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# Signaling Pathways Coupled to Activation of the Kinin B1 Receptor

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## 1. Introduction

### 1.1 The field of kinins and their receptors

Kinins are a group of bioactive peptides, which are formed by an endogenous enzymatic cascade consisting of precursor substrates called kininogens and the proteolytic kallikrein enzymes (kininogenases). So far, two kinin-releasing enzymes (kininogenases) have been characterized, plasma (hKB1/*LKLB1*) and tissue (hK1/*KLK1*) kallikreins (Fig. 1). Both enzymes are serine proteases that are found in glandular cells, neutrophils, and biological fluids. Kininogens are multifunctional proteins involved in cascade reactions during inflammation (Bhoola et al., 1992), and more recently in carcinogenesis (Bhoola et al., 1992; 2001; Leeb-Lundberg et al., 2005). Kinins namely bradykinin (BK) and kallidin (Lys-BK) are among the most potent pro-inflammatory vasoactive peptides generated during tissue injury and noxious stimulation. BK and Lys-BK undergo metabolic degradation at a variable rate by amino-, carboxy- and endopeptidases found in tissues and biological fluids. The most physiologically relevant enzymes are carboxypeptidase N (from plasma) and carboxypeptidase M (from cell membranes), which remove the carboxy-terminal Arg present at end of the kinin molecule, resulting in the formation of the active metabolites des-Arg<sup>9</sup>-BK (DBK) and des-Arg<sup>10</sup>-kallidin, known also as Lys-des-Arg<sup>9</sup>-BK (LDBK) (Fig. 1). Furthermore, neutral endopeptidase 24.11 (CD10, enkephalinase) and angiotensin I converting enzyme, act as dipeptidyl carboxypeptidases by removing the dipeptide Phe<sup>8</sup>-Arg<sup>9</sup> from the carboxy terminus end of BK or Lys-BK (Couture et al., 2004). It is important to mention that endopeptidase 24.15 and angiotensin I converting enzyme cleave the dipeptide Ser<sup>6</sup>-Pro<sup>7</sup> from bradykinin 1-7 (BK1-7) to produce bradykinin 1-5 (BK1-5), one of the final metabolites of BK and DBK that possesses the longer half-life of this peptide family. The half-life of kinins depends on the rate and site of destruction, most rapid in the circulation, but less so in the extracellular fluid space and by cells. Actually, in plasma the half-life of BK and LDBK is short (15 to 20 sec) whereas BK1-5, considered in the past an inactive fragment, is considered to have a half-life of 86 to 101 min (Shima et al., 1992; Murphey et al., 2000; 2006; Morinelli et al., 2002).

Kinins are positively charged peptides that influence tissues and cells by stimulating two pharmacologically distinct G protein-coupled receptors (GPCRs). Kinin receptors are

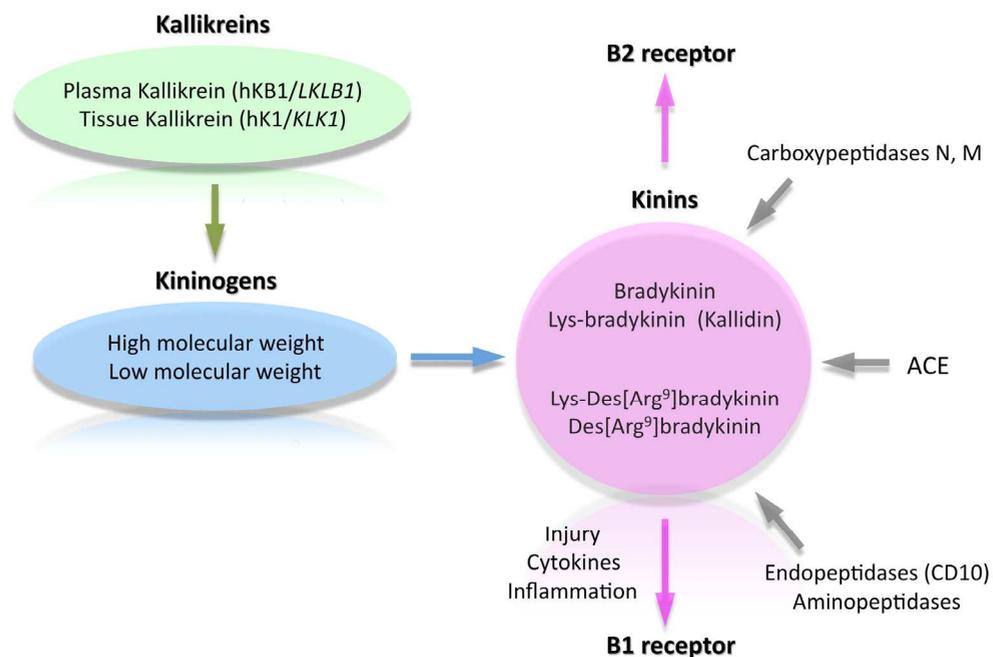


Fig. 1. Major components of the kinin system. The kininogenases plasma and tissue kallikreins hydrolyze either high or low molecular weight kininogens to release the kinin domain. Carboxypeptidases remove the Arg<sup>9</sup> from bradykinin or Lys-bradykinin to generate B1 receptor agonists.

situated on the plasma membranes of many cell types and are coupled principally to Gαq and Gαi, (Austin et al., 1997; Regoli et al., 2001). These receptors are designated as kinin B1 (B1R) or B2 (B2R) receptors (Fig.1). B2R is a preformed receptor, widely distributed and activated by the parent molecules BK and Lys-BK. B1R is activated by DBK and LDBK, two kinins that lack the Arg<sup>9</sup> at the carboxy-terminus of the parental BK or LBK molecule (Bhoola et al., 1992; 2001; Leeb-Lundberg et al., 2005).

B1R is generally expressed at low levels in normal tissues, but is strongly up-regulated during inflammation, tissue injury, cancer, exposure to bacterial endotoxins such as lipopolysaccharide, growth factors (epidermal growth factor and endothelial growth factor) cytokines (principally interleukin-1β, interleukin-8 and tumor necrosis factor-α) or even its own agonist, LDBK (Marceau et al., 1998; Yang et al., 2001). For this reason, there is an increase in the number of B1 binding sites in inflamed or carcinogenic tissue. Moreover, an up-regulation of the kinin B1R has been found in several tumors, immune-modulated disorders such as rheumatoid arthritis, transplant rejection, glomerulonephritis and in human fibrotic lung tissue.

Additional differences between both types of kinin receptors reside in the fact that the B2R is internalized and extensively recycled to the cell surface by its agonist, whereas the B1R is up-regulated in inflammatory disorders and cancer, and its expression is controlled by signaling pathways such as stress mitogen-activated protein and nuclear factor kappa B

(NF- $\kappa$ B) (Marceau et al., 2002). In fact, sequence analyses show the presence of a transcriptional regulatory site for NF- $\kappa$ B in the promoter region of the B1R (Bachvarov et al., 1996; Ni et al., 1998) a finding that may explain why expression of the B1R gene is tissue and cell specific (Yang et al., 2001).

The different cell types in which expression and activation of kinin receptors has been determined include endothelial, epithelial and neural cells, smooth muscle cells, neutrophils, lymphocytes, monocytes, keratinocytes, chondrocytes and fibroblasts. Depending upon the cell type involved, kinin receptors activate different intracellular signaling pathways, which regulate processes such as, cell proliferation, differentiation, migration, vascular permeability, contraction of smooth muscle, excitation of nerve endings and release of a variety of biologically active secondary mediators. In this chapter, we will focus primarily on the most important signaling pathways triggered after B1R stimulation.

## 2. Structure of kinin B1 receptor

In general, the information on the structure of kinin receptors has been obtained by chemical cross-linking experiments, mutagenesis approaches, classical pharmacological assays, and studies with domain-specific anti-receptor antibodies (Prado et al., 2002; Blaukat, 2004). All these approaches have shown that B1R belongs to the GPCR family and that it is integrated into the plasma membrane. The amino-terminus end is exposed to the extracellular space and contains (three) consensus sites of the Asn-Xaa-Ser/Thr type for N-glycosylation and the most of them are clustered on their N-terminal domains. It is noteworthy that the kinin-binding site is located at the amino-terminus end of the third extracellular loop. According to Kang et al., (2005) the presence of multiple bands observed for the recombinant expressed human B1R is strongly reminiscent of patterns of the partially glycosylated B2R. However, a B1R in which the N-terminal domain had been truncated to remove putative N-glycosylation sites migrated as a homogeneous specie (Kang et al., 2005). Furthermore, the treatment of the hemagglutinin-tagged human B1R, expressed in HEK293 cells, with N-glycosidase F resulted in the conversion of the receptor from a heterogeneous specie migrating at 35 to 45 kDa to a relatively homogeneous one migrating at 37 kDa. We have observed a similar electrophoretic pattern of the B1R protein in human neutrophils (Ehrenfeld et al., 2006), breast cancer cells (Molina et al., 2009), endothelial cells and fibroblasts (our group, unpublished data). It is postulated that glycosylation probably increases the hydrophilicity of the extracellular portions of B1R affecting or regulating ligand affinity, efficient G protein coupling, maturation (folding, stabilization), intracellular trafficking and receptor oligomerization or receptor degradation (Menke et al., 1994; MacNeil et al., 1995; Leeb-Lundberg et al., 2005). Even more, it has been demonstrated that kinin-mediated mitogenic signaling and prostate cell growth is blocked by B1 and B2 receptor antagonists indicating that these effects depend on both kinin receptors. These results provide evidence for the existence of B1R/B2R heterodimers in PC3 prostate cancer cells and demonstrate that antagonism of one receptor interferes with the ability of the other, possibly at the level of receptor-G $\alpha$ q protein coupling (Barki-Harrington et al., 2003). It is known that B1R can dimerize, oligomerize or heterodimerize with the B2R or other molecule. Actually, it is known that B1R heterodimerizes with membrane carboxypeptidase M, facilitating receptor signaling via carboxypeptidase M-mediated conversion of bradykinin or kallidin to a des-[Arg<sup>9</sup>]kinin. This critical interaction potentiates B1R signaling

and uncovers a new mode of GPCR activation by a cell surface peptidase (Zhang et al., 2011). Therefore, it is clear that knowledge of the molecular structure has been important in determining B1R function.

### 3. Classical signaling pathways activated by kinin B1 receptor

In general, B1R activates most of the signaling pathways activated by B2R (reviewed by Marceau et al., 1998 and Leeb-Lundberg et al., 2005). Although both kinin receptors are coupled to similar signal transduction pathways, the differences in patterns of signaling are due to different degrees of short-term regulation that include both receptor desensitization and internalization (Leeb-Lundberg et al., 2005). The B2R is stable in the absence of its agonist, but it is rapidly desensitized after ligand stimulation by a mechanism that includes recruitment of  $\beta$ -arrestin 2, internalization in a clathrin-dependent manner and recycling upon agonist treatment (Enquist et al., 2007). The B1R is not phosphorylated either under basal conditions or in response to agonist (Blaukat et al., 1999) and desensitizes slightly upon further stimulation with its agonist (Mathis et al., 1996). Actually, the B1R is constitutively internalized in the absence of agonist via a clathrin-dependent pathway, do not recruit  $\beta$ -arrestin 2, bind G protein-coupled receptor sorting protein and go to lysosomes for degradation. Binding of agonist to the B1R inhibits its constitutive internalization thereby reducing the rate of spontaneous clearance of receptors from the cell membrane and at the same time delaying B1R degradation (Enquist et al., 2007). This behavior is according with B1R up-regulation by cytokines and other inflammatory stimuli that result in a high number of receptors on the cell surface, available for ligand binding (Fig. 2).

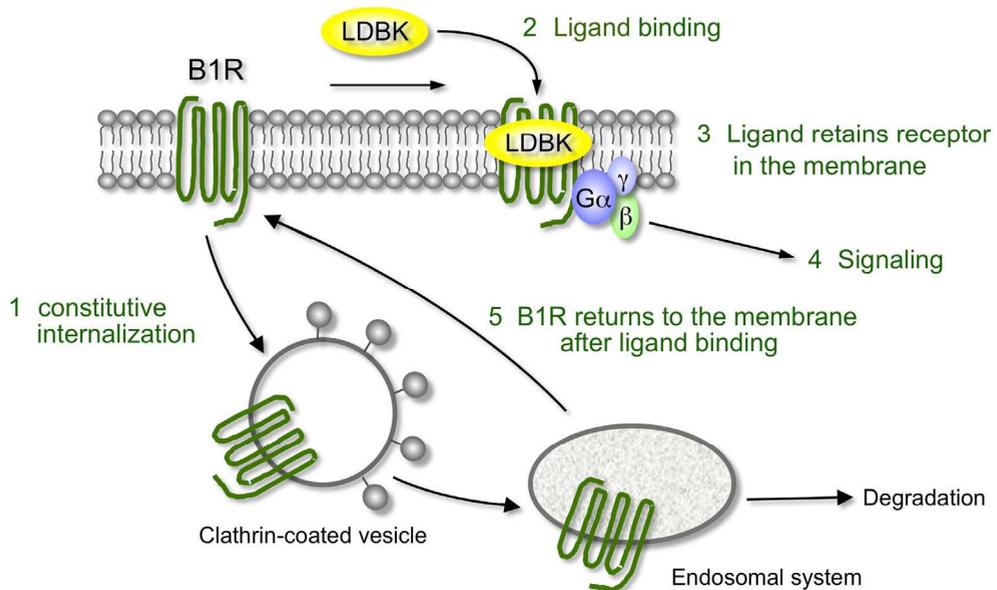


Fig. 2. Regulation of B1R levels on the cell membrane. The B1R is constitutively internalized, but the number of B1R molecules on the cell membrane increases after binding of its ligand.

It is known that B1R can be coupled to different pathways according to the cell type involved. Signaling of the B1R results in the activation of protein kinase C (PKC) and tyrosine kinase cascades, coordinated with activation of the mitogen-activated protein kinase (MAPK) pathway and NF- $\kappa$ B. B1R activation also stimulates phosphatidylinositol hydrolysis in smooth muscle cells leading to mobilization of intracellular Ca<sup>2+</sup>, phospholipase C or phospholipase A, and appears to induce biosynthesis and release of prostaglandins (Bhoola et al., 1992; 2001; Leeb-Lundberg et al., 2005). In vascular smooth muscle cells, B1R stimulation produces a significant dependence on extracellular Ca<sup>2+</sup> and a transient increase in phosphatidylinositol hydrolysis that is more sustained than that generated by the B2R (Tropea et al., 1993). Nevertheless, in the rat renal cortical collecting duct cells, keratinocytes, breast cancer cells and neutrophils B1R does not induce intracellular Ca<sup>2+</sup> mobilization (Ehrenfeld et al., 2006; Matus et al., 2008; Molina et al., 2009)

Another characteristic of B1R is its capacity to regulate the cell cycle. In some cases, B1R is proliferative as seen in fibroblasts, vascular smooth muscle cells, estrogen-sensitive breast cancer cells, and this response involves MAPK activation (Marceau and Tremblay, 1986; Christopher et al., 2001; Molina et al., 2009). In vascular smooth muscle cells, B1R is induced only in response to injury, regulates proliferation by pathways that include activation of cholera toxin-sensitive G $\alpha_q$ , PKC, Src kinase and MAPK. In estrogen-sensitive breast cancer cells, Molina et al., (2009) showed that nanomolar concentrations of the B1R agonist produced an increase in BrdU incorporation as measured in a proliferation assay. The use of inhibitors of MEK, the kinase which phosphorylates ERK1/2 MAPK, such as PD98059 or UO126, decrease the phosphorylation of ERK1/2 and completely abolished the incorporation of BrdU. At the same time, we demonstrated that this effect was dependent on epidermal growth factor receptor (EGFR) transactivation because the use of AG1478 blunted the activation of this MAPK. In addition, we have observed the proliferative effect of B1R stimulation on MDA-MB-231 estrogen-insensitive breast cancer cells (unpublished data). On the contrary, an antiproliferative effect has been observed in vascular smooth muscle cells, probably due to prolonged activation of MAPK and increased p27Kip1 activity (Dixon et al., 2002).

Studies on the involvement of B1R in cell migration have shown that in primary cultures of arterial smooth muscle cells, activation of B1R inhibited cell migration, an effect that involves activation of PI3K, but not nitric oxide or prostanoid release (Morissette et al., 2006). In contrast, activation of B1R induces the migration of human PC3 prostate cancer cells via activation of focal adhesion kinase (FAK), an important kinase involved in cytoskeletal reorganization and cell migration (Taub et al., 2003). On the other hand, studies performed in human neutrophils show that B1R stimulates leukocyte chemotaxis, though the pathways implicated in this process are still unknown (Ahluwalia & Perretti, 1996; Paegelow et al., 2002; Ehrenfeld et al., 2006). Moreover, it is known that release of the kinin moiety from kininogens sited on the neutrophil surface by enzymatic action of the classical tissue kallikrein (hK1) results in opening of junctions between the endothelial cells, thereby causing plasma extravasation (Stuardo et al., 2004). B1R modulates the release of matrix metalloproteases from both human neutrophils (Ehrenfeld et al., 2009) and breast cancer cells (Ehrenfeld et al., 2011), an event that may contribute to the extracellular matrix remodeling in processes such as inflammation, wound healing and cancer. Part of the effects produced by B1R in neutrophils and cancer cells include activation of ERK1/2 and p38 MAPK and transactivation of EGFR in breast cancer cells, an event that usually results in the activation of MAPK.

Phosphorylation of a 125-kDa protein, following stimulation of B1R expressed in human keratinocytes was identified as FAK (Yurko et al., 2001; Matus et al., 2008). The activation of this kinase after stimulation of B1R has also been observed in immortalized HaCaT keratinocytes (unpublished data) and in human PC3 prostate cancer cells (Taub et al., 2003). Moreover, we have shown that treatment of keratinocytes with herbimycin before stimulation with B1R agonists reduces FAK phosphorylation (Matus et al., 2008). Thus, it is likely that B1R activation may contribute to cell motility in several cell types. Another signaling pathway used by B1R includes activation of PKC. Overexposure of human keratinocytes to phorbol 12-myristate 13-acetate (PMA) or the preincubation with GF109203X, a potent and selective inhibitor of PKCs, demonstrated that phosphorylation of the EGFR was greatly reduced, corroborating the involvement of PKC (Matus et al., 2008).

#### **4. Mechanisms of EGFR transactivation by kinin B1 receptor**

It is known that various stress factors such as ultraviolet light and ionizing radiation can activate receptor tyrosine kinases (RTKs), like EGFR, in the absence of ligand. Also, activation of GPCRs, like the B1R, can activate RTKs by a mechanism that has been called "transactivation" by Alex Ullrich's group (Daub et al., 1996). They found that several GPCR agonists were able to activate the EGFR that in turn acts as a signal transducer for GPCRs. The EGFR belongs to a family of type I RTKs that comprise four members: EGFR (ErbB1/HER1), ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4) (reviewed by Liebmann, 2011). Activation of these receptors occurs by dimerization after ligand binding or by high receptor density on the cell membrane. When the ligand binds to its receptor, it induces the formation of homo and heterodimers activating the intrinsic tyrosine kinase domain that results in phosphorylation of specific tyrosine residues forming part of the cytoplasmic receptor tail. These residues serve as docking sites for a variety of signaling molecules (Endoh et al., 2009). Overexpression of EGFR is an indication of poor prognostic in multiple tumor types. EGFR autophosphorylation in sites such as Tyr845 are directly phosphorylated by Src kinase family (Wetzker & Böhmer, 2003) and GPCR agonists induce activation of Src, which directly phosphorylates EGFR by ligand-independent pathways (Biscardi et al., 1999; Tice et al., 1999). Furthermore, phosphorylation of EGFR at Tyr845 in the kinase domain is implicated in stabilizing the activation loop, maintaining the active state enzyme, and providing a binding surface for substrate proteins among which are the MAPK pathways (Cooper & Howell, 1993; Hubbard et al., 1994). EGFR and cytoplasmic tyrosine kinase c-Src cooperate in several cellular functions such as proliferation and apoptosis. Boerner et al., (2005) showed that c-Src-dependent phosphorylation of Tyr845 of EGFR is required for the DNA synthesis induced by activation of some G protein-coupled receptors in murine fibroblasts and breast cancer cells. The role of Tyr845 in DNA synthesis and cell proliferation was demonstrated by the microinjection of phosphoTyr845-containing peptide in these cells, which was able to ablate EGF-stimulate S-phase entry in both cell systems. This finding suggests that this residue is an important regulator of DNA synthesis induced by mitogens like EGF. Sato et al., (2003) using A431 carcinoma cells demonstrated that the expression of adaptor protein p52shc, or stimulation with EGF or H<sub>2</sub>O<sub>2</sub> leads to phosphorylation of EGFR on Tyr845. Phosphorylation in this residue was inhibited by PP2, but not by AG1478, and is associated with Src activation and phosphorylation of activators of transcription type 3 and 5 (STAT 3/5). This effect was inhibited by introduction of an

antibody against phosphorylated Tyr845 or by transfection of a dominant-negative for c-Src into the cells. Moreover, the co-incubation of purified c-Src and EGFR leads to phosphorylation of Tyr845 *in vitro*. Altogether, these results demonstrate that c-Src can directly phosphorylate EGFR on Tyr845. Similarly, different cell lines and animal studies have shown that MAPK and signal transducers like STAT-3 are important mediators of EGFR signaling after phosphorylation of different tyrosine residues in liver cells and hepatocellular carcinoma. Interestingly, it has been shown that homogenates of hepatocellular carcinomas present phosphorylation at Tyr845, but no EGFR phosphorylation at Tyr998, Tyr1045 or Tyr1068 (Kannangai et al., 2006). On the other hand, the SH2 domain of PLC $\gamma$  binds at phospho-Tyr992, resulting in activation of PLC $\gamma$ -mediated downstream signaling (Emlet et al., 1997). Phosphorylation of this residue was demonstrated in 38 of the 39 cases of esophageal carcinomas by immunohistochemistry with an anti-phosphoTyr992-EGFR antibody (Miyawaki et al., 2008). Immunoreactivity to phosphorylated Tyr992 was mainly associated with areas of severe dysplasia and microinvasive foci adjacent to invasive carcinoma, suggesting a role of phosphorylated Tyr992-EGFR in invasion. Moreover, phosphorylation of Tyr992, in responses of ionizing radiation in chinese hamster ovary or A431 cells, is related to activation of PLC $\gamma$  and increases when the phosphatase SHP2 is blocked (Sturla et al., 2005).

Phosphorylation of EGFR at Tyr1045 creates a major docking site for c-Cbl, an adaptor protein that leads to receptor ubiquitination and degradation following EGFR activation (Levkowitz, et al.,1999; Ettenberg, et al.,1999). The GRB2 adaptor protein binds activated EGFR at phospho-Tyr1068 (Rojas et al., 1996). Interestingly, Yamauchi et al.,(1998) demonstrated that binding of growth hormone to its receptor, which belongs to the cytokine receptor superfamily, activates Janus kinase tyrosine kinase, STAT proteins and MAPK that regulate expression of c-fos. This activation depends on phosphorylation of Tyr1068 by Janus kinase tyrosine kinase 2, providing docking sites for Grb2 and Shc protein adaptor and activating MAPK and gene expression. Phosphorylated Tyr1148 and Tyr1173 provide a docking site for the Shc scaffold protein and induce MAPK activation (Zwick, et al.,1999). Phosphorylation of these tyrosine residues is a key step for signaling activation and inhibition of intrinsic tyrosine kinase activity by highly potent and selective inhibitors such as AG1478 (tyrphostin), that block receptor phosphorylation and the subsequent signaling pathways triggered by EGFR (Fig. 3).

In several cell types, the EGFR transactivation induced by GPCRs is mediated by the release of EGFR ligands such as heparin-binding EGF-like growth factor (HB-EGF), transforming growth alpha (TGF- $\alpha$ ) and/or amphiregulin (Fig. 3). These ligands are generated by activation of the ADAM family (a disintegrin and metalloprotease) of zinc-dependent metalloproteases. Members of the ADAM family like ADAM10, ADAM12 and ADAM17 mediate GPCR-induced EGFR transactivation in different cell types (reviewed by Rozengurt et al., 2007). The mechanism by which members of the ADAM family are activated has been suggested to depend on the GPCR and the cell type involved. Activation can be through reactive oxygen species, PKC and Src, PI3K or ERKs (review by Ohtsu et al., 2006; Lemjabbar-Alaoui et al, 2011; Maretzky et al., 2011; Sun et al., 2010). In addition, different reports indicate that the use of Src inhibitors such as PP2 and GM6001, a broad spectrum metalloprotease inhibitor, partially block the MAPK or Akt pathways (Chen et al., 2011; Cramer et al., 2001; Mugabe et al., 2010; Stirnweiss et al., 2006) suggesting that GPCRs do not necessarily transactivate the EGFR to activate signaling pathways. Through

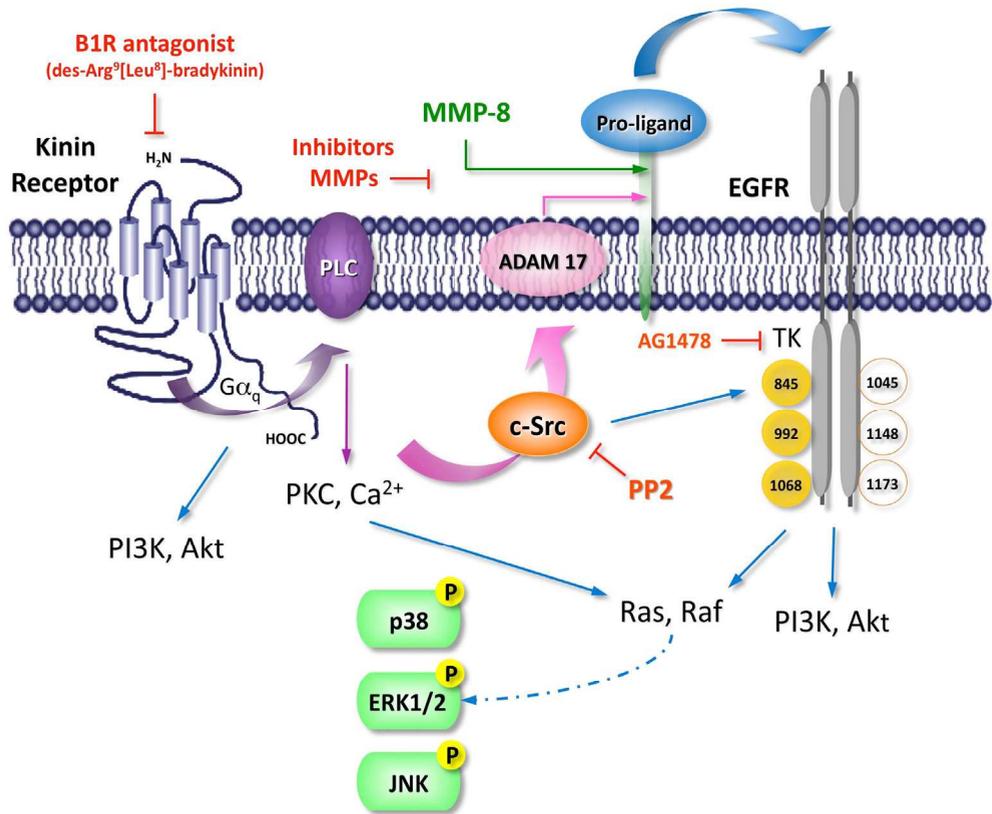


Fig. 3. Schematic representation of EGFR transactivation by a GPCR like the B1R. Stimulation of the B1R by its natural agonist may trigger specific signaling pathways and also produce activation of matrix metalloproteases (MMP-8, ADAM17) to release membrane-bound proligands that after being released will stimulate the EGFR. Stimulation of B1R also results in c-Src phosphorylation that may in turn produce phosphorylation of specific tyrosine residues (Tyr845, Tyr992, Tyr1068) of EGFR. Phosphorylation of other tyrosine residues such as Tyr1045, Tyr1148 and Tyr1173 has not been yet described. Transactivation of EGFR is used by the B1R to activate specific signaling pathways like p38, ERK1/2 and JNK MAPK. PLC, Phospholipase C.

transactivation, GPCRs can regulate cell growth, cell differentiation, survival and migration and play important roles in pathophysiological processes such as embryo development, wound healing and cancer progression.

Few studies have considered the transactivation of EGFR by either kinin B1 or B2 receptors. Studies performed in different cell types show that B2R activation triggers EGFR transactivation. The phosphorylation of ERK1/2 MAPK, produced by stimulation of COS-7 cells with a B2R agonist involves the independent activation of PKC and EGFR transactivation (Adomeit et al., 1999). Similar results in keratinocytes and renal IMCD-3 cells show that MAPK phosphorylation was partially and completely dependent on EGFR

transactivation, respectively (Mukhin et al., 2006 and Vidal et al., 2005). It has been shown that bradykinin can produce activation of p60Src and Src-dependent phosphorylation on Tyr845 of EGFR as well as recruitment of PLC $\gamma$  in primary cultures of rat adrenal chromaffin cells and PC12 cells (Hur et al., 2004). Moreover, Yang et al., (2005) demonstrated, using Western blot and [ $^3$ H]thymidine incorporation, that bradykinin induces proliferation of vascular smooth muscle cells mediated, at least in part, through activation of kinases of the Src family, EGFR transactivation, and PI3K-AKT pathways. Studies directed to elucidate the transactivation mechanism induced by B2R have shown that depending on the cell type, involve ADAM17 or metalloprotease 8 to cleave EGFR ligands (Dey et al., 2010; Methner et al., 2009). Reports regarding these events and the B1R are scarce. Matus et al., (2008) showed that stimulation of the kinin B1R in human keratinocytes produced the phosphorylation of tyrosine residues in a protein with a molecular mass of 170 kDa, that was later identified as EGFR. In these cells, the B1R induces the phosphorylation of Tyr845, Tyr992 and Tyr1068 residues on the EGFR molecule, an effect that was blocked by AG1478, a specific inhibitor of the EGFR tyrosine kinase activity. Similar results were obtained in the HaCaT keratinocyte cell line when EGFR transactivation by the B1R was analyzed (Matus et al., unpublished data).

Studies performed on estrogen-sensitive breast cancer cells strongly suggest that the proliferative effect induced by the B1R depends on the activity of EGFR and subsequent ERK1/2 MAPK phosphorylation (Molina et al., 2009). We have also reported that release of metalloproteases in MCF-7 and MDA-MB-231 breast cancer cells was blocked by AG1478, an observation that was confirmed by transfection of breast cancer cells with the dominant negative EGFR mutant HERCD533 (Ehrenfeld et al., 2011). The transactivation mechanism involved in EGFR activation by B1R agonists has not been elucidated yet, but work performed by our group in human HaCaT keratinocytes, MCF-7 breast cancer cell has visualized that B1R stimulation produces Src phosphorylation, that is blocked by the specific Src inhibitor, PP2 (Figs. 4 and 5). Interestingly, Src phosphorylates specifically the Tyr845 residue present in the active site of EGFR, therefore when we inhibited Src activity, phosphorylation of EGFR in Tyr845 was also inhibited (our group, unpublished data (Fig. 3).

## **5. Potential usefulness of currently available kinin antagonists and kinase inhibitors in pathological processes**

The importance of kinin receptors, especially the B1R, is illustrated by many publications that demonstrate their involvement in different pathological processes such as cancer, and especially breast cancer as mentioned early in the text. From a functional point a view, both B1 and B2 receptors are central players in the aetiology of pain, inflammation and cancer. Thus, the use of antagonists or inhibitors directed to specific intracellular pathways may be a useful approach to understand the mechanisms of particular pathological processes and then to promote them as useful pharmacological agents. Frequently, B2R is associated with the acute phase of inflammation and noniception, whereas the B1R after its up-regulation by inflammatory mediators is more relevant during chronic or persistent inflammation. For this reason, the use of antagonists of both peptidic and non-peptidic nature or blockade of kinase pathways triggered by activation of kinin receptors may become important clinical tools for treatment of persistent inflammation, cancer and pain, especially when no other therapy is available or provides beneficial effects (Campos et al., 2006).

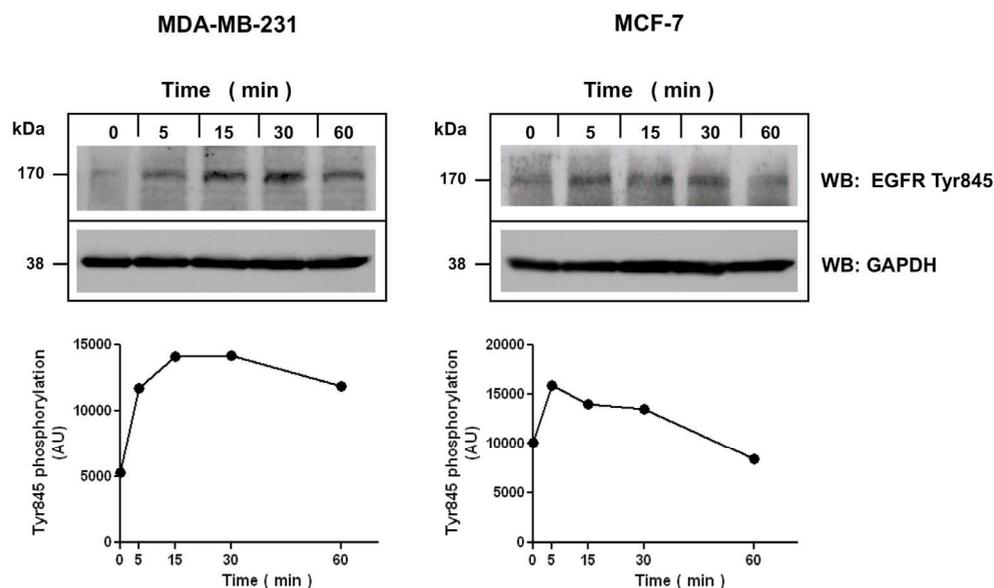


Fig. 4. The kinin B1R stimulation triggers transactivation of EGFR in estrogen-insensitive (MDA-MB-231) and estrogen-sensitive (MCF-7) breast cancer cells. Cells were cultured in DMEM-F12 or DMEM without phenol red, synchronized for 24 h and then stimulated with 10 nM Lys-des[Arg<sup>9</sup>]bradykinin for different periods of time. When stimulation was completed, the cells were homogenized in the presence of protease inhibitors, proteins separated by polyacrylamide gel electrophoresis and then transferred to immobilon membranes. Representative Western blots performed with a monoclonal antibody that detects phosphorylation on Tyr845 residue of the EGFR are shown. AU, arbitrary intensity units.

IL-4 is a cytokine with an important role in asthma and chronic obstructive pulmonary disease that promotes eosinophilic inflammation and mucus hypersecretion. In this regard, Bryborn et al.,(2004) demonstrated that long-term exposure to IL-4 increases contractile response of bronchial smooth muscle induced by B1 or B2 receptor agonists. This effect appears to be mediated via an up-regulation of B1R and MAPK pathways since the pre-treatment with SP600125, a c-Jun N-terminal kinase inhibitor or SB203580, a p38 MAPK inhibitor or PD98059, an inhibitor of ERK1/2 MAPK phosphorylation, inhibited that response. Similarly, IL-1 $\beta$  induces the up-regulation of B1R and B2R in mouse tracheal smooth muscle and increases the contractile responses induced by the respective kinin agonists (Zhang et al., 2007a). The effect was inhibited by SP600125 and TAT-TI-JIP, two JNK inhibitors, but not by inhibitors of ERK1/2 MAPK pathways (Zhang et al., 2007b). The importance of B1R in inflammatory reactions was also investigated by the group of Lin et al.,(2010) who demonstrated the involvement of B1R in cigarette smoke-induced airway inflammation by a mechanism that includes the participation of IL-1 $\beta$ . In fact, rat lung slices treated with total particulate matter of cigarette smoke for 24 h showed an enhanced expression of B1R and IL-1 $\beta$ . At the same time, Xu et al.,(2010) reported that nicotine induces airway hyperresponsiveness via transcriptional up-regulation of B1R and B2R and activation of JNK, but not ERK1/2 or p38 MAPK. The use of SP600125 a specific JNK inhibitor and

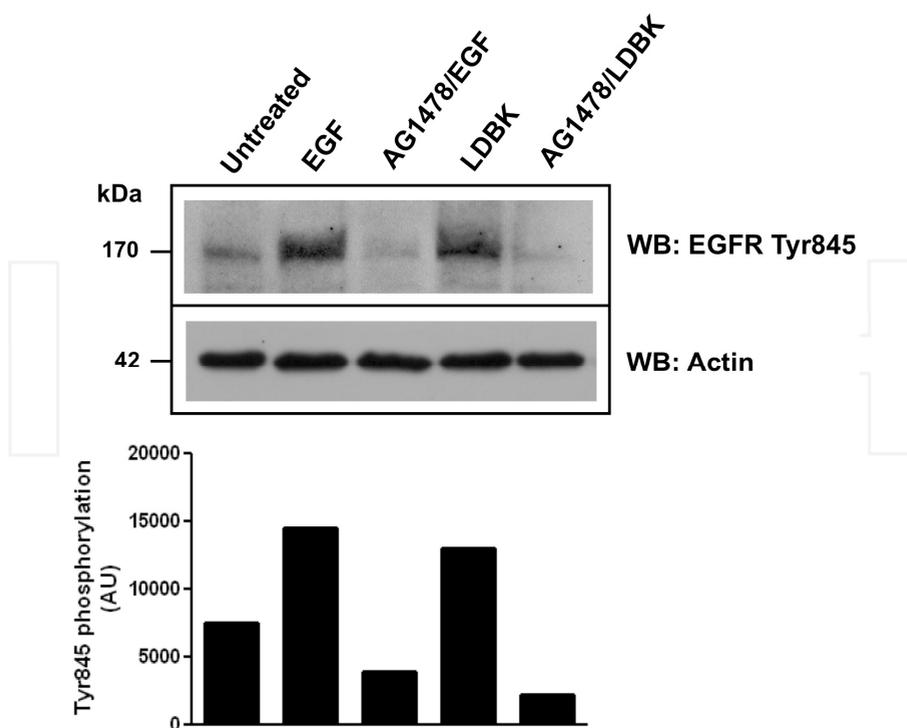


Fig. 5. Stimulation of the kinin B1R triggers transactivation of EGFR in human HaCaT keratinocytes. The epidermal cells were pre-incubated with 1  $\mu$ M of AG1478 for 30 min or directly stimulated with 100 nM Lys-des[Arg<sup>9</sup>]bradykinin for 15 min or 2.5 ng/ml EGF for 10 min. Proteins were separated by polyacrylamide gel electrophoresis and Western blots were performed using a monoclonal antibody that detects phosphorylation on Tyr845 residue of the EGFR molecule. A representative experiment is shown. AU, arbitrary intensity units.

YM976 a specific phosphodiesterase inhibitor abolished the nicotine-induced effects on kinin receptor-mediated contractions and reverted the enhanced receptor mRNA expression. Other reports demonstrate that the increased expression of B1R relays on oxidative stress, IL-1 $\beta$  and NF- $\kappa$ B, but not on cyclooxygenase or TNF- $\alpha$ . These results were obtained using the human alveolar epithelial A549 cell line treated with particulate matter. The increase of B1R mRNA was prevented by co-treatments with N-acetyl-l-cysteine (a potent antioxidant), diphenyleneiodonium (NADPH oxidase inhibitor), IL-1Ra (IL-1 receptor antagonist) and SN-50 (specific inhibitor of NF- $\kappa$ B activation), but not by pentoxifylline (TNF- $\alpha$  release inhibitor), indomethacin and niflumic acid (COX-1 and 2 inhibitors)(Talbot et al., 2011).

In the skin, the mRNAs for hK1 tissue kallikrein and B1R have been reported in both normal human skin and in biopsies of patients with some skin diseases (Schremmer-Danninger et al., 1995; 1998; 2004; Matus et al., 2008; Pietrovski et al., 2009). Furthermore, the expression of B1R mRNA and protein has been demonstrated in keratinocytes of normal human

epidermis, in primary cultures and in the immortalized HaCaT cell line (Matus et al., 2008). Additionally, the expression of B1R in skin may be enhanced when this organ is exposed to many noxious agents present in our environment. In cutaneous models of inflammation developed in mice, peptidic and non-peptidic kinin receptor antagonists have been effective in reducing cutaneous neurogenic inflammation (Pietrovski et al., 2009). Recently, Pietrovski et al., (2011) demonstrated that both B1R and B2R participate in the hyperproliferative and inflammatory responses of mouse skin suggesting that the use of kinin receptor antagonists may be useful in the treatment of some skin disorders.

Another disease in which the role of B1R has been considered is diabetes. Dias & Couture (2011) used the B1R antagonist, SSR240612 in a rat model of diabetes and insulin resistance, based on the hypothesis that B1R activates signaling pathways that lead to increased oxidative stress and promote insulin resistance (Dias et al., 2010). The use of such an experimental design reversed the effects of glucose on the expression of IL-1 $\beta$ , TNF- $\alpha$ , and macrophage CD68 that are linked to the deposition of adipose tissue. Given the association between chronic inflammation and insulin resistance, inhibition of B1R activity may contribute to increase sensitivity to insulin and to prevent obesity. In general, diabetes induces sensory polyneuropathy associated with pain (allodynia and thermal hyperalgesia), conditions that are difficult to treat. For this reason, Dias et al., (2007) tested the non-peptide and orally active B1R antagonist SSR240612 in a rat model of insulin resistance and tactile cold allodynia. Their results are the first to associate the B1R with allodynia, pain that was alleviated by treatment with SSR240612, probably by direct inhibition of B1R affecting the spinal cord and/or afferent sensory nerve excitation. Hawkinson et al., (2007) probed other B1R antagonist named benzamide B1R antagonist 7-chloro-2-[3-(9-pyridin-4-yl-3,9-diazaspiro[5.5]undecanecarbonyl)phenyl]-2,3-dihydro-isoindol-1-one (ELN441958). This antagonist reduces in a dose-dependent manner the carrageenan-induced thermal hyperalgesia in a rhesus monkey tail-withdrawal model.

In renal disease, B1R has been associated with tissue inflammation and renal fibrosis. Focal and segmental glomerulosclerosis is one of the most important causes of end-stage renal failure and up-regulation of B1R in podocyte injury has been demonstrated by Pereira et al., (2011). Despite this report, there are not additional studies that examine the use of specific antagonists or inhibitors for specific signaling pathways activated by the B1R in this disease. Respect to the participation of the kinin B1R in cancer, only a few reports indicates the importance of interrupting its intracellular signaling; the major efforts have been orientated to the B2R. Thus, many studies have tried to elucidate the importance of B2R in cancer and the consequence of using specific antagonists to reduce cell proliferation, enhance apoptosis or decrease the secretion of metalloproteases (Chan et al., 2002; Stewart et al., 2002; 2003; 2005; Jutras et al., 2010; Wang et al., 2010). Although one of these studies demonstrate that both PGE<sub>2</sub> and BK stimulate invasion of head and neck squamous carcinoma cells via EGFR, the treatment of these cells with the B2R antagonist CU201 resulted in growth inhibition (Thomas et al., 2006). The combination of CU201 with the EGFR small-molecule inhibitor erlotinib resulted in additive inhibitory effects on cell growth in the same type of cells *in vitro*.

## 6. Conclusion

Despite the high number of reports demonstrating the involvement of B1R in the regulation of various diseases, studies on the use of specific kinin antagonists and/or kinase inhibitors

as potential therapeutic drugs has been limited. Therefore, further validation of kinin receptors as important therapeutic targets is crucial. Even though EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib interfere with ATP binding and have been used in clinical trials, inhibiting tyrosine kinase activity and subsequently inhibiting signal transduction pathways triggered by EGFR such as MAPK, PI3K/Akt, STAT and PLC $\gamma$ /PKC. Activation of these pathways also influences cell proliferation, survival and the metastatic potential of tumor cells. Others EGFR tyrosine kinase inhibitors (PD15035, AG1478 and PKI 166) have been tested in animal model as mice with Type 2 Diabetic, mice with high-fat diet-fed or rats with diabetic nephropathy. Results of experiments indicate that these inhibitors restore ischemia-induced neovascularization and blood flow recovery in type 2 diabetic mice, improves glucose tolerance, insulin sensitivity and reduces subclinical inflammation in high-fat diet-fed mice and attenuates the development diabetic nephropathy in rats. (Advani et al., 2011; Choi et al., 2011; Prada et al., 2009).

Despite the success of inhibitors reported in clinical trials, some patients still develop resistance to anti-EGFR therapy. In addition, there are two mutations of EGFR that have been described. Mutations of the exon coding for tyrosine kinase domain (18 to 21) and truncating mutations (exons 2 to 7) that involves signaling pathways. The first group of mutations is involved in the failure of tyrosine kinase inhibitors whereas the second group of mutation leads to resistance of drugs (Colabufo, 2011). However, an alternative approach for overcoming resistance may be to simultaneously target additional pathways such as VEGF/VEGFR, and/or inhibit parallel signaling pathways that complement those activated by EGFR (e.g., mesenchymal-epithelial transition factor and mTOR) (Giaccone & Wang, 2011) and we suggest, by the evidence expose in this chapter, may include the B1R, or B2R. Furthermore, understanding of the intracellular signaling pathways initiated by kinin receptors linked to EGFR and their involvement in the causation and progression of most human neoplasias (e.g., non-small cell lung cancer, colorectal cancer, glioblastoma, breast cancer and squamous cell carcinomas) suggests that these signaling pathways could be used as targets for development of novel drugs that inhibit EGFR activation. Currently there are insufficient studies on the inhibition of EGFR transactivation triggered by B1R to elucidate whether the interaction between EGFR and B1R could provide novel molecular agents for prevention or treatment of human cancer.

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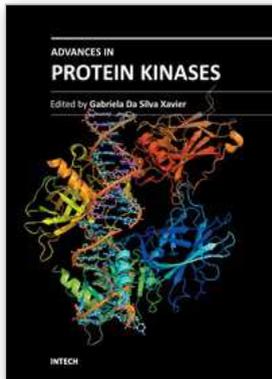
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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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