Differential Distribution of Bradykinin B2 Receptors in the Rat and Human Cardiovascular System

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Abstract—Bradykinin, a major vasodilator peptide, plays an important role in the local regulation of blood pressure, blood flow, and vascular permeability; however, the cellular distribution of the major bradykinin B2 receptor in the cardiovascular system is not precisely known. Immunoblot analysis with an anti-peptide antibody to the bradykinin B2 receptor or chemical cross-linkage with [125I]Tyr6-bradykinin revealed a band of 69±3 kDa at varying intensity in the homogenates of the endothelium and tunica media of the rat aorta and endocardium. Immunostaining showed that the B2 receptor is abundant in the endothelial linings of the aorta, other elastic arteries, muscular arteries, capillaries, venules, and large veins, where it localizes preferentially to the luminal face of the endothelial cells. In marked contrast, small arterioles (ie, the principal blood–pressure regulating vessels) of the mesenterium, heart, urinary bladder, brain, salivary gland, and kidney had a different staining pattern in which B2 receptor was prominent in the perivascular smooth muscle cells of the tunica media. A similar distribution pattern was found in mouse as well as in human tissues, indicating that the particular distribution pattern of the B2 receptor in arterioles is not a species-specific phenomenon. During development, the distribution of B2 receptor in the heart changes; for example, in the heart of newborn rats, the B2 receptor was abundant in the myocardium, whereas in the adult heart, the receptor was present in the endocardium of atria, atrioventricular valves, and ventricles but not in the myocardium. Thus, B2 receptors are localized differentially in different parts of the cardiovascular system: the arterioles have smooth muscle–localized B2 receptors, and large elastic vessels have endothelium-localized receptors. (Hypertension. 2001;37:110-120.)

Key Words: receptors, bradykinin □ endothelium □ endocardium □ nitric oxide synthase □ histochemistry □ antibodies

The variety of biological effects produced by kinins in mammals are mediated by two types of receptors: B1 and B2.1 Both kinin receptors belong to the superfamily of G protein–coupled receptors characterized by 7-transmembrane spanning helices, although they differ in their primary structures, regulation of expression, tissue distributions, and ligand profiles.2,3 The B1 receptor is stimulated by des-Arg9-bradykinin and blocked by des-Arg9-Leu9-bradykinin, whereas the B2 receptor is stimulated by bradykinin and inhibited by HOE140.4 Under physiological conditions, most biological effects produced by kinins are mediated through the B2 receptor, which appears to be constitutively expressed by many cell types.5

The known cardiovascular effects of kinins include the induction of hypotension, regulation of local blood flow, and increased vascular permeability.5 The hypotensive effects of kinins are mediated at least in part by the release of NO from endothelial cells, because endothelial denudation of large arteries abrogates their vasodilator response to kinins.6 In addition, autacoids, such as prostaglandin I2,7 are synthesized and released due to bradykinin stimulation. In other situations, the endothelium-derived hyperpolarizing factor may mediate the vasodilator effects of kinins.8 Recent experimental evidence indicates that kinins may also have cardioprotective effects during ischemia, although this issue has been disputed.9–11

Several components of the kinin-generating system have been localized in the cells of the cardiovascular system. For example, Nolly et al12 found a tissue kallikrein-like kininogenase in rat blood vessels. Kallikrein activity and kallikrein gene expression were found in the rat and human heart.13–15 Cultured rat vascular smooth muscle cells have been shown to contain tissue kallikrein, kininogen, and kininase activity.16 Moreover, kininogen immunoreactivity was found on human endothelial cells17 and neutrophils.18

Many previous studies have provided evidence for bradykinin receptors in the cardiovascular system, such as, in endothelial cells of the aorta,19–21 of the pulmonary artery,22
of postcapillary venular vessels, of coronary arteries, and of cerebral microvessels. Kinin receptors were also found in aortic smooth muscle cells, in heart valves, and in the myocardium. Most of these studies demonstrated the presence of B2 receptors by functional assays after second messenger release, by autoradiographic techniques, by Northern blot analyses, or by RT-PCR. However owing to the limited resolution inherent in these methods, the precise cellular distribution of the kinin receptors in the cardiovascular system has remained unknown. For example, the mRNA for the B2 receptor was found in the heart, whereas radioreceptor assays revealed the absence or presence of bradykinin-binding sites in the adult myocardium and neonatal cardiomyocytes. Autoradiographic studies demonstrated bradykinin-binding sites in rat heart valves but not in other portions of the heart. The reasons for these discrepancies are unknown.

We sought to study the distribution of the B2 receptor in the cardiovascular system of the rat. By applying complementary techniques with anti-receptor antibodies, radioligands, antiligand antibodies, and binding site-directed immunoglobulins, we identified and mapped the B2 receptor in the various portions of the vascular bed in vitro and in vivo. Our findings indicate that the principal bradykinin receptor is differentially distributed with respect to developmental stage (neonatal versus adult), vessel caliber (arterial versus arteriolar), and cellular face (luminal versus basolateral), suggesting that the distinct distribution of B2 receptors may contribute to the differential effects of kinins in the various parts of the cardiovascular system.

### Methods

#### Collection of Tissues and Crude Membrane Preparation

Adult Holtzman (Sprague-Dawley) rats (350 to 400 g; Charles River Laboratories) used throughout this study were killed while under ether anesthesia. The thoracic and abdominal portions of the aorta were removed and immediately washed in freshly prepared Hanks’ balanced salt solution (HBSS). The vessels were placed in cold HBSS, stripped of fat and surrounding connective tissues, and opened with scissors, and the endothelium was removed using a Teflon policeman. Endothelial crude membranes were prepared as detailed later. The tunica media was mechanically separated from the adventitia under a stereoscopic microscope and monitored with hematoxylin–eosin staining of corresponding tissue sections. Hearts were removed from the killed rats, washed in freshly prepared HBSS at room temperature, and placed in an ice-cold Petri dish. Heart compartments were opened, and heart valves were removed. The endocardium was scraped off, and the myocardium was prepared from the left ventricle wall by removing the inner and the outer layers. The tissues were homogenized in 1 mmol/L NaHCO3 from the left ventricle wall by removing the inner and the outer layers. The tissues were homogenized in 1 mmol/L NaHCO3 containing 0.1 mmol/L PMSF, 1 mmol/L EDTA, and 20 mmol/L captopril (Sigma), and 0.5 mmol/L PMSF. The homobifunctional cross-linker ethylene glycol bis(succinimidylsuccinate) was used at a final concentration of 0.25 mmol/L. Nonspecific binding was determined in the presence of 10 to 20 mmol/L HOE140 (Icatibant).

#### Autoradiography With [125I]Tyrα-Bradykinin

The bradykinin analog Tyrα-bradykinin (Sigma) was radioactively labeled with [125I]Nαl (specific activity 17 Ci/mg; Comisión de Energía Nuclear). Freshly removed hearts and vessels were rapidly frozen in liquid nitrogen, and sections of 15 to 20 μm were prepared in a cryostat at −30°C, mounted on slides precoated with polylysine (Sigma), and stored at −70°C. Before use, the slides were warmed to room temperature and washed with cold 50 mmol/L Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin (Sigma), 1 mmol/L EDTA, 1 mmol/L EGTA, and 20 μmol/L captopril.

### Antigens and Antisera Used in the Study

<table>
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<th>Antibody</th>
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<th>Dilution†</th>
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AS indicates antiserum.

*Protein sequences are from rat if not otherwise stated.

†Used for immunocytochemistry.

‡Anti-ED3, and anti-ID4 represent affinity-purified antibodies; monoclonal antibodies H32, H210, and MH01 were applied as whole ascites.

§HOE140, D-Arg-[Hyp3,Thi5,D-Tic7,Oic8]bradykinin; D-arginyl-arginyl-prolyl-4-hydroxyprolyl-glycyl-β-2-thienyl-seryl-o-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-(3aS,7aS)octahydroindole-2-carboxyl-arginine.
Light Microscopy Immunohistochemistry

Rat blood vessels and hearts were frozen in liquid nitrogen and maintained at −70°C or fixed in periodate-lysine-paraformaldehyde,

hyde and embedded in Histosec (Merck, Germany). For a compar-

ison of the distribution of B2 receptors among different species,

various tissues from mouse (SVE 129/J strain; Taconic) and human

origins were processed as described. The human tissues corre-

sponded to histologically normal segments obtained from organs

surgically removed due to inflammation or cancer and were kindly

provided by the Department of Pathology at the Regional Hospital

(Valdivia, Chile). The fixative for NO synthase (NOS)-III staining was 1% (wt/vol) paraformaldehyde in 0.1 mol/L borate buffer, pH 9.5.36 Tissue sections prepared from frozen materials and used for B2 receptor localization were fixed with cold acetone for 20 minutes.

For immunohistochemistry, the sections from frozen or embedded tissues were washed 3 times with 50 mmol/L Tris-HCl, pH 7.8, or with PBS for 5 minutes each and incubated overnight with the relevant antibody in the same buffer including 1% immunoglobulin-free bovine serum albumin (Sigma). Rabbit antibodies to peptides derived from the rat B2 receptor sequence were used as a mixture diluted 1:500 to 1:1000 or applied individually at 1:100 (Table). After an overnight incubation with the primary antibody, the sections were sequentially incubated with swine anti-rabbit immunoglobulin (1:30), and sections were mounted with hematoxylin for 5 seconds, dehydrated, and mounted with balsam. Bound peroxidase complex was visualized with 0.1% 3,3'-diaminobenzidine-HCl (Sigma), 0.03% (v/v) H2O2 in 50 mmol/L Tris-HCl, pH 7.8. The sections were counterstained with Harris’ hematoxylin for 5 seconds, dehydrated, and mounted with balsam. Alternative FITC-labeled swine anti-rabbit immunoglobulin (1:80; DAKO) was applied at 1:30, and sections were mounted with Mowiol (Polysciences Inc) containing 0.83% p-phenyleneediamine.

Ligand Probing of the B2 Receptor

To probe for ligand binding in situ, we used affinity-purified anti-peptide antibodies (anti-ED3a) to the amino-terminal portion of ED3 of the B2 receptor.37 Thoracic aortas were prepared as described and, cut into rings ~10 mm long, and the sections were incubated with 5 mmol/L [125I]Tyr0-bradykinin in HBSS for 2 hours at 4°C in the absence or presence of bradykinin, HOE140, des[Arg2]bradykinin (100 mmol/L each), or anti-ED3a or anti-ID4 (300 nmol/L each; Table). After incubation for 15 minutes at 4°C, the radioac-

tivity present in each ring was measured in a γ-counter (Packard). For autoradiography, the treated aortic rings were frozen in liquid N2, and 15-μm sections were prepared, dried, and exposed to a Biomax film (Kodak). For immunostaining, unfixed frozen sections were incubated overnight at 4°C with 300 nmol/L anti-ED3a in the presence or the absence of 100 mmol/L bradykinin, HOE140, or des[Arg2]bradykinin in HBSS containing 20 mmol/L captopril, followed by an FITC-labeled secondary antibody as described. For in vivo labeling of the receptors, the high-affinity antagonist HOE140 was applied in a single dose of 500 μg/kg body wt in 0.9% NaCl via injection into the tail vein of rats. For control, the vehicle alone was used. At 5 to 10 minutes after the injection, the rats were killed under ether anesthesia, and the hearts were fixed through immersion in periodate-lysine-paraformaldehyde as described. A portion of each sample was embedded in Histosec, and the remainder was rapidly frozen and stored in liquid nitrogen. Tissue sections were incubated with antibodies to HOE140 (Table) and further processed for immunohistochemistry as described.

Figure 1. Vascular bradykinin B2 receptor probed by immunoblotting, chemical cross-linking, and ligand blotting. Lanes 1 to 4, crude membrane proteins (100 μg) from endothelium (E) and tunica media (M) of rat aorta were subjected to SDS-PAGE under reducing conditions, followed by Western blotting with antisera AS351 to ED1 of the B2 receptor (B2R) (lanes 1 and 3) or preimmune serum (lanes 2 and 4); lanes 5 to 8, membrane proteins were chemically cross-linked with 10 mmol/L [125I]Tyr0-bradykinin (Bk) in the absence (lanes 5 and 7) or presence (lanes 6 and 8) of a 1000-fold molar excess of HOE140, sepa-

rated, and autoradiographed; lanes 9 to 12, crude membrane proteins were cross-linked with HOE140, separated, and probed by antisera AS255 to HOE140 (lanes 9 and 11) or preimmune serum (lanes 10 and 12); lanes 13 and 14, crude membrane proteins (100 μg) from endothelium (lane 13) and tunica media (lane 14) of rat aorta were subjected to 6% SDS-PAGE and Western blotting with an antibody to human von Willebrand factor (vWF) (from rabbit; DAKO). Sera were used at 1:100 through-

out. Relative molecular masses of marker proteins (left), B2 receptor (filled arrowhead), and von Willebrand factor (open arrowhead) are indicated on the margins.

Immunogold Electron Microscopy

Aortic rings and atria fixed with periodate-lysine-paraformaldehyde for 1 hour13 were incubated with a mixture of anti-peptide antibodies (1:200) directed to ED1 of the B2 receptor (Table) in PBS, pH 7.4, supplemented with 1% immunoglobulin-free bovine serum albumin. Controls were performed with preimmune rabbit serum at 1:200. The tissue sections were washed and incubated with anti-rabbit immunoglobulin-coupled to gold particles of 10 nm (DAKO) or 30 nm (Amersham International). The washed sections were postfixed with 3% glutaraldehyde followed by 1% osmium tetroxide and embedded.18

Results

Biochemical Characterization of the Vascular B2 Receptor

Crude membrane preparations from the endothelium and tunica media of the adult rat aorta were subjected to SDS-PAGE and Western blotting with an anti-peptide antiserum to the rat B2 receptor (Figure 1). A major band of 69±3 kDa was observed for both the endothelium (Figure 1, lane 1) and media (lane 3), although the intensities differed between the 2 sites. Preimmune serum applied at the same dilution failed to produce significant staining (lanes 2 and 4), thus demonstrat-

ing the specificity of the antibody. To monitor the completeness of the separation of endothelium and media, we applied an antibody to von Willebrand factor and found that only the endothelial preparation was positive for this marker protein (lanes 13 and 14). In a parallel experiment, B2 receptors from the same membrane preparations were chemically cross-linked to [125I]Tyr0-bradykinin by ethylene glycol bis(succinimidylsuccinate) and subjected to SDS-PAGE. The corresponding autoradiography revealed a major band of 69

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kDa each for endothelium and media (lanes 5 and 7). A 1000-fold molar excess of the unlabeled B₂ antagonist, HOE140, completely displaced the radioligand (lanes 6 and 8), thus proving the specificity of the ligand-receptor interaction.

**Distribution of B₂ Receptors in the Rat Aorta**

Identification of B₂ receptors at the cellular level was made with 2 independent techniques. Immunohistochemistry with antibodies to the various intracellular and extracellular domains of the B₂ receptor revealed a strong immunoreactivity of the aortic endothelium (Figure 2a). Less intense staining was seen for the smooth muscle cells interspersed in the tunica media (Figure 2a), whereas the tunica externa was free of staining except for vasa vasorum, which showed a strong labeling (not shown). Application of the corresponding preimmune serum or preabsorption of the antibodies with their corresponding antigens failed to produce a significant immunostaining (Figures 2j and 2k). Autoradiography with [125I]Tyr⁴-bradykinin revealed a similar distribution pattern for bradykinin-binding sites localized in the endothelium and in the media layer (Figure 3a). Specific labeling

**Figure 2.** Visualization of B₂ receptor in major blood vessel types. A mixture of antibodies to the B₂ receptor (AS276-83) was applied at 1:1000 (a, c, d, e, g, and h). For comparison, monoclonal antibodies H32 and H210 to rat NOS-III were used at 1:1000 to 1:2000 (b and f). Rat aorta (a and b); mesenteric artery (c); skin capillaries (d); mesenteric arteriole (e and f); urinary bladder venule (g); vena cava (h); and consecutive sections of a renal radial arteriole immunostained with AS276-83 (i), by replacement of AS276-83 with nonimmune rabbit serum (j), or preabsorbed AS276-83 (k) incubated with a molar excess of the same peptides used for immunization. E indicates endothelium; M, media. Arrows point to immunoreactive smooth muscle cells of the tunica media. Original magnification: a, b, c, and h x 800; d, f, and g, x1300; e x 500; i, j, and k, x1300. Scale bar 25 μm.
was abrogated by a 1000-fold molar excess of unlabeled bradykinin (Figure 3b) but not of the B₂ receptor agonist des[Arg⁹]bradykinin (not shown). Densitometric analyses revealed that bradykinin-binding sites were more dense in the endothelial layer than in the tunica media (Figure 3, insets). Hence, the aortic endothelium is rich in B₂ receptors, whereas the smooth muscle cells interspersed in the tunica media of the aortic wall have a lower density of B₂ receptors.

Immunoprobing for Kinin-Binding Sites

Autoradiographic studies with radiolabeled bradykinin are complicated by the fact that other high-affinity binding sites for bradykinin, such as ACE or endopeptidase EP24.15, present in the same tissues may produce false-positive staining. To test this possibility, we used frozen sections prepared from nonfixed aorta and incubated them with 300 nmol/L anti-ED3₃ in the presence or the absence of 100 μmol/L bradykinin, HOE140, or des[Arg⁹]bradykinin overnight at 4°C (Figure 4, top). A strong immunostaining of the endothelial layer was seen with anti-ED3₃ in the absence of competing ligands (Figure 4a), whereas coincubation with 100 μmol/L HOE140 (Figure 4b) or bradykinin (Figure 4c), but not with des[Arg⁹]bradykinin (Figure 4d), markedly reduced the specific staining. The failure of anti-ED3₃ to stain smooth muscle cells in the media likely reflects the lower affinity of the monospecific antibody to a single sequence segment as opposed to a polyvalent antiserum used for other immunolocalizations in this study. Collectively, these data stress the capacity of our anti-peptide antibodies to specifically detect the B₂ receptor in the various layers of the rat aorta in situ.

Differential Distribution of B₂ Receptors in Blood Vessels

Immunovisualization of B₂ receptors in blood vessels other than aorta revealed that large muscular arteries such as the carotid, mesenteric artery (Figure 2c), iliac and femoral arteries, vena cava (Figure 2h), small capillaries (Figure 2d), and venules (Figure 2g) from various organs show a rather uniform pattern, that is, prominent staining of the endothelial cell layer and little, if any, staining of the media and externa. Unexpectedly, an “inverse” immunostaining pattern was seen for small arterioles: immunoreactivity was most prominent in the muscularis surrounding the arterioles, and only a thin rim of immunostaining was visible at the arteriolar endothelium (Figures 2e and 2i). The replacement of the B₂ receptor antiserum by nonimmune serum and the use of an excess of the same peptides used for immunization, during the incubation of the antiserum, demonstrated the specificity of the immunostaining pattern observed in arterioles (Figures 2j and 2k). To confirm this strikingly differential staining pattern, we followed the distribution pattern of NOS-III (i.e., the major effector enzyme of the B₂ receptor in the cardiovascular system). NOS-III was invariably present in the endothelial layer of the aorta and of small arterioles, whereas the media of vessels of varying caliber was free of immunoreactivity (Figures 2b and 2f), thus demonstrating the selectivity of our antibody probes. We made qualitatively similar observations through autoradiography using [¹²⁵I]Tyr⁴-bradykinin (not binding of the receptor, thereby preventing bradykinin attachment to its cognate receptor.⁷ Rings of thoracic aorta were incubated with 5 nmol/L [¹²⁵I]Tyr⁴-bradykinin in the presence or absence of anti-ED3₃. At a 300 nmol/L concentration of the antibody (60-fold molar excess over radioligand), anti-ED3₃ effectively displaced [¹²⁵I]Tyr⁴-bradykinin from the aortic binding sites (Figure 4, bottom and middle). Similarly, 100 μmol/L bradykinin or HOE140 displaced the radioligand, whereas control antibody to an intracellular domain ID4 of B₁ receptor or des[Arg⁹]bradykinin had no effect (Figure 4, bottom).

This finding prompted the question of whether anti-ED3₃ could reversibly bind to B₂ receptors in situ and thereby provide a unique specificity control that selectively probes for intact (“functional”) kinin-binding sites. To test this possibility, we used frozen sections prepared from nonfixed aorta and incubated them with 300 nmol/L anti-ED3₃ in the presence or the absence of 100 μmol/L bradykinin, HOE140, or des[Arg⁹]bradykinin overnight at 4°C (Figure 4, top). A strong immunostaining of the endothelial layer was seen with anti-ED3₃ in the absence of competing ligands (Figure 4a), whereas coincubation with 100 μmol/L HOE140 (Figure 4b) or bradykinin (Figure 4c), but not with des[Arg⁹]bradykinin (Figure 4d), markedly reduced the specific staining. The failure of anti-ED3₃ to stain smooth muscle cells in the media likely reflects the lower affinity of the monospecific antibody to a single sequence segment as opposed to a polyvalent antiserum used for other immunolocalizations in this study. Collectively, these data stress the capacity of our anti-peptide antibodies to specifically detect the B₂ receptor in the various layers of the rat aorta in situ.

Figure 3. Autoradiography of the B₂ receptor in rat aorta. In a paired experiment, serial sections of aorta were incubated with 5 nmol/L [¹²⁵I]Tyr⁴-bradykinin in the absence (a) or presence (b) of 5 μmol/L unlabeled Tyr⁴-bradykinin. Insets, Densitometric tracings performed on the sections; arrows indicate the tracing direction. Exposure times (4 weeks) and intensity scales are identical for the 2 panels. E indicates endothelium; M, media. Original magnification: a and b ×700. Scale bar 25 μm.
shown), although the low-resolution power of this method did not allow an unequivocal identification of the labeled cells. Hence, B2 receptor appeared to be prominent in the endothelium of the entire vascular system of Holtzman rats, with the notable exception of resistance vessels, where B2 receptors were abundant in the muscularity.

Distribution Pattern of B2 Receptor in Rat, Mouse, and Human Arterioles
Given this striking pattern of B2 receptor distribution in rat mesenteric arterioles, we asked whether our finding reflects a more generalized phenomenon. Indeed, rat urinary bladder arterioles produced a similar staining pattern as mesenteric arterioles (Figure 5a). In addition, immunostaining of human breast (Figure 5c), myometrium (Figure 5d), and skin (Figure 5g), as well as mouse salivary gland (Figure 5f), showed essentially the same arteriolar distribution for the B2 receptor: intense staining of the tunica media and minor staining of the endothelium. Controls with preimmune serum (Figure 5e) were negative. Hence, the characteristic distribution pattern of the B2 receptor in rat arterioles is also found in other mammalian arterioles, although the intensity of staining may vary. In some cases, smooth muscle cells of the arteriolar wall that express immunoreactive B2 receptors were intermingled with cells devoid of any specific staining (Figure 5c). By contrast, large distributing arteries of human thyroid exhibited the typical distribution pattern of B2 receptors in rat aorta: prevalent staining of the endothelium with minor staining of the smooth muscle cells interspersed in the vessel wall (Figure 5b). Thus, the “inverse” distribution pattern of B2 receptors in arterioles is a general phenomenon across organs and species.

Localization of B2 Receptors in the Heart
Previous studies have produced conflicting results regarding the presence of B2 receptors in the rat heart.28,31,38 Our initial Western blotting experiments revealed a weak band of ≈69 kDa indicative of the B2 receptor in ventricular and valvular endocardium, whereas the myocardium of the adult heart was virtually free of immunoreactivity, as were controls with preimmune sera (data not shown). Immunofluorescence staining demonstrated B2 receptors in endocardium of ventricles and atria (Figures 6a and 6b), in the endothelium of coronary arteries and veins (not shown), and, less prominently, in heart capillaries (Figure 6b). B2 receptors were also present in the endocardium of tricuspid and mitral valves (Figure 6c). Autoradiography with [125I]Tyr0-bradykinin confirmed these findings (Figure 6d). Hence, the adult rat heart shows a distinct localization of B2 receptors prominent in the endocardium and scarce or even absent in the myocardium. Because recent work had demonstrated bradykinin-binding sites of cultured neonatal myocytes,28 we wondered whether the newborn heart would differ in its expression pattern of B2 receptors. Immunostaining clearly revealed positive immunoreactivity for both the endothelial cells of the endocardium and the cardiomyocytes of the myocardium of the newborn heart (Figure 7a; preimmune serum control, Figure 7b), indicating that the expression of the B2 receptor may vary during development and growth of the rat heart.
In Vivo Labeling of B2 Receptors

B2 receptors are subject to rapid sequestration, swift desensitization, and possibly extensive downregulation. Therefore, we asked whether the relative localizations of the receptor demonstrated in vitro are congruent with the patterns observed in vivo. We took advantage of the facts that (1) the principal B2 antagonist HOE140 binds very tightly to the B2 receptor, (2) unbound HOE140 is rapidly cleared from the plasma via glomerular filtration, and (3) antibodies to HOE140, a kinin-like peptide, do not cross-react with the endogenous ligand bradykinin. Crude membrane preparations from the endothelium and tunica media of the rat aorta were chemically cross-linked with HOE140, and the resultant products were analyzed by SDS-PAGE and Western blotting. A major band of 69 kDa was observed for both endothelium (Figure 1, lane 9) and media (lane 11), whereas an isotype-matched control antibody failed to produce significant staining (lanes 10 and 12), demonstrating the specificity of the detection method. HOE140 was administered at 500 mg/kg of rat for 10 minutes via the tail vein; thereafter, the animals were killed. Immunohistochemistry of the heart atrium (Figure 7c) and ventricles (Figure 7e) with anti-HOE140 revealed patterns of immunostaining that were almost indistinguishable from those obtained with anti-receptor antibodies. No specific immunostaining was observed with isotype-matched nonimmune mouse immunoglobulin (Figure 7d). Likewise, when rats were injected with vehicle alone, we did not observe a specific tissue staining (not shown). Collectively, these findings indicate that the cellular distribution of the B2 receptors in vitro adequately reflects the situation in vivo.

Subcellular Distribution of B2 Receptors

Autoradiography and immunocytochemistry at the light microscopy level do not reveal the details of a differential receptor distribution at the cellular level. Indeed, previous studies of the B2 receptor in the rat distal nephron had demonstrated that this receptor is abundant on both the basolateral face and the luminal side of tubular cells, and studies with recombinant kinin receptors revealed that the bulk of the receptor protein resides in a perinuclear compartment that overlaps the endoplasmic reticulum of overexpressing cells. Therefore, we used immunoelectron microscopy with anti-peptide antibodies to the extracellular domains of the B2 receptor and gold-labeled secondary antibodies to probe for the receptor on the various faces of endothelial cells. The vast majority of B2 receptors were associated with the luminal face of the plasma membrane of nonpermeabilized aortic endothelial cells (Figure 8), whereas the abluminal side exposed the receptor at much lower frequency. We have made similar observations for endothelial cells of the endocardium (data not shown).

Discussion

Cardiovascular functions are regulated by numerous factors, such as the sympathetic nervous system, endocrine hormone systems, and paracrine autacoid systems. In recent years, the role of local mediators, in particular those released by endothelial cells, has attracted significant attention. Due to its strategic anatomic position, the endothelium can respond to mechanical forces, such as shear stress, and to mediators from other cells, such as leukocytes, by releasing contracting...
and/or relaxing factors, which in turn modulate the activity of the vascular smooth muscle cells. Accumulating evidence suggests that many of the endothelium-mediated effects are strictly regulated with respect to time and space; that is, the signals are transient and local rather than sustained and systemic. One prototypic system that intimately interacts with the endothelium is the kallikrein-kinin system. Endothelial cells expose specific docking sites that serve to assemble the constituents of the kinin-generating pathway on the endothelium, thereby allowing the circumscribed release of short-lived kinins. The findings indicate that the structural characterization and spatial mapping of the terminal effectors of the system, kinin receptors, are critical for a deeper understanding of the mechanisms that limit the cardiovascular effects of bradykinin with respect to time and space.

Using site-directed antibodies, we demonstrate that the bradykinin B₂ receptor is differentially distributed in the rat cardiovascular system. The most unexpected finding is the variation in B₂ receptor distribution in the muscularis of arterial vessels with caliber: elastic, and muscular arteries abundantly express B₂ receptors in their endothelial lining and have much less or even no receptor in the smooth muscle cells of their walls. In marked contrast, small arterioles express large numbers of B₂ receptors in the smooth muscle cells of their tunica media and only a few receptors in their endothelial layer. Many studies have demonstrated the presence of bradykinin B₂ receptors in cerebral, renal, and coronary arteries and in muscular arterioles of vertebrate species; however, owing to the limitations inherent to receptor autoradiography, intracellular signaling, and/or ligand binding studies, none of them have revealed the precise in situ distribution of kinin receptors.

The presence of B₂ receptors in the smooth muscle cells of the arteriolar wall may well explain the endothelium-independent relaxation observed for omental and coronary arterioles. In accord with these findings, pharmacological studies have demonstrated that vasodilator effects of kinins in large-bore vessels are primarily mediated by endothelial NO, whereas the inhibition of NO formation by N\textsuperscript{G}-methyl-L-arginine has only a minor effect on bradykinin action in arterioles. Because smooth muscle cells of the arteriolar vessel wall do not express NOS-III (endothelial NOS), it is tempting to speculate that bradykinin triggers a NO-independent signaling cascade or cascades in these perivascular smooth muscle cells. This notion is in line with previous findings that the effects of bradykinin on small arterioles are at least in part mediated by signaling pathways that bypass endothelial cells. The differential distribution of B₂ receptors in the resistance vessels may also help explain the biphasic response of renal afferent arterioles to increasing concentrations of bradykinin.

### Figure 6

**Distribution of the B₂ receptor in rat heart.** Top, Immunohistochemical detection using 300 nmol/L affinity-purified anti-ED3N (a, b, and c). Bottom, Autoradiography using 5 nmol/L [\textsuperscript{125}I]Tyr\textsuperscript{5}-bradykinin (d). Cp indicates capillary. *Cardiomyocytes of the ventricles. Arrows point to endocardium of ventricle (a), atrium (b and d), and valve (c). Arrowheads identify coronary arterioles. Inset, Morphological appearance of the same arterioles present in d. Original magnification: a, b, and c \( \times 800 \); d \( \times 50 \). Scale bar 15 µm (a, b, and c), 300 µm (d).
An issue not solved in the present study is the considerable variation in B2 receptor expression levels during cardiac growth and development,\textsuperscript{28,30,31} which may have important (patho)physiological consequences. Functional studies have demonstrated the presence of bradykinin B2 receptors in cultured neonatal cardiomyocytes\textsuperscript{28} and the adult heart of the rat,\textsuperscript{31} whereas others failed to detect kinin receptors in adult cardiomyocytes.\textsuperscript{30} Our results clearly show the presence of B2 receptors in the heart of newborn rats; thus, the finding of kinin receptor expression in cultured neonatal cardiomyocytes is not an in vitro artifact.\textsuperscript{28,31} Because functional studies revealed only small copy numbers of B2 receptors in the adult heart,\textsuperscript{31} it is possible that the density of kinin receptors is too low to be detected through our immunological approach. Our findings seem to indicate that the myocardial B2 receptor is downregulated during growth and development of the rat heart. This observation correlates with the frequently seen “loss” of B2 receptors in the first few passages of human umbilical vein endothelial cell culture (W. Müller-Esterl, unpublished results). The B2 receptor gene could be the target of an extensive transcriptional regulation,\textsuperscript{52} and differential modulation of mRNA and/or protein stability may further contribute to the observed phenomena.

Differential B2 receptor expression may have important implications for the application of B2 receptor agonists in brain tumor therapy.\textsuperscript{62} Owing to the large number and/or efficient coupling of B2 receptors present in the endothelium lining of newly formed vessels, novel kinin receptor agonists such as RMP-7 have been developed\textsuperscript{63} that transiently and selectively open the blood-brain barrier, thereby promoting the extravasation of coadministered anticancer drugs en route to tumor tissues.\textsuperscript{64} It is tempting to speculate that the overexpression of B2 receptors in newly formed tumor vessels may translate into differential susceptibility to kinin agonists and therefore enhanced vulnerability to coadministered cytostatic drugs.\textsuperscript{65}

In summary, we have shown that the distribution of the bradykinin B2 receptor is affected by 3 factors: age (newborn

**Figure 7.** Distribution of B2 receptor in neonatal heart and in vivo tracing of HOE140 in adult heart. Top, Immunostaining of neonatal rat heart with unfixed frozen sections incubated overnight with AS276-83 (a) or the corresponding preimmune serum (b) at 1:500 each. Bound antibody was visualized by an FITC-labeled secondary antibody. Bottom, Rats were intravenously injected with 500 μg/kg body wt HOE140 and killed after 10 minutes. HOE140 bound to B2 receptors was identified by monoclonal antibody MHO1 (1:100) to HOE140. Arrows point to the endocardium (c). Cp indicates capillaries; V, venules (e). For control, an isotype-matched nonimmune mouse immunoglobulin was applied (d). Original magnification: a and b ×1000; c and d ×200; e ×500. Scale bar 20 μm (a and b); 25 μm (c, d, and e).

**Figure 8.** Immunoelectron microscopy of endothelial B2 receptors. A mixture of anti-peptide antisera to the extracellular domains of the B2 receptor was applied to rat aorta endothelium at 1:1000, followed by a gold-labeled (30 nm) secondary antibody. Inset, Control; the first antibody was replaced by nonimmune serum. Original magnification ×33,000. Scale bar 300 μm. Arrows point to immunogold clusters at the luminal side.
versus adult heart), vessel type (arteries versus arterioles), and cell polarity (luminal versus basolateral). Our results may help to underpin future studies that address the effects of kinins on growth, development, and protection of the heart; the beneficial effects of ACE inhibitors in hypertension and cardiac ischemia; and the role of kinins in blood-brain barrier opening and their applications to tumor therapy.

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