# Autoradiographic and immunological techniques to analyse kinin receptor distribution in rat and human tissues

CARLOS D FIGUEROA*,[1] and WERNER MÜLLER-ESTERL [2]

[1] Instituto de Histología y Patología, Universidad Austral,Valdivia, Chile
[2] Institute for Physiological Chemistry and Pathobiochemistry, University of Mainz, Germany

*Kinins are vasoactive peptides that have traditionally been associated with vascular tone regulation. Nevertheless, the availability of adequate probes directed to kinin receptors has resulted in the identification of these proteins in organs and cells outside the cardiovascular system. The sensitivity, specificity and simplicity of the techniques developed to visualize kinin receptors have allowed the identification of the specific cells that express these receptors under physiological conditions and the investigation of the possible modifications that they may undergo during the onset and development of experimentally induced or diseased-related pathological conditions.*

***Key terms:*** *autoradiography, bradykinin B2 receptor, chemical cross-linking, HOE140 kinin antagonist, kallikrein, kinin receptors, kinins.*

## INTRODUCTION

Kinin receptors have so far been classified in two different subtypes, B1 and B2 (Regoli & Barabe, 1980; Bhoola *et al*, 1992). Several agonists such as bradykinin and kallidin (lysyl-bradykinin), generated through the enzymic action of kallikreins on kininogens, activate the B2 receptor which appears to mediate the majority of the physiological effects induced by these peptides. Unlike the constitutively expressed B2 receptor, the B1 receptor mRNA and the corresponding protein are rapidly up-regulated during the inflammatory response of the rabbit to lipopolysaccharide and is activated by des[Arg$^{10}$]-kallidin and des[Arg$^{9}$]-bradykinin. The B1 receptor seems to be normally absent, but its expression becomes evident under inflammation probably stimulated by one of its endogenous triggers, interleukin-1β (de Blois *et al*, 1991). In a similar manner, B1 receptors are induced under stressful conditions like those produced when tissues, such as the rabbit aorta, are maintained for prolonged periods of time in an organ bath incubator (Regoli & Barabe, 1980; Regoli *et al*, 1981).

Although the B1 type of kinin receptors was first characterized by pharmacological means, it was the B2 receptor to be cloned firstly (McEachern *et al*, 1991) and to be most extensively studied. On the other hand, information concerning the kinin B1 receptor was for many years almost entirely obtained from pharmacological experiments. Typically the experiments analyzed the contractile properties of various tissues to des[Arg$^{9}$]-bradykinin after their incubation for 3 to 5 h in oxygenated organ baths and after injecting laboratory animals with

* **Correspondence to:** Dr Carlos D Figueroa, Instituto de Histología y Patología, Universidad Austral, Casilla 567, Valdivia, Chile. Phone: (56-63) 221-241. Fax: (56-63) 221-604. E-mail: cfiguero@valdivia.uca.uach.cl

noxious stimuli such as lipopolysaccharide or interleukin-1β.

Based on their vasodilator properties, kinins have been proposed as regulators of local organ blood flow, vascular permeability and systemic vascular resistance (for recent reviews see Bhoola *et al*, 1992; Müller-Esterl, 1993; Margolius, 1995). The hypotensive effects produced by kinins on the vascular bed are mediated by the release of prostaglandin $PGI_2$ and nitric oxide from endothelial cells since ablation of the endothelium from large arteries blunts the kinin-relaxing response (Whorton *et al*, 1982; Cherry *et al*, 1982; Busse & Lamontagne 1991; for recent review see Linz *et al*, 1995). In addition to their vascular effects, kinins provoke pain, contract smooth muscle cells of the respiratory tree and function as mediators of water and electrolyte excretion (Croxatto *et al*, 1977; Bhoola *et al*, 1992; Margolius, 1995).

A major thrust of the ongoing research in the kinin field has addressed the precise sites of kinin action. Classical methods analysed the binding of radiolabeled bradykinin to cell membranes isolated from various mammalian tissues (Innis *et al*, 1981). More recently, specific immunological probes for the kinin B2 receptor have been developed (Müller-Esterl, 1997) which allow an extensive biochemical characterization of the receptor and the study of its cellular distribution. In this article we describe the relevant techniques presently available to visualize kinin receptors on tissue sections, and summarize the results obtained by us and other groups to map the B2 receptor in rat and human tissues.

## TECHNIQUES THAT ALLOW THE IDENTIFICATION OF KININ RECEPTORS

### *Autoradiography of kinin receptors*

Autoradiography has widely been used to identify and characterize peptide and non-peptide receptors in different species. The *in vitro* binding of a radiolabeled probe to tissue sections attached to glass slides is a powerful alternative to identify kinin receptors. The advantages of this approach are several: *i)* identically prepared sections can be used to compare receptor visualization for various ligands and to evaluate nonspecific binding; *ii)* localization of receptors is feasible for peptides (kinins) which are rapidly metabolized *in vivo* or fail to cross blood-tissue barriers; *iii)* binding conditions can be optimized by manipulating ligand concentrations, ionic strength, temperature and pH; *iv)* sections are easily washed to suppress nonspecific binding.

The best probe for kinin radiolabeling is the commercially available analogue $Tyr^0$-bradykinin (Sigma Chemicals, USA) that can be radiolabeled with [$^{125}$I]-iodine by the chloramine-T method or the Iodogen technique (Estrada *et al*, 1991). The radiolabeled peptide is easily separated from the unlabeled fraction by high performance liquid chromatography.

For autoradiography, tissue samples are rapidly frozen on aluminum foil floated onto liquid nitrogen, and then secured to cryostat pedestals with cryomatrix (Shandon, USA). Sections of 15 μm thickness are placed onto slides precoated with polylysine (Sigma Chemicals, USA) or gelatine, and stored in plastic boxes over a desiccant at -70°C until use. Before incubation, the sections are warmed to room temperature, freeze dried and washed with 0.05 M Tris-HCl buffer, pH 7.6 containing 0.1% bovine serum albumin, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol bis-(β-aminoethyl ether) tetraacetic acid (EGTA), and 20 μM captopril (Sigma). Incubation with 1-3 nM [$^{125}$I]$Tyr^0$-bradykinin dissolved in the same buffer supplemented with 1 mM dithiothreitol, 140 μg/ml bacitracin (Sigma Chemicals, USA) and DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA), is performed overnight at 4°C. After incubation, the sections are washed with cold buffer (3 x 1 min) and distilled water (1 x 1 min), cold dried rapidly with a fan and exposed to Kodak Biomax photographic film for 72 h at -20°C. Specificity of the binding is assessed by incubating consecutive sections with the radioligand in the presence of 10,000-fold excess of unlabeled B2 agonists such as $Tyr^0$-bradykinin, bradykinin or kallidin, the B2 antagonist

HOE140 (Icatibant$^R$, Hoechst-Marion-Roussel, Germany) or a B1 agonist such as des[Arg$^{10}$]-kallidin.

### *Immunohistochemistry*

*In vitro* autoradiography provides an excellent low power view of receptor sites in tissues, however the cellular and subcellular locations of receptors cannot be fully discriminated. In contrast, the quality and resolution provided by immunohistochemical techniques has produced important information about kinin receptor localization. The availability of the cDNA sequences for kinin receptors has made possible the synthesis of peptide segments to generate antibodies that crossreact with the receptor protein.

Peptide segments selected from the corresponding cDNA sequences of the rat and human B2 receptor (McEachern *et al*, 1991; Hess *et al*, 1992) following the criteria of hydrophilicity, antigenicity and relative location in the receptor protein have been used to raised polyclonal antibodies that recognize the B2 receptor protein in various rat and human tissues (for a review, see Müller-Esterl, 1997). An indirect ELISA technique with the unmodified peptides as the capture antigens (Kaufmann *et al*, 1993) was employed to follow the titer of the anti-peptide sera and to establish their specificity. Further, these antibodies were fully characterized by Western blotting (Abd-Alla *et al*, 1996) and immunohistochemistry (Figueroa *et al*, 1995; Tables I and II).

Visualization of kinin B2 receptors on tissue sections can be achieved by using frozen material or tissue fixed with periodate-lysine-paraformaldehyde (Figueroa *et al*, 1995; El-Dahr *et al*, 1997). Tissue is obtained under ether anesthesia and fixed in periodate-lysine-paraformaldehyde buffered at pH 7.4 for 24 h at room temperature (McLean & Nakane, 1974). The fixed samples are dehydrated in ethanol and embedded. Sections prepared from frozen material are fixed with acetone or directly used without fixation.

Immunostaining is performed by the peroxidase/anti-peroxidase (PAP) method or by immunofluorescence. Briefly, embedded sections are dewaxed, rehydrated, washed with 0.05 M Tris-0.15 M NaCl, pH 7.8 and treated with absolute methanol and 10% (v/v) $H_2O_2$ for 15 min each to block remnant pseudoperoxidase activity. Acetone fixed (20 min) or nonfixed frozen sections are washed three times with 0.1 M phosphate, 0.15 M NaCl, pH 7.4 (phosphate buffered saline, PBS) and then directly incubated with antibodies diluted in the same buffer supplemented with 1% immunoglobulin-free bovine serum albumin as described below. Antipeptideantibodies (Table I) may be used individually or as a pool combination of the various antisera. Dilution of antisera in buffer containing 1% immunoglobulin-free bovine serum albumin usually gives an acceptable ratio between specific staining and background. Incubation with the B2 receptor antisera may be performed overnight at 22°C in a water bath that is used as a moist chamber or at 4°C. The secondary antibody at a dilution of 1:80 and the PAP complex at 1:100 are applied for 30 min each. The peroxidase activity is visualized by incubating the sections in 0.1% (w/v) 3,3'-diaminobenzidine, 0.03% (v/v) of $H_2O_2$ for 15 min in the dark. For immunofluorescence samples are incubated with the first antibody overnight, washed with PBS, followed by a fluoresceinlabeled immunoglobulin.

Controls for the immunostaining procedure are prepared by omission of the first antibody or by its replacement with non-immune rabbit serum at the same dilution. Alternatively the sections are incubated with the pool mixture of anti-peptide antisera in the presence of an excess of the authentic peptides (100 to 200 μg/ml) used for immunization. The concentration of peptides necessary to give an antigen excess is determined empirically using the lowest concentration (highest dilution) of antiserum that will produce staining. After overnight competition the sections are washed and immunostained as described above.

### *In vivo labeling of B2 receptors by the peptide antagonist HOE140*

Ever since its first description (Hock *et al*, 1991; Wirth *et al*, 1991), the major B2

**Table I**

Anti-peptide antisera raised against intracellular and extracellular domains of the rat B2 receptor and antibodies to HOE140

| Peptide sequence[a] | Domain | Antibody |
|---|---|---|
| TIANNFDWLFGEVLC | ED2[b] | AS276[c] |
| DRYLAVKTMSMGRMC | ID2[b] | AS277 |
| CMGESVQMENSMGTLR | ID4 | AS278 |
| SAHNGTFSEVNC | ED1 | AS279 |
| LHKTNCTVAE | ID1 | AS280 |
| KRFRKKSREVYQAISRK | ID4 | AS281 |
| KDYSDEGHNVTACVISY | ED3 | AS282 |
| DTLLRLGVLSGC | ED4 | AS283 |
| MLNVTLQGPTLNGTFAQSKCPQVEWLGWLNTIQ | ED1 | AS251 |
| HOE140[d] | — | AS255 |
| | — | MHO1, MHO2[e] |

[a] Standard one-letter-code for amino acids used
[b] ED, putative extracellular domain; ID, putative intracellular domain
[c] AS, antiserum raised in rabbit
[d] HOE140: D-Arginyl-arginyl-prolyl-4-hydroxyprolyl-glycyl-§-2-thienylalanyl-seryl-D-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-[3aS,7aS]-octahydroindol-2-carboxyl-arginine (Hock *et al*, 1991)
[e] Monoclonal antibodies (Abd-Alla *et al*, 1993)

receptor antagonist HOE140 (D-Arg$^0$-[Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]-bradykinin) has been widely used to study the role of kinins under physiological and pathophysiological conditions. Likewise, the *in vivo* binding of HOE140 to B2 receptors, and its subsequent probing by an anti-HOE140 antibody, has been used to identify the cells that express bradykinin B2 receptors on its surface (Vio *et al*, 1996; El-Dahr *et al*, 1997). One of the major advantages of using HOE140 to trace B2 receptors is due to the fact that this molecule is a synthetic compound thus minimizing the possibility of cross-reactivity of the anti-antagonist antibodies with other cellular components.

*In vivo* labeling is carried out by injecting a single dose of 500 µg/Kg body weight of HOE140 dissolved in sterile 0.9% sodium chloride into the tail vein of rats. A control group is simultaneously injected with the vehicle alone. Fifteen minutes after the injection, the rats are sacrificed under ether anesthesia and the tissue fixed by immersion in periodate-lysine-paraformaldehyde as described above. Part of the samples can be embedded in Histosec (Merck, Germany) for conventional immunohistochemistry whereas the rest may be rapidly frozen using liquid nitrogen. To identify the cells labeled by the antagonist, sections from both frozen (15 µm thick) and embedded material (5 µm thick) of treated and control animals, respectively, are incubated with the monoclonal antibody MHO1 (culture supernatant, 1:100) or MHO2 (ascitic fluid, 1:5,000) or the polyclonal antibody (AS255, 1:250-1:500) raised against HOE140 (Table I; Figueroa *et al*, 1995; Vio *et al*, 1996; El-Dahr *et al*, 1997). Additional controls are carried out during the immunohistochemical procedure by omission or replacement of the anti-antagonist antibodies with non immune rabbit serum or isotype-matched mouse immunoglobulins.

### *Western blotting and chemical cross-linking*

The immunochemical characterization of the kinin B2 receptor has been carried out on partially purified plasma membranes prepared from human foreskin fibroblasts or cells transfected with the B2 receptor cDNA (Abd-Alla *et al*, 1993; 1996), on partially purified myometrial membranes (Herzig & Leeb-Lundberg, 1995) or on rat renal membranes isolated on sucrose gradients

(Figueroa *et al*, 1996a). For cell membrane preparation tissues from ether anesthetized rats are removed and immediately placed in an ice-cold petri dish containing 1 mM sodium bicarbonate, 0.2 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.1 μg/ml antipain (Sigma Chemicals, USA). Organs are cut into small pieces with a scissor and the tissue fragments are homogenized using a teflon pestle. The resultant suspension is centrifuged at 1,200 x g for 10 min. The supernatant is discarded, and the pellet is resuspended in freshly prepared 69% (w/w) sucrose. The final sucrose concentration after adding the pellet is 44% (w/w). The mixture (approx. 14 ml) is placed in a centrifuge tube and 7 ml of 42.3% (w/w) sucrose are added on top (Bockaert *et al*, 1973). The cell membranes isolated by flotation are washed with 1 mM bicarbonate and immediately used or resuspended in 50 mM Tris-HCl pH 7.4 containing protease inhibitors (as above) and 0.25 M sucrose to be aliquoted and stored at –70°C until used. Cell membranes from cultured cells are isolated by performing a high speed centrifugation step.

For subsequent Western blotting, the isolated membranes (100 μg of protein per lane) are resuspended in SDS sample buffer and run in a 12.5% (w/v) polyacrylamide gel. The proteins are electrotransferred onto nitrocellulose strips that are incubated overnight with an anti-peptide antibody directed to the extracellular domain-1 of the B2 receptor (Abd-Alla *et al*, 1996; Figueroa *et al*, 1996a, 1997; Müller-Esterl, 1997). The bound antibody can be detected by using a [$^{125}$I]-labeled secondary antibody, by the peroxidase/anti-peroxidase method or by chemiluminescence.

When chemical cross-linking experiments are performed, approximately 100 μg of protein is incubated for 1 h at 4°C with 10 nM [$^{125}$I] Tyr$^{0}$-bradykinin in 50 mM HEPES pH 7.0 in the presence of 5 mM EDTA, 20 μM captopril and 0.5 mM phenylmethanesulfonyl fluoride. A cross-linking agent such as ethylene glycol bis(succinimidyl succinate) is added from a 100 mM freshly prepared stock solution to a final concentration of 0.25 mM. After incubating the mixture for 10 min the cross-linking reaction is quenched by the addition of 1 M Tris pH 8.0. To assess non-specific binding the reaction is performed in the presence of 10-20 μM of a kinin antagonist or agonist.

## CELLULAR DISTRIBUTION OF KININ RECEPTORS

### *Autoradiographic detection of B2 receptor binding sites.*

Binding of [$^{3}$H]-bradykinin to crude membrane preparations of Guinea pig ileum, colon and duodenum and of estrous rat uterus was first reported by Innis *et al* (1981). Their observations were extended by numerous other studies demonstrating the existence of bradykinin binding sites in the brain and the spinal cord (Steranka *et al*, 1988; Fujiwara *et al*, 1989; Privitera *et al*, 1991; Lopes *et al*, 1993; Lopes *et al*, 1995), in the kidney (Tomita & Pisano, 1984; Manning & Snyder, 1989; Bascands *et al*, 1989), in the lung (Mak & Barnes, 1991), and in the uterus (Innis *et al*, 1981) (Table II). Further, using *in vitro* autoradiography and the radioiodinated B2 receptor antagonist 3-4-hydroxyphenylpropionyl-D-Arg$^{0}$-[Hyp$^{3}$, Thi$^{5}$, D-Tic$^{7}$, Oic$^{8}$]-bradykinin ([$^{125}$I]HPP-HOE140), Murone *et al* (1996) observed intense binding in the lamina propria of the villi of ileum, in the inner third of the myometrium and over epithelial cells of uterine glands, in the endothelium and muscularis of pulmonary arteries and in smooth muscle cells of bronchioles. Using the same radioligand, Murone *et al* (1997) demonstrated the distribution of B2 receptors in many brain regions which had not been previously reported. They described the highest density of B2 binding sites in the pleoglial periaqueductal gray, oculomotor and trochlear nuclei, and in the circumventricular organs. Moderated densities occurred in the substantia nigra, the posterior thalamic and subthalamic nuclei, zona incerta, the red and pontine nuclei and in discrete layers of the superior colliculus. In the hindbrain, moderate levels of B2 receptors were found in the nucleus of the

## Table II

Cellular distribution of bradykinin B2 receptors

| **Organ** (cell type, segment) | **Technique** | **References** |
|---|---|---|
| **Kidney** (collecting duct cell, connecting tubule cell, distal straight tubule cell, glomerulus, interstitial cells) | immunohistochemistry | Figueroa *et al*, 1995 |
| | mRNA expression | Ma *et al*, 1994 |
| | autoradiography | Manning & Snyder, 1989; Figueroa *et al*, 1995 |
| | receptor binding | Tomita & Pisano, 1984; Bascands *et al*, 1989; Fredrick *et al*, 1985 |
| | *in vivo* labeling | Vio *et al*, 1996; El-Dahr *et al*, 1997 |
| **Vas deferens** (smooth muscle cells, epithelial cells) | immunohistochemistry | Figueroa *et al*, 1996a |
| **Heart** (neonatal cardiocytes) | receptor binding | Minshall *et al*, 1995 |
| **Uterus** (smooth muscle cells, glands, surface epithelium) | immunohistochemistry | Figueroa *et al*, 1997 |
| | mRNA expression | McEachern *et al*, 1991 |
| | autoradiography | Murone *et al*, 1996 |
| **Blood vessels** (endothelial cells, smooth muscle cells) | mRNA expression | Ma *et al*, 1994 |
| | receptor binding | Whorton *et al*, 1982; Cherry *et al*, 1982 |
| **Urinary bladder** (epithelial cells) | immunohistochemistry | Figueroa *et al*, 1997 |
| **Brain** (hypothalamus, midbrain and brainstem) | autoradiography | Privitera *et al*, 1991; Murone *et al*, 1996; Murone *et al*, 1997 |
| | mRNA expression | Ma *et al*, 1994 |
| | immunohistochemistry | Qadri *et al*, 1998 (personal communication) |
| **Pituitary**, adrenal gland | mRNA expression | Ma *et al*, 1994 |
| | immunohistochemistry | Qadri *et al*, 1998 (personal communication) |
| **Salivary glands** (striated duct, granular tubule) | immunohistochemistry | Figueroa *et al*, 1996a |
| **Spinal cord** | autoradiography | Lopes *et al*, 1993, 1995; Murone *et al*, 1997 |
| **Lung** | autoradiography | Mak & Barnes, 1991 |
| **Skeletal muscle** | mRNA expression | Ma *et al*, 1994 |
| | immunohistochemistry | Figueroa *et al*, 1996b |
| **Testis, prostate, breast, intestine and stomach** | mRNA expression | McEachern *et al*, 1991; Ma *et al*, 1994 |
| **Skin** (keratinocytes) | autoradiography | Schremmer-Danninger *et al*, 1995 |

solitary tract and in the spinal trigeminal, inferior olivary, cuneate and vestibular nuclei. Laminae I, X and dorsal root ganglia displayed the most striking binding densities in the spinal cord, while the remainder of the dorsal and ventral horn displayed a low diffuse density of binding (Table II).

***B2 receptor mRNA.***

Initial studies on the expression of the rat B2 receptor gene performed by McEachern *et al* (1991) indicated that the B2 kinin receptor mRNA is present in several tissues and organs such as uterus (highest level), kidney, ileum, heart, lung, testis and brain. A similar distribution was observed in human tissues except that the kidney was the most abundant source of the B2 receptor (Hess *et al*, 1992). Other studies using human tissues and reverse transcription-polymerase chain reaction (RT-PCR; Ma *et al*, 1994) have revealed that the B2 receptor message is also present in pancreas, salivary gland, colon, prostate, adrenal glands, skeletal muscle, stomach, breast, ovary, bronchus, pituitary, hippocampus, cerebellum, hypothalamus, and in most of the large human arteries and veins (Table II).

***Immunoreactivity for B2 receptors and for receptor-bound HOE140.***

Antipeptide antibodies directed to sequences of the predicted intra- and extracellular domains of the B2 receptor (Abd-Alla *et al*, 1996) have proven to be ideal probes to precisely localize the B2 receptor in the kidney (Figueroa *et al*, 1995, 1996a; El-Dahr *et al*, 1997), uterus (Figueroa *et al*, 1997), skeletal muscle cells (Figueroa *et al*, 1996b), and in renal cell lines such as Madin-Darby canine kidney cells (Figueroa *et al*, 1997).

When used on tissue sections prepared from rat kidney, the staining patterns produced by the various antisera have revealed a receptor distribution essentially congruent with the autoradiographic visualization of the renal B2 receptor (Figueroa *et al*, 1995; Fig 1). With the aid of anti-peptide antibodies and the peroxidase/anti-peroxidase system we have been able to detect immunoreactivity for the renal B2 receptor in collecting ducts, connecting tubules, distal straight tubules, and straight portions of the proximal tubules of the rat kidney. Microlocalization of the bradykinin B2 receptor mRNA by RT-PCR on microdissected nephron segments has confirmed this pattern of distribution (Marin-Castaño *et al*, 1996). Subcellularly, collecting ducts and connecting tubules express the B2 receptor in the basal infoldings and the luminal membranes of the tubular cells (Figueroa *et al*, 1995). Notably kinins had been shown to stimulate $PGE_2$ release from cortical collecting ducts stimulated on the apical face (García-Pérez & Smith, 1984) and to inhibit vasopressin-stimulated water permeability and sodium resorption only when the peptide is applied to the basal side (Schuster *et al*, 1984; Tomita *et al*, 1985). Studies employing an enriched fraction of renal plasma membranes showed by immunoprinting with anti-peptide antibodies that the rat B2 receptor has a molecular mass of 69 ± 3 kDa (Figueroa *et al*, 1996a). An identical result is obtained using cross-linking of the renal B2 receptor to radiolabeled bradykinin (Fig 2). These results are in good agreement with other reports where B2 receptors of 69 kDa were found in human foreskin HF-15 fibroblasts (Abd-Alla *et al*, 1993) and Madin-Darby canine kidney cells (Figueroa *et al*, 1997; Fig 3) and of 65 kDa in bovine myometrial membranes (Herzig & Leeb-Lundberg, 1995).

A similar labeling of renal nephron segments has been found when single or multiple injections of HOE140 were applied to adult rats (Vio *et al*, 1996) or new-born rats (El-Dahr *et al*, 1997). The binding of HOE140 revealed by monoclonal/polyclonal antibodies to the antagonist, was strong and uniform in the cortex and less intense in the outer and inner medulla (Fig 4). The decoration of distal nephron segments by anti-HOE140 antibodies was undetectable when kidney samples were taken 2 h after administration of the antagonist (Vio *et al*, 1996). No immunoreactivity for HOE140 was observed in the kidneys of the saline-

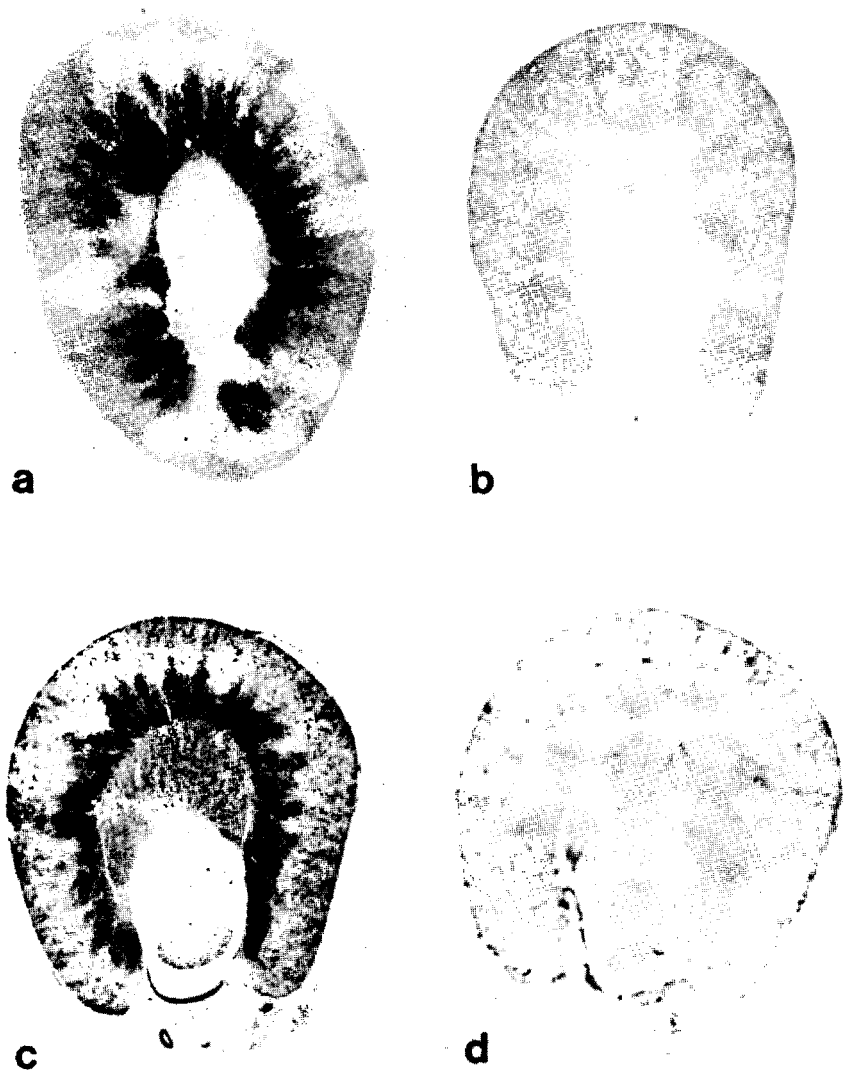


**Fig 1.** Visualization of the renal kinin B2 receptor by autoradiography (a, b) and by anti-peptide antibodies (c, d). **a.** Whole kidney sections were incubated with 1 nM [$^{125}$I]Tyr$^{0}$-bradykinin. **b.** 1 nM [125I]Tyr$^{0}$-bradykinin in the presence of 20 μM HOE140. **c.** a mixture of eight different anti-peptide antisera, AS276 through AS283, at a dilution of 1:1000 followed by the peroxidase/anti-peroxidase technique. **d.** Same mixture of anti-peptide antibodies in the presence of an excess (100 μg/ml) of the authentic peptides used for immunization. Magnification for all figures x 8.

injected rats or when the antibodies directed to HOE140 were replaced by non-immune serum or were preabsorbed with HOE140. Interestingly, for many cells the antagonist was also located intracellularly (Vio *et al*, 1996) suggesting the internalization of the compound, a finding similar to that reported for the angiotensin II antagonists saralasin and Dup753 (Conchon *et al*, 1994).

The HOE140-labeled nephron segments of the cortex included cortical collecting ducts, connecting tubules and proximal tubules whereas no labeling was found in the glomeruli. HOE140 immunoreactivity was associated with both the luminal and the basolateral cell membranes of distal nephron segments such as collecting ducts and connecting tubules, including those

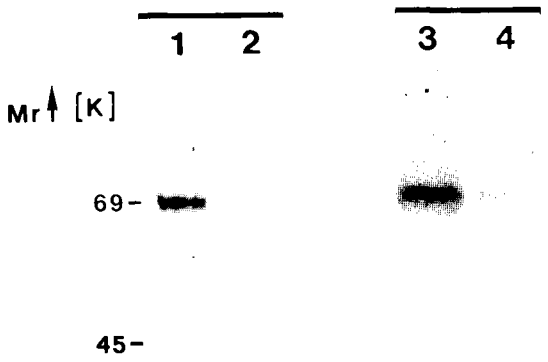


Fig 2. Immunochemical characterization of the renal kinin B2 receptor by Western blotting and by chemical cross-linking. Western blotting: Renal membrane proteins (100 μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoprinted with an anti-peptide antibody directed to extracellular domain-1 (lane 1) of the B2 receptor (AS251, 1:100) or with pre-immune serum at the same dilution (lane 2). Bound immunoglobulins were visualized by the peroxidase/anti-peroxidase method. Chemical cross-linking: Renal membrane proteins (100 μg) were incubated with the ligand 10 nM [$^{125}$I]Tyr$^{0}$-bradykinin alone (lane 3) or in the presence of a 1000-fold molar excess of HOE140 (lane 4), separated by polyacrylamide gel electrophoresis and autoradiographed on Kodak Biomax film.

tubules located immediately under the kidney capsule. In proximal tubules the immunoreactivity was mainly associated with the luminal side and particularly prominent in the reabsorption droplets. Staining was also observed in isolated collecting duct cells of the outer and inner medulla. In these cells, the immunoreactivity was often spread over the entire cytoplasm of collecting duct cells.

Outside the kidney, immunohistochemical techniques have identified the presence of B2 receptor-immunoreactivity in various mammalian tissues including vas deferens (Fig 5f), salivary gland, uterus, urinary bladder, blood vessels, skeletal muscle, adrenal gland, pituitary and skin (Fig 5b). In the rat brain anti-peptide antibodies have demonstrated presence of B2 receptors in the hypothalamus, midbrain and brainstem all sites consistent with the proposed roles of bradykinin to modulate body fluid homeostasis, pain and motor activity (Table II). The staining patterns produced by immunohistochemistry at these sites are very similar to those revealed by autoradiography (Fig 5).

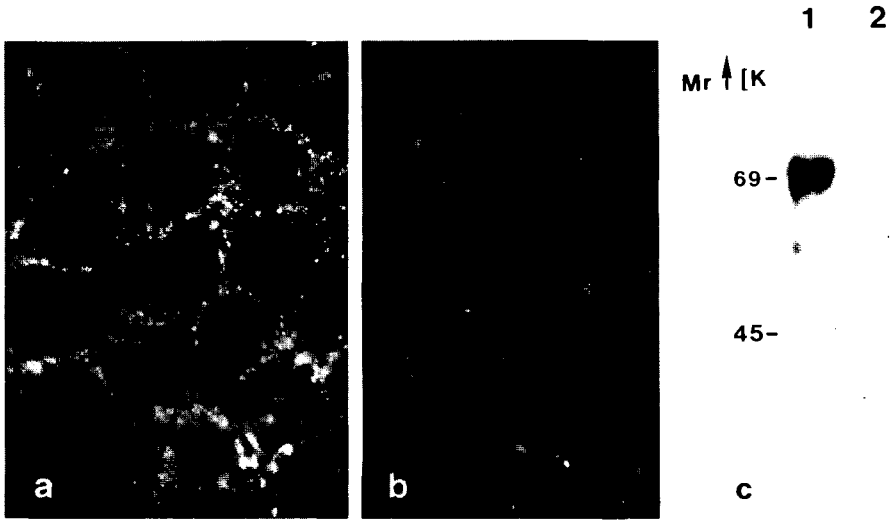


Fig 3. Identification of the kinin B2 receptor in Madin-Darby canine kidney (MDCK) cells by immunofluorescence and Western blotting. Immunofluorescence: MDCK cells were grown on coverslips, washed and fixed with paraformaldehyde-lysine-periodate mixture for 15 min at room temperature and then incubated with anti-peptide sera (a) or non immune serum (b) followed by a fluorescein labeled antibody. x 1500. Western blotting: Cell membranes prepared from MDCK cells (100 μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis, immunoprinted with the anti-peptide antibody AS251 (1:1000; lane 1) or with pre-immune serum at the same dilution (lane 2). Bound antibodies were detected by chemiluminescence.

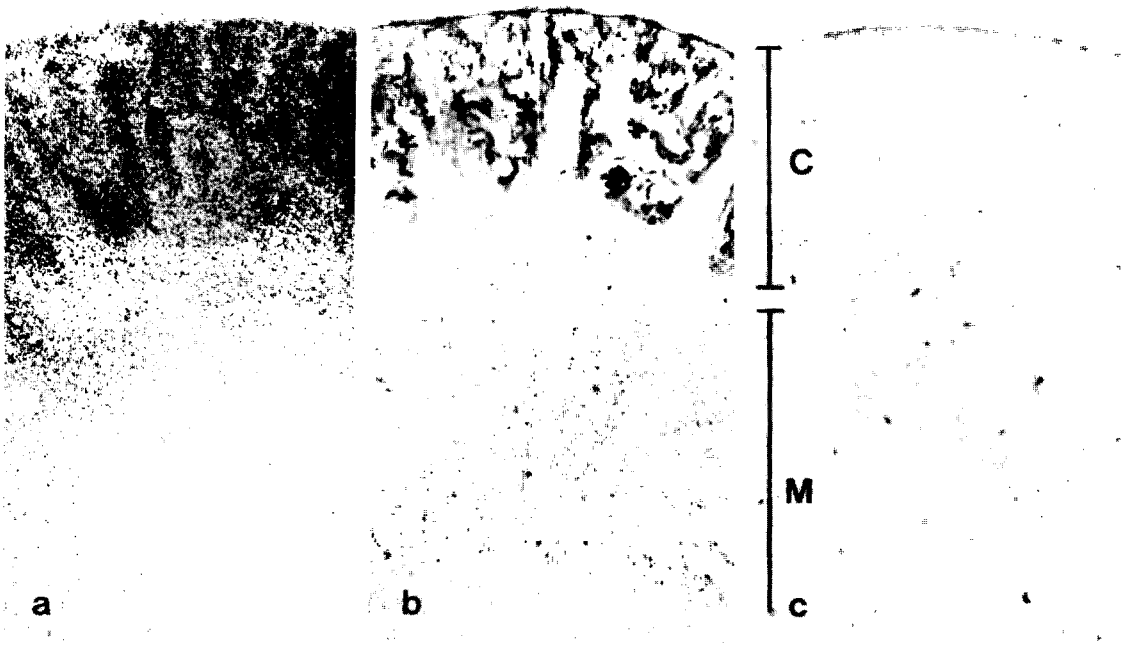


**Fig 4.** Low power localization of renal B2 receptor by autoradiography and *in vivo* labeling with HOE140 on day 8 of postnatal life. **a.** Autoradiography using [$^{125}$I]Tyr$^{0}$-bradykinin as ligand. **b.** Immunohistochemistry using anti-HOE140 antibodies to detect the *in vivo* bound B2 antagonist. c. Section prepared from a rat injected with 0.9% sterile saline and then immunostained with anti-HOE140 antibodies applied at the same dilution used in (b). All figures x 50. C= cortex; M= medulla.

## ONTOGENY OF THE RENAL B2 RECEPTOR

Several lines of evidence suggest that the kallikrein-kinin system may play an important role in kidney growth and development. In fact, the developing rat kidney expresses all the essential components of this system, *i.e.*, tissue kallikrein, kininogens, kininases and kinin receptors. During the newborn period the B2 receptor gene expression is enhanced (El-Dahr *et al*, 1996) and chronic pharmacological blockade of the B2 receptor compromises fetal metanephrogenesis in the developing but does not affect the adult kidney of salt-loaded rats (El-Dahr *et al*, 1996).

It is important to consider that after birth, the rat kidney is still under development and from day 1 to day 7, the formation of new nephron units is observed. The presence of the B2 receptor during kidney development has been assessed by analysis of its messenger RNA, its capacity to bind radiolabeled bradykinin, its recognition by anti-peptide antibodies and its capacity to bind *in vivo* the B2 antagonist HOE140. Autoradiography and immunohistochemistry revealed the existence of B2 receptors from day 1 though a gradual increase in the intensity of the labeling and a changing pattern of receptor distribution was observed with aging. Between 1 to 12 days after birth, the B2 receptor was mainly located in the renal cortex where it localized in collecting ducts and in distal nephron segments that in some cases could be clearly recognized as connecting tubules. At day 1 and 3, B2 receptor immunoreactivity was also associated to the luminal membrane of immature tubular structures of the developing cortex. The daily injection of HOE140 into newborn rats resulted in the *in vivo* labeling of renal structures that were similar to those visualized by autoradiography or by immunohistochemistry (Fig 4).

## CONCLUSIONS

Several lines of evidence converge at the conclusion that the kinin B2 receptor is

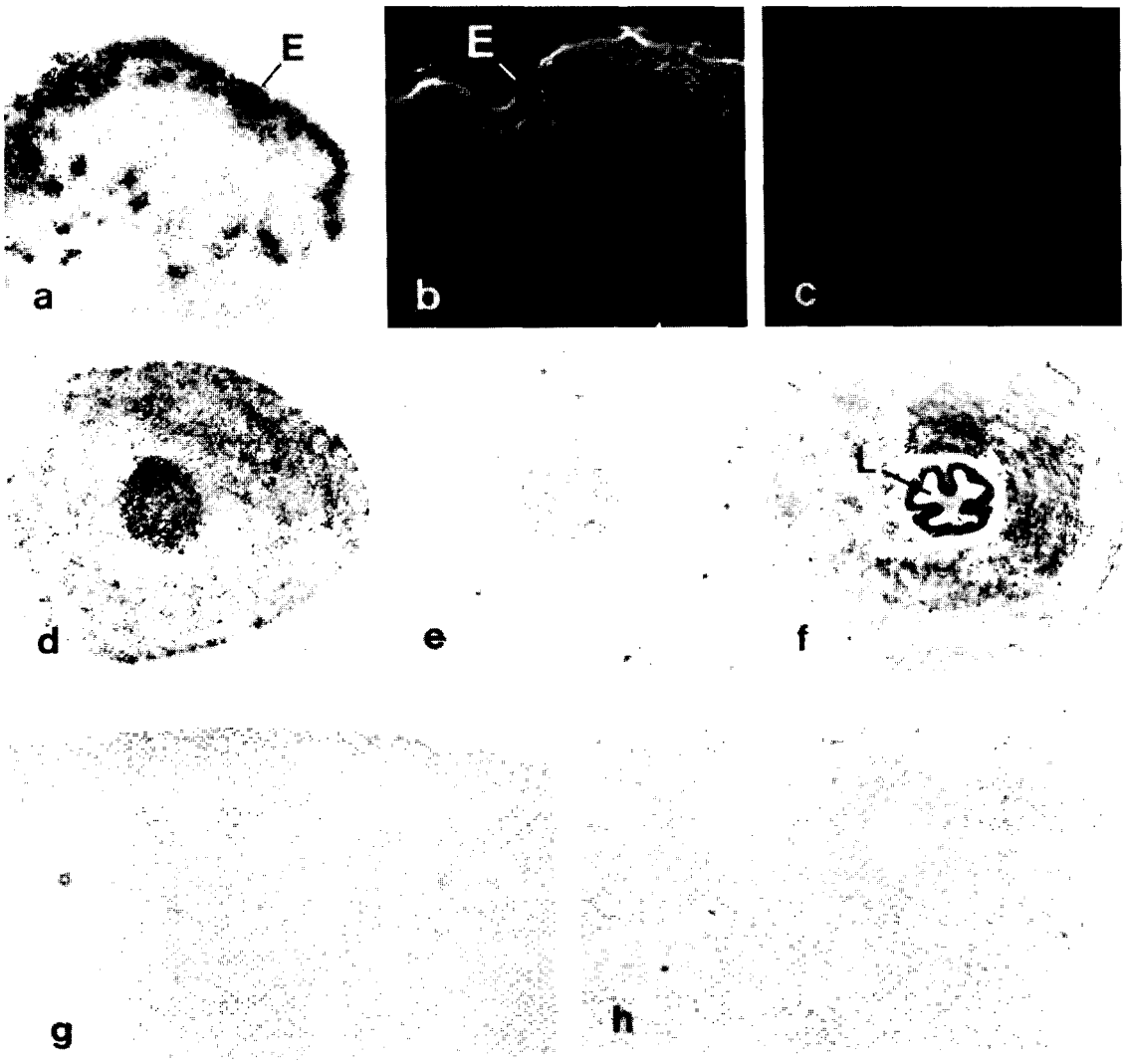


**Fig 5.** Visualization of the kinin B2 receptor in skin, vas deferens and liver by autoradiography and immunohistochemistry. **a-c.** Human skin sections were incubated with [$^{125}$I]Tyr$^{0}$-bradykinin (a), with a mixture of eight anti-peptide antibodies at 1:500 (b) and with pre-immune serum at the same dilution (c). E= epidermis. x 100. **d-f.** Rat vas deferens sections incubated with [$^{125}$I]Tyr$^{0}$-bradykinin (d), with the same radioligand in the presence of an excess of HOE140 (e) and with a mixture of anti-peptide antibodies at 1:500. L= lumen. x 150. **g-h.** Rat liver sections processed for autoradiography (g) and for immunohistochemistry (h). x 100.

almost ubiquitously distributed in the rat and human body suggesting that kinins may participate in multiple physiological and/or pathological conditions beyond their well established roles in the cardiovascular system. The presence of B2 receptors in the form of functional binding sites and of immunoreactive structural protein from the first day after birth and their presence at the site where nephrogenesis is taking place suggest that kinins may modulate kidney development.

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge the contributions of C Lizama, U Novoa and P Ehrenfeld (Valdivia), and B Welsch and Dr A Maidhof (Mainz) for their help with the preparation and characterization of anti-peptide antibodies and ligand conjugates. We also thank Dr B Schölkens (Hoechst-Marion-Roussel, Frankfurt-Am-Main, Germany) for donating HOE140; Dr Carlos B González (Instituto de Fisiología,

Universidad Austral, Valdivia) and Dr Samir El-Dahr (Tulane University, New Orleans, USA) for their contribution on the localization and ontogeny of kinin receptors.

This work was supported by grants 1970622 from FONDECYT (Chile), by the *Deutsche Forschungsgemeinschaft*, the *Fonds der Chemischen Industrie* and the Volkswagen Foundation (Germany).

## REFERENCES

ABD-ALLA S, BUSCHKO J, QUITTERER U, MAIDHOF A, HAASEMANN M, BREIPOHL G, KNOLLE J, MÜLLER-ESTERL W (1993) Structural features of the human bradykinin B2 receptor probed by agonists, antagonists, and anti-idiotypic antibodies. J Biol Chem 268: 17277-17285

ABD-ALLA S, QUITTERER U, GRIGORIEV S, MAIDHOFF A, HAASEMANN M, JARNAGIN K, MÜLLER-ESTERL W (1996) Extracellular domains of the bradykinin B2 receptor involved in ligand binding and agonist sensing defined by anti-peptide antibodies. J Biol Chem 271: 1748-1755

BASCANDS JL, PECHER C, CABOS G, GIROLAMI JP (1989) B2 kinin receptor-like binding in rat glomerular membranes. Biochem Biophys Res Commun 154: 99-104

BHOOLA KD, FIGUEROA CD, WORTHY K (1992) Bioregulation of kinins: Kallikreins, kininogens, and kininases. Pharmacol Rev 44: 1-80

BOCKAERT J, ROY C, RAJERISON R, JARD S (1973) Specific binding of [$^3$H]lysine-vasopressin to pig kidney plasma membranes. Relationship of receptor occupancy to adenylate cyclase activation. J Biol Chem 248: 5922-5931

BUSSE R, LAMONTAGNE D (1991) Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors. Naunyn-Schmiedeberg's Arch Pharmacol 344: 126-129

CHERRY PD, FURCHGOTT RF, ZAWADZKI JV, JOTHIANANDAN D (1982) Role of endothelial cells in relaxation of isolated arteries by bradykinin. Proc Natl Acad Sci USA 79: 2106-2110

CONCHON S, MONNOT C, TEUSCH B, CORVOL P, CLAUSER E (1994) Internalization of the rat AT and AT receptors: pharmacological and functional requirements. FEBS Lett 349: 365-370

CROXATTO HR, ALBERTINI R, CORTHORN J, ROBLERO J, ROSAS R (1977) Kallikrein and kinins in hypertension. In: GENEST J, KOIRE E, KUCHEL O (eds) Hypertension. Physiopathology and Treatment. New York: McGraw-Hill. pp 364-373

DE BLOIS D, BOUTHILLIER J, MARCEAU F (1991) Pulse exposure to protein synthesis inhibitors enhances vascular responses to des-Arg$^9$-bradykinin: possible role of interleukin-1. Br J Pharmacol 103:1057-1066

EL-DAHR SS, DIPP S, YOSIPIV IV, MÜLLER-ESTERL W, SCICLI AG (1996) Developmental activation and segment-specific co-expression of kininogen and bradykinin (BK) B2 receptors during rat nephrogenesis. Pediatr Res 39: 360 (Abstract)

EL-DAHR SS, FIGUEROA CD, GONZALEZ CB, MÜLLER-ESTERL W (1997) Ontogeny of bradykinin B2 receptors in the rat kidney: Implications for segmental nephron maturation. Kidney Int 51: 739-749

ESTRADA EF, BARRA V, CAORSI CE, TRONCOSO S, RUIZ-OPAZO N, GONZALEZ CB (1991) Identification of the $V_1$ vasopressin receptor by chemical cross-linking and ligand affinity blotting. Biochemistry 30: 8611-8616

FIGUEROA CD, GONZALEZ CB, GRIGORIEV S, ABD-ALLA S, HAASEMANN M, JARNAGIN K, MÜLLER-ESTERL W (1995) Probing for the bradykinin B2 receptor in rat kidney by anti-peptide and anti-ligand antibodies. J Histochem Cytochem 43: 137-148

FIGUEROA CD, MARCHANT A, MENZEL D, NOVOA U, GONZALEZ CB, MÜLLER-ESTERL W (1996a) Rat bradykinin B2 receptor: Immunochemical characterization and immunovisualization in epithelial and smooth muscle cells. Immunopharmacology 33: 85-89

FIGUEROA CD, DIETZE G, MÜLLER-ESTERL W (1996b) Immunolocalization of bradykinin B2 receptors on skeletal muscle cells. Diabetes (suppl 1) 45: S24-S28

FIGUEROA CD, NOVOA U, VALDES G, CORTHORN J, MÜLLER-ESTERL W (1997) Localization of the bradykinin B2 receptor in uterus, bladder and Madin-Darby canine kidney cells. Immunopharmacology 36: 127-133

FREDRICK MJ, ABEL FC, RIGHTSEL WA, MUIRHEAD EE, ODYA CE (1985) B2 bradykinin receptor-like binding in rat renomedullary interstitial cells. Life Sci 37: 331-338

FUJIWARA Y, MANTIONE CR, VAVREK RJ, STEWART JM, YAMAMURA HI (1989) Characterization of [$^3$H]bradykinin-binding sites in guinea pig central nervous system: possible existence of B2 subtypes. Life Sci 44: 1645-1649

GARCIA-PEREZ A, SMITH WL (1984) Apical-basolateral membrane asymmetry in canine cortical collecting tubule cells. J Clin Invest 74: 63-73

HERZIG MCS, LEEB-LUNDBERG LMF (1995) The agonist binding site on the bovine bradykinin B2 receptor is adjacent to a sulfhydryl and is differentiated from the antagonist binding site by chemical cross-linking. J Biol Chem 270: 20591-20598

HESS JF, BORKOWSKI JA, YOUNG GS, STRADER CD, RANSOM RW (1992) Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. Biochem Biophys Res Commun 184: 260-268.

HOCK FJ, WIRTH K, ALBUS U, LINZ W, GERHARDS HJ, WIEMER G, HENKE ST, BREIPHOL G, KÖNIG W, KNOLLE J, SCHÖLKENS BA (1991) HOE140, a new potent and long acting bradykinin-antagonist: in vitro studies. Br J Pharmacol 102: 769-773.

INNIS RB, MANNING DC, STEWART JM, SNYDER S (1981) [$^3$H]Bradykinin receptor binding in mammalian tissue membranes. Proc Natl Acad Sci USA 78: 2630-2634

KAUFMANN J, HAASEMANN M, MODROW S, MÜLLER-ESTERL W (1993) Structural dissection of the multidomain kininogens. Fine mapping of the target epitopes of antibodies interfering with their functional properties. J Biol Chem 268: 9079-9091

LINZ W, WIEMER G, GOHLKE P, UNGER T, SCHÖLKENS B (1995) Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. Pharmacol Rev 47: 25-49

LOPES P, KAR S, TOUSIGNANT C, REGOLI D, QUIRION R, COUTURE R (1993) Autoradiographic

localization of [$^{125}$I-Tyr$^{8}$]-bradykinin receptor binding sites in the guinea pig spinal cord. Synapse 15: 48-57

LOPES P, KAR S, CHRETIEN L, REGOLI D, QUIRION R, COUTURE R (1995) Quantitative autoradiographic localization of [$^{125}$I-Tyr$^{8}$]-bradykinin receptor binding sites in the rat spinal cord: Effects of neonatal capsaicin, noradrenergic deafferentation, dorsal rhizotomy and peripheral axotomy. Neuroscience 68: 867-881

MA JX, WANG DZ, WARD DC, CHEN L, DESSAI T, CHAO J, CHAO L (1994) Structure and chromosomal localization of the gene (BDKRB2) encoding human bradykinin B2 receptor. Genomics 23: 362-369

MAK JCW, BARNES PJ (1991) Autoradiographic visualization of bradykinin receptors in human and guinea pig lung. Eur J Pharmacol 194: 37-43

MANNING DC, SNYDER SH (1989) Bradykinin receptors localized by quantitative autoradiography in kidney, ureter, and bladder. Am J Physiol 256: F909-F915

MARGOLIUS HS (1995) Kallikrein and kinins. Some unanswered questions about system characteristics and roles in human disease. Hypertension 26: 221-229

MARIN-CASTAÑO ME, PRADDAUDE F, BOMPART G, GIROLAMI JP, BASCANDS JL (1996) RT-PCR microlocalization of bradykinin B2 receptor mRNA in microdissected rat nephron segments. Immunopharmacology 33: 171-173

McEACHERN AE, SHELTON ER, BHAKTA S, OBERNOLTE R, BACH C, ZUPPAN P, FUJISAKI J, ALDRICH RW, JARNAGIN K (1991) Expression cloning of a rat B2 bradykinin receptor. Proc Natl Acad Sci USA 88: 7724-7728

McLEAN IW, NAKANE PK (1974) Periodate-lysine-paraformaldehyde fixative: A new fixative for immunoelectron microscopy. J Histochem Cytochem 22: 1077-1083.

MINSHALL RD, NAKAMURA F, BECKER RP, RABITO SF (1995). Characterization of bradykinin B2 receptors in adult myocardium and neonatal cardiomyocytes. Circ Res 76:773-780.

MÜLLER-ESTERL W (1993) The realm of the kinins. Biomed Progr 6: 49-53

MÜLLER-ESTERL W (1997) Immunological probes for the bradykinin B2 receptor. A toolbox. In: FARMER S (ed) The Kinin System. London: Academic Pr. pp 99-110

MURONE C, PERICH RB, SCHLAWE I, CHAI SY, CASLEY D, MacGREGOR DP, MÜLLER-ESTERL W, MENDELSOHN FAO (1996) Characterization and localization of bradykinin B2 receptors in the guinea-pig using a radioiodinated HOE140 analogue. Eur J Pharmacol 306: 237-247

MURONE C, PAXINOS G, McKINLEY MJ, OLDFIELD BJ, MÜLLER-ESTERL W, MENDELSOHN FAO, CHAI SY (1997) Distribution of bradykinin B2 receptors in sheep brain and spinal cord visualized by in vitro autoradiography. J Comp Neurol 381: 203-218

PRIVITERA PJ, DAUM PR, HILL DR, HILEY CR (1991) Autoradiographic visualization and characteristics of [$^{125}$I] bradykinin binding sites in guinea pig brain. Brain Res 577: 73-79

REGOLI D, BARABE J (1980) Pharmacology of bradykinin and related kinins. Pharmacol Rev 32: 1-46

REGOLI D, MARCEAU F, LAVIGNE J (1981) Induction of B1 receptors for kinins in the rabbit by a bacterial lipopolysaccharide. Eur J Pharmacol 71: 105-115

SCHREMMER-DANNINGER E, HEINZ-ERIAN P, TÖPFER-PETERSEN E, ROSCHER AA (1995) Autoradiographic localization and characterization of bradykinin receptors in human skin. Eur J Pharmacol 283: 207-216

SCHUSTER VL, KOKKO JP, JACOBSON HR (1984) Interaction of lysyl-bradykinin and antidiuretic hormone in the rabbit cortical collecting tubule. J Clin Invest 73: 1659-1667

STERANKA LR, MANNING DC, DEHAAS CJ, FERKANY JW, BOROSKY SA, CONNOR JR, VAVREK RJ, STEWART JM, SNYDER SH (1988) Bradykinin as a pain mediator: Receptors are localized to sensory neurons and antagonist have analgesic actions. Proc Natl Acad Sci (USA) 85: 3245-3249

TOMITA K, PISANO JJ (1984) Binding of [$^{3}$H] bradykinin in isolated nephron segments of the rabbit. Am J Physiol 246: F732-F737

TOMITA K, PISANO JJ, KNEPPER MA (1985) Control of sodium and potassium transport in the cortical collecting duct of the rat. J Clin Invest 76: 132-136

VIO CP, VELARDE V, MÜLLER-ESTERL W (1996) Cellular distribution and fate of the bradykinin antagonist HOE140 in the rat kidney. Colocalization with the bradykinin B2 receptor. Immunopharmacology 33: 146-150

WHORTON AR, YOUNG SL, DATA JL, BARCHOWSKY A, KENT RS (1982) Mechanism of bradykinin-stimulated prostacyclin synthesis in porcine aortic endothelial cells. Biochem Biophys Acta 712: 79-87

WIRTH K, HOCK FJ, ALBUS U, LINZ W, ALPERMANN HG, ANAGNOSTOPOULOS H, BREIPOHL G, KÖNIG W, KNOLLE J, SCHÖLKENS BA (1991) HOE 140 a new potent and long acting bradykinin-antagonist: *in vivo* studies. Br J Pharmacol 102: 774-777