Norepinephrine- and Phorbol Ester-induced Phosphorylation of α1a-Adrenergic Receptors

FUNCTIONAL ASPECTS*

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Maximal adrenergic responses in Rat-1 fibroblasts expressing α1a-adrenergic receptors are not blocked by activation of protein kinase C. In contrast, activation of protein kinase C induces the phosphorylation of α1b-adrenergic receptors and blocks their actions. The effect of norepinephrine and phorbol esters on α1a-adrenergic receptor phosphorylation and coupling to G proteins were studied. Both stimuli lead to dose-dependent receptor phosphorylation. Interestingly, protein kinase C activation affected to a much lesser extent the actions of α1a-adrenergic receptors than those of the α1b subtype (norepinephrine elicited increases in calcium in whole cells and [35S]GTPgS binding to membranes). Basal phosphorylation of α1a-adrenergic receptors was much less than that observed with the α1b subtype. The carboxyl terminus seems to be the main domain for receptor phosphorylation. Therefore, chimeric receptors, where the carboxyl-terminal tails of α1a and α1b adrenergic receptors were exchanged, were constructed and expressed. α1a-Adrenoreceptors wearing the carboxyl tail of the α1b subtype had a high basal phosphorylation and displayed a strong phosphorylation in response to norepinephrine and phorbol esters. Our results demonstrate that stimulation of α1a-adrenergic receptor, or activation of protein kinase C, leads to α1a-adrenergic receptor phosphorylation. α1a-Adrenoreceptors are affected to a much lesser extent than α1b-adrenergic receptors by protein kinase C activation.

Phosphorylation of G protein-coupled receptors is considered the initial step in the process of desensitization. The paradigm of desensitization is based mainly on exhaustive studies performed with the Gs-coupled β2-ARs (1–5). Accordingly, when agonist-occupied receptors activate heterotrimeric G proteins, the released Gβγ complexes recruit soluble GRKs (particularly GRK-2) to the site of receptor activation (2). Receptors are phosphorylated by these enzymes and bind arrestin proteins that stabilize the uncoupled state of the receptors (3). Arrestins act as bridges that bind to clathrin molecules, initiating 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 (4, 5). Second messenger-activated kinases, such as PKA (protein kinase A) and PKC, also promote receptor phosphorylation, eliciting heterologous desensitization by a parallel process that does not require receptor activation and in which arrestins do not seem to participate (1).

G protein-coupled receptor phosphorylation is associated with desensitization, particularly with homologous desensitization (1, 6). Some heterologous stimuli lead to receptor phosphorylation and consequent desensitization. For example, α1b-ARs are phosphorylated and desensitized after their activation by agonists, as a result of a cross-talk with endothelin ET receptors or activation of PKC by phorbol esters (7–11). However, this same receptor is phosphorylated without evidencing desensitization by a cross-talk with B2 bradykinin receptors (12) or when active GRK-5 are expressed (9). Few examples exist in which desensitization takes place with no detectable receptor phosphorylation; these include studies with the parathyroid hormone receptor (13) and the luteinizing hormone/chorionic gonadotropin receptor (14).

Differential regulation within a family of receptors is frequently associated with differential susceptibility of members to be modified by phosphorylation. The actions of epinephrine and norepinephrine (NE), for example, are mediated through nine known adrenergic receptors. Subtypes of α2- and β-ARs seem to be subject to desensitization according to their susceptibility as kinase substrates (15–17). The information on α1-AR phosphorylation/desensitization is far less complete.

α1-ARs couple to the phosphoinositide turnover/calcium mobilization pathway (18, 19) through Gαq11, GTP-binding proteins (20). Activation of other signal transduction processes such as modulation of mitogen-activated protein kinase and phosphoinositide 3-kinase activities has also been reported (21, 22). These receptors participate in a plethora of physiological actions, including autonomic neurotransmission and the control of cardiovascular, respiratory, genitourinary, and gastrointestinal functions. They modulate metabolic pathways, smooth muscle contraction, water and electrolyte metabolism, and vascular tone. Long term effects of α1-AR stimulation include expression of c-fos and c-jun proto-oncogenes and cell growth and proliferation (20–24). The potential participation of α1-ARs in several pathologic states such as hypertension (25, 26) and

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† To whom correspondence should be addressed. Tel.: 525-622-5612; Fax: 525-622-5613; E-mail: agarcia@ifisiol.unam.mx.
‡ The abbreviations used are: AR, adrenergic receptor; DMEM, Dulbecco’s modified Eagle’s medium; GRK, G protein-coupled receptor kinase; PKC, protein kinase C; NE, norepinephrine; TPA, tetradecanoyl phorbol acetate; GST, glutathione S-transferase; ORF, open reading frame; bp, base pair(s).
cardiac (27, 28) and prostatic (29, 30) hypertrophy further emphasizes their importance. Therefore, knowledge of these receptors and their regulation is of interest from basic, clinical, and therapeutic perspectives.

Three α_{1a}-ARs have been cloned, i.e. the α_{1a}-AR, α_{1b}-AR, and α_{1d}-ARs (Refs. 31–34; see also Ref. 35). When transfected into Rat-1 fibroblasts, these receptors are differentially regulated by activation of PKC (18). Thus, activation of PKC with TPA markedly inhibited the actions mediated through the α_{1b}-AR and α_{1d}-ARs, whereas those mediated through α_{1a}-ARs were only marginally altered (18). The α_{1a}-AR was the first to be cloned (31) and the most frequently studied. As mentioned before, there is information on α_{1a}-AR desensitization/phosphorylation (7–12) whereas no studies on the possible phosphorylation of the other subtypes have been reported.

α_{1a}-ARs seem to be responsible for phenylephrine-promoted hypertrophy in cultured neonatal rat myocytes (28). Other studies have reported the presence of α_{1a}-AR as the predominant subtype present in the human prostate and have suggested the use of specific α_{1a}-AR antagonists in the treatment of benign prostatic hypertrophy (29, 30). In our opinion, further knowledge of the molecular events that regulate α_{1a}-AR may contribute to the understanding of the pathogenesis of such conditions and potentially to new approaches to alleviate them.

In this work, we show that α_{1a}-ARs are phosphorylated as a consequence of agonist binding or PKC activation. Interestingly, these events affect α_{1a}-ARs to a very limited extent, which is in marked contrast to the desensitization that has been observed with α_{1b}-ARs.

**EXPERIMENTAL PROCEDURES**

**Materials**—(-)-Norepinephrine, lysophosphatidic acid, TPA, staurosporine, GDP, GDP-[γ-35S]S, and protease inhibitors were obtained from Sigma. Bo 31–8290 and bisindolylmaleimide I were from Calbiochem. Pertussis toxin was purified from vaccine concentrates from Wyeth Laboratories. Fura-2/AM was from Molecular Probes. DNA purifications were done with the QIAquick PCR purification kit (Qiagen). Sepharose coupled to bovine serum albumin, pH 7.4, and the most frequently studied. As mentioned before, there is information on α_{1a}-AR desensitization/phosphorylation (7–12) whereas no studies on the possible phosphorylation of the other subtypes have been reported.

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α₁a-adrenoceptor phosphorylation for 24 h. For the experiments using only cells expressing α₁a-ARs, fibroblasts were maintained in phosphate-free DMEM for 1 h and then incubated in 3 ml of the same medium containing [35S]Pi (0.2 mCi/ml) for 3–5 h at 37 °C. In the experiments in which the phosphorylation of α₁a- and α₁b-ARs were compared, the amount of radioactive phosphate was 0.1 mCi/ml. Labeled cells were stimulated with NE or TPA as indicated, and then they were washed with ice-cold phosphate-buffered saline and solubilized with 1.0 ml of ice-cold solubilization buffer containing 50 mM NaF, 1 mM NaVO₄, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM p-serine, 1 mM p-threonine, and 1 mM p-tyrosine. The plates were maintained on ice for 1 h. Then the extracts were centrifuged at 12,700 × g for 15 min at 4 °C, and the supernatants were immunoprecipitated as described above. At least three independent experiments were performed for each treatment. Receptor phosphorylation was detected with a Molecular Dynamics PhosphorImager and quantified with ImageQuant software. Data were within the linear range of detection of the apparatus and were plotted using Prism 2.01, GraphPad software.

Chimeric and α₁b-adrenoceptors—cDNAs for wild type bovine α₁a-AR in pBluescript and hamster α₁a-AR in pSP65, containing 5' and 3' nontranslated regions, were generous gifts from Dr. Robert Lefkowitz (Duke University). Chimeric α₁a-AR- and α₁b-AR were constructed by interchanging their carboxyl-terminal tails. A conserved Pvu II site located in the coding sequence of the seventh transmembrane segment of both cDNAs was used for the ligation of fragments. cDNAs in the original plasmids were digested with EcoRI and PvuII, and the 5' cDNA of the α₁a-AR was ligated with the 3' cDNA of the α₁b-AR and vice versa; chimeric cDNAs were cloned into pcDNA3 for expression in Cos-1 cells. Fragments used for the construction of chimeric receptors were obtained from digestions in original plasmids. From the α₁a-AR cDNA these fragments were: (a) a 1136-bp EcoRI-PvuII fragment containing the 5' nontranslated sequence and the information for the amino-terminal 319 amino acids; and (b) a 1456-bp PvuII-EcoRI fragment containing the coding sequence for the 147 amino acids from the carboxyl terminus and the 3' nontranslated sequence. Fragments from the α₁b-AR cDNA, obtained by partial digestion, were: (a) a 1038-bp EcoRI-PvuII fragment containing the 5' nontranslated sequence plus the information for the amino-terminal 341 amino acids; and (b) a 1069-bp PvuII-EcoRI fragment containing the information for the carboxyl-terminal 174 amino acids and the 3' nontranslated sequence. Because inefficient expression (binding sites) was obtained with plasmids that contained 3' and 5' noncoding sequences, these regions from wild type and chimeric receptors were eliminated by polymerase chain reaction. Oligonucleotides were: 5'-AAGATATCGAATTCATGAACCGATCTGGAATTC-3' (sense) corresponding to bases 1–21 from the hamster α₁b-AR ORF, containing additional restriction sites for EcoRV and EcoRI, 5'-GAATTCCGCGCCCTAAAGTGCCCGGGTGCCAG-3' (antisense) corresponding to bases 1527–1545 from the hamster α₁a-AR ORF, with additional NotI and EcoRI restriction sites; 5'-AAGATATCGAATTCATGAGATTTCTTTCCGGAAT-3' (sense) corresponding to bases 1–21 from the bovine α₁a-AR ORF, with EcoRV and EcoRI sites; and 5'-GAATTCCGCGCCCTAAAGTGCCCGGGTGCCAG-3' (antisense) corresponding to bases 1380–1401 of the bovine α₁a-ORF, with NotI and EcoRI sites. The initial constructions on pcDNA3 were used on polymerase chain reactions with the convenient combination of oligonucleotides, and the resulting products were digested with EcoRI-NotI and ligated with pcDNA3 digested with the same enzymes. These new constructions were sequenced using an ABI Prism 310 genetic analyzer from Perkin-Elmer. Transient transfection of wild type and chimeric receptors into Cos-1 cells was performed using DEAE-dextran (40). Cells at 50–70% confluence (seeded the previous day) were transfected with 2 μg of plasmid for each 10-cm Petri dish. Experiments with transient transfected cells were performed 48–72 h after transfection. Transfection efficiency (60–80%) was determined in parallel dishes transfected with pCH110 and evaluated by the activity of β-galactosidase. The expression levels reached by each receptor and affinity were determined by [3H]prazosin binding and were as follows: α₁a-AR (Bₘax = 1104 ± 378 fmol/mg protein; Kᵦ = 0.51 ± 0.15 nM), α₁b-AR (Bₘax = 2863 ± 1053 fmol/mg protein; Kᵦ = 0.33 ± 0.12 nM), α₁a-AR (Bₘax = 2933 ± 171 fmol/mg protein; Kᵦ = 0.51 ± 0.13 nM), and α₁b-AR (Bₘax = 2358 ± 290; KD = 0.46 ± 0.12) (values are the means ± S.E. from 4–5 experiments performed in triplicate). Receptor phosphorylation studies with Cos-1 cells were performed as described above using 0.1 mCi/ml [3H]Pi. The antibody against the corresponding carboxyl terminus present in each receptor was used for immunoprecipitation, i.e. anti-α₁a antibody for the experiments using the wild type α₁a- and the chimeric α₁a-ARs and the anti-α₁b antibody for the wild type α₁b- and chimeric α₁b-ARs. No cross-immunoprecipitation was observed.

![Table](https://via.placeholder.com/150)

**RESULTS**

Functional Consequences of PKC Activation on α₁a-AR-induced [Ca²⁺]ᵢ mobilization and G Protein Coupling—We previously reported a differential blockade in the action of α₁a- and α₁b-ARs by phorbol esters (18). To obtain further information on the effect of PKC stimulation on α₁a-AR activity, agonist-induced [Ca²⁺]ᵢ mobilization and G protein coupling to this receptor were studied. It can be observed (Fig. 1, upper left panel; see also Ref. 18) that a marked [Ca²⁺]ᵢ mobilization was induced by 10 nM NE and that this effect was not inhibited by preincubation with 1 μM TPA for 15 min. Neither did shorter incubations with 1 μM TPA (1 and 5 min) affect the response to 10 μM NE (data not shown). A slight displacement to the right in the concentration-response curve to NE was observed in cell pretreated with 1 μM TPA for 15 min (the EC₅₀ changed from 130 to 240 nM) (Fig. 1, lower left panel). The repetitive addition of 10 μM NE resulted in gradual decreases of [Ca²⁺]ᵢ, but this effect seems to be at least partially because of calcium depletion (18). The effect of preincubation with 1 μM TPA on the NE-induced [Ca²⁺]ᵢ was also studied in cells expressing α₁b-ARs. It was observed that TPA markedly reduced the effect of NE and increased the EC₅₀ from 190 nM in the controls to 1790 nM in the cells treated with the phorbol ester (Fig. 1, right panels). This finding is in agreement with previous findings and confirms that the susceptibility of these receptors to desensitization by PKC activation differs greatly (18).

To test whether agonist stimulation of α₁a-ARs or activation of PKC with phorbol esters affects the coupling of these receptors to GTP-binding proteins, we determined the ability of NE to stimulate the binding of [35S]GTPγS to membranes from control, NE-stimulated, or TPA-stimulated cells. Fig. 2, left
In vitro agonist-stimulated [35S]GTPγS binding to membranes from cells preincubated with norepinephrine or TPA. Rat-1 fibroblasts expressing α1a-ARs (left panel) or α1b-ARs (right panel) were incubated in the absence of agonist (filled circles), in the presence of 10 μM norepinephrine (filled squares) for 5 min, or in the presence of 1 μM TPA (open circles) for 15 min, and membranes were prepared. The means are plotted, and vertical lines represent the S.E. of 12–15 determinations using membranes obtained from three different cultures. The mean vehicle-stimulated [35S]GTPγS binding was normalized to 100% for each group. In cells expressing α1a-ARs (left panel), the effects of preincubation with NE and TPA were statistically significant at 100 nM and 1 μM NE (p < 0.05). In cells expressing α1b-ARs (right panel), the effects of NE and TPA were statistically significant at 100 nM and greater (p < 0.01).

The same treatments applied to cells expressing α1b-ARs gave very different results. As shown in Fig. 2, right panel, cell treatment with NE or TPA markedly decreased maximal in vitro NE-stimulated [35S]GTPγS binding and also altered the EC50 values (125 nM in control membranes, 700 nM in membranes from NE-treated cells, and 700 nM in membranes from TPA-treated cells). None of these treatments modified the response to 1 μM lysophosphatidic acid, used as control (data not shown). Overnight treatment of the cells with pertussis toxin blocked the effect of lysophosphatidic acid but had no influence on the action of 10 μM NE (data not shown).

To compare the phosphorylation of α1a-ARs and α1b-ARs under identical labeling conditions (0.1 mCi of [32P]Pi for 3 h) in simultaneous experiments, and as shown in Fig. 4, right panel, the phosphorylation observed was much more intense in the α1b-AR.

NE-stimulated α1a-AR phosphorylation took place very rapidly, reaching its maximum at 1 min (Fig. 5, upper panel) and rapidly decreasing afterward; but it remained ~2–3-fold above basal level for 1 h. The effect was dependent on the concentration of NE used (EC50 ~ 500 nM, Fig. 6). The action of TPA was also concentration-dependent (EC50 ~ 25 nM, Fig. 6) but had a very different time course, with the effect of TPA being much slower (t1/2 ~ 10 min) and sustained for 1 h (Fig. 5).

Effect of Protein Kinase C Inhibitors on the α1a-AR Phosphorylations Induced by Norepinephrine and TPA—Preincubation for 30 min with 300 nM staurosporine, 300 nM Ro 31–8220, or 1 μM bisindolylmaleimide I markedly decreased but did not abolish the effects of NE and TPA (Fig. 7).

Photoaffinity Labeling, Immunoprecipitation, and Phosphorylation of Chimeric α1x-ARs—To compare the phosphorylation characteristics between α1a- and α1b-ARs, both subtypes were independently transfected into Cos-1 cells. Phosphorylation of these receptors was similar to that observed in Rat-1 fibroblasts (see Fig. 4), i.e. α1a-AR phosphorylation was much less
Fig. 4. Effects of norepinephrine and TPA on $\alpha_{1a}$-AR and $\alpha_{1b}$-AR phosphorylation. Left panel, Rat-1 fibroblasts expressing $\alpha_{1a}$-ARs were metabolically labeled with $[^{32}P]P_i$ (0.2 mCi/ml) and incubated for 5 min in the absence of any agent (B) or with 10 $\mu$M NE or 1 $\mu$M TPA. A representative autoradiograph of immunoprecipitated $\alpha_{1a}$-ARs is shown. The positions of pre-stained molecular mass markers are indicated (kDa). As a reference, $[^{125}I]$arylazidoprazosin-labeled $\alpha_{1a}$-AR was electrophoresed in the same gel. The graph shows the results of seven independent experiments (done at least in duplicate). The means are plotted, and vertical lines represent the S.E. of four independent experiments. Right panel, cells expressing $\alpha_{1a}$-ARs (first three columns) or $\alpha_{1b}$-ARs (last three columns) were metabolically labeled with $[^{32}P]P_i$ (0.1 mCi/ml). The means are plotted, and vertical lines represent the S.E. of four experiments performed in parallel with the two cell lines. Representative autoradiographs are shown.

Fig. 5. Time courses of the effects of norepinephrine and TPA on $\alpha_{1a}$-AR phosphorylation. Rat-1 fibroblasts expressing $\alpha_{1a}$-ARs were metabolically labeled with $[^{32}P]P_i$ and incubated for the times indicated with 10 $\mu$M NE (upper panel) or 1 $\mu$M TPA (lower panel). The means are plotted, and vertical lines represent the S.E. of four independent experiments in each case. Basal phosphorylation was subtracted, and the highest value was considered as 100% in each experiment. Representative autoradiographs for the effects of norepinephrine and TPA are shown. Phosphorylation of $\alpha_{1b}$-ARs immunoprecipitated from nonstimulated (basal) cells is shown in lane B.

Fig. 6. Dose-response curves for the effects of norepinephrine and TPA on $\alpha_{1a}$-AR phosphorylation. Rat-1 fibroblasts expressing $\alpha_{1a}$-ARs were metabolically labeled with $[^{32}P]P_i$ and incubated with the indicated concentrations of NE for 1 min or TPA for 15 min. Basal phosphorylation was subtracted, and the highest value was considered as 100% in each experiment. The means are plotted, and vertical lines represent the S.E. of four (NE) or three (TPA) independent experiments. Representative autoradiographs for the effects of norepinephrine and TPA are shown below the respective graphs. Phosphorylation of $\alpha_{1b}$-ARs immunoprecipitated from nonstimulated (basal) cells is shown in lane B.

Fig. 7. Effect of protein kinase C inhibitors on norepinephrine- and TPA-induced $\alpha_{1a}$-AR phosphorylation. Rat-1 fibroblasts expressing $\alpha_{1a}$-ARs were metabolically labeled with $[^{32}P]P_i$, preincubated for 30 min with 300 nM staurosporine (+ ST), 300 nM Ro 31–8220 (+ Ro), or 1 $\mu$M bisindolylmaleimide I (+ Bm I) and incubated with 10 $\mu$M NE or 1 $\mu$M TPA. The means are plotted, and vertical lines represent the S.E. of four independent experiments. A representative autoradiograph is shown. The effect of the protein kinase C inhibitors on the actions of NE and TPA were statistically significant (p < 0.001).

Fig. 8. Effects of norepinephrine and TPA on wild type and chimeric $\alpha_{1a}$-ARs phosphorylation. Upper panels, schematic representation of wild type (left) and chimeric $\alpha_{1a}$-ARs with interchanged carboxyl-terminal tails (right). Black and gray lines represent sequences from $\alpha_{1a}$-AR and $\alpha_{1b}$-AR, respectively. Zig-zag regions correspond to putative transmembrane domains. The extracellular amino-terminal domains and extracellular loops are those in the upper part of the scheme, and intracellular domains are in the lower part. Lower panels, phosphorylation of wild type and chimeric receptors transfected into Cos-1 cells is shown below the respective schemes. Cells were metabolically labeled with $[^{32}P]P_i$ and incubated for 5 min in the absence of any agent (B), with 10 $\mu$M NE, or with 1 $\mu$M TPA. Autoradiographs are representative of five independent experiments. Arrows indicate the receptors.

Intense than that of the $\alpha_{1b}$-ARs, but in both cases NE and TPA were able to increase it (Fig. 8, left panel). The phosphorylation sites of the $\alpha_{1b}$-AR have been mapped to its carboxyl-terminal tail (10, 42). Therefore, to determine the influence of the carboxyl-terminal tail in the phosphorylation characteristics of $\alpha_{1a}$- and $\alpha_{1b}$-ARs, chimeric receptors with exchanged tails were constructed. Interestingly, the origin of the carboxyl-terminal tail determined the phosphorylation characteristics of these receptors. Whereas wild-type $\alpha_{1a}$-ARs showed little basal phos-
The opposite was detected in 1b-ARs wearing the 1a-AR carboxyl-terminal tail (Fig. 8, right panel).

**Discussion**

We previously reported that the maximally stimulated activity of 1a-ARs was unaffected by phorbol ester-mediated activation of PKC in whole cells (18). In the present experiments, measuring [Ca\(^{2+}\)], we observed a barely detectable decrease in sensitivity to NE, with no change in maximal effect, in cells treated with TPA. However, a more clear shift to the right, also with no change in the maximum, was observed when 1b-AR-stimulated \([^{35}S]GTP\_yS\) binding was studied. This finding is in marked contrast with what was observed in cells expressing 1b-ARs in both parameters. These data indicate that PKC activation induced a marked desensitization of the 1a subtype and affected 1a-ARs to a much lesser extent. We provide data indicating that even when activation of PKC does not lead to clear receptor desensitization in whole cells, TPA and NE induce phosphorylation of 1b-ARs. When the phosphorylation data are compared with those obtained with cells expressing the 1a-ARs, the differences are very dramatic. First, the basal phosphorylation of the receptors is markedly different; 1b-ARs basal labeling is very strong, whereas that of 1a-ARs was observed with great difficulty. In fact, we had to increase the amount of radioactive phosphate used during the labeling period by 4-fold (as compared with the amount used in our previous studies with 1b-ARs (11, 12)) to observe clearly the basal labeling. The efficiencies of immunoprecipitation of photoaffinity-labeled receptors were very similar (compare the present data with those shown in Ref. 11). However, the density of receptors in the line of Rat-1 fibroblasts that express the 1b subtype (~2 pmol/mg membrane protein) (11, 18) is greater than that found in cells expressing the 1a subtype (1.5 pmol/mg membrane protein). Nevertheless, this does not seem to explain the difference in basal receptor labeling. In fact, the data with chimeric receptors strongly suggest that the properties (sequence/conformation) of carboxyl-terminal tails determine the susceptibility of these receptors to be phosphorylated. This finding is consistent with the localization of the phosphorylation site of the hamster 1a-ARs in the carboxyl terminus (42).

Second, although the effects of NE and TPA are strong when compared with the weak basal 1a-AR labeling (i.e. ~5- and ~10-fold increases), the intensity of the signal is clearly less than that observed in the 1b-ARs. This was also evidenced in the studies with the chimeric receptors and again emphasizes the importance of the carboxyl-terminal tails. It is also clear that the time courses of the 1a-AR phosphorylations induced by NE and TPA differ markedly. NE induced a very rapid receptor phosphorylation that also diminished very fast. In contrast, TPA induced a relatively slow effect on receptor phosphorylation that was maintained for up to 60 min. These contrasting time courses suggested that phorbol ester-sensitive PKC isoforms might not be involved in the rapid effect of NE. However, the use of PKC inhibitors strongly suggested that PKC could be involved in agonist-induced 1a-AR phosphorylation. Nevertheless, the PKC inhibitors did not block all of the agonist-mediated effect. Current ideas suggest that GRKs play a major role in homologous desensitization of several G protein-coupled receptors, including 1a- and 1b-ARs (2, 3, 6, 9). It is therefore very likely that the NE-induced 1a-AR receptor phosphorylation could involve some isoform(s) of GRK and PKC, but this possibility remains to be defined experimentally.

Most evidence associates phosphorylation of G protein-coupled receptors to desensitization. Certainly one of the most surprising findings in the present work was that, despite clearly observing TPA-induced 1a-AR phosphorylation, there was great difficulty in detecting receptor desensitization in whole cells. It is possible that receptor reserve and signal amplification steps could have contributed to such difficulty. However, even in the experiments on adrennergically mediated \([^{35}S]GTP\_yS\) binding to membranes, treating cells with NE or TPA did not decrease the maximal binding but only reduced receptor sensitivity to NE. The difference from what is observed with 1b-ARs is very clear.

NE, TPA, and activation of endothelin ETA receptors elicit a strong phosphorylation of 1a-ARs that correlates with desensitization (7–11). However, even in the case of 1b-ARs, not all stimuli that result in phosphorylation of these receptors desensitize them. For example, activation of B2 bradykinin receptors, or coexpression of GRK-5, increases the phosphorylation of 1b-ARs without any clear negative effect on their function (9, 12).

On the other hand, recent examples have indicated that G protein-coupled receptors can be desensitized without being phosphorylated (13, 14). However, in this family of receptors, no information is available regarding phosphorylation events that do not result in desensitization. Current ideas suggest that in a family of receptors, diversity could often be explained in terms of differential distribution and/or differential regulation. In the case the ARs, members of the \(\beta\) and \(\alpha\) families show variation in their abilities to be desensitized, and in these cases, that variation coincides to differential phosphorylation (15–17). In the case of 1a-ARs, there are clear differences between 1a- and 1b-ARs in their ability to be desensitized and susceptibility to phosphorylation.

The resistance to inactivation may have physiological significance. Persistent activity of 1a-ARs has been detected in several cellular contexts in which these receptors are endogenously expressed. An elegant study by Rokosh et al. (28) showed that the three members of the 1a-AR family are coexpressed in neonatal rat cardiac myocytes. Interestingly, chronic stimulation with phenylephrine induced down-regulation of 1b-ARs and 1a-ARs, whereas the content of 1a-ARs increased and correlated with the cellular hypertrophy elicited by phenylephrine (28). Certainly, this complex phenomenon may involve various processes. Numerous studies have revealed the participation of 1a-ARs in the etiology of benign prostate hypertrophy (29, 30, 43). Some indications of vascular tone modulation by 1a-ARs have also been reported (25). However, whereas the participation of 1a-ARs in cellular events related to hypertrophy and vascular tone is unquestionable, the intracellular events that regulate these situations are still under investigation. When the different 1a-AR subtypes were expressed in Rat-1 fibroblasts, 1a-ARs showed better coupling efficiency for NE-induced \([Ca^{2+}]_i\) mobilization and \([^{3}H]\)inositol phosphate production than the 1b or 1a-AR subtype (18). Recent reports indicate that even if the phosphoinositide turnover/calcium mobilization pathway is a major signal transduction mechanism employed by 1a-ARs, other signaling pathways, including activation of phosphoinositide 3-kinase and mitogen-activated protein kinase are stimulated by these receptors (22). Interestingly, differences in the intracellular processes that lead to phosphoinositide 3-kinase activation by 1a-AR and 1b-AR subtypes have been observed, and the 1a-AR subtype seems to be unable to elicit activation of this kinase (22). It is currently unknown to what extent these differences are related to susceptibility to desensitization/phosphorylation.

In summary, our data indicate that: 1) receptor activation or stimulation of TPA-sensitive PKC isoforms induces phosphorylation of 1a-ARs, 2) NE-induced receptor phosphorylation takes place much faster than that induced by TPA, 3) 1a-ARs...
are much less sensitive (or more resistant) to desensitization and are phosphorylated to a lesser extent than $\alpha_\text{1b}$-ARs, and 4) chimeric $\alpha_{1\text{a}}/\alpha_{1\text{b}}$-AR receptors provided further evidence on the importance of the carboxyl-terminal tails for receptor phosphorylation.

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