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Lysophosphatidic Acid Modulates α_{1b}-Adrenoceptor Phosphorylation and Function: Roles of Gi and Phosphoinositide 3-Kinase

PATRICIA CASAS-GONZÁLEZ, JOSÉ VÁZQUEZ-PRADO, and J. ADOLFO GARCÍA-SÁINZ

Departamento de Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México City, México

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ABSTRACT

The effect of lysophosphatidic acid on the phosphorylation and function of α_{1b}-adrenoceptors transfected into rat-1 fibroblasts was studied. This phospholipid mitogen increased in a concentration-dependent fashion (EC_{50} ~50 nM) the phosphorylation of these adrenoceptors. Lysophosphatidic acid-induced α_{1b}-adrenoceptor phosphorylation was relatively rapid ($t_{1/2}$ ~1 min), intense (2.5-fold), and sustained for at least 60 min. The effect of lysophosphatidic acid was blocked by pretreatment with pertussis toxin. The α_{1b}-adrenoceptor phosphorylation induced by lysophosphatidic acid was not blocked by genistein, a tyrosine kinase inhibitor, but it was inhibited by inhibitors of protein kinase C (bisindolylmaleimide I, staurosporine, and Ro 31-8220) and phosphoinositide 3-kinase (wortmannin and LY 294002). The ability of norepinephrine to increase cytosol calcium concentration was markedly decreased in cells previously challenged with lysophosphatidic acid. Norepinephrine-induced [^{35}S]GTPγS binding in membrane preparations was used as an index of the functional coupling of the α_{1b}-adrenoceptors and G proteins. Norepinephrine-stimulated [^{35}S]GTPγS binding was markedly decreased in membranes from cells pretreated with lysophosphatidic acid. This effect of lysophosphatidic acid was blocked by pretreatment with wortmannin or staurosporine. Our data indicate that: 1) activation of lysophosphatidic acid receptors induce phosphorylation of α_{1b}-adrenoceptors; 2) this effect is mediated through pertussis toxin-sensitive G proteins, phosphatidylinositol 3-kinase, and protein kinase C; and 3) the phosphorylation of α_{1b}-adrenoceptors induced by the lipid mitogen is associated to adrenoceptor desensitization.

G protein-coupled receptors play a central role in cellular communication mediating the response to numerous hormones and neurotransmitters. There are intense interactions among the different receptor signaling pathways ("cross talk"), and such interactions play cardinal roles in determining cell responsiveness.

Control of receptor function is a key event in the adaptation of cells to changes in the internal milieu of an organism and to the overall homeostasis. Different cellular processes with different time frames seem to be involved. This includes modulation of receptor coupling to G proteins, receptor internalization, recycling to the plasma membrane, and degradation and regulation of expression (Lefkowitz et al., 1998). An initial event in the control of receptor function seems to be the phosphorylation of the receptors themselves. Three groups of protein kinases are the major modulators of G protein-coupled receptors: 1) second messenger-activated kinases, such as protein kinase A and protein kinase C (PKC) (Clark et al., 1988; Houslay, 1991), 2) members of the G-protein receptor kinase (GRK) family (Ferguson et al., 1997; Krupnick and Benovic, 1998), and 3) some receptors with tyrosine kinase activity (Hadcock et al., 1992).

It is generally accepted that homologous desensitization, in which only the receptors that are activated reduce their responsiveness, involves receptor phosphorylation by GRKs (Ferguson et al., 1997; Krupnick and Benovic, 1998). Accordingly, agonist-occupied receptors activate heterotrimeric G proteins, release G$\beta\gamma$ complexes, and recruit soluble GRKs (particularly GRK-2) (Pitcher et al., 1998). These enzymes phosphorylate the receptors, which bind β-arrestin molecules, stabilizing the uncoupled state of the receptor (Krupnick and Benovic, 1998). β-Arrestin acts as a bridge binding to clathrin molecules. This initiates the internalization of phosphorylated receptors into vesicles where specific phosphatases remove the phosphates and allow the dephosphorylated receptors to return to the cell surface, completing the cycle of activation-desensitization-resensitization (Fergu-

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ABBREVIATIONS: PKC, protein kinase C; AR, adrenoceptor; LPA, lysophosphatidic acid; DMEM, Dulbecco's modified Eagle's medium; GRK, G protein-coupled receptor kinase; PI3K, phosphoinositide 3-kinase; $[Ca^{2+}]_i$, cytosol calcium concentration; NE, norepinephrine; TPA, tetradecanoyl phorbol acetate; PDK1, phosphoinositide-dependent protein kinase-1.

son et al., 1997; Krupnick and Benovic, 1998). Second messenger-activated kinases, such as protein kinase A and PKC, and receptors with endogenous tyrosine kinase activity also promote receptor phosphorylation, eliciting heterologous desensitization by a parallel process that does not require receptor activation (Clark et al., 1988; Hadcock et al., 1992; Pitcher et al., 1998).

The activity of α_{1b}-adrenoceptors (α_{1b}-ARs) is tightly regulated. Most evidence suggests that when activated by agonists GRKs phosphorylate α_{1b}-ARs (Lattion et al., 1994; Diviani et al., 1996); GRK-2 and GRK-3 seem to be the isoforms that mainly participate in this effect (Diviani et al., 1996). Pharmacological activation of PKC with phorbol esters blocks α_{1b}-adrenergic actions in cells that naturally express this receptor (Corvera and García-Sáinz, 1984; Leeb-Lundberg et al., 1985; Corvera et al., 1986). Likewise, this effect has been observed in cells transfected with this receptor, and this is associated to receptor phosphorylation (Lattion et al., 1994; Diviani et al., 1997; Vázquez-Prado et al., 1997; Medina et al., 1998). On a more physiological context, it has been recently observed that activation of endothelin ET_A receptors induce phosphorylation of α_{1b}-ARs and uncoupling of these receptors from G proteins (Vázquez-Prado et al., 1997). The effect of endothelin is mediated by pertussis toxin-insensitive G proteins and involves activation of PKC and an as yet unidentified tyrosine kinase; no role of phosphoinositide 3-kinase (PI3K) was observed in this effect of endothelin (Vázquez-Prado et al., 1997).

Lysophosphatidic acid (LPA) is a water-soluble phospholipid that is released by cells such as platelets during activation. It is a mitogen for many cells and evokes other actions such as contraction, secretion, adhesion, or chemotaxis (Moolenaar et al., 1997; Goetzl and An, 1998). The actions of LPA are mediated through G protein-coupled receptors (Fukushima et al., 1998; Chun et al., 1999). These receptors seem to couple to Gq and Gi (van Corven et al., 1989). LPA inhibits adenylyl cyclase (van Corven et al., 1989) and increases cytosol calcium concentration ($[Ca^{2+}]_i$) (Hordijk et al., 1994; An et al., 1998), generation of inositol phosphates (Hordijk et al., 1994), phosphorylation of mitogen-activated protein kinase (Hordijk et al., 1994), and protooncogene expression (van Corven et al., 1993; Chuprun et al., 1997). Interestingly, many of the actions of LPA seem to involve heterotrimeric Gi proteins (van Corven et al., 1989, 1993; Carr et al., 1994; Hordijk et al., 1994; Chuprun et al., 1997). The effect of LPA was studied in rat-1 fibroblasts stably expressing α_{1b}-ARs; it was observed that LPA induces α_{1b}-AR phosphorylation and G protein uncoupling. Such action of LPA involves pertussis toxin-sensitive G proteins and PI3K and represents a new process for the modulation of this AR.

Experimental Procedures

Materials. (−)-Norepinephrine (NE), LPA (1-oleoyl), endothelin-1, tetradecanoyl phorbol acetate (TPA), GTPγS, GDP, staurosporine, wortmannin, and protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was purified from vaccine concentrates as described previously (Sekura et al., 1983; García-Sáinz et al., 1992). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, antibiotics, and other reagents used for cell culture were from Life Technologies (Gaithersburg, MD). [^{35}S]GTPγS (1250 Ci/mmol) and [^{32}P]P_i (8500–9120 Ci/mmol) were from New England Nuclear (Boston, MA). Bisindolylmaleimide I, LY 294002, and Ro 31-8220 were obtained from Calbiochem (San Diego, CA). Sepharose-coupled protein A was from Upstate Biotechnology (Lake Placid, NY). Fura-2/AM was from Molecular Probes (Eugene, OR), and genistein was from Research Biochemicals International (Natick, MA).

Cell Lines and Culture. Rat-1 fibroblasts transfected with the hamster α_{1b}-AR (Cotecchia et al., 1988), generously provided to us by Drs. R. J. Lefkowitz, M. G. Caron, and L. Allen (Duke University, Durham, NC), were cultured in glutamine-containing high-glucose DMEM supplemented with 10% fetal bovine serum, 300 μg/ml of the neomycin analog G-418 sulfate, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B at 37°C under a 95% air, 5% CO_2 atmosphere as described previously (Vázquez-Prado et al., 1997). For all the experiments, confluent cells were serum-deprived overnight in unsupplemented DMEM.

α_{1b}-AR Phosphorylation. Rat-1 cells expressing the α_{1b}-ARs were incubated in phosphate-free DMEM for 1 h and then labeled in the same medium containing [^{32}P]P_i (50 μCi/ml) for 3 h at 37°C as described previously (Vázquez-Prado et al., 1997). In brief, after treatment with inhibitors and/or agonists, cells were washed with ice-cold PBS and lysed for 1 h on ice in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS, 50 mM NaF, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM phosphoserine, 1 mM phosphothreonine, and protease inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, 500 μg/ml bacitracin, and 50 μg/ml soybean trypsin inhibitor). Cell lysates were centrifuged at 12,700*g* for 15 min, and the supernatants were incubated overnight at 4°C with the anti-α_{1b}-AR antiserum (Vázquez-Prado et al., 1997) and protein A-Sepharose. After five washes with 50 mM HEPES, 50 mM NaH_2PO_4, 100 mM NaCl, pH 7.4, 1% Triton X-100, 0.05% SDS, 100 mM NaF followed by a final wash with 50 mM Tris, 150 mM NaCl, pH 7.4, the immune complexes were denatured by boiling in SDS-sample buffer containing 5% β-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. The gels were dried and exposed for 3 to 24 h at −70°C using Kodak X-Omat X-ray films and intensifying screens. The level of receptor phosphorylation was assessed in the same gels with a Molecular Dynamics (Sunnyvale, CA) PhosphorImager and Imagequant software.

$[Ca^{2+}]_i$ Measurements. Confluent fibroblasts were incubated overnight in DMEM without serum and antibiotics. Cells were loaded with 5 μM Fura-2/AM in Krebs-Ringer-HEPES containing 0.05% BSA, pH 7.4 for 1 h at 37°C. Cells were detached by gentle trypsinization, and fluorescence measurements were carried out as described previously (Vázquez-Prado et al., 1997) with an Aminco-Bowman (Urbana, IL) Series 2 Spectrometer with the excitation monochromator set at 340 and 380 nm, a chopper interval set at 0.5 s, and the emission monochromator set at 510 nm. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985) using the software provided by AMINCO-Bowman; traces were directly exported to the graphs.

Membrane Preparation and [^{35}S]GTPγS Binding. Confluent cells were stimulated in the absence (control) or presence of the different agonists for 5 min at 37°C. The reaction was terminated by washing with ice-cold PBS, and cells were scraped with 1 ml of ice-cold buffer (50 mM Tris, 150 mM NaCl, pH 7.5, 5 mM EDTA, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, plus protease inhibitors as in the lysis buffer). Membranes were prepared, and [^{35}S]GTPγS binding was performed as described by Wieland and Jakobs (1994) with minor modifications (Vázquez-Prado et al., 1997). Briefly, membranes were resuspended in binding buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 1 μM GDP, and 0.1% BSA). Binding was performed at 25°C for 30 min in a volume of 250 μl of binding buffer containing 0.2 nM [^{35}S]GTPγS. The reaction was initiated by the addition of membranes (25 μg of protein/tube) and terminated by

rapid filtration through Whatman GF/C filters followed by three washes of the filters with ice-cold buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4). The filters were dried, and the radioactivity was measured by liquid scintillation. Nonspecific binding was determined in the presence of unlabeled GTPγS (30 μM) and represented 10% of total binding. Statistical analysis between comparable groups was performed using ANOVA with Bonferroni's post-test.

Results

As shown in Fig. 1, LPA (1 μM) induced a relatively rapid increase in α_{1b}-AR phosphorylation ($t_{1/2}$ ~1 min, maximum ~15 min). Such increased phosphorylation was sustained up to 60 min (Fig. 1). The effect of LPA was concentration-dependent with an EC_{50} of ~50 nM and a maximum increase in phosphorylation of 2.5-fold (Fig. 2).

To get additional insight on the mechanism of this action of LPA, the effect of pertussis toxin was tested. Cells were preincubated with pertussis toxin (300 ng/ml for 3 h or 100 ng/ml for 24 h, which resulted in identical data) under conditions that essentially inactivated all pertussis toxin-sensitive G proteins (Vázquez-Prado et al., 1997), and the effects of NE, LPA, and TPA on α_{1b}-AR phosphorylation were tested. It can be seen in Fig. 3 that the actions of NE and TPA were not affected at all by the pretreatment with pertussis toxin, which is in agreement with previous data (Vázquez-Prado et al., 1997). In contrast, the α_{1b}-AR phosphorylation induced by LPA was almost completely inhibited.

Further differences became evident through the use of kinase inhibitors. Staurosporine (300 nM), a relatively selective inhibitor of PKC; genistein (10 μM), a protein tyrosine kinase inhibitor; and wortmannin (100 nM), a selective PI3K inhibitor, were tested. None of these agents at the concentrations tested altered basal receptor phosphorylation (data not shown). Similarly and in agreement with previous data (Vázquez-Prado et al., 1997), none of these agents altered in any way the α_{1b}-AR phosphorylation induced by NE (Fig. 4). In contrast, the effect of LPA was not altered by genistein, but it was almost completely abolished by either staurosporine or wortmannin (Fig. 4). Wortmannin is unable to alter the α_{1b}-AR phosphorylation induced by endothelin (Vázquez-Prado et al., 1997) or TPA (data not shown). To further substantiate these findings, the effect of several inhibitors of these kinases was studied. The PKC inhibitors bisindolylmaleimide I (IC_{50} ~80 nM), staurosporine (IC_{50} ~125 nM), and Ro 31-8220 (IC_{50} ~185 nM) blocked in concentration-dependent fashion the effect of LPA on α_{1b}-AR phosphorylation (Fig. 5). Similarly, wortmannin (IC_{50} ~4 nM) and LY 294002

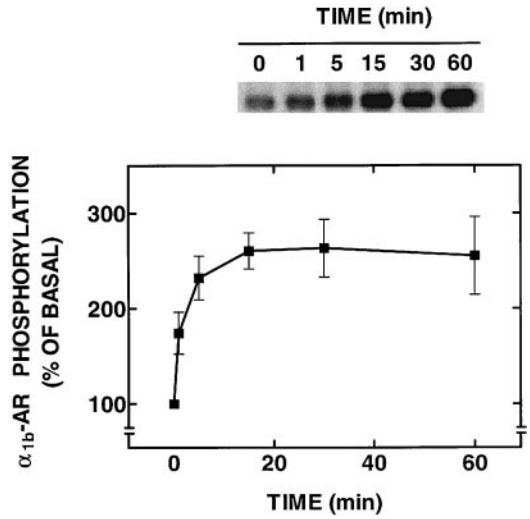

Fig. 1. Time course of the effect of 1 μM LPA on α_{1b}-AR phosphorylation. Cells were incubated in the absence or presence of LPA. Plotted are the means and vertical lines representing the S.E.M. of 15 determinations using five different cell cultures. A representative autoradiograph is shown.

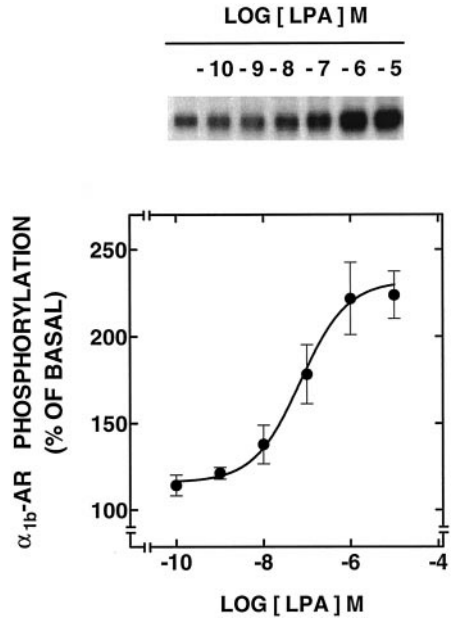

Fig. 2. Concentration-response curve for the effect of LPA on α_{1b}-AR phosphorylation. Cells were incubated with the indicated concentrations of LPA. Plotted are the means and vertical lines representing the S.E.M. of 15 determinations using five different cell cultures. A representative autoradiograph is shown.

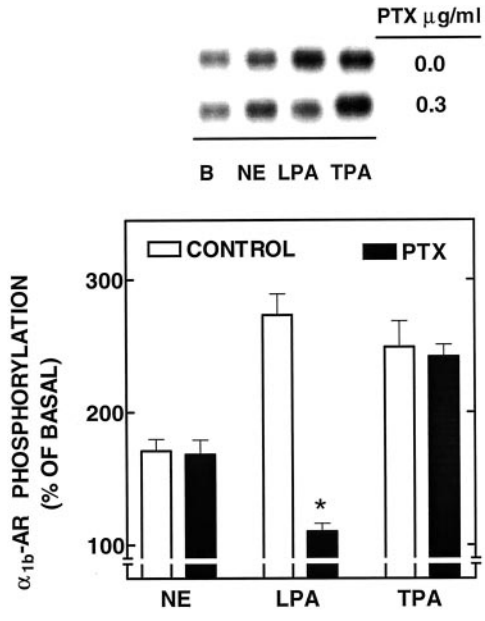

Fig. 3. Effect of pertussis toxin on α_{1b}-AR phosphorylation. Cells were preincubated in the absence (open bars) or presence of 0.3 μg/ml pertussis toxin (PTX; solid bars). Cells were incubated for 5 min in the absence of agonist (B) or in the presence of 10 μM NE, 1 μM LPA, or 1 μM TPA. Plotted are the means and vertical lines representing the S.E.M. of 15 determinations using five different cell cultures. Representative autoradiographs are shown. *$P < .001$ versus LPA-CONTROL.

(IC_{50} ~60 nM), which are inhibitors of PI3K, blocked the effect of LPA on α_{1b}-AR phosphorylation (Fig. 6).

To determine if α_{1b}-AR phosphorylation induced by LPA has any functional consequence, two parameters were studied: the increase in $[Ca^{2+}]_i$ induced by NE in whole cells and the α_{1b}-AR-mediated stimulation of [^{35}S]GTPγS binding to membranes. It can be seen in Fig. 7 that NE and LPA were able to induce marked increases in $[Ca^{2+}]_i$. However, when NE was added to the cells after stimulation by LPA the adrenergic effect was markedly inhibited. These data are consistent with desensitization of the adrenergic action. However, this effect was not selective because after the initial action of LPA, the effect of a second stimulation by the same agent or by endothelin resulted in decreased responses (Fig. 7). These data suggest that depletion of calcium stores is taking place and that the action of LPA on α_{1b}-adrenergic-mediated increase in $[Ca^{2+}]_i$ cannot be explained only on the basis of receptor desensitization. Therefore, a more direct experimental approach, such as the adrenergic-mediated [^{35}S]GTPγS binding, which is an index of α_{1b}-AR-G protein interaction, was studied.

In these studies, cells were incubated in the absence of any agent (control) or in the presence of either 10 μM NE or 1 μM LPA for 5 min. Membranes were obtained, and the effects of NE or LPA on [^{35}S]GTPγS binding in vitro were studied. It can be observed that in membranes from control cells NE and LPA stimulated [^{35}S]GTPγS binding (Fig. 8). In cells incubated with NE, the effect of the adrenergic agonist but not that of LPA on [^{35}S]GTPγS binding was inhibited (Fig. 8). In cells treated with LPA, the in vitro effect of NE was essentially abolished, but the effect of LPA was not altered (Fig. 8). In cells incubated with pertussis toxin, the effect of LPA on [^{35}S]GTPγS binding was markedly inhibited but that of NE was not affected (data not shown). In addition, it was observed that preincubation with wortmannin or staurosporine blocked the effect of LPA on NE-stimulated [^{35}S]GTPγS binding (Fig. 8). These data clearly indicate that the α_{1b}-AR phosphorylation induced by LPA is associated to G protein uncoupling (desensitization).

Discussion

These results indicate that LPA induces α_{1b}-AR phosphorylation and receptor-G protein uncoupling. The increase in receptor phosphorylation induced by this phospholipid is similar in magnitude to that observed with endothelin and larger than that induced by NE (Vázquez-Prado et al., 1997). The time course of the effect of LPA markedly differs with those previously observed with NE and endothelin (Vázquez-Prado et al., 1997). The actions of these latter agents were faster, reaching their maximum at 5 min and rapidly declining to nearly basal levels at 60 min (Vázquez-Prado et al., 1997).

The ability of pertussis toxin to block this action of LPA is consistent with the involvement of G proteins of the Gi subfamily in mediating many of the actions of this phospholipid (van Corven et al., 1989, 1993; Carr et al., 1994; Hordijk et al., 1994; Chuprun et al., 1997). The data are particularly interesting because they show the modulation of a Gq/11-coupled receptor, such as the α_{1b}-AR (Wu et al., 1992, 1995), by a receptor coupled to pertussis toxin-sensitive G proteins, which are likely to be of the Gi subfamily. The pertussis toxin sensitivity clearly indicates that the effect of LPA differs

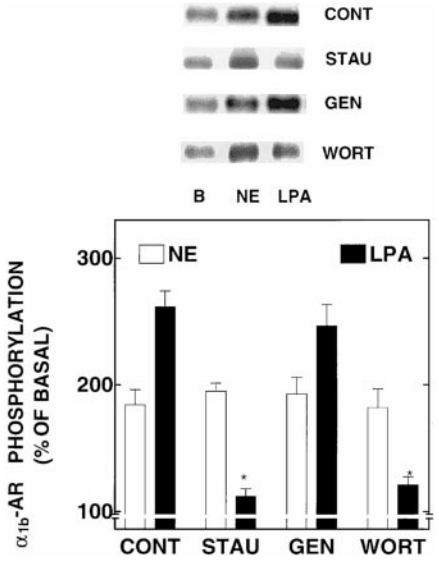

Fig. 4. Effect of staurosporine, genistein, and wortmannin on LPA- and NE-induced α_{1b}-AR phosphorylation. Cells were preincubated for 30 min in the absence of any agent (CONT) or presence of 300 nM staurosporine (STAU), 10 μM genistein (GEN), or 100 nM wortmannin (WORT). Cells were then incubated with 10 μM NE (open bars) or 1 μM LPA (solid bars) for 5 min. Plotted are the means and vertical lines representing the S.E.M. of 15 determinations using five different cell cultures. Representative autoradiographs are shown. *$P < .001$ versus LPA-CONT.

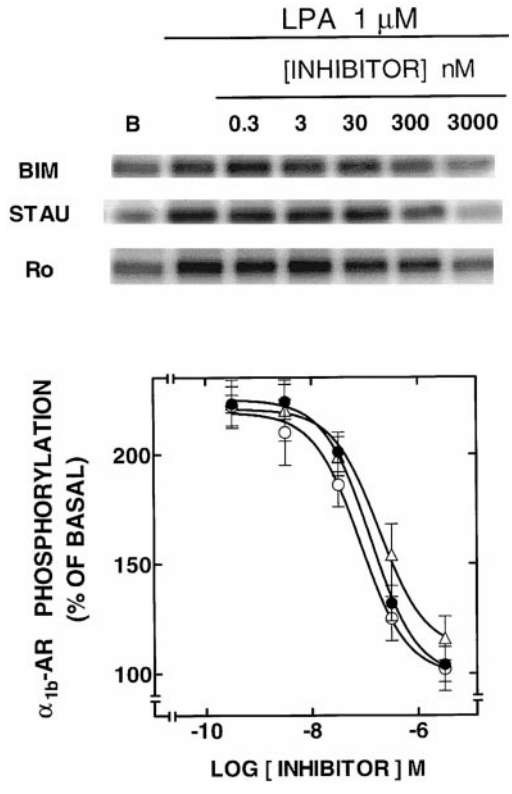

Fig. 5. Effect of PKC inhibitors on LPA-induced α_{1b}-AR phosphorylation. Cells were preincubated for 30 min in the presence of different concentrations of bisindolylmaleimide I (BIM; open circles), staurosporine (STAU; solid circles), or Ro 31-8220 (Ro; open triangles). After this preincubation, 1 μM LPA was added, and the incubation continued for 5 min. Plotted are the means and vertical lines representing the S.E.M. of four to seven determinations using different cell cultures. Representative autoradiographs are shown. B, basal.

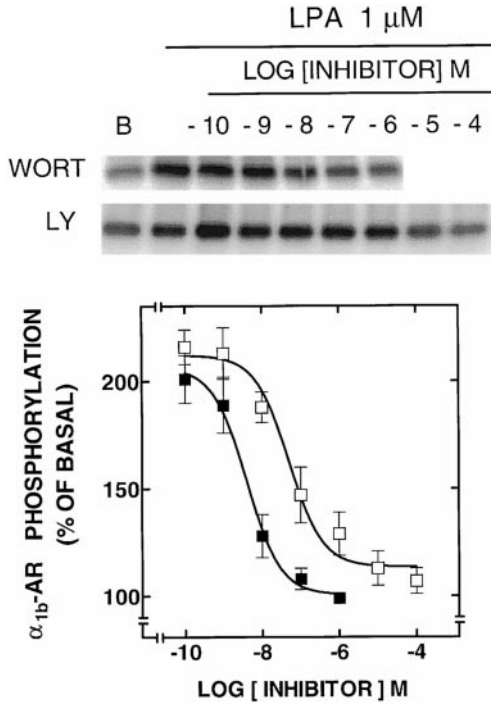

Fig. 6. Effect of PI3K inhibitors on LPA-induced α_{1b}-AR phosphorylation. Cells were preincubated for 30 min in the presence of different concentrations of wortmannin (WORT; solid squares) or LY 294002 (LY; open squares). After this preincubation, 1 μM LPA was added, and the incubation continued for 5 min. Plotted are the means and vertical lines representing the S.E.M. of four to seven determinations using different cell cultures. Representative autoradiographs are shown.

from both that due to receptor activation, putatively mediated through GRKs (Lattion et al., 1994; Diviani et al., 1996), and that induced by direct pharmacological activation of PKC. Previously, it was shown that the α_{1b}-AR phosphorylation induced by endothelin is mediated through pertussis toxin-insensitive G proteins (Vázquez-Prado et al., 1997). This is the first example of a Gi-coupled receptor modulating the phosphorylation state and function of α_1-ARs.

It should be mentioned that α_{1b}-AR phosphorylation induced by endothelin was partially blocked by genistein or staurosporine and completely blocked when both protein kinase inhibitors were present, which suggested participation of PKC and protein tyrosine kinase(s) in this effect (Vázquez-Prado et al., 1997). These data suggest that α_{1b}-AR phosphorylation induced by LPA did not involve protein tyrosine kinases but PKC and, interestingly, PI3K. This further emphasizes the differences in the cross talk between α_{1b}-ARs and endothelin receptors (Gq/11-coupled) and LPA receptors (Gi-coupled).

The role of PKC in modulating α_{1b}-AR function has been extensively documented. In fact, in a very elegant work, the PKC phosphorylation sites of the hamster α_{1b}-AR were recently identified as Ser^{394} and Ser^{400} located at the carboxyl terminus (Diviani et al., 1997). The role of PI3K is particularly interesting. PI3K is a family of enzymes, which has been grouped into several classes. In class IA PI3K isoforms, the adaptor p85 subunit interacts with phosphorylated tyrosine motifs of receptors with intrinsic tyrosine kinase activity, whereas PI3Kγ (class IB isoform) interacts with heterotrimeric G proteins via the p101 protein; such interactions seem to control PI3K activity (Nietgen and Durieux, 1998; Wymann and Pirola, 1998).

The mechanism through which PI3K stimulates PKC activity likely involves a direct interaction with the phosphoinositides generated by PI3K. Phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate have been reported to activate novel PKC (δ, ϵ, and η) and atypical PKC (ζ and λ) isoforms (Nakanishi et al., 1993; 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 (PDK1) (Allesi and Cohen, 1998; Stephens et al., 1998) may also participate in the control of PKC. PDK1, which binds with high affinity to phosphatidylinositol 3,4,5-trisphosphate, phosphorylates the activation loop sites of PKCζ and PKCδ in vitro and in a PI3K-dependent manner in vivo (Chow et al., 1998; Le Good et al., 1998). Interestingly, classical PKCα, -βI, and -βII bind directly to PDK1 coexpressed in HEK 293 cells (Le Good et al., 1998), raising the possibility of general control of the PKC family by PDK1.

The nearly complete inhibition of receptor phosphorylation observed in the presence of PI3K inhibitors (wortmannin and LY 294002) or PKC inhibitors (bisindolylmaleimide I, staurosporine, and Ro 31-8220) suggests that these enzymes may act sequentially in the same linear signaling pathway. In accord with current ideas, our data suggest that: 1) LPA activates its receptors that interact and activate pertussis toxin-sensitive G proteins, which are likely of the Gi family; 2) this allows the activation of PI3K, which 3) leads to activation of PKC via interaction with either 3-phosphoinositides or phosphorylation by PDK1; and 4) PKC catalyzes the phosphorylation of α_{1b}-ARs.

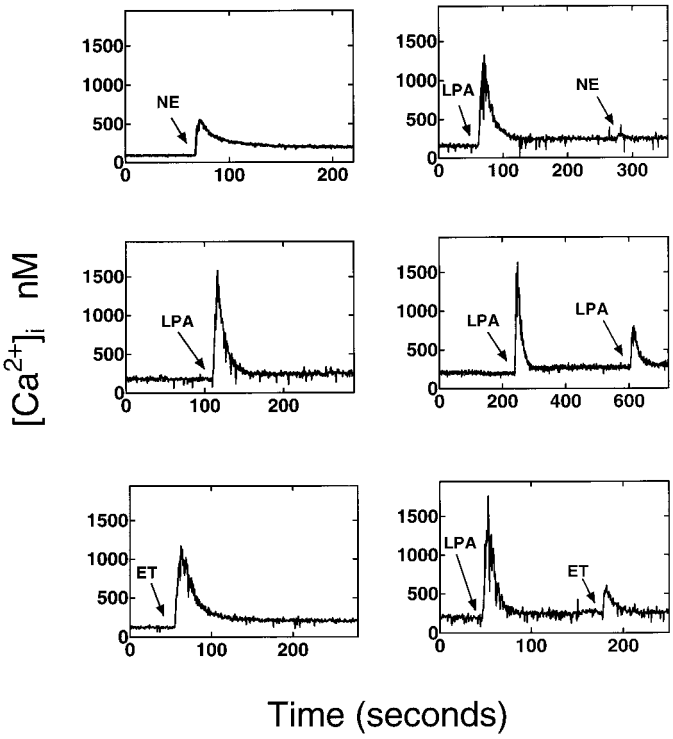

Fig. 7. Effect of LPA on the NE-induced $[Ca^{2+}]_i$ increase. Cells were loaded with Fura-2/AM, and $[Ca^{2+}]_i$ was recorded. Where indicated, 10 μM NE, 1 μM LPA, or 10 nM endothelin-1 (ET) was added. Traces are representative of three to four experiments using different cultures.

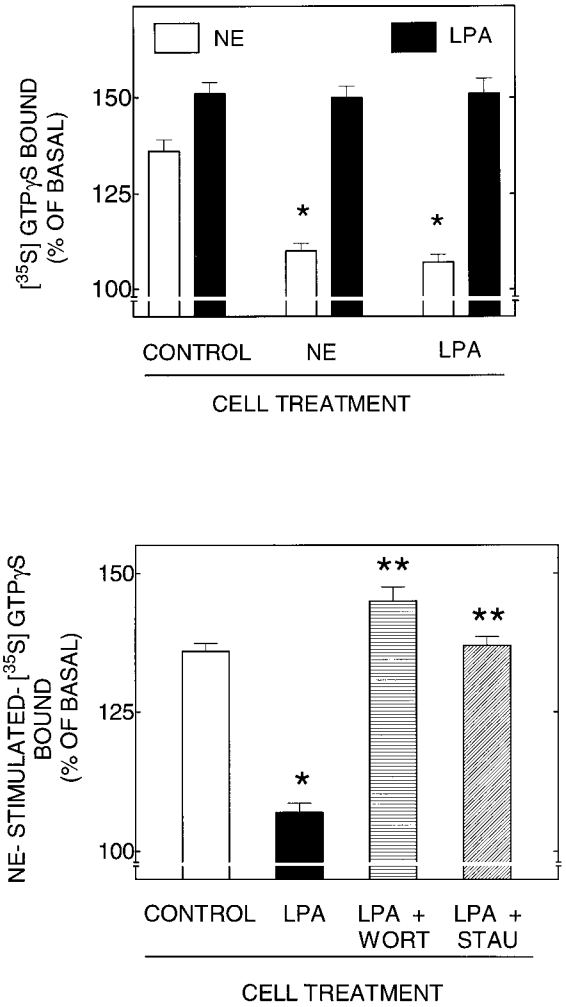

Fig. 8. In vitro agonist-stimulated [^{35}S]GTPγS binding to membranes. Top, cells were incubated for 5 min in the absence of any agent (CONTROL) or presence of 10 μM NE or 1 μM LPA, and membranes were obtained. In vitro [^{35}S]GTPγS binding to membranes was performed in the absence of any stimulus (basal) or presence of either 10 μM NE (open bars) or 1 μM LPA (solid bars). Plotted are the means and vertical lines representing the S.E.M. of 20 to 25 determinations using membranes obtained from three different cell cultures. *$P < .001$ versus NE-CONTROL. Bottom, cells were incubated for 30 min. Where indicated, 100 nM wortmannin (WORT) or 1 μM staurosporine (STAU) was present; during the last 5 min, no agent (CONTROL) or 1 μM LPA was added. In vitro [^{35}S]GTPγS binding to membranes was performed in the absence of any stimulus (basal) or presence of 10 μM NE. Plotted are the means and vertical lines representing the S.E.M. of 10 to 12 determinations using membranes obtained from two different cell cultures. *$P < .001$ versus CONTROL; **$P < .001$ versus LPA.

In summary, our data indicate that LPA induces α_{1b}-AR phosphorylation through a PI3K- and PKC-dependent pathway. Such phosphorylation is associated to receptor-G protein uncoupling. The data indicate that the function of α_{1b}-ARs is regulated by diverse processes including homologous modulation, i.e., due to activation by agonist and putatively GRKs, and heterologous modulations by receptors coupled to Gq/11, such as the endothelin ET_A receptors, and by receptors coupled to Gi, such as LPA receptors. A key role of PKC is evident in heterologous modulations.

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Send reprint requests to: J. Adolfo García-Sáinz, M.D., Ph.D., Inst. Fisiología Celular, UNAM, Ap. Postal 70–248, México, D. F. 04510. E-mail: agarcia@ifisiol.unam.mx