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Review

α_1-Adrenoceptors: function and phosphorylation

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Abstract

This review focuses on α_1-adrenoceptor phosphorylation and function. Most of what is currently known is based on studies on the hamster α_{1B}-adrenoceptor. It is known that agonist stimulation leads to homologous desensitization of these receptors and current evidence indicates that such decrease in receptor activity is associated with receptor phosphorylation. Such receptor phosphorylation seems to involve G protein-receptor kinases and the receptor phosphorylation sites have been located in the carboxyl tail (Ser^{404}, Ser^{408}, and Ser^{410}). There is also evidence showing that in addition to desensitization, receptor phosphorylation is associated with internalization and roles of β-arrestins have been observed. Direct activation of protein kinase C leads to receptor desensitization/internalization associated with phosphorylation; the protein-kinase-C-catalyzed receptor phosphorylation sites have been also located in the carboxyl tail (Ser^{394} and Ser^{400}). Activation of G_q-coupled receptors, such as the endothelin ET_A receptor induces α_{1B}-adrenoceptor phosphorylation and desensitization. Such effect involves protein kinase C and a yet unidentified tyrosine kinase. Activation of G_i-coupled receptors, such as the lysophosphatidic acid receptor, also induces α_{1B}-adrenoceptor phosphorylation and desensitization. These effects involve protein kinase C and phosphatidyl inositol 3-kinase. Interestingly, activation of epidermal growth factor receptors also induces α_{1B}-adrenoceptor phosphorylation and desensitization involving protein kinase C and phosphatidyl inositol 3-kinase. A pivotal role of these kinases in heterologous desensitization is evidenced.

Keywords: α_1-Adrenoceptor; Receptor phosphorylation; Protein kinase C; Phosphatidylinositol 3-kinase; G protein receptor kinases; Desensitization

1. Introduction

Adrenoceptors are a heterogeneous group of hormone/neurotransmitter receptors that mediate the central and peripheral actions of the natural adrenergic amines, adrenaline, and noradrenaline. These receptors constitute a subfamily of the seven transmembrane domains/G-protein-coupled receptors, and have been divided into three major types based on their affinities for agonists and antagonists, their coupling to signaling pathways, and their amino acid sequences. The major types are the α_1-, the α_2- and the β-adrenoceptors (Hieble et al., 1995). Three receptor isoforms have been cloned of each of these three major types (Hieble et al., 1995).

It is well-known that α_1-adrenoceptors are mainly coupled to $G_{q/11}$ to stimulate phospholipase C activity. This enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the subsequent formation of inositol 1,4,5-trisphosphate and diacylglycerol. These molecules act as second messengers mediating intracellular Ca^{2+} release and activation of protein kinase C, respectively. Nevertheless, it is now clear that these α_1-adrenoceptors can also be coupled to other classes of G proteins and, therefore, are capable of modulating different signaling pathways. Reviews on the structure, subtypes, tissue distribution and signaling of these receptors have been published recently (Graham et al., 1996; García-Sáinz et al., 1999c; Zhong and Minneman, 1999).

Usually, when cells are exposed to an agent, their subsequent responsiveness is decreased or blunted. This biological phenomenon is called desensitization. Different cellular processes, with different time frames, seem to be involved. These include modulation of receptor function, receptor internalization, recycling to the plasma membrane, degradation and regulation of expression (Lefkowitz, 1998; Lefkowitz et al., 1998). We will restrict our

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review to the initial events, which are associated to receptor phosphorylation.

Phosphorylation of receptors with endogenous protein tyrosine kinase activity is associated to signaling turn-on and dephosphorylation with turning-off (Carpenter, 1987; Yarden and Ullrich, 1988). Similarly, dephosphorylation of receptors with guanylyl cyclase activity seems to be involved in desensitization (Schulz et al., 1989; Potter and Hunter, 1999). In contrast, phosphorylation of G-protein-coupled receptors is associated to signaling turn-off/desensitization and receptor dephosphorylation with resensitization (Premont et al., 1995; Ferguson et al., 1997).

Two major types of desensitization have been distinguished: homologous and heterologous desensitizations. In the homologous type, reduced responsiveness is observed exclusively in the agent (a related agonist) that originally stimulated the cells. In heterologous desensitization a decreased responsiveness is observed in an agent or agents unrelated to the initial stimulus. Certainly, this classification is only operational and both desensitization processes may occur simultaneously in cells. Nevertheless, it has interesting mechanistic implications. In homologous desensitization hormone/neurotransmitter receptors seem to be the molecular targets of the process, whereas in heterologous desensitization, receptors and other distal signaling devices can be affected.

The present review focuses on α_1-adrenoceptor phosphorylation and function. Reference will be made to what it is known in β_2-adrenoceptor phosphorylation, since these receptors have been studied to a much bigger extent. As indicated above, α_1-adrenoceptors are heterogeneous and three subtypes have been already cloned, i.e., α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors (Hieble et al., 1995). Most of what it is known on the phosphorylation of this group of receptors is based on data on the hamster α_{1B} subtype. We will concentrate on this subtype, and at the end of the review, we will address the differences that are likely to exist among the three subtypes, in the regulation of their function by phosphorylation (Vázquez-Prado and García-Sáinz, 1996; Vázquez-Prado et al., 1997, 2000).

2. Homologous desensitization

2.1. General aspects

It is generally accepted that homologous desensitization involves receptor phosphorylation by G-protein-coupled receptor kinases (Ferguson et al., 1997; Krupnick and Benovic 1998). G protein receptor kinases are a family of at least six serine/threonine protein kinases that phosphorylate G-protein-coupled receptors only in the agonist-bound state. Accordingly, receptors occupied by agonist activate heterotrimeric G proteins, releasing $G\beta\gamma$ complexes. Such membrane-bound $G\beta\gamma$ heterodimers and phosphatidylinositol bisphosphate bind to the carboxyl terminal domain of soluble G protein receptor kinases (particularly G protein receptor kinase 2) that targets the kinase to the receptor (Pitcher et al., 1998). These enzymes phosphorylate the receptors, which markedly increases the receptor affinity for arrestin molecules (Fig. 1). The binding of arrestin proteins to the receptors sterically interdicts the receptor–G protein interaction, stabilizing the uncoupled state of the receptor (Krupnick and Benovic, 1998).

In addition, β-arrestin binds with clathrin molecules with high affinity, directly and stoichiometrically (Goodman et al., 1996). This initiates the internalization of phosphorylated receptors into vesicles. Dynamin, a GTPase that regulates the formation and internalization of clathrin-coated vesicles is essential for agonist-promoted sequestration of some G-protein-coupled receptors such as the β_2-adrenoceptor (Zhang et al., 1996). At least two distinct endocytic pathways seem to exist with different requirements for β-arrestin and dynamin (Zhang et al., 1996). Caveolae are vesicular structures in cells that participate in transcytosis, and signaling molecules associate with them, suggesting that these structures may participate in organizing signaling pathways. Caveolin, an integral membrane protein, is the major structural component of caveolae. Interestingly, caveolin interacts with G protein receptor kinase 2 and modulates its kinase activity (Carman et al., 1999).

Receptor phosphorylation by G protein receptor kinases is a cardinal event that triggers receptor desensitization and internalization. Interestingly, however, recent evidence suggests that G protein receptor kinases may also inhibit receptor signaling through phosphorylation-independent processes (Dicker et al., 1999). Thus, it has been shown, using kinase-negative mutants of G protein receptor kinases and truncated receptors, that binding of G protein receptor kinases to the receptors inhibits signaling; such binding constitutes a first step in homologous desensitization (Dicker et al., 1999). Similarly, there is evidence that arrestins may bind and desensitize some G-protein-coupled receptors in the absence of receptor phosphorylation; receptor activation seems to trigger such processes (Mukherjee et al., 1999).

2.2. α_{1B}-Adrenoceptor homologous desensitization

Using hamster DDT_1 MF-2 cells, it was first observed that noradrenaline promoted desensitization and phosphorylation of α_{1B}-adrenoceptors (Leeb-Lundberg et al., 1987). These authors also observed that the agonist induced a marked decrease in the number of surface receptors, i.e., induced receptor internalization (Leeb-Lundberg et al., 1987). It has been observed that noradrenaline markedly increases receptor phosphorylation using the hamster α_{1B}-adrenoceptor transfected into rat-1 fibroblasts (Lattion et al., 1994; Diviani et al., 1996, 1997; Vázquez-Prado et al., 1997). Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitiza-

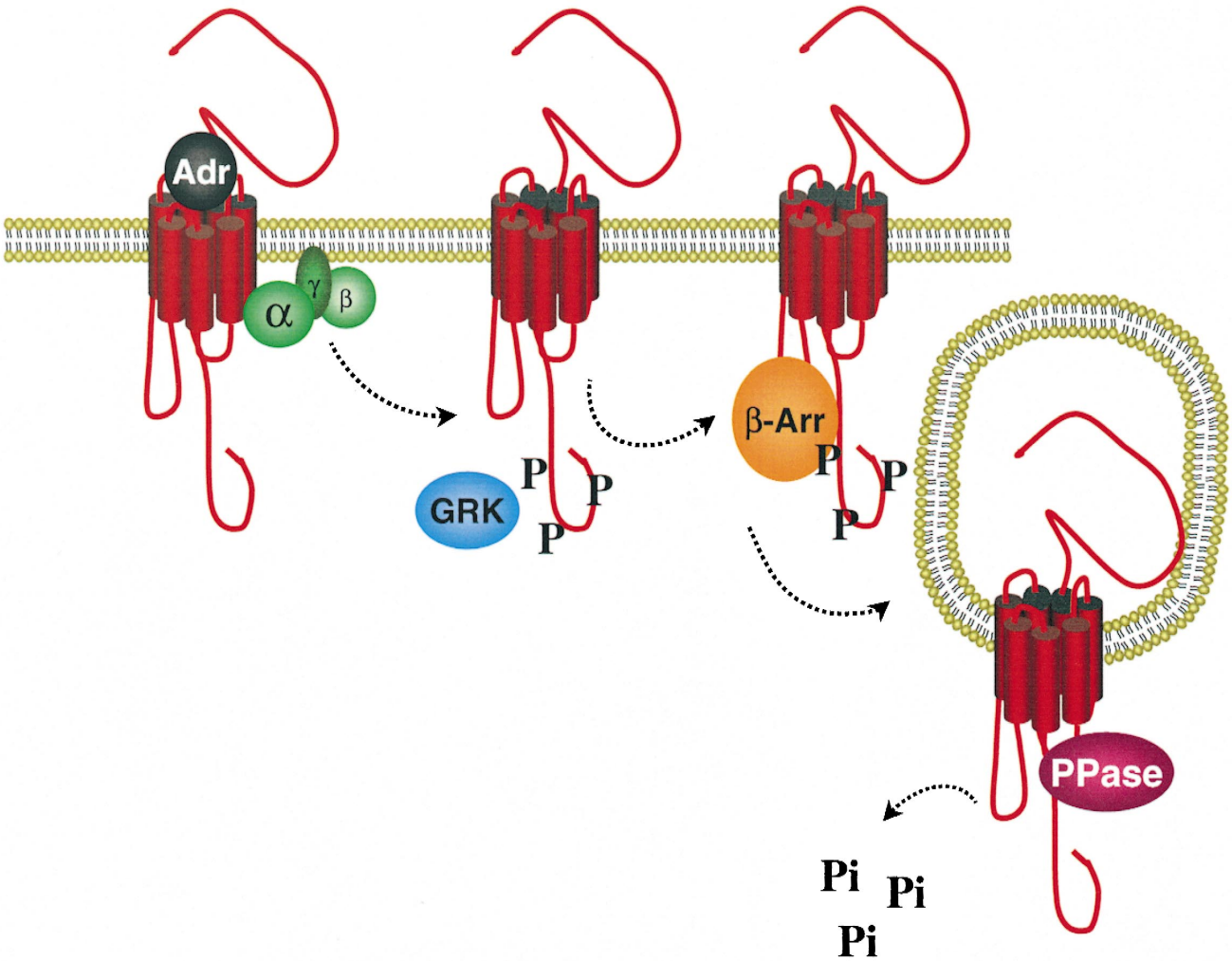

Fig. 1. Model for agonist-induced α_{1B}-AR phosphorylation. GRK, G protein receptor kinase; β-Arr, β-arrestin; PPase, protein phosphatase; Adr, adrenaline.

tion, which indicated that such domain plays a very important regulatory function in the α_{1B}-adrenoceptors (Lattion et al., 1994). In addition, these authors observed that such agonist-dependent phosphorylation was not affected by inhibitors of protein kinase C (Lattion et al., 1994). Latter studies by Cotecchia and her group showed that co-expression of the α_{1B}-adrenoceptor and G protein receptor kinases 2 or 3 increased the agonist-induced phosphorylation of the receptor, and that a dominant negative G protein receptor kinase 2 (K220R) mutant impaired agonist-induced α_{1B}-adrenoceptor phosphorylation (Diviani et al., 1996). Furthermore, they showed that co-expression of α_{1B}-adrenoceptors and these G protein receptor kinases attenuated the adrenergic action on phosphoinositide hydrolysis (Diviani et al., 1996). Surprisingly, however, it has been observed that α_{1B}-adrenoceptor-mediated effects are little affected by G protein receptor kinase 2 or the dominant negative mutant G protein receptor kinase 2 (K220R) at low levels of G protein receptor kinase expression in FRTL-5 cells (Iacovelli et al., 1999). In this study, the action of the thyroid-stimulating hormone receptor was greatly affected by expression of G protein receptor kinases, suggesting that different receptors may be under different levels of control by these protein kinases (Iacovelli et al., 1999). It is also worth noticing that co-expression of α_{1B}-adrenoceptor and G protein receptor kinase 5 or 6 increased basal- but not agonist-induced phosphorylation of the receptor, and that such phosphorylations did not affect receptor-mediated actions in whole cells (Diviani et al., 1996).

In a very elegant study using site-directed mutagenesis, Diviani et al. (1997) characterized the phosphorylation sites involved in G protein receptor kinase-mediated desensitization of α_{1B}-adrenoceptors, located in the carboxyl tail (Ser^{404}, Ser^{408}, and Ser^{410}). All these studies clearly indicated that when the α_{1B}-adrenoceptors are activated by agonists, G protein receptor kinases phosphorylate the receptors and that such phosphorylation is associated with

desensitization and receptor internalization. Surprisingly, there are great differences in agonist-induced internalization of α_{1B}-adrenoceptors, probably depending on the cell type. Thus, in human embryonal kidney 293 (HEK 293), cells stably expressing hamster α_{1B}-adrenoceptors, it was observed that noradrenaline induced a rapid and striking internalization of cell surface receptors as visualized by confocal inmmunofluorescence microscopy (Fonseca et al., 1995). In contrast, the agonist-stimulated α_{1B}-adrenoceptor internalization that is observed in rat-1 fibroblasts is much slower and involves only a relatively small proportion of the total surface receptors (Lattion et al., 1994; Vázquez-Prado et al., 1997). In DDT_1 MF-2 cells, agonists induce $\sim$ 30% α_{1B}-adrenoceptor internalization in 30 min (Leeb-Lundberg et al., 1987; Cowlen and Toews, 1988). Interestingly, in these cells, agonists induced the sequestration of the receptors to an intracellular compartment that remains associated to the plasma membrane (Cowlen and Toews, 1988). It has been observed that surface-sorting and agonist-promoted internalization of α_{1B}-adrenoceptors are two independent processes involving different components of the cellular endocytic machinery. Basal surface-sorting was sensitive to brefeldin A, an inhibitor of vesicular transport, and that agonist-promoted internalization was sensitive to actin depolymerization agents such as cytochalasin D and mycaloide B (Hirasawa et al., 1998). Real-time optical monitoring of agonist-mediated internalization of α_{1B}-adrenoceptors tagged with the green fluorescent protein, in mouse αT3 cells, indicated that the receptor internalized and redistributed in intracellular compartments, within minutes of exposure to agonists (Awaji et al., 1998). It is surprising that although protein kinase C activity does not seem to play a major role in agonist-mediated phosphorylation of α_{1B}-adrenoceptors (Lattion et al., 1994), it seems to do so in receptor internalization (Fonseca et al., 1995; Awaji et al., 1998).

Using constitutively active α_{1B}-adrenoceptors, it has been observed that when different receptor domains are mutated, divergent effects on receptor phosphorylation and internalization take place; it was concluded that agonist-independent activity of α_{1B}-adrenoceptor mutants does not necessarily correlate with any of these parameters (Mhaouty-Kodja et al., 1999).

Expression of arrestin proteins attenuated α_{1B}-adrenoceptor-mediated phosphoinositide hydrolysis and a dominant negative arrestin mutant inhibited adrenoceptor internalization, in agreement with what has been observed with other G-protein-coupled receptors (Diviani et al., 1996; Mhaouty-Kodja et al., 1999).

All the previously mentioned α_{1B}-adrenoceptor phosphorylation studies have been performed using the hamster receptor endogenously expressed in DDT_1 MF-2 cells or receptors transfected into model cells. We have recently observed that the human α_{1B}-adrenoceptors are also phosphorylated in response to agonist stimulation (García-Sáinz et al., 1999a).

3. Heterologous desensitization

3.1. General aspects

It has been observed that many G-protein-coupled receptors are desensitized via feedback regulation by second-messenger-stimulated kinases, such as protein kinase A and protein kinase C. This type of desensitization is heterologous, since in principle, any stimulant that can increase cyclic AMP or diacylglycerol has the potential to induce the phosphorylation and desensitization of any G-protein-coupled receptor containing the consensus phosphorylation sites for protein kinase A or protein kinase C. Certainly, other proteins involved in signaling and containing such consensus sites can also be phosphorylated, and, as a consequence, their function can be modified. Similarly, a single receptor type can be phosphorylated and desensitized by multiple protein kinases (Nambi et al., 1985).

In addition to second-messenger-activated kinases, some receptors with endogenous protein tyrosine kinase activity have been shown to induce the phosphorylation in tyrosine residues of G-protein-coupled receptors, such as β_2-adrenoceptors, and lead to desensitization (Hadcock et al., 1992; Baltensperger et al., 1996; Karoor and Malbon, 1996).

In the case of α_{1B}-adrenoceptors, there is ample evidence that protein kinase C plays a key role in heterologous desensitization/phosphorylation, and this will be reviewed in the following sections. Nevertheless, it is important to mention that it has been observed in vitro that protein kinase A can phosphorylate α_{1B}-adrenoceptors purified from DDT_1 MF-2 cells (Bouvier et al., 1987) on the third intracellular loop of the mouse α_{1B}-adrenoceptor fused to glutathione-S-transferase (Alonso-Llamazares et al., 1997). However, there is no evidence suggesting that such protein-kinase-A-mediated phosphorylation might take place in vivo and less is known on its possible functional consequences.

3.2. α_{1B}-Adrenoceptor phosphorylation / desensitization by phorbol esters

It was first shown using rat hepatocytes that activation of protein kinase C with phorbol esters blocked α_{1B}-adrenoceptor action (Corvera and García-Sáinz, 1984; Corvera et al., 1986; García-Sáinz et al., 1985). Leeb-Lundberg et al. (1985) confirmed this finding and showed that phorbol esters induced α_{1B}-adrenoceptor phosphorylation using DDT_1 MF-2 cells. Using purified α_{1B}-adrenoceptor from these cells it was observed that protein kinase C induced receptor phosphorylation and that interestingly, such phosphorylation was further increased by noradrenaline in vitro (Bouvier et al., 1987). The ability of phorbol esters to induce the phosphorylation of this adrenoceptor has been studied using rat-1 fibroblast expressing the hamster α_{1B}-adrenoceptor (Lattion et al., 1994; Diviani et al., 1997; Vázquez-Prado et al., 1997). Experiments using α_{1B}-

adrenoceptor truncated at the carboxyl terminus showed that the receptor increased its phosphorylation state in response to phorbol esters, but only slightly. These data indicated that the carboxyl tail contained the major sites for protein-kinase-C-mediated phosphorylation/desensitization (Lattion et al., 1994). This was later characterized by Diviani et al. (1997) who identified the sites as Ser^{394} and Ser^{400}. The phosphoinositide hydrolysis observed in response to agonists of the truncated α_{1B}-adrenoceptor was still impaired by phorbol esters, although to a very limited extent (15%). Moreover, as indicated, this was associated to a small level of phorbol-ester-induced phosphorylation (Diviani et al., 1997). These data suggest that a yet uncharacterized site exists in α_{1B}-adrenoceptors, in a domain separate from the carboxyl tail, which can be phosphorylated by protein kinase C, and plays an apparent minor role in receptor desensitization (Diviani et al., 1997).

Recently, we have observed that the human α_{1B}-adrenoceptor expressed in mouse fibroblasts is also phosphorylated in response to activation of protein kinase C by phorbol esters (García-Sáinz et al., 1999a). Such phosphorylation is associated with blockade of α_{1B}-adrenoceptor-mediated increases in cytosol Ca^{2+} and phosphoinositide hydrolysis in whole cells; phosphorylation is also associated to inhibition of adrenergic-stimulated [^{35}S]GTPγS binding in membranes, which suggests receptor-G protein uncoupling (García-Sáinz et al., 1999a). The actions of phorbol esters were blocked by protein kinase C inhibitors and by overnight treatment with phorbol esters that leads to protein kinase C downregulation (García-Sáinz et al., 1999a). Yang et al. (1999) have studied the human α_{1B}-adrenoceptors expressed in rat-1 fibroblasts and observed that treatment for 24 h with phorbol esters did not change receptor density or cause functional desensitization.

The action of active phorbol esters on α_{1B}-adrenoceptors seems to involve several processes. It has been observed that the blockade of the receptor function is very rapid, which is consistent with receptor phosphorylation and uncoupling from G proteins. Such uncoupling has been detected as a loss in the ability of GTP and hydrolysis-resistant analogues to modulate agonist-binding affinity, i.e., conversion of the receptors to the low affinity state (Jagadeesh and Deth, 1988; Beeler and Cooper, 1993) and also as a decreased α_{1B}-adrenoceptor agonist-mediated [^{35}S]GTPγS binding (García-Sáinz et al., 1999a; Alcántara-Hernández et al., 2000; Vázquez-Prado et al., 2000). It has also been observed that activation of protein kinase C with phorbol esters leads to receptor internalization in cells that endogenously express α_{1B}-adrenoceptors, such as hepatocytes (Lynch et al., 1985; Beeler and Cooper, 1995) and in transfected cells, such as HEK 293 cells (Fonseca et al., 1995) or mouse αT3 cells (Awaji et al., 1998). In DDT_1 MF-2 cells, it has been observed that phorbol 12-myristate 13-acetate did not induce receptor internalization by itself. However, when the cells were incubated with this phorbol ester and noradrenaline or adrenaline, there was a marked receptor internalization (bigger than that induced by the agonists alone) and redistribution of these receptors into intracellular vesicles (light vesicle fraction) (Cowlen and Toews, 1988).

3.3. α_{1B}-Adrenoceptor phosphorylation / desensitization via receptors coupled to G_q

Receptors coupled to G_q increase phosphoinositide hydrolysis, generating diacylglycerol and inositol trisphosphate. Such diacylglycerol can activate protein kinase C and putatively induce α_{1B}-adrenoceptor phosphorylation and desensitization. We observed that activation of endothelin ET_A receptors endogenously expressed in rat-1 fibroblasts stably expressing transfected α_{1B}-adrenoceptors induced a marked and rapid phosphorylation and desensitization of these adrenoceptors (Vázquez-Prado et al., 1997). Interestingly, such effect was only partially blocked by either staurosporine, a protein kinase inhibitor, and genistein, a protein tyrosine kinase inhibitor, but it was completely blocked when both inhibitors were present (Vázquez-Prado et al., 1997). Surprisingly, phosphoaminoacid analysis revealed phospho-serine and phosphothreonine in the α_{1B}-adrenoceptors but no phospho-tyrosine (Vázquez-Prado et al., 1997). It has been observed that activation of G_q-coupled receptors, such as the endothelin ET_A receptor, can activate (transactivate) EFG receptors (Daub et al., 1996). A Ca^{2+}-dependent phosphorylation cascade seems to participate in transactivation of epidermal growth factor (EGF) receptors by G_q-coupled receptors (Zwick et al., 1997; Selbie and Hill, 1998). However, in our experiments, intracellular Ca^{2+} seems to play a small role in the α_{1B}-adrenoceptor phosphorylation induced by endothelin (García-Sáinz et al., 1999b) and it was insensitive to wortmannin (Vázquez-Prado et al., 1997). These data suggest that transactivation of EGF receptors does not play a major role in this effect of endothelin (see below). Our data suggest that protein kinase C phosphorylates the α_{1B}-adrenoceptors and that a yet unidentified protein tyrosine kinase, which does not seem to phosphorylate the receptor directly, participates in a phosphorylation cascade independent of protein kinase C (Fig. 2).

In addition to endothelin, it has been observed that bradykinin can also induce α_{1B}-adrenoceptor phosphorylation (Leeb-Lundberg et al., 1987; Medina et al., 1998). The receptor phosphorylation induced by bradykinin in rat-1 cells stably expressing α_{1B}-adrenoceptors was of small magnitude as compared to that induced by endothelin and no evidence of desensitization was observed in whole cells (Medina et al., 1998).

3.4. α_{1B}-Adrenoceptor phosphorylation / desensitization via receptors coupled to G_i

Lysophosphatidic acid is a water-soluble phospholipid that is a mitogen for many cells, including fibroblasts. The

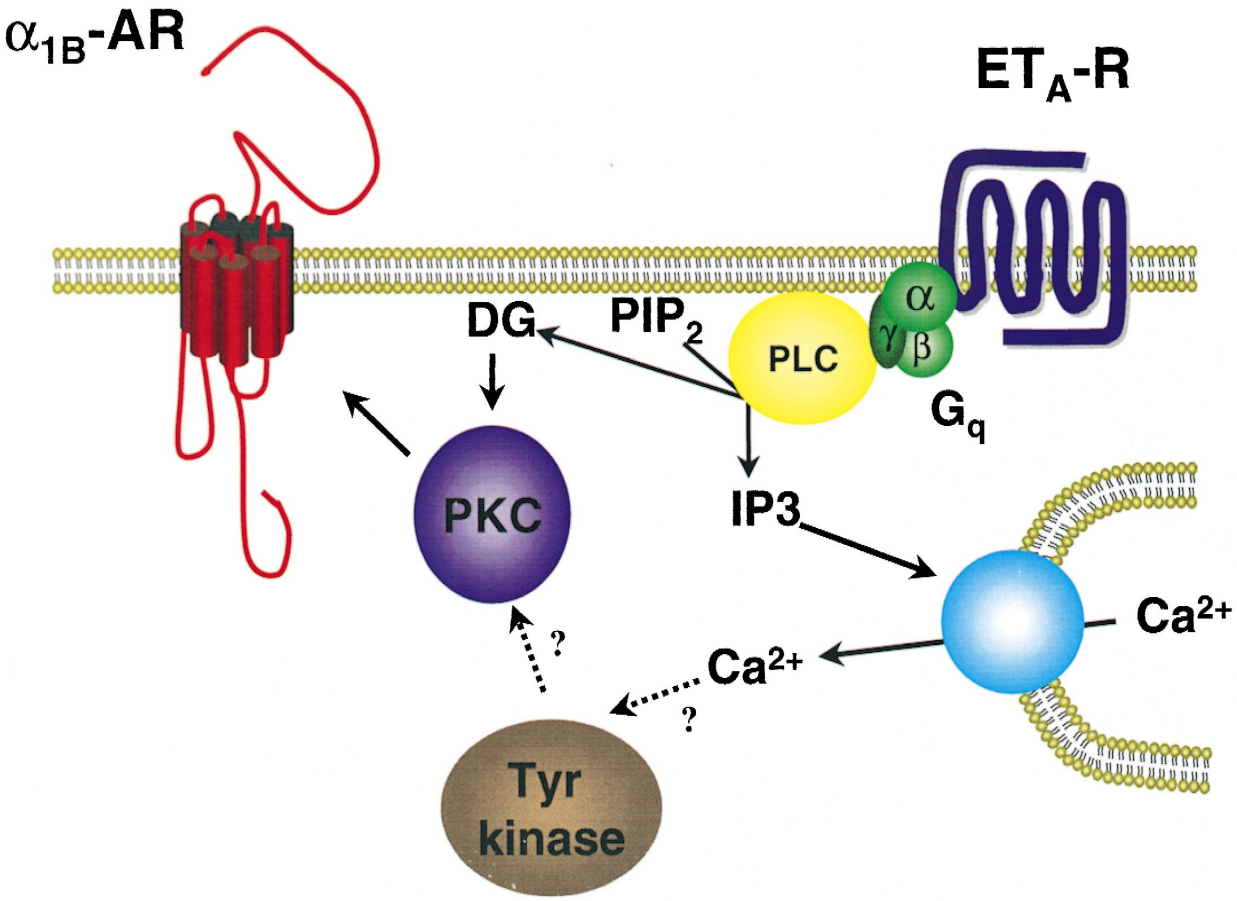

Fig. 2. Model for the effect of a G_q-coupled receptor on α_{1B}-AR phosphorylation. α_{1B}-AR, α_{1b}-adrenoceptor; ET_A, endothelin ET_A receptor: PLC, phospholipase C; PIP2, phosphatidyl inositol 4,5-bisphosphate; DG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; Tyr kinase, protein tyrosine kinase; PKC, protein kinase C.

actions of lysophosphatidic acid are mediated through seven transmembrane domain receptors (Fukushima et al., 1998; Chun et al., 1999) mainly via heterotrimeric G_i proteins (Van Corven et al., 1993; Carr et al., 1994; Hordijk et al., 1994; Chuprun et al., 1997). These receptors are endogenously expressed in rat-1 fibroblasts stably expressing transfected α_{1B}-adrenoceptors. Lysophosphatidic acid induced a rapid, intense and sustained α_{1B}-adrenoceptor phosphorylation (manuscript submitted for publication). In contrast to the pertussis toxin insensitivity of the α_{1B}-adrenoceptor phosphorylation induced by endothelin (Vázquez-Prado et al., 1997) the effect of lysophosphatidic acid was blocked by pretreatment with pertussis toxin (manuscript submitted for publication), suggesting a role of G_i proteins. This α_{1B}-adrenoceptor phosphorylation induced by lysophosphatidic acid was not blocked by genistein, a tyrosine kinase inhibitor, but it was inhibited by staurosporine, a protein kinase C inhibitor, and wortmannin, a selective phosphatidylinositol 3-kinase inhibitor (manuscript submitted for publication). These data suggest that protein kinase C and phosphatidylinositol 3-kinase mediate such phosphorylation. Interestingly, each of these inhibitors was able to essentially block the action of lysophosphatidic acid, indicating that these kinases do not act in parallel, but rather in a sequential action. A model for the receptor phosphorylation induced by lysophosphatidic acid is presented in Fig. 3. The model suggests that: (a) lysophosphatidic acid activates its receptors, which interact and activate pertussis-toxin-sensitive G proteins, likely of the G_i family; (b) this allows the activation of phosphatidylinositol 3-kinase, which (c) leads to activation of protein kinase C, and (d) protein kinase C catalyzes the phosphorylation of α_{1B}-adrenoceptors. The role of phosphatidylinositol 3-kinase is particularly interesting. Phosphatidylinositol 3-kinase is a family of enzymes, which has been grouped into several classes (Nietgen and Durieux, 1998; Wymann and Pirola, 1998). In Class IA phosphatidylinositol 3-kinase isoforms, the adaptor p85 subunit interacts with phosphorylated tyrosine motifs of receptors with intrinsic tyrosine kinase activity. Phosphatidylinositol 3-kinase γ (Class IB isoform) interacts with heterotrimeric G proteins via the p101 protein. Such interactions seem to control phosphatidylinositol 3-kinase activity (Nietgen and Durieux, 1998; Wymann and Pirola, 1998). The mecha-

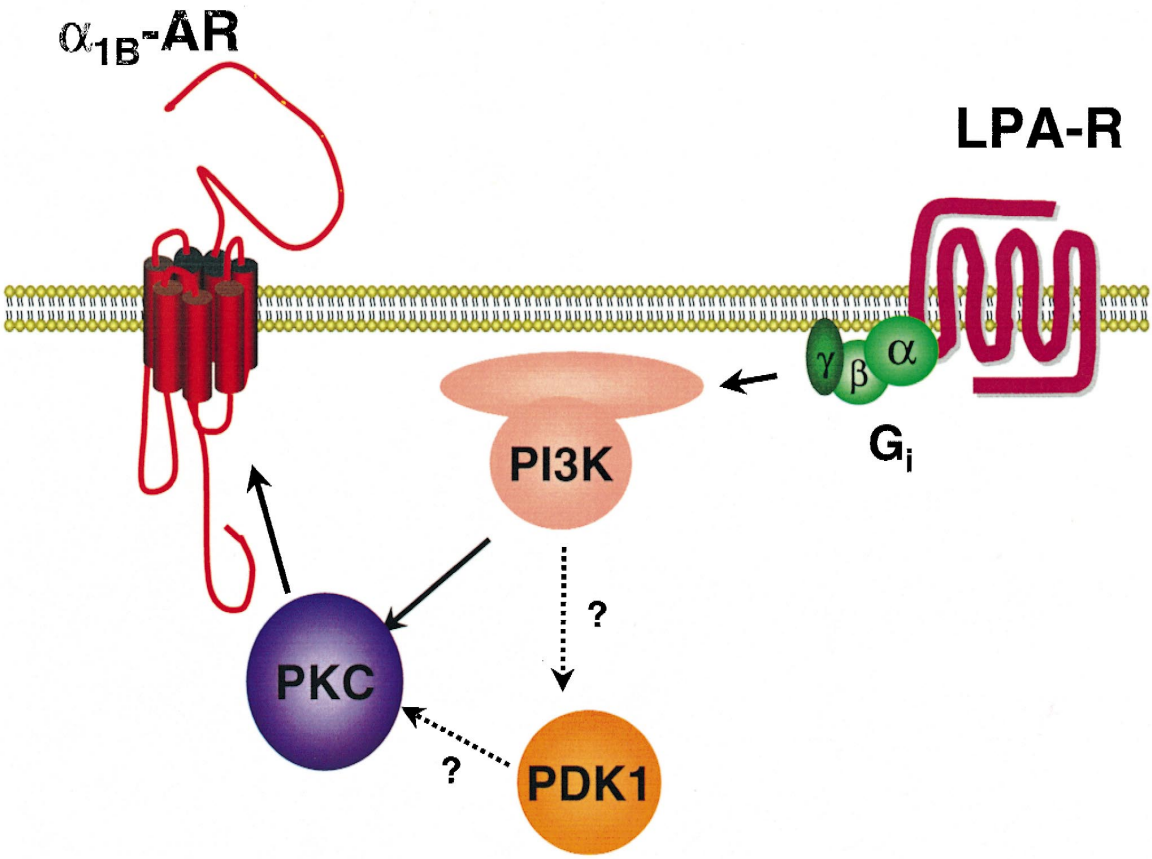

Fig. 3. Model for the effect of a G_i-coupled receptor on α_{1B}-AR phosphorylation. α_{1B}-AR, α_{1B}-adrenoceptor; LPA-R, lysophosphatidic acid receptor; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent protein kinase-1; PKC, protein kinase C.

nism through which phosphatidylinositol 3-kinase stimulates protein kinase C activity likely involves a direct interaction with the phosphoinositides generated by phosphatidylinositol 3-kinase. Phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate have been reported to activate novel protein kinase C (δ, ε and η) and atypical protein kinase C (ζ and λ) isoforms (Nietgen and Durieux, 1998; Wymann and Pirola, 1998; Rameh and Cantley, 1999). An intermediary kinase, such as the recently identified phosphoinositide-dependent protein kinase-1 or PDK1 (Allesi and Cohen, 1998; Stephens et al., 1998), may also participate in the control of protein kinase C. As mentioned above, protein-kinase-C-mediated α_{1B}-adrenoceptor phosphorylation has been extensively documented.

Lysophosphatidic-acid-induced α_{1B}-adrenoceptor phosphorylation has functional consequences. The ability of noradrenaline to increase cytosol Ca^{2+} concentration was markedly decreased in cells previously challenged with lysophosphatidic acid, and noradrenaline-induced [^{35}S]GTPγS binding was markedly decreased in membranes from cells pretreated with lysophosphatidic acid (manuscript submitted for publication). Therefore, our data indicate that lysophosphatidic acid induces α_{1B}-adrenoceptor phosphorylation through a phosphatidylinositol 3-kinase and protein kinase C-dependent pathway, and that such phosphorylation is associated with receptor-G protein uncoupling.

3.5. α_{1B}-Adrenoceptor phosphorylation / desensitization via receptors with endogenous tyrosine kinase activity

Growth factor receptors, such as epidermal growth factor receptor and platelet-derived growth factor receptor, have intrinsic tyrosine kinase activity. Agonist binding to these receptors induces their dimerization and autophosphorylation in multiple tyrosine residues within their intracellular domains. The autophosphorylated receptors provide specific binding sites for SH2 domain-containing adaptor proteins such as Shc and Grb2, and receptor-associated enzymes such as Src family members, protein tyrosine phosphatases, phospholipase Cγ and phosphatidylinositol 3-kinase (Ullrich and Schlessinger, 1990; Wymann and Pirola, 1998).

Information on the possible crosstalk from growth factor receptors to G-protein-coupled receptors is limited. Nevertheless, as mentioned before, Malbon and coworkers (Hadcock et al., 1992; Baltensperger et al., 1996; Karoor

and Malbon, 1996) have provided ample evidence that the β_2-adrenergic receptor (β_2-adrenoceptor) is cross-regulated by activation of the insulin receptor.

The effect of EGF on the phosphorylation of α_{1B}-adrenoceptor has been studied in our laboratory. EGF increased the phosphorylation of these adrenoceptors and this effect was blocked by tyrphostin AG 1478 ([4-(3-chloroanilino)-6,7-dimetoxyquinazoline]), inhibitor of the intrinsic tyrosine kinase activities of the EGF receptors (manuscript submitted for publication). As observed in the α_{1B}-adrenoceptor phosphorylation induced by lysophosphatidic acid, the action of EGF was blocked by wortmannin and staurosporine, indicating that phosphatidylinositol 3-kinase and protein kinase C are involved (manuscript submitted for publication). Probably, a sequence of events similar to those postulated for the action of lysophosphatidic acid could be operating. A model is presented in Fig. 4. Noradrenaline-stimulated [^{35}S]GTPγS binding was markedly decreased in membranes from cells pretreated with EGF, which suggests that the phosphorylation induced by the growth factor has functional consequences (manuscript submitted for publication).

4. Role of protein phosphatases in α_{1B}-adrenoceptor phosphorylation

The phosphorylation state of a phosphoprotein results from the balance between the activities of the protein kinases and protein phosphatases that act on it. However, little is known about the role(s) of protein phosphatases in receptor phosphorylation and function. It has been suggested that endocytosis via clathrin-coated vesicles is crucial for resensitization of some G-protein-coupled receptors (Zhang et al., 1997). Receptors proceed from these vesicles to endosomes where they are dephosphorylated and resensitized by a mechanism that is proposed to involve a conformational change in the receptor brought about by acidification of the edosomal compartment (Zhang et al., 1997). In this regard, Pitcher et al. (1995) have shown that a latent oligomeric form of protein phosphatase 2A actively dephosphorylates the β_2-adrenoceptor in vitro, and Shih et al. (1999) reported that protein phosphatases 2A and 2B are associated with β_2-adrenoceptors. It has also been observed that okadaic acid, an inhibitor of protein phosphatases, alters β_2-adrenoceptor-mediated

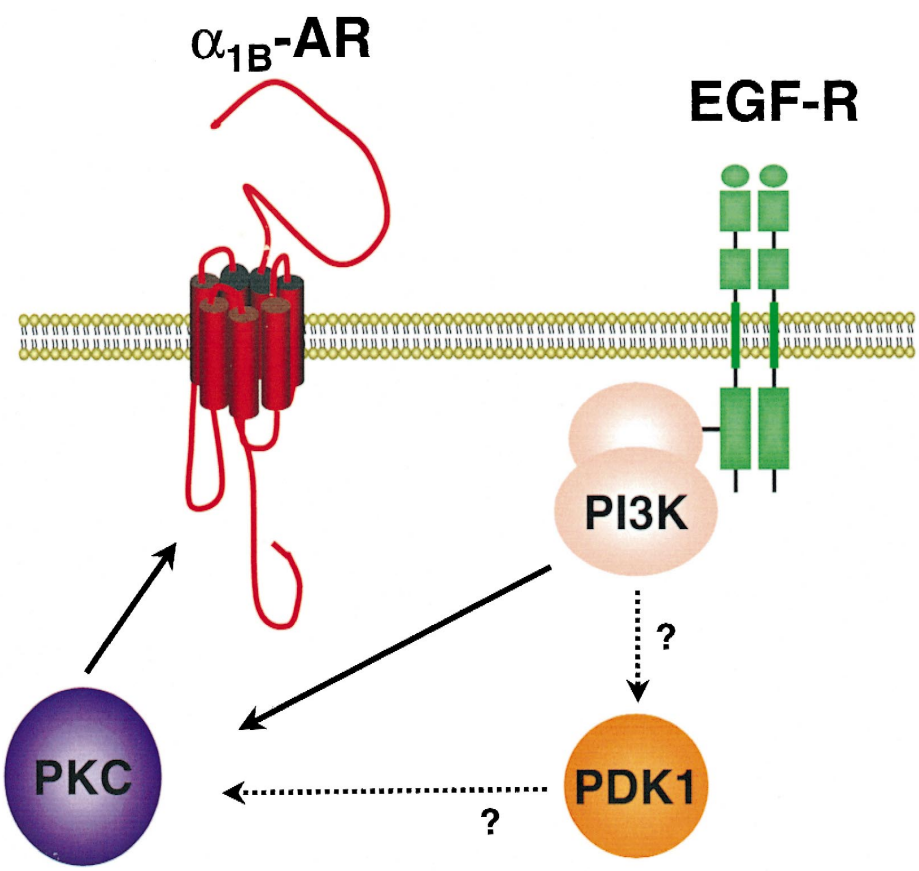

Fig. 4. Model for the effect of EGF on α_{1B}-adrenoceptor phosphorylation. α_{1B}-AR, α_{1B}-adrenoceptor; EGF-R, EGF receptor; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent protein kinase-1; PKC, protein kinase C.

stimulation of cyclic AMP accumulation (Clark et al., 1993). Inhibition of the serine/threonine protein phosphatase, calcineurin, enhances desensitization and phosphorylation of adipocyte β_1-adrenoceptor (Bahouth et al., 1996).

We have recently explored the possible role(s) of protein phosphatases in receptor phosphorylation, through the use of cell-permeant inhibitors, and observed that okadaic acid and other inhibitors such as calyculin A, tautomycin and cypermethrin induced marked α_{1B}-adrenoceptor phosphorylation (Alcántara-Hernández et al., 2000). The effect of okadaic acid can be blocked by inhibitors of protein kinase C (Alcántara-Hernández et al., 2000). Surprisingly, the intense adrenoceptor phosphorylation induced by okadaic acid was associated to a very small decrease in noradrenaline-stimulated [^{35}S]GTPγS binding and it did not alter the noradrenaline-stimulated increases in either intracellular Ca^{2+} or phosphoinositide hydrolysis (Alcántara-Hernández et al., 2000).

5. Different α_1-adrenoceptor subtypes

Differential regulation within a family of receptors is frequently associated with the susceptibility of members to be modified by phosphorylation. Subtypes of α_2- and β-adrenoceptors seem to be subject to desensitization according to their susceptibility as kinase substrates (Liggett, 1998; Liggett et al., 1993; Kurose and Lefkowitz, 1994). The information on α_1-adrenoceptors phosphorylation/desensitization is far less complete.

When transfected into Rat-1 fibroblasts, these receptors are differentially regulated by activation of protein kinase C (Vázquez-Prado and García-Sáinz, 1996). Thus, activation of protein kinase C with phorbol esters markedly inhibited the actions mediated through α_{1B}- and α_{1D}-adrenoceptors whereas those mediated through α_{1A}-adrenoceptors were not altered (Vázquez-Prado et al., 1996). As reviewed above, there is information on α_{1B}-adrenoceptor desensitization/phosphorylation but information is needed on the possible phosphorylation of the other subtypes. To the best of our knowledge, there is no information on the possible phosphorylation of α_{1D}-adrenoceptors.

We recently studied the phosphorylation of α_{1A}-adrenoceptors stably expressed in rat-1 fibroblasts. These receptors are phosphorylated in response to noradrenaline and when protein kinase C is activated by phorbol esters (Vázquez-Prado et al., 2000). This was surprising, since there is very little effect of active phorbol esters on the adrenergic modulation of cellular parameters (α_{1A}-adrenoceptor-mediated increases in intracellular Ca^{2+} concentration or phosphoinositide hydrolysis) (Vázquez-Prado et al., 2000). However, a small effect of active phorbol esters was observed on noradrenaline-stimulated [^{35}S]GTPγS binding. It should be mentioned, however, that the α_{1A}-adrenoceptor phosphorylation detected was much less than that observed for the α_{1B}-adrenoceptors (Vázquez-Prado et al., 2000).

6. Final remarks

Both α_{1A}-adrenoceptors and α_{1B}-adrenoceptors are substrates of protein kinases. However, the α_{1B} subtype seems to be a much better substrate than the α_{1A} subtype, and the former is desensitized to a much greater extent than the latter. It is also clear that not all α_1-adrenoceptor phosphorylations result in desensitization at a cellular level. In some cases, such as those observed by us with bradykinin (Medina et al., 1998) for α_{1B}-adrenoceptors and with phorbol esters for α_{1A}-adrenoceptors (Vázquez-Prado et al., 2000), the absence of cellular desensitization could be due to an inefficient phosphorylation. However, in other cases, such as those observed for the α_{1B} subtype with okadaic acid (Alcántara-Hernández et al., 2000) or with transfection with G protein receptor kinase 5 and 6 (Diviani et al., 1996), receptor phosphorylation was very intense but no consequences at a cellular level were clearly observed. It is possible that the site(s) phosphorylated could differ and/or that other component(s) of the signaling complex need(s) also to be altered by the treatments in order to observe repercussions at a cellular level. Certainly, receptor reserve and signal amplification steps could mask the consequences of receptor phosphorylation and this is probably much more important when transfected models with high levels of expression are used as compared to natural systems.

The changes incorporated to the receptor molecules by phosphorylation (bulk, charge, hydration, etc.) likely affect their ability to productively interact with G proteins. However, a major aspect is the receptor interaction with other proteins. In the case of homologous desensitization, G protein receptor kinases themselves (Dicker et al., 1999) or β-arrestins (Krupnic and Benovic, 1998; Pitcher et al., 1998) interact with the receptors and constitute a very substantial sterical impediment for the effective interaction of receptors with G proteins. In the case of heterologous desensitizations, it is not completely clear if kinases, arrestins, or other molecules play such role.

Certainly a major future trend is the identification of the isoforms of protein kinase C and phosphatidylinositol 3-kinase that participate in heterologous desensitizations. These kinases now appear as major players in the regulation of receptor function. There are no pharmacological agents with sufficient selectivity to address these aspects. Therefore, molecular biological approaches probably will have to be used. Once these basic aspects are clarified, opportunities for drug design and pharmacological intervention will appear. This may have therapeutical potential.

The study of receptor dephosphorylation seems also of major interest and a future challenge. Receptor internalization seems to be required for receptor dephosphorylation/resensitization of some G-protein-coupled receptors,

but it is not clear if this is a general requirement. There are several pathways for receptor endocytosis and differences may also exist in the receptor dephosphorylation processes. The recent finding that protein kinases and phosphatases form dynamic complexes with membrane receptors and the role of anchoring proteins in these processes (Shih et al., 1999; Westphal et al., 1999) add further interest from a biochemical and cell biological perspectives, and certainly future possibilities for pharmacological intervention.

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