as endothelial nitric oxide synthase and cytosolic phospholipase A_2, may account for the production of nitric oxide (Drummond and Cocks, 1995a; Pruneau *et al.*, 1996) and eicosanoids (Levesque *et al.*, 1995b) mediated by B_1Rs. The secondary mediators are also shared with B_2Rs in many experimental systems, and are of considerable importance for the in vivo pharmacology of B_1Rs (see below, Section IV.). NO and PGs act in an autocrine or paracrine manner, thus extending the signaling mechanisms that kinins may activate in receptive tissues (notably to cyclic guanosine 3′,5′-monophosphate and cyclic AMP).

E. Distinct Nucleotide/Amino Acid Sequences

The two kinin receptor subtypes have been further defined on molecular basis after the recent isolations and characterizations of their genes. A BK receptor was first cloned from rat uterus, using a *Xenopus* oocyte expression assay and shown to have the pharmacological profile of a B_2R subtype (McEachern *et al.*, 1991). Subsequently, the human B_2R has been cloned from the fibroblast cell line CCD-16Lu by PCR and cDNA screening (Hess *et al.*, 1992), its genomic organization studied (Kammerer *et al.*, 1995; Ma *et al.*, 1994) and its localization mapped to chromosome 14q32 (Ma *et al.*, 1994; Powell *et al.*, 1993). The murine and rabbit B_2R genes were also characterized (Bachvarov, *et al.* 1995; McIntyre *et al.*, 1993). The deduced amino acid sequences of the B_2Rs from the four species studies have shown extensive similarity (80 to 84%), consistent with being orthologs of the same gene.

The B_1R became a defined molecular entity since the cloning and sequencing of the corresponding human cDNA. That the human embryonic cell line IMR-90 expresses B_1Rs has been known for some time, and receptor stimulation leads to metabolic effects such as collagen and DNA synthesis (Goldstein and Wall, 1984). Menke *et al.* (1994) observed that these cells bind [^{3}H]Lys-des-Arg^9-BK and that the binding is vigorously up-regulated by pretreating the cells with IL-1β, consistent with current regulation studies (see below, Section III.). These stimulated cells were an enriched source for the isolation of mRNA further used for the expression cloning of the human B_1R cDNA. Different molecular weight mRNA fractions were consecutively injected in *X. laevis* oocytes and the photoprotein aequorin was used as an indicator of the ability of the B_1R agonist Lys-des-Arg^9-BK to mediate Ca^{2+} mobilization in the injected oocytes. A 1307-bp clone that included a 1059 nucleotide open reading frame encoding a 353-amino acid protein was thus isolated and proposed to encode the B_1R (Menke *et al.*, 1994; predicted primary structure, fig. 1). This discovery was followed by the full analysis of the cDNA of the B_1R (1.4 kb), considerably smaller than that

coding for B_2R (over 4 kb), and by the determination of the corresponding genomic organization and chromosomal localization (14q32 between markers D14S265 and D14S267) (Bachvarov *et al.* 1996, 1998b; Chai *et al.*, 1996; Yang and Polgar, 1996; fig. 5). The rabbit and mouse homologues of the B_1R gene were also isolated and characterized (MacNeil *et al.*, 1995; Pesquero *et al.*, 1996) as there is 68 to 78% homology between the B_1Rs of the three species studied. A rat nucleotide sequence with a similar level of homology is proposed to encode the rat B_1R (GenBank sequence U66107), but its pharmacological profile is not reported yet. The cloned human B_1R also exhibits the seven transmembrane structure typical for G protein coupled receptors, although its amino acid sequence identity with the B_2R type of BK receptor is only about 36%. Nevertheless, they are the closest relatives based on the degree of similarity, followed by angiotensin receptors.

The predicted intracellular carboxy tail of the human B_1R is longer than that of their rabbit, mouse or rat counterparts (fig. 1). Perhaps significantly, the only effective antiserum to human B_1R to date has been derived from a rabbit immunized with the 16-mer C-terminal peptide (underlined in fig. 1; Hess *et al.*, 1996). These antibodies detected human recombinant B_1Rs expressed under the control of a viral promoter in transfected COS cells (immunofluorescence, Western blot; Hess *et al.*, 1996). Four immunoreactive bands were identified in the Western blot experiments, corresponding to 39, 31, 25, and 20 kDa. Because the predicted molecular weight of the human B_1R without carbohydrate is 40.4 kDa, it is quite possible that all four bands correspond to proteolytic cleavage fragments. All the published immunohistochemistry of the B_1R (see Section IV.) can be traced back to this antiserum.

Few studies of the structure-function relationship have been performed on the B_1R, and they are always comparative to the B_2R, for which a putative binding pocket involving transmembrane (TM) domain VI and the adjacent extracellular residues has been character-

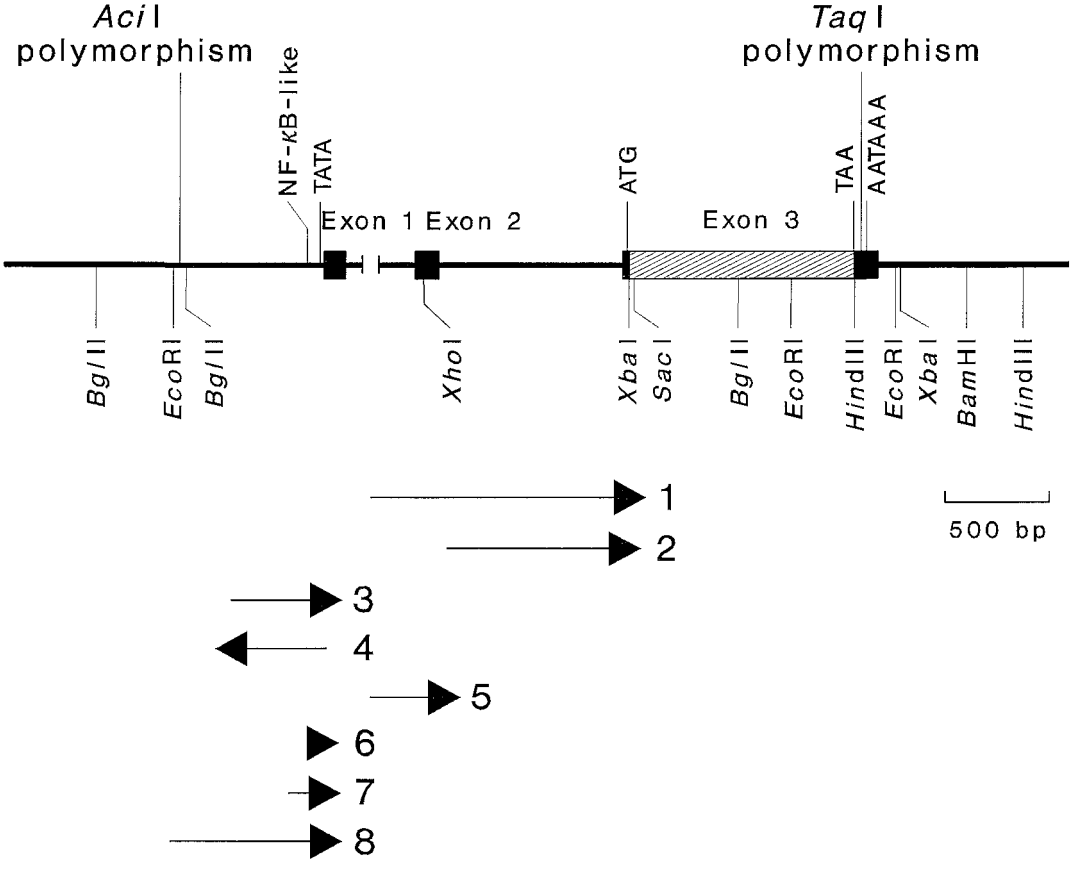

FIG. 5. Schematic structure of the human B_1 receptor gene (Bachvarov *et al.*, 1996; Yang and Polgar, 1996). The hatched region represents the uninterrupted coding sequence. Intron 1 length has been estimated to be 6 to 7 kb. Motifs of functional significance are indicated (start and stop codons, the polyadenylation signal, the TATA box in the 5′ flanking sequence, an important NF-κB-like site). Two polymorphic sites that can be studied by restriction length polymorphism are indicated (*Aci*I, *Taq*I). Some of the fragments tested for promoter activity in constructions involving a reporter gene are indicated by numbered lines terminated with an arrowhead pointing to the right (sense orientation), or the left (antisense orientation) : (1) a 1.9-kb fragment 5′ of the coding sequence and spanning exon 2 (Chai *et al.*, 1996); (2) a 0.87-kb fragment in the same region; (3 and 4) 0.45-kb fragments 5′ of and partially overlapping exon 1; (5) a 0.92-kb fragment starting in intron 1 and spanning exon 2 (Yang and Polgar, 1996); (6) a 0.14-kb gene fragment (−111/+34 relative to the transcription initiation site) containing a consensus TATA box (Ni *et al.*, 1998); (7) a 226-nt gene fragment (−139/+86); (8) the most active promoter fragment tested (−735/+86); a postulated negative control region in the core promoter is located 5′ relative to fragment 8 (D. R. Bachvarov, R. Drouin, M. Angers, J.-F. Larrivée, M. Bachvarova, and F. Marceau, in preparation).

ized (discussed by Leeb *et al.*, 1997). The similarity between the two receptor subtypes is significantly higher in these regions. Leeb *et al.* (1997) have constructed chimeric human B_1 and B_2Rs in which TM-VI domains have been exchanged and have shown that TM-VI in both receptor subtypes are important for the ability of these receptors to discriminate between their subtype-selective agonists. Substitution of B_1 TM-VI into the B_2R dramatically reduced the affinity of the B_2R-selective agonist BK. This affinity was fully restored when two residues, Tyr^{259} and Ala^{263} near the extracellular surface of the chimeric B_1R-TM-VI (see fig. 1) were replaced with the corresponding residues in the wild-type B_2R, which are Phe^{259} and Thr^{263}. Conversely, substitution of the B_2 TM-VI into the B_1R reduced the high affinity binding of the B_1-selective agonist Lys-des-Arg^9-BK. Interestingly, the latter chimera retained a functional response ($[Ca^{2+}]_i$ increase) to a high concentration of Lys-des-Arg^9-BK and to Lys-BK, the latter peptide confirming its status as a B_1 and B_2R agonist. TM-VI is probably less important for ligand docking in the B_1R than in the B_2R. Recent experiments have focused on TM-III exchange between the two kinin receptors, as this domain is presumably facing the TM-VI docking domain (Fathy *et al.*, 1997; L. M. F. Leeb-Lundberg, personal communication). Introduction of B_1R TM-III into the B_2R sequences decreased the affinity of the B_2R selective agonist BK, whereas the substitution of B_2R TM-III into the B_1R decreased the affinity of the selective ligand Lys-des-Arg^9-BK. High affinity for BK was restored when Lys^{118} in B_1R TM-III (numbering as in B_1R) was replaced with the corresponding wild-type B_2R residue (Ser^{111}). These observations and complementary analyses suggest that Lys^{118} in the B_1R structure repels the positive charge of the ligand C-terminal Arg^9, thus providing a possible explanation for the fundamental feature of the B_1R pharmacological profile: selectivity for des-Arg^9-BK homologues. These types of experiments further show the surprising structural compatibility of the two types of human kinin receptors, already suggested by the recent development of promiscuous antagonist ligands (see above, Section II.B.).

There is no genetic support for the existence of B_1 or B_2R subtypes in any given species, although fairly large differences of pharmacological profiles exist from one species to another. Experiments based on cloned and highly homologous receptors expressed in heterologous systems show conclusively that these pharmacological discrepancies are species differences (table 2).

F. Targeted Disruption of Receptor Genes

Perhaps it can be postulated that the ultimate form of receptor antagonism is the targeted disruption ("knockout") of receptor genes by homologous recombination. In mammalian species this technology, based on manipulations of embryonic stem cells, is currently restricted to the mouse. The B_2R knockout mice fail to respond to BK (smooth muscle, afferent nerve stimulation, etc.), indicating that no other B_2R subtype or variant responsive to that peptide exists in this species (Borkowski *et al.*, 1995). These animals develop normally and may not exhibit important hemodynamic changes. However, if overloaded with dietary NaCl, they develop severe hypertension with end organ damage (Alfie *et al.*, 1996). The relative importance of renal deficit in B_2R knockout mice is an important novel aspect of the kallikrein-kinin system, further exemplified by a polymorphism in the human B_1R gene (see below, Section III.C.). There is ample evidence that cytokine or postinjury regulation of the B_1R gene expression is not disrupted in B_2R knockout mice (Asonganyi *et al.*, 1996; Cuthbert *et al.*, 1996; Rupniak *et al.*, 1997; Seabrook *et al.*, 1997; see Section IV.). Such results are important, because both kinin receptor genes lie close to each other on human chromosome 14. This clustering may not represent a functionally coordinated locus comparable to those of globins or serum albumin homologues, for instance.

The B_1R knockout mice have been produced quite recently (Bader *et al.*, 1997; J. B. Pesquero, personal communication). These animals develop normally, are normotensive, but fail to functionally respond to des-Arg^9-BK (e.g., contractility of the mouse isolated stomach). Further, basal and LPS-induced expression of B_1R mRNA is absent (RT-PCR, reverse transcriptase polymerase chain reaction). Further experiments will be needed to exploit this murine strain in different physiopathological conditions. In this context, the creation of a double B_1/B_2R knockout strain is extremely intriguing.

G. Distinct Regulatory Profile

The transcriptional activity of the receptor genes is regulated by both tissue-specific and physiological factors. This may be relevant for receptor classification, and refers to the promoter function of distinct genes. Although there is some evidence that B_2R expression is transcriptionally regulated in cultured cells (e.g., modest positive regulation by cyclic AMP, PDGF and IL-1; Bathon *et al.*, 1992; Dixon, 1994; Dixon *et al.*, 1996; Schmidlin *et al.*, 1997; suppression by tumor necrosis factor-α; Sawutz *et al.*, 1992), these receptors are constitutively expressed in a wide variety of tissues. One of the few systematic efforts to document B_2R regulation concerns a sexual dimorphism of B_2R expression in several organs of the rat, the females expressing more of the corresponding mRNA (detected using RT-PCR; Madeddu *et al.*, 1997). Ovariectomy reduced the hypotensive effect of BK and estrogen treatment restored the hemodynamic effect of the kinin; however, hemodynamic responses to some agonists for other receptors followed a similar pattern (Madeddu *et al.*, 1997). Based on hemodynamic responses to des-Arg^9-BK, the B_1Rs were not regulated by estrogen in these studies. In some chronic inflammation models, B_2R function may be depressed (several examples will be given in Section IV.), but it is

not known whether this is caused by transcriptional repression or ligand-mediated down-regulation. In contrast, a large body of evidence shows that the B_1R is generally absent from normal tissues and animals (with some exceptions), but is rapidly induced after certain types of injuries in many species (Marceau, 1995). Molecular and genetic techniques have recently confirmed this receptor up-regulation. The regulatory mechanisms of gene expression are a recent focus of the research on B_1Rs, which is covered in detail in the next section.

III. Immunological and Molecular Analysis of B_1 Receptor Regulation by Tissue Injury

A. Postisolation B_1 Receptor Induction in Tissues

Smooth muscle contractility measured using organ bath methodology has been historically important to discover and characterize the B_1Rs. The distinct pharmacological profile of the kinin B_1R was initially derived from the analysis of kinin-induced contractility of the rabbit aorta (Regoli *et al.*, 1977). On this preparation, exogenous kinins exert a contractile effect that develops from an initial null level but increases in magnitude as a function of the in vitro incubation time (Bouthillier *et al.*, 1987). In this sense, the rabbit isolated aorta is a model for many other smooth muscle preparations derived from normal animals and used to illustrate the phenomenon of postisolation B_1R induction. Many such systems from various animal species are reviewed elsewhere (Marceau, 1995). To cite just a few recently documented examples, the rat portal and pig renal veins also demonstrate this phenomenon based on contractility (Campos and Calixto, 1994; Rizzi *et al.*, 1997). It is now clear that nonmuscle cellular elements can also exhibit the postisolation induction of B_1R: the vascular endothelial and colonic epithelial cells acquire such a responsiveness in vitro from a null or low initial level in the bovine or porcine isolated coronary artery and rat isolated colon, respectively (Drummond and Cox, 1995a; Pruneau *et al.*, 1996; Teater and Cuthbert, 1997). In such preparations, the de novo synthesis of B_1R is supported by the selectivity of the regulated behavior; similar responses mediated by other receptor types (including B_2Rs in some preparations) are much more stable as a function of time. Characteristically, the use of metabolic inhibitors also support the postisolation B_1R induction. Inhibition of RNA synthesis with actinomycin D, of protein synthesis with cycloheximide, anisomycin or puromycin, or of protein maturation with brefeldin A or tunicamycin, specifically prevent the development of a responsiveness to exogenous B_1R agonists without displaying toxic effects on other types of responses, or an acute inhibitory effect on responses to kinin that had been allowed to develop without metabolic inhibitors (Deblois *et al.*, 1991; Drummond and Cox, 1995a; Pruneau *et al.*, 1996; Teater and Cuthbert, 1997). Brefeldin A is a selective inhibitor of the translocation of newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus. A membrane receptor with transmembrane domain(s) would follow such a relatively slow maturation process and accordingly, the drug completely and selectively prevented des-Arg^9-BK-induced contraction in the rabbit isolated aorta (Audet *et al.*, 1994). The B_1R contains up to three putative but conserved N-glycosylation sites (fig. 1), which may be of importance for receptor assembly, stability or function. This is supported by the partial inhibitory effect of tunicamycin on the development of responses to des-Arg^9-BK in rings of rabbit aorta (Audet *et al.*, 1994). The biochemical effects of some of the metabolic inhibitors have been validated in assays involving the incorporation of [^{35}S]methionine (Deblois *et al.*, 1991) or [^{3}H]leucine into the protein fraction and of [^{3}H]mannose into the glycoprotein fraction of rabbit aortic tissue (Audet *et al.*, 1994).

The isolation-induced B_1R induction also occurs in human tissue preparations. Rings of human coronary arteries develop in vitro as a function of time the capacity to respond to des-Arg^9-BK resulting in an endothelium-dependent relaxation that is competitively antagonized by [Leu^8]des-Arg^9-BK, but not by Hoe 140 (Drummond and Cocks, 1995b). The human isolated ileum and umbilical vein develop responsiveness to B_1R agonists as a function of time, as evidenced using a full range of agonists and antagonists for kinin receptors, and this increase of responsiveness was prevented by treatment with a protein synthesis inhibitor (Gobeil *et al.*, 1996b; Sardi *et al.*, 1997; Zuzack *et al.*, 1996). In all these systems, B_2Rs were present, but in a preformed and stable manner, and mediated qualitatively similar effects.

The rabbit aortic contractility has also been exploited to show that cytokines, corticosteroids and drugs inhibitory for mitogen-activated protein (MAP) kinases influence the up-regulation of B_1Rs. Some inflammatory cytokines and growth factors increase the rate of sensitization to B_1R agonists in rabbit aortic rings: IL-1β, IL-2, epidermal growth factor (EGF), and oncostatin M have a similar effect on the rabbit aorta (Bouthillier *et al.*, 1987; Deblois *et al.*, 1988, 1991; Levesque *et al.*, 1995b), and interferon-γ on the bovine isolated mesenteric artery (De Kimpe *et al.*, 1994). The most potent stimulants increase both the maximal effects and the apparent potencies of B_1R agonists. However, the induction of B_1R-mediated responses is still prevented by cycloheximide in cytokine-stimulated aortic rings (Audet *et al.*, 1994). Glucocorticoids (dexamethasone and others), but not an estrogen, suppressed extensively the up-regulation of the B_1R-mediated responses, either under the spontaneous or cytokine-stimulated conditions (Deblois *et al.*, 1988). This also applies to the isolation-induced up-regulation of B_1R in the human umbilical vein (Sardi *et al.*, 1997). These findings are suggestive for a gene regulated by the cytokine network and immu-

nopathology. However, the cytokines regulating the process are very diverse as far as their receptor structure and signal transduction system pathways are involved. It was recently observed that freshly isolated rabbit aortic rings from normal animals show high MAP kinase activities (p38, ERK and SAPK/JNK pathways) relative to untreated cultured cells of various types, and these activities were further up-regulated by exogenous IL-1β (SAPK/JNK) or EGF (ERK) (Larrivée *et al.*, 1998). An inhibitor of the p38 MAP kinase, SB 203580, selectively inhibited by ~75% the spontaneous sensitization to des-Arg^9-BK over 6 h. SB 203580 also significantly reduced the development of the response to des-Arg^9-BK as stimulated by IL-1 or EGF (by about 50% at 6 h). Both the spontaneous and IL-1β-stimulated up-regulation of responsiveness to des-Arg^9-BK were strongly inhibited by the MEK1 inhibitor PD 98059 (by about 40%); however, the drug did not affect des-Arg^9-BK-induced responses in tissues treated with EGF. There is no specific inhibitor of the SAPK/JNK MAP kinase pathway to date, thus it cannot be excluded that this pathway also regulates B_1R expression. The protein kinase inhibitors failed to inhibit protein synthesis or to acutely block the contractile effect of des-Arg^9-BK, suggesting that they do not influence B_1R transduction mechanisms. Thus, protein kinase inhibitors reveal the role of cell injury-controlled MAP kinase pathways, and singularly of the p38 pathway, in the induction of B_1R (Larrivée *et al.*, 1998). These pathways conceivably may serve as mediators between cytokine receptor activation or direct cell injury (hyper- or hypoosmolarity, nutrient deprivation, oxidative stress or hypoxia) and the activation of the multiple transcription factors. The isolation-induced paradigm of B_1R induction has provided useful insights into the implicated immunological/pathological mechanisms and has indicated a minimal time (about 1 to 2 h) for the functional appearance of the response. The use of metabolic inhibitors, introduced in isolated organ pharmacology to study B_1R regulation, has revealed the reactivity of such preparations and has subsequently been important for the analysis of other systems (e.g., the cytokine induction of NO synthase or arachidonate metabolism in isolated blood vessels; Beasley *et al.*, 1991; Petitclerc *et al.*, 1994).

B. In Vivo Paradigms for the Induction of the B_1 Receptors and Their In Vitro Correlates

An in vivo paradigm for B_1R induction which has been particularly studied is the induction of cardiovascular responses to the corresponding agonists by a treatment with bacterial LPS in rabbits and other animals (see below Sections IV.A.). The evidence for transcriptional regulation of the receptor and its immunological basis will be reviewed here. Rabbits receiving intravenous injections with a sublethal dose (10 to 40 μg) of LPS exhibit dose-related hypotensive responses to exogenous intraarterial des-Arg^9-BK (Regoli *et al.*, 1981) or Lys-des-Arg^9-BK (Drapeau *et al.*, 1991b) after 5 h (see also fig. 3). By contrast, control animals do not respond significantly to these agents (fig. 3A). LPS pretreatment does not modify the basal blood pressure or the hypotensive responses to BK (mediated by B_2Rs) or to other agonists, and is not effective if given immediately before hemodynamic assessment, indicating a minimal lag time, probably necessary for protein synthesis and maturation. Tissues removed from LPS-pretreated animals (e.g., vascular strips, fig. 3D) exhibit functional responses to B_1R agonists within the first hour of in vitro incubation, suggesting that the response was acquired in vivo before death. Des-Arg^9-BK is a vasodilator of the coronary arteries only in hearts removed from LPS-pretreated animals and perfused in vitro (Langendorff technique; Regoli *et al.*, 1981). The B_1R mRNA up-regulation in heart tissue accordingly precedes this responsiveness in LPS-treated animals (Marceau *et al.*, 1997; fig. 3B). Northern blot experiments performed on poly(A$^+$) RNA derived from freshly isolated aortic smooth muscle of rabbits pretreated or not with LPS produced results virtually identical with those presented in figure 3B (J. F. Hess, D. R. Bachvarov, and F. Marceau, unpublished data). This model suggests that the whole cardiovascular system of the rabbit can be sensitized to B_1R agonists without modifying the preexisting population of B_2Rs or of receptors for other agents. The transcriptional activation of the B_1R gene is supported in this model by the kinetics of the corresponding mRNA in rabbit tissues after LPS injection, preceding the functional responses. LPS induction of cardiovascular responses has been observed in other species (see Section IV.A.) and supportive molecular evidence of de novo formation of B_1Rs has been generated. Autoradiography of [^{3}H]Lys-des-Arg^9-BK (6 nM) bound to frozen sections of porcine pulmonary arteries and thoracic aortas has revealed that the specific binding signal associated with the media SMCs is increased at least 3-fold by a pretreatment with LPS (Schremmer-Danniger *et al.*, 1996). RT-PCR has been used to study the expression of the B_1R in the mouse (Pesquero *et al.*, 1996). This highly sensitive and nonlinear amplification technique revealed B_1R transcripts in several organs of the normal mouse, but these signals were markedly increased by LPS treatment, notably in the heart, lung, and liver.

LPS, a complex polymer usually in the form of high molecular weight aggregates, is derived from Gram-negative bacteria and is capable of recruiting numerous host effector systems and modifying gene expression in various cell types. Notably, LPS is an inducer of cytokine synthesis in leukocytes and other cell types. An alternate cytokine inducer and pyrogen, the muramyl-dipeptide, is a synthetic LMW activator of cytokine production and can substitute for LPS to induce cardiovascular sensitivity to des-Arg^9-BK in rabbits (Bouthillier *et al.*, 1987). Intravenously injected human recombinant IL-1β is also active in this respect (Deblois *et al.*, 1991). Bridg-

ing the gap between the in vivo model and the molecular receptor populations, binding assays of radiolabeled B_1R agonists to rabbit cultured vascular SMCs have confirmed that IL-1, EGF, and LPS up-regulate the B_1R population (B_{max}), without influencing receptor affinity (Galizzi *et al.*, 1994; Levesque *et al.*, 1995a; Schneck *et al.*, 1994). In these studies, a relatively high background of B_1Rs in the cells may be a culture-induced artifact, perhaps derived from MAP kinase stimulation by serum in the medium or by stress. Nevertheless, we have observed that exogenous IL-1 stimulates B_1R B_{max} by a factor very similar to the potentiating effect of IL-1 on phospholipase C by des-Arg^9-BK in rabbit aortic SMCs (fig. 4A and B). Other studies, based on different culture conditions, have demonstrated a more dynamic regulation of the receptor population by cytokines and LPS. The rabbit B_1R cloning by subtraction hybridization took advantage of the presence of the B_1R in a cDNA library constructed from cultured aortic SMCs, from which a library derived from fresh aortic tissue was subtracted, thus enriching the receptor cDNA (MacNeil *et al.*, 1995).

Another important in vivo paradigm for B_1R up-regulation is the mediation of hyperalgesia in various models of persistent or chronic inflammation based on the rat or mouse (see below, Section IV.C.). The hyperalgesia models in rats, based on various primary irritants or noxious stimuli, can also reveal the B_1R-mediated loss of mechanical or thermal tolerance when the rats receive injections of recombinant cytokines such as IL-1β, IL-2, IL-8 (Davis and Perkins, 1994b), or nerve growth factor (Rueff *et al.*, 1996). The natural IL-1 receptor antagonist, IRA, prevented the hyperalgesia produced by intra-articular injection of IL-1, IL-2, or IL-8, suggesting that the effect of some cytokines may be ultimately linked to the autocrine/paracrine production of IL-1 (Davis and Perkins, 1994b). Isolated and cultured mouse superior cervical ganglia expressed B_1R (detected as a functional response, i.e., depolarization after exposure to Lys-des-Arg^9-BK in the presence of captopril) only in tissues treated in vitro with IL-1β or IL-8, but not leukemia inhibitory factor (Seabrook *et al.*, 1997). The same report showed B_1R transcripts in neural tissue by in situ hybridization, although the functional correlates were not developed.

In summary, immunological analysis of B_1R up-regulation in vivo and in vitro indicates that inflammatory cytokines play an important role in transcriptional activation of the corresponding gene, with a prominent place for IL-1. However, an obligatory role of IL-1 in B_1R expression under all experimental conditions is not proven. For instance exogenous IRA, a competitive antagonist of IL-1α or -β at the level of IL-1 membrane receptors, failed to inhibit the spontaneous sensitization to des-Arg^9-BK as a function of time in rabbit aortic rings, although IRA was effective to suppress the potentiating effect of exogenous IL-1β on this process (Petitclerc *et al.*, 1992). More direct forms of cellular stress (isolation of tissues and incubation in nutrient-poor physiological media; long term cell culture), typically applied in the absence of leukocytes, may bypass the need for cytokines and their receptors, by activating downstream stress-sensitive MAP kinases (Larrivée *et al.*, 1998). In addition, biochemical responses to B_1R agonists that are distant from the receptors, such as PGI_2 synthesis (Galizzi *et al.*, 1994) or DNA synthesis (fig. 4D), may be amplified to a greater extent by IL-1 or other cytokines than expected on the basis of B_1R B_{max} changes, possibly caused by various postreceptor interactions between the kinin and cytokine signaling pathways. Similarly, an immediate synergistic effect of EGF with des-Arg^9-BK-induced contractile effect is observed in the rabbit isolated aorta, and this is not likely to be mediated by a B_1R up-regulation (Deblois *et al.*, 1992), although long-term induction of B_1Rs by EGF has been shown (Schneck *et al.*, 1994). The molecular basis of an EGF receptor-B_1R interaction is not determined yet.

C. Controversies and Exceptional Situations

The inducible behavior of the B_1R, whose expression is restricted to immunopathology, is a substantially documented concept, but important exceptions may exist. In addition to the significant species-dependent differences in ligand affinities, major discrepancies in the regulatory function of the B_1R may exist at least in the dog and the cat. Hypotension, natriuresis and renal vasodilator responses were observed in normal dogs injected with des-Arg^9-BK (Lortie *et al.*, 1992; Nakhostine *et al.*, 1993). Some tissues isolated from the dog also exhibit an immediate and stable response to B_1R agonists (reviewed by Marceau, 1995). Complex hemodynamic effects are also produced by des-Arg^9-BK in the feline pulmonary circulation (DeWitt *et al.*, 1994). In each case, the B_2R coexisted and the identity of each receptor type was also validated with the use of appropriate antagonists. It is tempting to speculate that a difference in the B_1R gene promoter of the order Carnivora can explain the constitutive expression of cardiovascular B_1Rs. The guinea pig gallbladder is a smooth muscle preparation which acquires a contractile response to des-Arg^9-BK in a time- and protein synthesis-dependent manner (Cabrini and Calixto, 1997). However, this response is antagonized by Hoe 140, not by [Leu^8]des-Arg^9-BK, prompting these authors to conclude that the response to des-Arg^9-BK is mediated by inducible B_2Rs. Molecular biology approaches are needed to clarify whether the canine and feline constitutive B_1Rs are the real species homologues of the genetically defined B_1Rs, whether the guinea pig receptors responsive to des-Arg^9-BK are B_2Rs, and whether immunopathology can regulate the expression of all these receptors.

In such animal species as the pig and rats, some reports suggest a minimal but measurable hemodynamic responsiveness to exogenous des-Arg^9-BK (Sie-

beck *et al.*, 1989; Madeddu *et al.*, 1997), although it is clear that treatments with bacterial products can upregulate these responses (Lagneux and Ribuot, 1997; Siebeck *et al.*, 1989; Tokumasu *et al.*, 1995). Whether a low population of B_1Rs plays a physiological role in normal animals, at least in some organs, is not a trivial issue. Stomach strips from apparently normal mice exhibit an immediate and constant contractile response to B_1R agonists (Allogho *et al.*, 1995), and exogenous des-Arg^9-BK is inflammatory when injected into the pleural space of naive mice, but not when injected into the paw (Vianna and Calixto, 1998). Further, the B_1R antagonist [Leu^8]des-Arg^9-BK reduces the glomerular filtration rate and the urine concentration in normal rats infused with saline (Fenoy and Roman, 1992). Whether tissue-specific constitutive B_1Rs can be invoked in these examples could not be excluded. However, subclinical infectious disease in "control" animals is another possible explanation for a "baseline" B_1R population. For example, in pigs, spontaneous infections are common and this can predict the level of cardiovascular response to des-Arg^9-BK (Siebeck *et al.*, 1996). A systematic effort will be needed to clarify these issues.

D. Analysis of the Promoter Function of the B_1 Receptor Gene

The body of evidence summarized above shows that the B_1R is a strongly regulated gene. The analysis of the promoter region of the human B_1R gene has been initiated using constructions involving a reporter gene (luciferase or chloramphenicol acetyl transferase) transfected in the human cell lines (IMR-90, HEK-293 or HepG2) or rat aortic SMCs (Chai *et al.*, 1996; Ni *et al.*, 1998; Yang and Polgar, 1996; D. R. Bachvarov, R. Drouin, M. Angers, J.-F. Larrivée, M. Bachvarova, and F. Marceau, in preparation). DNA sequencing and analysis had revealed several potential regulatory sites in the noncoding parts of the genes, in fact too many to make useful predictions. Some of the gene fragments tested for promoter activity are indicated in fig. 5. An initial study showed a weak promoter activity in the 5′ region of the coding sequence, amounting to a maximal of about 10% of that of a positive control, the Rous sarcoma virus promoter (maximal activity in the 1.9-kb fragment indicated as 1 in fig. 5; Chai *et al.*, 1996). This fragment spans exon 2, a fact that was not known at this time. Recent experiments showed that this fragment does not confer regulatory effects to IL-1 (Ni *et al.*, 1998). Knowledge of the 3-exon structure prompted Yang and Polgar (1996) to cover more systematically potential regulatory domains (fragments 2 to 5, fig. 5). Although fragment 2 promoted some expression of the reporter gene in SV40-transformed IMR-90 cells, fragment 3 (located 5′ to exon 1) was about 10-fold more active. Fragment 4, the antisense counterpart of fragment 3, as well as fragment 5, covering a part of exon 1 and exon 2, were essentially inactive. This comparative study established the predominant promoter activity of the region located 5′ relative to exon 1.

A 2.6-kb fragment localized upstream from the first intron of the receptor gene has been recently studied for promoter activity (Ni *et al.*, 1998). Different fragments of this region, cloned in front of a reporter gene (firefly luciferase), exhibited promoter activity in several cell lines, but a regulated behavior only in some lines. Successive deletions indicated that a 0.14-kb 5′ flanking fragment (6 in fig. 5) was sufficient for transcriptional activity and inducibility by IL-1, TNF-α and LPS and suppression by dexamethasone and by a putative antioxidant inhibitor of nuclear factor κB (NF-κB) (Ni *et al.*, 1998). An NF-κB-like binding sequence at -64 to -55 relative to the major transcription initiation has been found; its mutagenesis abolished most of the regulatory effects. Another group studied the 4.2-kb fragment upstream of exon 1 (D. R. Bachvarov, R. Drouin, M. Angers, J.-F. Larrivée, M. Bachvarova, and F. Marceau, in preparation). The promoter activity of this zone was addressed using chloramphenicol acetyl transferase reporter gene constructs. Transient transfection of reporter gene constructs in IMR-90 cells indicated that a 226-nt gene fragment (-139 to $+86$ relative to the transcription initiation site; fragment 7 in fig. 5) containing a consensus TATA box, was sufficient to direct transcription in the cell line IMR-90, a cell line spontaneously expressing the B_1R. The corresponding antisense fragment was not active. Promoter activity was not observed in four constructs lacking the TATA box which is located 21 to 28 bp upstream of the major transcription initiation site, indicating its functional importance. Promoter activity was not importantly modulated by IL-1 or dexamethasone in IMR-90, by contrast to the findings of Ni *et al.* (1998) in SMCs. The presence of IL-1 responsive element(s) in the region studied was indirectly shown by transient transfections using the human embryonal kidney cell line HEK-293, which expresses the IL-1 receptor type I gene only at a very low level. Cotransfection experiments, using a construct expressing the human IL-1 receptor type I gene together with promoter constructs, doubled the promoter activity of fragments as short as the 226-nt fragment 7, confirming the presence of IL-1-responsive motif(s) in the region studied. Further experiments in IMR-90 cells based on several constructions of various length identified a positive and a negative control regions, localized upstream from the TATA box (fig. 5). The most active fragment, indicated as 8 in fig. 5, exhibited about two-thirds of the activity of a cytomegalovirus promoter in transfected IMR-90 cells, and spanned a polymorphic site of potential importance (see below, Section III.E.). A negative control region was located upstream to the maximally active promoter fragment 8.

Thus, the promoter region of the human B_1R bears the characteristics of an eukaryotic inducible promoter with a functional TATA box, and contains additional positive

and negative control elements. Evidence of tissue-specific and cytokine regulatory control was also obtained. At this time, it cannot be excluded that regulatory elements lie outside the studied regions, for instance in the large intron 1.

Induction of B_1R in IMR-90 cells by immunostimulants may involve a stabilization of the corresponding mRNA, in addition to transcriptional stimulation, suggesting a role for of a posttranscriptional regulator (Zhou *et al.*, 1998). IL-1 doubled the mRNA half-life to 2 h; protein synthesis inhibitors were even more potent to increase B_1R mRNA stability in these cells (Zhou *et al.*, 1998). Perhaps related to these findings, protein synthesis inhibitors applied as a "pulse" of 1 to 3 h on rabbit aortic rings were found to paradoxically increase the subsequent up-regulation of B_1R-mediated responses (Deblois *et al.*, 1991).

E. Allelic Polymorphisms of the B_1 Receptor in the Human Population and Human Disease

Human genetic studies have shown that a transmissible low urinary kallikrein excretion is associated with a positive history of hypertension (Berry *et al.*, 1989). Investigations on genetic polymorphisms of kinin receptors have been initiated. Of the described B_2R gene polymorphisms identified, only one has been shown to be of potential clinical significance: an exon 1 polymorphism in which alleles differ by a 9-bp deletion, designated (−) versus the complete sequence, designated (+) (Lung *et al.*, 1997). The (−) allele, presumably more stable relative to the action of RNases, appears to confer a higher level of expression and is always present in the most symptomatic cases of C1 inhibitor deficiency (hereditary angioedema with angioedema crises, 21 patients examined). Thus, the B_2R (−) allele is proposed to modulate in a dominant manner the phenotype (penetrance) of the basic genetic defect in this disorder, the C1 inhibitor deficiency. The latter plasma component is an endogenous inhibitor of plasma kallikrein activity (table 1).

Minor variations in the coding sequence of the human B_1R have been reported (see fig. 1 legend); whether these are genetic polymorphisms or the result of methodological errors in DNA sequencing has not resolved. Based on the genomic structure of the human B_1R for kinins, the presence of possible allelic polymorphisms of this gene was investigated using both restriction fragment length polymorphism and single strand conformation polymorphism methods (Bachvarov *et al.*, 1998b). It was reasoned that the protective effect of the kallikrein-kinin system on the kidney and cardiovascular system could be altered if the polymorphic alleles were functionally different, leading to decompensation and end organ damage with the less functional alleles, as is seen in an extreme model, i.e., the knockout mice for the B_2R gene submitted to a NaCl overload (Alfie *et al.*, 1996). Two B_1R gene polymorphisms were found and the frequencies of the corresponding allele pairs were determined in healthy volunteers and inpatients with a history of end stage renal failure (Bachvarov *et al.*, 1998b). An $A^{1098} \rightarrow G$ polymorphism has been identified in exon 3 in a minority of volunteer blood donors. This polymorphism is located 35 nucleotides downstream of the stop codon and 14 nucleotides upstream of the polyadenylation signal, and appeared to be clinically neutral. The A → G substitution introduces an additional *Taq*I restriction site (fig. 5). A second, and more frequent polymorphism consists of a single base substitution ($G^{-699} \rightarrow C$) in a positive control region of the promoter (position relative to the major transcription initiation site; this generates an additional *Aci*I restriction site, fig. 5). This polymorphism is significantly less frequent in the population of renal failure patients (33.3% prevalence in 102 healthy volunteers, versus 20.6% in 287 diseased individuals, $P = 0.014$) and determines in IMR-90 cells a significantly increased activity of the promoter function in constructions involving both versions of gene fragment 8 (fig. 5) cloned in front of a reporter gene. The altered prevalence of this allele was also found in several etiological subgroups of uremic patients. Thus, the polymorphism of the B_1R promoter may be a marker of prognostic significance for the preservation of renal function in diseased individuals. The hypothetical influence of the relative B_1R overexpression determined by the C allele could not be linked to a specific etiology, but may rather be related to nonspecific compensatory mechanism(s), as with resistance to ischemic damage, maintenance of glomerular filtration, etc. It is not currently known whether the alleles are associated with negative clinical aspects (e.g., amplified role of kinins as inflammatory and pain mediators).

IV. Physiopathological Relevance of B_1 Receptor Up-Regulation

The conservation of the B_1R structure (fig. 1) and regulatory mechanisms in several mammalian species suggests that this system affords a selective advantage in stressful situation such as infection or cardiovascular or renal disease. Cytokine and MAP kinase regulation of this gene are likely to make the B_1R up-regulation a rather nonspecific tissue response to many intense stressful situations (e.g., 42°C heat shock applied to anesthetized rats, Lagneux and Ribuot, 1997). In experimental pathology, the contribution of the kinin B_1R has been very generally overlooked, until recently. Part of the problem may be related to the less-than-ideal properties of the available peptide B_1R antagonists (discussed above). However, typical of inflammatory mediators, the pharmacological blockade of the B_1R system in inappropriate forms of inflammation (e.g., autoimmune, allergic) is emerging as a useful therapeutic intervention, based on animal models. Practically nothing is known about the clinical pharmacology of the B_1R. Investigators have initiated the immunohistochemical or

in situ hybridization detection of the B_1R in human tissue sections. Although these results are very interesting, their relevance to receptor population is not always clear, because most adult "normal" tissue donors are either clinically ill defined or objectionable (e.g., post-traumatic death that was probably preceded by an episode of cardiovascular shock; Raidoo *et al.*, 1997).

A. Circulation

The in vivo induction of B_1Rs in the rabbit by a LPS injection described above, is based on functional and molecular evidence (fig. 3; Section III.B.). As for the physiopathological implications, this model exhibits selectivity, as several other treatments recruiting immunological effectors failed to induce the state of responsiveness to the selective B_1R agonist des-Arg^9-BK (e.g., in vivo activation of circulating neutrophils with N-formyl-Met-Leu-Phe; Bouthillier *et al.*, 1987). The dominant vasoconstrictor effect of B_1R agonists in large conduction vessels (the rabbit aorta, the human umbilical vein, etc.), conveniently used for in vitro pharmacological studies (see above), may only be important in a limited number of cases; for instance, both B_1 and B_2Rs have been hypothetically involved in postpartum umbilical vessel closure (Abbas *et al.*, 1998). Such vasoconstriction may not be predictive for the response of smaller muscular or resistance vessels, where vasorelaxation mediated by various secondary released autacoids (eicosanoids, nitric oxide) may overshadow contractile effects (as seen in the perfused rabbit heart, the rabbit isolated carotid and mesenteric arteries; Regoli *et al.*, 1981; Churchill and Ward, 1987; Pruneau and Bélichard, 1993). The mechanism of the hypotension induced by the metabolically stable B_1 agonist Sar[D-Phe^8]des-Arg^9-BK has been studied in LPS-pretreated rabbits. The duration, but not the amplitude, of the hypotensive episodes were reduced by indomethacin (Drapeau *et al.*, 1991b) or diclofenac (Audet *et al.*, 1997), suggesting a modulating effect of secondarily released prostaglandins. The mechanism of the prolonged hypotension caused by the stable B_1R agonist, Sar-[D-Phe^8]des-Arg^9-BK, appears to be complex in LPS-pretreated rabbits: a fall of peripheral vascular resistance accounts for the early response, but the prolonged hypotension associated with persistent receptor stimulation is explained by a fall of cardiac output (Audet *et al.*, 1997). Pharmacological evidence showed that PG-stimulated sympathetic nervous system activation follows B_1R activation in this system, comparable to the Lys-des-Arg^9-BK-induced depolarization of IL-1-pretreated mouse sympathetic ganglia in vitro, where a prostanoid intermediate is suspected (Seabrook *et al.*, 1997). As mentioned above, induction of cardiovascular responsiveness to des-Arg^9-BK by bacterial endotoxin may also apply for the pig (Siebeck *et al.*, 1989, 1996) and the rat (Lagneux and Ribuot, 1997; Nicolau *et al.*, 1996; Tokumasu *et al.*, 1995), but no detailed hemodynamic analysis is available in these species. Dog blood vessels express constitutive B_1Rs mediating hypotension when des-Arg^9-BK is injected intravascularly. Vasodilation (such as in the coronary vasculature), fall of peripheral resistance and increase of cardiac output were observed during these episodes (Bélichard *et al.*, 1996; Lamontagne *et al.* 1996). Bélichard *et al.*, (1996) performed their experiments under ganglionic blockade with hexamethonium and, accordingly, did not observe manifestations of sympathetic activation. However, Lamontagne *et al.* (1996) monitored des-Arg^9-BK-induced tachycardia that could be prevented by propranolol. These authors agree that at least part of the vasodilator effect of des-Arg^9-BK is dependent on nitric oxide, as N^G-nitro-L-arginine inhibits the coronary vasodilator effect or part of the hypotension that follows intraarterial injection of the B_1R agonist; by comparison, prostanoids seem unimportant. The same two effector systems of hemodynamic B_1R-mediator response, NO-dependent vasodilation superimposed to vasoconstriction of sympathetic origin, are acting in a concurrent manner during the infusion of des-Arg^9-BK into the cat pulmonary circulation (DeWitt *et al.*, 1994).

Because either B_1 or B_2R activation in vivo results in hypotension, one can wonder about the role of kinins in septic shock, a cardiovascular condition that can be reproduced by injecting large doses of LPS (typically 500 μg/kg or more in rodents or rabbits). Low doses of LPS up-regulate B_1Rs, but do not necessarily produce much of the corresponding agonist(s), as infusions of B_1R antagonists do not change the baseline blood pressure (Drapeau *et al.*, 1993). However, there is evidence for a massive consumption of kininogen and Hageman factor in rabbits injected with large doses of LPS (Erdös and Miwa, 1968). This situation is therefore a possible field of application for the kinin antagonists developed in the last decade. However, B_2R antagonists have been disappointing in treating septic shock in animals and humans, despite some favorable hemodynamic effects in the early phase of the pathology (Fein *et al.*, 1997; Félétou *et al.*, 1996). A proper evaluation of a very good B_1R antagonist has not been reported in this condition. An adverse effect of [Leu^8]des-Arg^9-BK, combined with a B_2R antagonist, has even been suggested in short term septicemia in the pig, but the effect of the B_1R antagonist alone has not been reported (Siebeck *et al.*, 1996). It is likely that B_1R-mediated vasodilation and sympathetic system stimulation, as observed in several animal species, represents useful cardiovascular compensatory mechanisms during the initial phase of systemic sepsis, for instance by increasing tissue oxygen delivery and improving lung circulation (Siebeck *et al.*, 1996). On this basis, a pharmacotherapeutic intervention with a B_1R agonist has been advocated in systemic sepsis (Siebeck *et al.*, 1997). Decompensation in this condition is a relatively late event, in which excessive B_1R stimulation may also participate. This may be illustrated by the

counterproductive drop of cardiac output in the presence of a persistent low peripheral resistance in LPS-pretreated, anesthetized rabbits injected with Sar-[D-Phe^8]des-Arg^9-BK (fig. 3C), and is possibly associated with a decreased venous return. The intense and prolonged fall of cardiac output was also observed when Sar-[D-Phe^8]des-Arg^9-BK was infused in this model (A. Audet, F. Rioux, and F. Marceau, unpublished data). Cardiovascular complications of sepsis may respond differently to drugs as a function of the duration of the pathology and of the species, and a systematic approach to this problem could help to clarify the potential of B_1R antagonists in this condition.

The expression of kinin receptors by cardiomyocytes and cardiac conduction tissue is further supported by a PG-independent negative chronotropic effect of BK mediated only by B_2Rs in the canine sinus node (Ribuot *et al.*, 1993). Both B_1 and B_2R stimulation can prolong the action potential duration in a preparation of rat ventricular muscle (Gouin *et al.*, 1996). The B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK was used in this demonstration. Whether postisolation up-regulation of B_1R has occurred in the latter system is a possibility that has not been rigorously tested. A significant decrease in heart rate was measured during the prolonged hypotension induced by Sar-[D-Phe^8]des-Arg^9-BK in the rabbit (Audet *et al.*, 1997), and it is not excluded that a direct effect of the peptide on the heart contributed to the observed drop of cardiac output.

ACE inhibitors have been very successful in clinical practice for treatment of hypertension and heart failure. The contribution of kinins and B_2Rs to the therapeutic and side effects of cardiovascular drugs which block ACE (such as captopril, enalapril, and others) has attracted much interest. These inhibitors not only repress the activation of angiotensin I into angiotensin II, but also have the capacity to potentiate endogenous kinins if they are formed in sufficient amounts during a pathological state (Linz *et al.*, 1995; Marceau, 1997). However, the clinical evidence for this phenomenon is still limited. A privileged experimental approach to test this hypothesis in animals has been to combine the administration of a B_2R antagonist with that of an ACE inhibitor to observe a more or less complete antagonism of the therapeutic effect of the latter drug. Kinin contribution assessed in that manner varies from small to important, depending on the animal model of cardiovascular pathology (Marceau, 1997). The possibility that B_1Rs also participate in the tissue effects of ACE inhibitors has not been systematically tested. The B_1Rs are not up-regulated 3 to 20 h after large intravenous doses of enalaprilat, enalapril or captopril in normal rabbits (based on Northern blot detection of B_1R transcripts in the heart or on in vivo hemodynamic analysis; Marceau *et al.*, 1997; Deblois *et al.*, 1991). However, these drugs were not planned to be given to normal individuals, and the pathological context (e.g., end organ damage in hypertension) may locally modify the receptor population. It is of considerable interest that BK increases in the effluent of rat isolated hearts perfused with Krebs buffer during a reperfusion period that followed ischemia; in this case the des-Arg^9-BK immunoreactivity increases only when ACE was concomitantly blocked using ramiprilat (Lamontagne *et al.*, 1995). This suggests that the ischemic pathology and the pharmacotherapy of cardiovascular disease with ACE inhibitors can cooperate to produce B_1R agonists. In view of the possible regulation of B_1R population in such situation (see below), there is clearly an opportunity to assess the importance of the duality of receptor types for kinins. Virtually nothing is known about B_1R regulation in hypertension, cardiac failure and their complications. However, it is interesting to note that cardiac failure is associated with a high production of inflammatory cytokines in humans (Lommi *et al.*, 1997).

Angioplasty applied to the rabbit carotid artery, a form of vascular lesion, is associated with both the proliferation of SMCs and the acquisition of a contractile response to the B_1R agonist des-Arg^9-BK (Pruneau *et al.*, 1994). Although no causal relationship is proven between the two findings, B_1R stimulation leads to DNA synthesis in cytokine treated rabbit aortic SMCs (fig. 4D). Only cells pretreated with cytokines, such as IL-1β, EGF, or oncostatin M, exhibit this stimulatory effect of kinins (Levesque *et al.*, 1995b). However, B_1R stimulation mediates the suppression of DNA synthesis in PDGF-stimulated rat mesenteric artery SMCs (Dixon and Dennis, 1997). It has been recently observed that the immunoreactivity of the human B_1R is highly increased in all cell types that compose atheromatous plaques in tissue section of large arteries (endothelial cells, foamy macrophages, infiltrating leukocytes, proliferating SMCs; Raidoo *et al.*, 1997). Immunoreactive B_2Rs were also somewhat overexpressed in these lesions, but this staining was consistently inferior to that of the B_1Rs. Kinin receptor up-regulation may be determined by the inflammatory nature of atherosclerosis, but its precise role (contributing or protective) in the development of human ischemic disease is difficult to predict, because of the conflicting nature of the in vitro animal evidence.

Ischemia is a basic pathological process caused by a local circulatory deficit and there is ample evidence that it activates the kallikrein-kinin system (e.g., see Lamontagne *et al.*, 1995). Several of the MAK kinases, more notably p38, are activated by experimental ischemia or ischemia/reperfusion in rat and dog organs (Yin *et al.*, 1997). These signaling molecules may determine B_1R induction after tissue injury (discussed above, Section III.A.). Various experimental settings based on rat isolated cardiac tissue have been used to document indirectly ischemia-induced B_1R up-regulation by their capacity to modulate positively or negatively norepinephrine release or to preserve endothelium-dependent vasodilation

(Bouchard *et al.*, 1998; Chahine *et al.*, 1993; Feng *et al.*, 1997; Foucart *et al.*, 1997). The understanding of the observed relatively rapid B_1R induction (30–50 min) would benefit from the use of metabolic inhibitors and from molecular approaches. Further indirect evidence of B_1R induction by ischemia in the rat is derived from a model of cerebral artery occlusion (Relton *et al.*, 1997). In this system, administration of a B_2R antagonist reduced the cerebral infarct size under some experimental conditions, a finding disputed by other investigators (Campbell *et al.*, 1997). However, the B_1R antagonist B9858 exerted no direct effect on the pathology, but this peptide, as well as Lys-[Leu^8]des-Arg^9-BK, significantly attenuated the beneficial effect of the B_2R antagonist (Relton *et al.*, 1997). It was concluded that B_1 and B_2R have differential effects on ischemic brain insult, with a rather protective effect for the B_1Rs (Relton *et al.*, 1997).

B. Inflammation, Neurogenic Inflammation

The investigators who first had access to synthetic BK have shown initially that this relatively simple peptide reproduced the cardinal signs of inflammation when injected into animal tissues, including inflammatory swelling (edema) (Elliot *et al.*, 1960). Exudation of protein-rich fluid from the circulation is largely determined by vascular mechanisms involving the physical separation of endothelial cells, particularly at the level of postcapillary venules. Local vasodilation produced by kinins (inflammatory hyperemia) further facilitates exudation. Consistent with the regulatory patterns of kinin receptor subtypes, kinins stimulate B_2Rs when injected in normal tissues. For instance, an assessment of the acute edema produced by kinin injection into the rat paw, based on a full set of agonist and antagonist peptides, showed mediation of the edema formation by B_2Rs without a significant involvement of B_1Rs (Whalley *et al.*, 1984). Thus, a specific B_2R antagonist, such as FR 167344, exerts some antiinflammatory activity in acute animal models associated with the activation of the kallikrein-kinin system (Asano *et al.*, 1997).

However, systemic treatment with a relatively low dose of LPS sensitized in 24 h the rat paw to the inflammatory effect of exogenous des-Arg^9-BK, with some loss in the effect of B_2R stimulation (Campos *et al.*, 1996). Similarly, inflammation in more sophisticated immunopathological models in the rat also evidences B_1R upregulation. A shift from B_2 to B_1R mediation for kinin-induced exudation has been observed during the course (1 to 5 days) of antigen-induced chronic arthritis in the affected joint (Cruwys *et al.*, 1994). A full set of agonists (BK, des-Arg^9-BK) and antagonists (Hoe 140, [Leu^8]des-Arg^9-BK) further supported this conclusion. Moreover, the latter B_1R antagonist became progressively more effective to reduce the basal inflammatory plasma extravasation in this model, although the relative anti-inflammatory efficacy of Hoe 140 declined, suggesting that endogenous B_1R agonist(s) were active at the site of the maturing lesion (Cruwys *et al.*, 1994). Bacterial peptidoglycan-induced arthritis in the rat is also a form of immunological hypersensitivity; in this model, infusion of either a B_1 or a B_2R peptide antagonist reduced the inflamed paw volume and diameter, the combination of both types of antagonists being optimal 72 h after the sensitization (Blais *et al.*, 1997a). Chronic infection with the attenuated mycobacterium strain BCG determines a persistent (10 weeks) state of sensitivity to exogenous des-Arg^9-BK, as assessed by paw edema formation after local injection (Campos *et al.*, 1997). The edema response to a B_2R agonist was not changed in this model. Interestingly, the repeated administration of the B_2R agonist [Tyr^8]-BK in the rat paw sensitized the animal to des-Arg^9-BK, while the edema caused by [Tyr^8]-BK exhibited tachyphylaxis (Campos *et al.*, 1995). This suggests that intense B_2R stimulation may trigger directly or indirectly B_1R up-regulation to amplify tissue responses to kinins during an inflammatory reaction.

Streptozotocin-injected mice eventually develop an insulin-dependent form of diabetes mellitus; [Leu^8]des-Arg^9-BK, but not Hoe 140, could prevent this if administered twice daily from day 3 though day 13 (Zuccolo *et al.*, 1996). The inhibitor of tissue kallikrein, aprotinin, is also active in this respect. The major therapeutic end point was the glycemia at day 13, which failed to increase in animals treated with the B_1R antagonist. These fascinating results may be related to an anti-inflammatory effect of the B_1R antagonist, as the insulin deficiency is the complication of a chemically induced inflammation of the Langherans' islets. It would be desirable to further study several aspects of this model, including peptide doses that were high. Rabbit blood vessels have been shown to exhibit an immediate contractile response to des-Arg^9-BK if isolated from animals submitted to inflammatory treatments (immune complex-induced arthritis, Farmer *et al.*, 1991; or some surgical procedures as simple as a sham intervention; Davies and Hagen, 1994). As discussed above, the early response of isolated tissues to a B_1R agonist is suggestive for an in vivo B_1R formation in this animal (as in fig. 3D). Thus, an increasing body of evidence suggests that B_1Rs are up-regulated in various models of inflammation. However, this is generally an assumption that has not been substantiated using molecular approaches in most models.

Acute phase proteins are plasma components, usually of hepatic origin, whose concentration is modified by intense or systemic inflammation or sepsis. In rodents, a major acute phase reactant is T-kininogen, a kininogen homologue containing the Ile-Ser-BK sequence and corresponding to a gene lacking in humans. Carrageenin-induced acute inflammation in the rat paw is a rapidly evolving paw edema partially prevented by administration of Hoe 140, not of a B_1R antagonist (Raymond *et al.*, 1996). However, this form of local inflammation is followed by the production of T-kininogen (systemic in-

flammation) which is further increased by treatment with either Hoe 140 or Lys-[Leu^8]des-Arg^9-BK, suggesting a negative feedback between kinin receptor stimulation and kininogen production (Raymond *et al.*, 1996). Precisely the same systemic inflammatory reaction has been observed in the course of peptidoglycan-induced arthritis in the rat (Blais *et al.*, 1997a). In the latter model, the local content of immunoreactive BK and des-Arg^9-BK increased 4- to 5-fold in the inflamed paw, but it is not clear whether peripherally formed kinins can reach an hepatic site of action via the circulation. An alternate, but not proven possibility, is the existence of an hepatic kallikrein-kinin system that would include both B_1 and B_2Rs and would be perhaps activated from a distant site by cytokines. More experiments need to be done to explain this type of observation.

Leukocytes are inflammatory and immunological effectors of prime importance, and their functions are diversified. There is no extensive published evidence of important effects of kinins on these cells, at least in humans. However, some incomplete or preliminary in vitro observations are intriguing, as they suggest that B_1Rs may be expressed by phagocytic cells and lymphocytes. Carl *et al.* (1996) observed that freshly isolated human neutrophils respond to BK or Lys-des-Arg^9-BK by an elastase secretion mediated by B_2 and B_1Rs, respectively; thus, the Lys-des-Arg^9-BK-induced protease secretion was capable of increasing the permeability of an endothelial layer maintained in vitro only in the presence of neutrophils. The second system includes circulating lymphocytes, which seem to exhibit in vitro a chemokinetic response to kinins via the stimulation of B_1Rs (McFadden and Vickers, 1989). These observations are of great potential interest if several experimental parameters familiar to leukocyte biologists can be controlled (e.g., purity of cell preparations, postisolation activation, subtyping of lymphocytes) and if rigorous pharmacological and genetic experiments confirm the kinin receptor expression in these cells. B_1R can contribute in vivo to leukocyte recruitment, but this appears to be through an indirect mechanism (Ahluwalia and Perretti, 1996). Polymorphonuclear leukocytes converge in 6 day-old air pouches under the skin of mice when further locally treated with murine IL-1β (4 h). The leukocyte accumulation is partially prevented by [Leu^8]des-Arg^9-BK, but not by Hoe 140. Conversely, des-Arg^9-BK increased the migration into IL-1-treated pouches. The effect of the kinin on leukocyte accumulation was also inhibited by antagonists of the neuropeptides substance *P* and CGRP related peptide (Ahluwalia and Perretti, 1996), indicating that des-Arg^9-BK-stimulated sensory nerve afferents probably released locally neuropeptides that, in turn, determined the chemotactic response. There is also strong pharmacological evidence that the inflammatory effect of des-Arg^9-BK injected in the pleural space of mice is mediated by neurokinins and calcitonin-gene related peptide (Vianna and Calixto, 1998). Thus, B_1Rs may play a role in neurogenic inflammation. Stimulation of nervous afferents by kinins is examined in the next section.

C. Pain, Hyperalgesia, and Fever

BK is one of the few mediators of inflammation that directly stimulates afferent nerves. This is caused by the presence of B_2R on neural elements, notably in the sensory ganglions and dorsal layers of the spinal cord (Dray *et al.*, 1988). The autonomic nervous system is also affected by BK. At least in the rat, BK produces part of its inflammatory effect and recruits the hypothalamic-pituitary-adrenal axis to release corticosteroids via the stimulation of sympathetic postganglion (Green *et al.*, 1997). The expression of B_2R in the neurones is evidenced by several approaches, including systems based on cultured cells (Naruse *et al.*, 1992), in which BK-induced depolarization is a distant effect from calcium signaling. Although exogenous kinins produce pain perception stimulating B_2Rs in the blister base of human skin (Whalley *et al.*, 1987) or in rat tissues (Steranka *et al.*, 1988), the analgesic potential of B_2R antagonists has proved to be limited to very acute inflammatory situations (see below). Several studies have now shown that B_1R antagonists surpass B_2R antagonists in preventing or reversing inflammatory hyperalgesia in various models based on the rat and the mouse. Perkins *et al.* (1993) first described the antinociceptive effect of [Leu^8]des-Arg^9-BK in rats submitted to Freund's adjuvant-induced hyperalgesia in the rat knee or ultraviolet ray-induced hyperalgesia in the rat paw. This peptide (1 to 10 nmol/kg intravenously) proved to be effective for about 2 to 3 h. The time course of these experimental pathologies revealed that the B_2R antagonist Hoe 140 was effective at the beginning, but that the B_1R ligand progressively became the best analgesic in a few days after the lesion initiation. These kinetics are consistent with the B_1R up-regulation by tissue injury; in addition, exogenous des-Arg^9-BK exacerbated the pain. Later, it was found that IL-1, IL-2, IL-8, nerve growth factor, the neuropeptide substance P, the afferent nerve stimulant capsaicin and repeated injections of BK can all produce, after some hours-days of latency, inflammatory hyperalgesia that was favorably influenced by [Leu^8]des-Arg^9-BK in models involving the measurement of mechanical or thermal hyperalgesia (Davis and Perkins, 1994a,b, 1996; Khasar *et al.*, 1995; Perkins and Kelly, 1993; Rueff *et al.*, 1996; Sufka and Roach, 1996; Tonussi and Ferreira, 1997). Mice exhibited a [Leu^8]des-Arg^9-BK-induced reduction of pain induced by formalin injection (licking behavior) only in LPS-pretreated animals (Campos *et al.*, 1995). Exogenous des-Arg^9-BK also exacerbated formalin pain in LPS-treated mice. Related to the idea of latency in the induction of B_1Rs, a late phase of formalin-induced pain in the mouse may be partially dependent on B_1Rs. B_2R knockout mice exhibit this phase, which responds to [Leu^8]des-Arg^9-BK, and also the slow developing hyper-

algesia to Freund's adjuvant (Rupniak *et al.*, 1997). A sophisticated behavioral approach (place preference paradigm) shows that [Leu^8]des-Arg^9-BK is preferred against Hoe 140 as an analgesic by rats subjected to adjuvant-induced inflammation, and that the former peptide, unlike opioids, has no potential for abuse (no positive reinforcement) (Sufka and Roach, 1996). In models based on rats, exogenous des-Arg^9-BK may further increase the hyperalgesia if it is not already at maximal level under the effect of the inflammatory stimulus. However, it became recently apparent that very low doses of the agonist may be analgesic through the release of endogenous opioid peptides (Davis and Perkins, 1997).

As mentioned above (Section III.B.), administration of the exogenous IL-1 receptor antagonist prevented not only the hyperalgesia caused by IL-1, but also that caused by IL-2, IL-8, capsaicin or substance *P* (Davis and Perkins, 1994b, 1996), suggesting that some of these factors are dependent on the local IL-1 production before triggering the hypothetical B_1R up-regulation. These observations do not necessarily mean that nerve terminals acquire B_1Rs in tissue injury context; the soma of these neurons can be located distantly from the localized inflammatory site. Pharmacological evidence suggests that B_1Rs may rather control PG production locally (Davis and Perkins, 1994b), and some PGs are well-known hyperalgesic substances, as recently shown by the high threshold of pain perception in knockout mice for the PGI_2 receptor (Murata *et al.*, 1997). The analgesic profile of B_1R antagonists resembles that of nonsteroidal antiinflammatory drugs (Rupniak *et al.*, 1997), which are agents known to inhibit the fatty acid cyclooxygenase necessary for PG production. Thus, a chain of mediators (a cytokine, a kinin, eicosanoids) may be involved in these reactions. Repeated attempts have generally failed to show that neural elements (e.g., dorsal root ganglion neurones) can acquire functional responses to B_1R agonists (Davis *et al.*, 1996). According to this interpretation, peripheral B_1Rs located on nonneural cells (e.g., fibroblasts, endothelial cells, etc.) may produce PGs as secondarily mediators, which, in turn, would sensitize the nerve endings in the periphery. However, negative results are limited by the experimental conditions employed. Seabrook *et al.* (1997) showed that a mouse sympathetic ganglion treated in vitro with IL-1β could depolarize in response to Lys-des-Arg^9-BK. In addition, mRNA transcripts coding for B_1R were detected in mouse sensory dorsal root ganglia, and somewhat overexpressed in tissues from $B_2R^{-/-}$ animals, though without detectable function. Cultured rat dorsal root ganglia could acquire binding sites corresponding to B_1Rs in addition to the preexisting B_2Rs, but this process was very slow, taking 2 days (Von Banchet *et al.*, 1996). In this study, the experimental approach was rather unusual (inhibition of gold-labeled BK binding by a panel of peptides), but the cell type identity was unambiguous. Thus, neural expression of B_1Rs now appears to be possible.

Fever is another reaction to sepsis and inflammation in which a cytokine messenger is believed to affect a neural center with a PG intermediate. Could the kinins assume an intermediate position in this mediation chain, as in the inflammatory hyperalgesia? The answer seems to be positive: intravenously administered LPS causes a fever reaction in the rat which is initially inhibited by intracerebroventricular (i.c.v.) injection of Hoe 140, but, after 2.5 h, [Leu^8]des-Arg^9-BK is more effective (Coelho *et al.*, 1997). LPS pretreatment 24 h before testing is no longer pyrogenic, but abrogates the pyrogenic effect of i.c.v. BK, and confers a pyrogenic effect to des-Arg^9-BK administered by the same route (Coelho *et al.*, 1997). It should be noted that previous studies involving i.c.v. injection of LPS in the rat failed to demonstrate B_1R induction (Walker *et al.*, 1996). Nevertheless, the intermediate role of kinins was supported by the antipyretic effect of Hoe 140 in this study. The origin of the discrepancy is not clear, but may be related to different time frames, drug dosages and route of administration for LPS.

D. Nephrology and Urology

The importance of tissue kallikrein of renal origin is well documented and generally seen as nephroprotective, natriuretic, and diuretic (Majima and Katori, 1995; Margolius *et al.*, 1995), a suggestion confirmed by the natriuretic deficit of $B_2R^{-/-}$ mice and the altered frequency of a B_1R gene polymorphism in patients with a history of end stage renal failure (see above, Section III.E.).

The use of selective B_1 and B_2R antagonists in volume-loaded rats suggests, that the natriuretic and vasodilator effect of endogenous kinins is mediated by B_2R, whereas B_1R blockade reduces the glomerular filtration rate (Fenoy and Roman, 1992). In normal dogs, natriuresis and renal vasodilator responses were observed after injection with the B_1R agonist des-Arg^9-BK (Lortie *et al.*, 1992). In this case, the B_2Rs coexist and mediate effects that are partially overlapping (vasodilation). The dog may be an exceptional species, as renal B_1Rs are preformed (see above). The status of kinin receptors in the human kidney is now beginning to be explored. In situ hybridization with a riboprobe specific for B_1R indicates that the B_1R is distributed in epithelial cells of the parietal layer of Bowman's capsule and the thin segment of Henle's loop; thus, the B_1R seems to be colocalized in the nephron with the B_2R, kallikrein, kallistatin, and LMW-kininogen (Wang *et al.*, 1996). Tissue sections were derived from kidneys of adults with unknown clinical history, making impossible any statement about B_1R regulation. However, the B_1R mRNA was shown to be grossly overexpressed in cells forming a malignancy of the human kidney (Wang *et al.*, 1996). Thus the B_1R has the potential to be present both in the

kidney vasculature and in renal parenchymal cells, and probably to be regulated by inflammation and cytokines.

The relevance of B_1R up-regulation is more documented in the urinary tract. The urinary bladder isolated from the rat or the rabbit acquires a contractile response to B_1R agonists in a time- and protein-synthesis dependent process (Butt *et al.*, 1995; Marceau *et al.*, 1980; Roslan *et al.*, 1995). More importantly, chemical inflammation of the bladder promotes the B_1R up-regulation in vivo in rats (Marceau *et al.*, 1980; Roslan *et al.*, 1995). Although the mechanisms of this induction are still unclear, it seems that inflammatory mediators as well as protein synthesis are involved. It is tempting to speculate that the hypercontractile state of the infected or inflamed bladder is dependent on this up-regulation. In the mucosa-free rabbit urinary bladder it has been shown that des-Arg^9-BK can evoke contractions largely via activation of B_1Rs, which have similar properties, including time-dependent induction, to B_1Rs in the rabbit isolated blood vessels (Butt *et al.*, 1995).

E. Others

1. Fibrosis. Fibrosis is a postinflammatory pathological process affecting the lung and many other tissues. There are no drug known to be clearly effective in controlling fibrosis in humans (Goldstein and Fine, 1995). Kinin B_1Rs are perhaps related to fibrosis and B_1R antagonists may be worth trying to prevent it. The regulation of B_1R expression by cytokines and chronic inflammation would make a role for these receptors plausible if the relevant cell types were permissive. Immunohistochemistry of transbronchial biopsies in several patients and in control subjects reveals that the human B_1R is densely expressed in the fibrotic zones of lungs of patients with sarcoidosis or progressive systemic sclerosis, but undertectable in control lungs (Nadar *et al.*, 1996). The human embryonic fibroblasts IMR-90 exhibit mitotic and collagen synthesis responses to des-Arg^9-BK (Goldstein and Wall, 1984). The original observations of Appleton *et al.* (1994) suggest that the B_1R agonist des-Arg^9-BK is the strongest agonist to contract myofibroblasts in granulation tissue from the rat (relevance for retraction of scar tissue).

2. Gastroenterology. The intestinal SMCs at all levels contain B_2Rs that generally mediate contraction (Regoli and Barabé, 1980). However, the genetic program leading to the expression of B_1Rs after tissue injury is active in these tissues, as in isolated strips of human colon (Couture *et al.*, 1981) or ileum (Zuzack *et al.*, 1996). Thus, a responsiveness to des-Arg^9-BK or Lys-des-Arg^9-BK is acquired in vitro in a time- and protein synthesis-dependent process.

Manning *et al.* (1982) have shown that BK and Lys-BK (not des-Arg^9-BK) bind to B_2Rs present on the enterocyte and, via secondarily released PGs, increase the secretion of chloride and water thus linking kinins to diarrhea, a typical consequence of enteritis. The PG release by BK has been confirmed in a line of human colonic adenocarcinoma (Cuthbert *et al.*, 1985) and freshly isolated colonic mucosae preparations from rats or rabbits also show a functional, PG-dependent electrical response in the Ussing chamber consistent with the presence of basolateral B_2Rs (Phillips and Hoult, 1988). As for the smooth muscle preparations, incubation of the isolated mucosal preparations for several hours reveals an up-regulation of functional responses to the B_1R agonist des-Arg^9-BK (Cl^- secretion) in the mouse colonic epithelium, even in B_2R knockout animals (Cuthbert *et al.*, 1996). The process of injury-induced B_1R is further confirmed by a model of chronic inflammation initiated by an acetic acid enema in rat. Eight days after treatment, the colonic mucosa isolated from these animals exhibits an altered conductivity response to kinins, the response to BK (B_2R agonist) being depressed, and that to des-Arg^9-BK (B_1R agonist), being sharply up-regulated (Kachur *et al.*, 1986). Although these responses are consistent with differential kinin receptor population regulation, those to the unrelated peptide substance *P* were unaffected by inflammation. Recently the kinin receptor status in normal and inflamed human pyloric gastric mucosa has been estimated by immunohistochemical studies (Bhoola *et al.*, 1997). Control antrum tissue showed strong immunoreactivity for B_2Rs with positivity noted along the luminal border, at the base of the mucous and stem cells, and there was no B_1R immunolocalization. However, biopsies from patients with gastritis showed a decrease in immunolabeling of the B_2Rs and an induction of the B_1Rs especially in regenerating epithelial cells. This initial study of the identification of kinin receptors on gastric mucosal cells indicates a possible role for kinin B_1Rs in gastritis.

V. Conclusions

Although many receptor populations are regulated, the B_1R is a peculiar example of a G protein-coupled receptor that is inducible. The regulation process was partly characterized before the molecular definition of this receptor, because large changes in the pharmacological responses allowed indirect monitoring of the receptor induction. More recent molecular approaches have essentially confirmed that the B_1R gene is transcriptionally induced in tissue injury situations. This system remarkably integrates different humoral components of the inflammatory reactions, such as the cytokines, the blood proteolytic cascades and the secondary activation of eicosanoid synthesis. Use of kinin antagonists for both receptors in recent years supported such a sequential involvement of these mediators in phenomena such as inflammatory hyperalgesia, fever and, perhaps, hepatic regulation of acute phase reactants. This could be ultimately explained by the local coexpression of key components of the kallikrein-kinin systems, including kininogen, tissue kallikrein and both receptor

types, by various cell types (vascular, renal cells, etc.) in responding organs.

Twenty years after the description of the B_1R pharmacological profile, it is now clear that some tissue effects of kinins cannot be antagonized with B_2R ligands such as Hoe 140 and FR 173657, especially in diseased individuals. The cells types, second-messengers and pharmacological effects under the control of B_1 or B_2Rs are largely identical, with some possible exceptions. The specific role of B_1R induction may be to amplify the tissue effects of kinins as a function of time via the synthesis of novel receptor molecules that are resistant to tachyphylaxis and stimulated by the abundant des-Arg^9-metabolites of kinins. The therapeutic consequences of this are just beginning to be appreciated, with the recent demonstration of the better analgesic effect of the B_1R antagonists in chronic inflammatory pain, relative to the B_2R antagonists.

We have mentioned above several avenues for additional research on the B_1R. Notably, knowledge of the structure-function relationship of the receptor protein is relatively undeveloped. Most pathological models have not used molecular approaches suitable to describe the B_1Rs in terms of cell localization and gene regulation. The genetic definition of B_1 and B_2Rs would be helpful in additional animal species where the pharmacological profile (guinea pig) or the regulation (dog, cat) might be different, compared with more familiar species. Progress in the analytical biochemistry of kinins in health and disease, with special reference to Lys-des-Arg^9-BK, is also needed. The physiopathological roles of B_1 and B_2Rs should be more widely investigated, notably in relation with leukocytes and ischemia. Finally, nonpeptide B_1R antagonists of great selectivity and clinical pharmacology studies on the kinin B_1R are needed to evaluate the clinical relevance of pharmacological interventions on the B_1R.

VI. Addendum

The B_1R receptor promotor was recently analyzed by transient DNA transfections of constructions in SV-40 transformed IMR-90 cells (Yang *et al*, 1998). This study confirms that the promotor functions in a cell type specific manner, with weak stimulatory effect of LPS, TNF-α or phorbol ester on transcription, but with a significant effect of BK. Furthermore, a negative regulatory region (−682 to −604) and an enhancer (−548 to −448) were defined in a relatively distal portion of the promotor. The enhancer may bind AP-1 and other unidentified factors.

Chemical inflammation of the rat urinary bladder is associated with a decrease of micturition threshold when the bladder is progressively inflated; a viscero-visceral hyper-reflexia is the cause of this phenomenon. Kinin receptor antagonists were shown recently to inhibit this hyper-reflexia: Hoe 140 prevents or reverses it, whereas [Leu^8]des-Arg^9-BK only reverses the hyper-reflexia when administered at least 5 h after the induction of local inflammation (Jaggar *et al*, 1998). This suggests that endogenous kinins are mediators of the afferent part of the reflex, with a temporal shift of mediation from B_2 to B_1Rs.

Patients with inflammatory bowel disease were found to exhibit allele frequency alterations similar to but more profound than those observed in patients with renal failure (promotor B_1R polymorphism; Bachvarov *et al*, 1998a).

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