Acute Agonist-mediated Desensitization of the Human α₁a-Adrenergic Receptor Is Primarily Independent of Carboxyl Terminus Regulation

IMPLICATIONS FOR REGULATION OF α₁a-AR SPLICE VARIANTS

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Despite important roles in myocardial hypertrophy and benign prostatic hyperplasia, little is known about acute effects of agonist stimulation on α₁a-adrenergic receptor (α₁a-AR) signaling and function. Regulatory mechanisms are likely complex since 12 distinct human α₁aAR carboxyl-terminal splice variants have been isolated. After determining the predominance of the α₁aAR isoform in human heart and prostate, we stably expressed an epitope-tagged α₁a-AR cDNA in rat-1 fibroblasts and subsequently examined regulation of signaling, phosphorylation, and internalization of the receptor. Human α₁a-AR-mediated inositol phosphate signaling is acutely desensitized in response to both agonist and phorbol 12-myristate 13-acetate (PMA) exposure. Concurrent with desensitization, α₁aARs in [³²P]labeled cells are rapidly phosphorylated in response to both NE and PMA stimulation. Despite the ability of PKC to desensitize α₁aARs when directly activated with PMA, inhibitors of PKC have no effect on agonist-mediated desensitization. In contrast, involvement of GRK kinases is suggested by the ability of GRK2 to desensitize α₁aARs. Internalization of cell surface α₁aARs also occurs in response to agonist stimulation (but not PKC activation), but is initiated more slowly than receptor desensitization. Significantly, deletion of the α₁aAR carboxy terminus has no effect on receptor internalization or either agonist-induced or GRK-mediated receptor desensitization. Because mechanisms underlying acute agonist-mediated regulation of human α₁aARs are primarily independent of the carboxyl terminus, they may be common to all functional α₁a-AR isoforms.

α₁a-Adrenergic receptors (α₁aARs) are G protein-coupled receptors (GPCR) that mediate sympathetic nervous system responses such as smooth muscle contraction and myocardial inotropy (1). NE stimulation of α₁aARs predominantly activates Gα_{q} and results in membrane polyphosphoinositide hydrolysis by activation of phospholipase Cβ; the resultant second messengers IP_{3} and DAG mobilize intracellular calcium and activate protein kinase C (PKC), respectively (2). Three α₁AR subtypes (α₁a, α₁b, and α₁d) have been cloned and pharmacologically characterized in several expression systems (for review, see Ref. 2). Clinically, activation of α₁aARs has been implicated in the dynamic component of benign prostatic hyperplasia leading to bladder outlet obstruction and in the development of myocardial hypertrophy (3–5). Notwithstanding the importance of α₁ARs in several pathophysiological states, surprising little is known about mechanisms underlying α₁AR expression and function. Transcriptional mechanisms unique to α₁aARs have been shown to be important in maintaining full α₁AR responsiveness to agonist in rat neonatal myocytes where long term (24–72 h) NE stimulation leads to up-regulation α₁AR mRNA and receptor protein expression, concurrent with down-regulation of α₁bAR and α₁dAR subtypes (5, 6). While important, such long-term studies do not examine acute regulation of α₁aAR signaling in response to agonist stimulation, giving rise to the question whether α₁aARs are subject to acute processes such as agonist-induced desensitization and receptor phosphorylation.

Desensitization (dampening of receptor responsiveness to continued agonist exposure) is thought to involve a number of inter-related yet distinct mechanisms occurring at the receptor and more broadly in the signal transduction pathway (7). Receptor desensitization may occur within seconds to minutes of agonist stimulation, and is generally considered to result from receptor uncoupling from downstream effectors due to receptor phosphorylation. Phosphorylation of activated GPCRs by G protein-coupled receptor kinases (GRKs) leads to increased binding of β-arrestin to the receptor complex, uncoupling receptors from G proteins and, in some cases, leading to internalization of the phosphorylated receptors. Phosphorylation, desensitization, internalization, and down-regulation (decreased expression) of the α₁bAR subtype in response to either agonist or PMA exposure have been extensively demonstrated in native and cell models (8–12). Significantly, these processes are mediated through the carboxyl terminus of the α₁bAR. Truncation of its carboxyl terminus significantly decreases agonist- and PMA-mediated phosphorylation, desensitization, and internalization of the α₁aAR, and recent studies have pinpointed critical serine residues within that receptor

NE, norepinephrine; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; PBS, phosphate-buffered saline; IP, inositol phosphate.

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The abbreviations used are: α₁-AR, α₁a-adrenergic receptors; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; 1a-Adrenergic receptors (α₁a-ARs) are G protein-coupled receptors (GPCR) that mediate sympathetic nervous system responses such as smooth muscle contraction and myocardial inotropy (1). NE stimulation of α₁aARs predominantly activates Gα_{q} and results in membrane polyphosphoinositide hydrolysis by activation of phospholipase Cβ; the resultant second messengers IP_{3} and DAG mobilize intracellular calcium and activate protein kinase C (PKC), respectively (2). Three α₁AR subtypes (α₁a, α₁b, and α₁d) have been cloned and pharmacologically characterized in several expression systems (for review, see Ref. 2). Clinically, activation of α₁aARs has been implicated in the dynamic component of benign prostatic hyperplasia leading to bladder outlet obstruction and in the development of myocardial hypertrophy (3–5). Notwithstanding the importance of α₁ARs in several pathophysiological states, surprising little is known about mechanisms underlying α₁AR expression and function. Transcriptional mechanisms unique to α₁aARs have been shown to be important in maintaining full α₁AR responsiveness to agonist in rat neonatal myocytes where long term (24–72 h) NE stimulation leads to up-regulation α₁AR mRNA and receptor protein expression, concurrent with down-regulation of α₁bAR and α₁dAR subtypes (5, 6). While important, such long-term studies do not examine acute regulation of α₁aAR signaling in response to agonist stimulation, giving rise to the question whether α₁aARs are subject to acute processes such as agonist-induced desensitization and receptor phosphorylation.

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region that are involved in each of these regulatory processes (8, 11–13).

In contrast to the α1AR, current knowledge regarding agonist-mediated regulation of the α1AR subtype is severely lacking. One study of bovine α1ARs recently suggested that the α1aAR, similar to the α1b subtype, is subject to agonist-induced desensitization and phosphorylation (14). Mechanisms underlying regulation of α1ARs, however, are potentially more complex than those of the α1b subtype. Unlike α1b and α1dARs which are each expressed as a single isoform, several distinct α1a isoforms have been isolated in addition to the original “wild type” α1aAR, 12 in humans and 4 in rabbit (15–18). Although several of these variants give rise to non-functional truncated polypeptides with only six transmembrane domains, there are four fully functional α1aAR isoforms that exhibit ligand binding and signaling characteristics essentially identical to those of the wild type receptor (15–17). It is of particular interest that all functional α1aAR splice variants are identical except at the distal ends of their carboxyl terminal, where each differs in sequence and length. The study of α1AR regulation in endogenous systems may prove difficult, however, since several α1aAR carboxyl-terminal splice variants are concurrently expressed in every tissue studied thus far (15–17). Additionally, expression of severely truncated α1aAR isoforms can decrease signaling by full-length receptors, possibly through interference with trafficking and membrane localization of full-length receptors (17).

To accurately characterize acute agonist-mediated regulation of human α1aAR signaling, we directly profiled expression levels of functional full-length α1aAR splice variants in human heart and prostate and identified α1a-1 as the predominant α1aAR isoform in these tissues. We then stably expressed the α1a-1AR as an epitope-tagged fusion protein in rat-1 fibroblasts to examine effects of acute (≤30 min) NE and PMA stimulation on α1aAR signaling and to subsequently explore the roles of PKC and several GRK family members, receptor phosphorylation, and receptor internalization in acute regulation of α1aARs. In parallel we constructed, expressed, and tested a fully functional carboxyl-terminal truncated α1aAR (whose sequence is common to all functional α1aAR splice variants) to characterize any regulatory features that may be independent of carboxyl-terminal variation. Our findings suggest that acute agonist-mediated regulation of human α1ARs is primarily independent of the carboxyl terminus and could therefore be common to all functional α1aAR isoforms.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Invitrogen (Grand Island, NY). Bovine serum albumin, [3H]dihydroalprenolol, and [3H]propranolol were from PerkinElmer Life Sciences (Boston, MA). Dowex 1–20 were obtained from Bio-Rad Laboratories (Hercules, CA). PMA and phenolamine were from Sigma. Nor epinephrine, prazosin, and 5-methylurapidil were from Research Biochemical International (Natick, MA). Bisindolylmaleimide I was from Calbiochem (La Jolla, CA). The cDNA for GRK2 cloned into pcDNA1 was provided by Robert Lefkowitz (I). The cDNA for GRK2 cloned into pcDNA3 containing the wild type α1AR to generate full-length α1AR splice variant constructs. Digestion of each splice variant construct with EcoRV and PstI provided isoform-specific fragments that were subsequently ligated into pGEM7z+ (Promega, Madison, WI). Modified sequences of each PCR construct were verified by dyeoxy DNA sequencing using the fno1 DNA Sequencing System (Perkin–Elmer). The probe for RNase protection assays was conducted as previously described (13).

Construction of HA-tagged α1AR and Carboxyl Terminus Truncated Mutants—To facilitate phosphorylation studies, sequence of the hemagglutinin (HA) epitope was added to the amino terminus of the human α1aAR cDNA using PCR mutagenesis. The 5’ (sense) 59-mer oligonucleotide (5'-AAAAGAATTCATGACCATAAGCCGAGCAGCCTCACTGTCGCTTCTCTCGGGAATG-3') contained a synthetic EcoRI restriction endonuclease site, a 5-basesequence of the HA epitope (YPYDVPDYA, bold italics) immediately downstream of the α1aAR start codon, and 19 bases corresponding to bp 4 to 22 of the α1aAR (underlined). The 3’ (antisense) primer was 5’-GGGACCGCTTACAGAAGATGCTTGGAAGG-3’, corresponding to bases 796 to 763 of the α1aAR receptor (GenBank accession number 4501960). The resulting PCR product was digested with EcoRI and Eco47III and subcloned into the mammalian α1AR expression vector pcDNA3:α1AR; the final construct was called pcDNA3:HA-α1a. A carboxyl-terminal-truncated α1AR mutant (pcDNA3:HA-T348) was generated by introducing a stop codon after Arg294 of α1aAR, in pcDNA3:HA-α1a using the Transformer Site-directed Mutagenesis Kit (Clontech, Palo Alto, CA). Modified sequences of each construct were verified by dyeoxy DNA sequencing.

Cell Culture, Stable Transfection, and α1AR Ligand Binding—Cells were grown in monolayers and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO2 at 37 °C for 30 min to determine total cellular receptor content. Phenolamine (10−3 M) was used to determine nonspecific prazosin binding. Following binding, cells were washed three times with ice-cold PBS containing 0.1% bovine serum albumin, and scraped in 1 ml of water. 1H C was counted in 7 ml of scintillation mixture using a Wallac 1410 Liquid Scintillation Counter (Wallac, Gaithersburg, MD). Measurement of Total Inositol Phosphate Production—Rat-1 cells stably expressing either HA-α1a or HA-T348 receptors were labeled with [3H]inositol for 20–24 h with 2.5 μCi/ml in DMEM supplemented with 3% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). After labeling, cells were washed twice with PBS and then with a mixture of PBS and 5% fetal bovine serum at 37 °C for 15 min. Pretreated cells were exposed to various drugs during this incubation as indicated. 

Saturation binding and competition analysis of transfected cell membranes was performed with the radiolabeled α1AR antagonist [125I]HEAT (300 and 120 pM for saturation and competition binding, respectively) as previously described (20). The α1AR agonists norepinephrine and oxymetazoline, and antagonists prazosin and phentolamine were utilized for competition analysis. Resultant curves were fit using noniterative regression analysis with PRISM software (Graphpad, San Diego, CA).

Ligand binding on intact cells was performed as described by Lattion (8) with a few modifications. Cell monolayers grown in 12-well plates were washed once with DMEM and treated with drugs, washed once again with DMEM and then incubated with 2 nm [3H]prazosin in 0.5 ml of DMEM at each of 4 °C for 12–15 h to determine cell surface binding or at 37 °C for 30 min to determine total cellular receptor content. Phenolamine (10−3 M) was used to determine nonspecific prazosin binding. Following binding, cells were washed three times with ice-cold PBS containing 0.1% bovine serum albumin, and scraped in 1 ml of water; 1H C was counted in 7 ml of scintillation mixture using a Wallac 1410 Liquid Scintillation Counter (Wallac, Gaithersburg, MD). Measurement of Total Inositol Phosphate Production—Rat-1 cells stably expressing either HA-α1a, or HA-T348 receptors were labeled with [3H]inositol for 20–24 h with 2.5 μCi/ml in DMEM supplemented with 3% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). After labeling, cells were washed twice with PBS and then with a mixture of PBS and 5% fetal bovine serum at 37 °C for 15 min. Pretreated cells were exposed to various drugs during this incubation as indicated. 

Cells were then quickly washed with PBS, placed in PBS with 20 mM LiCl and immediately stimulated by NE addition for the indicated times. NE was added to the cells from >100 stocks in 10 mM acetic acid while only acetic acid was added to unstimulated cells. Inositol phosphates were extracted as described by Martin (21) and separated using Dowex
AG1-X8 anion exchange (formate) columns. After washing the columns twice with water, total inositol phosphates were eluted in 1 M ammonium formate, 0.1 M formic acid, and combined with 15 ml of scintillation mixture; \(^{3}H\) was quantitated using a liquid scintillation counter.

Final GRK-mediated desensitization assays were performed in transiently transfected cells, since GRK transfection in stable cell lines resulted in unexpected accelerated cell death (a condition not seen in any other assay). All final IP assays were normalized for \(\alpha_{1a}\)AR density and cell count at the time of assay. GRK overexpression was confirmed by Western analysis. Rat-1 or COS-7 cells were plated at 40,000 cells per well in 12-well plates, grown overnight, and transfected with 0.25 \(\mu\)g of pcDNA containing \(\alpha_{1a}\)ARs or HA-T348 with or without 0.25 \(\mu\)g of pcDNA containing GRK2 or GRK6; the total transfected DNA was kept at 0.75 \(\mu\)g (per 40,000 cells) by adding pcDNA3. Following 18 h of transfection, cells were washed with Hank’s balanced salt solution, labeled with \(^{32}\)P[H]inositol in 10% fetal bovine serum, and assayed for total inositol phosphate production in DMEM essentially as described for stable cells.

**Photoaffinity Labeling of \(\alpha_{1a}\)ARs—**Membranes from cells expressing either HA-\(\alpha_{1a}\), or HA-T348 receptors, prepared for ligand binding as described above, were resuspended at a concentration of 1 \(\mu\)g of total protein/\(\mu\)l. 175 \(\mu\)g of total protein were incubated with 6 \(\mathrm{nmol}\) \(^{125}\)I-Iodosozidoprazosin (200 \(\mu\)l total volume) in the dark at room temperature for 60 min in the presence or absence of prazosin (1 \(\mu\)M). Following incubation, samples were UV-irradiated for 10 min, centrifuged at 14,000 \(\times\) g for 10 min, and resuspended in 6 \(\times\) SDS-PAGE sample buffer. Proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel (Protegel, National Diagnostics). \(^{125}\)I-Labeled receptors were detected by exposure to X-Omat AR film (Kodak) for 24–48 h.

**\(^{32}\)P Labeling and Immunoprecipitation of \(\alpha_{1a}\)ARs in Cells—**Equal numbers of rat-1 fibroblasts stably expressing either HA-\(\alpha_{1a}\), or HA-T348 receptors were grown to confluency in 6- or 12-well cluster plates. Cell monolayers were washed three times with phosphate-free DMEM, then incubated in the same medium containing \(^{32}\)P (0.2 mCi/ml) for 2 h at 37 °C. Different drugs were added during this incubation as indicated under “Results.” Following incubation, cells were washed three times with ice-cold PBS, solubilized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1 mM Na$_{2}$VO$_{4}$, 10 mM Na$_{3}$PO$_{4}$, 10 mM NaF, and Complete Protease Inhibitor Mixture (Roche Molecular Biochemicals)), and centrifuged at 14,000 \(\times\) g for 10 min at 4 °C. To reduce background, solubilized proteins were incubated with protein G-agarose (Roche Molecular Biochemicals) for 1 h at 4 °C. After removal of nonspecific precipitated material, the supernatant was incubated with 3F10 rat monoclonal antibody (Roche Molecular Biochemicals) against the HA epitope tag at 4 °C. After 1 h, protein G-agarose beads were added to the sample mixture and incubation was continued overnight. Isolated immune complexes were washed twice with ice-cold lysis buffer, twice with high salt buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate), and once with low salt buffer (50 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, 0.05% sodium deoxycholate). After removal of buffer, immune complexes were resuspended in 6 \(\times\) SDS sample buffer and were separated by electrophoresis on a 10% SDS-PAGE gel. After autoradiography, \(^{32}\)P was quantitated with a PhosphorImager and analyzed using ImageQuant gel image analysis software.

**Statistical Analysis—**Results are expressed as mean ± S.E. Statistical significance was analyzed by two-factor ANOVA (where appropriate) followed by a two-tailed unpaired \(t\) test, with \(p < 0.05\) considered to be significant.

**RESULTS**

**Profile of Human \(\alpha_{1a}\)AR Isoform Expression in Prostate and Heart**

To focus our studies on the full-length functional \(\alpha_{1a}\)AR isoform that is predominantly expressed in human prostate and heart, we directly examined expression of the four full-length \(\alpha_{1a}\)AR splice variants in these tissues, as well as in SK-N-MC cells (the only currently available human cell line that endogenously expresses \(\alpha_{1a}\)ARs, albeit at very low levels). RNase protection assays (without prior PCR amplification) were performed using \(\alpha_{1a}\)AR probes designed to contain both isoform-specific carboxyl-terminal sequences (WT \(\alpha_{1a}-1\), \(\alpha_{1a}-2\), \(\alpha_{1a}-3\), and \(\alpha_{1a}-4\)) and sequences common to all functional \(\alpha_{1a}\)AR isoforms (Fig. 1A). With each probe, levels of individual isoform mRNA expression (isoform-specific fragment) were quantitated relative to all other functional \(\alpha_{1a}\) isoforms (common sequence fragment). As shown in Fig. 1, B and C, the \(\alpha_{1a}-1\) (wild type \(\alpha_{1a}\)) predominates in human prostate (81 ± 2%), heart (89 ± 4%), and SK-N-MC cells (85 ± 1%). \(\alpha_{1a}-4\) mRNA represents 6–11% of the remaining \(\alpha_{1a}\)AR mRNA pool, whereas \(\alpha_{1a}-2\) and \(\alpha_{1a}-3\) are rare in all tissues/cells studied (3–9% combined). Because of its overwhelmingly predominant expression, we subsequently focused our studies on regulation of the wild type \(\alpha_{1a}-1\) receptor (hereafter referred to as \(\alpha_{1a}\)AR).

**Construction, Stable Expression, and Characterization of HA-\(\alpha_{1a}\)AR and Carboxyl-Truncated Mutant HA-T348**

To facilitate phosphorylation studies of the wild type human \(\alpha_{1a}\)AR, we constructed two specialized mutant \(\alpha_{1a}\)ARs: 1) an amino terminus HA epitope-tagged \(\alpha_{1a}\)AR (HA-\(\alpha_{1a}\)), and 2) a mutated HA-\(\alpha_{1a}\) in which the carboxyl terminus was truncated after amino acid 348, eliminating the last 118 amino acids of the \(\alpha_{1a}-1\)AR (HA-T348). HA-T348 retains expression of the palmitoylated Cys$_{145}$ (22), but is truncated well upstream of the \(\alpha_{1a}\)AR carboxyl-terminal splice site present in all func-
Acute Desensitization of Human $\alpha_{1a}$-Adrenergic Receptor

We next examined the rate of total IP accumulation following NE stimulation as a function of time in rat-1 fibroblasts expressing HA-$\alpha_{1a}$ or HA-T348. Total IP production rates of both full-length and truncated receptors are identical (Fig. 2). Stimulation of cells with $10^{-5}\text{ M}$ NE induces a rapid rise in IP levels for 2 min, after which point the rate of accumulation decreases. This not only suggests that desensitization of $\alpha_{1a}$AR occurs at this early time point but also implies that the carboxyl-terminal tail is not required for acute desensitization. In addition, signaling by both receptors continues to rise at an identical, robust, and nearly linear rate through at least 30 min of receptor stimulation. Continued accumulation of total IPs throughout this time period indicates that the agonist-sensitive pool of $[\text{H}]$inositol-labeled membrane lipids is not rapidly depleted by agonist treatment in these experiments. It also suggests that non-acute mechanisms of desensitization do not predominate over this period.

**Time Course of Acute Agonist- and PMA-induced $\alpha_{1a}$AR Desensitization**

To address the issue of whether or not human $\alpha_{1a}$ARs undergo acute desensitization in response to agonist stimulation, we examined the ability of HA-$\alpha_{1a}$-expressing rat-1 fibroblasts to respond to a subsequent challenge with NE after initial pretreatment with NE. Cells were treated with $10^{-5}\text{ M}$ NE for 2 to 30 min in PBS without LiCl (to allow recycling of IPs generated during pretreatment), washed, and then restimulated with $10^{-5}\text{ M}$ NE in the presence of LiCl. NE-stimulated IP responses of pretreated cells were compared with those of untreated cells to determine both the time course and extent of HA-$\alpha_{1a}$ desensitization. Desensitization of HA-$\alpha_{1a}$ to subsequent NE stimulation occurs within 2 min of NE agonist pretreatment, reducing the IP response to ~60–70% that of naive cells (Fig. 3A, Table II). Longer periods of pretreatment with NE result in reduced, but continued receptor responsiveness; even after 30 min of NE stimulation, signaling by HA-$\alpha_{1a}$ persists at 50% naive levels. Significantly, parallel experiments involving the carboxyl-truncated $\alpha_{1a}$AR, HA-T348, revealed that both the rate and extent of agonist-induced desensitization are essentially identical to the corresponding wild type responses (Fig. 3A, Table II). Thus, even very short NE pretreatment periods can induce nearly maximal acute desensitization and this behavior is observed even in the HA-T348 receptor lacking the carboxyl terminus. The modest amount of additional desensitization that occurs between 5 and 30 min also appears similar in the full-length and truncated receptor.

**Table I** Pharmacological characteristics of HA-$\alpha_{1a}$ and HA-T348 compared to wild type $\alpha_{1a}$AR

| Characteristic | HA-$\alpha_{1a}$ | HA-T348 | $\alpha_{1a}$AR WT |
|----------------|-----------------|---------|-------------------|
| **Agonists**   |                 |         |                   |
| Norepinephrine | $4.7 \pm 0.10$  | $4.6 \pm 0.18$ | $4.8 \pm 0.03$    |
| Oxymetazoline  | $7.7 \pm 0.27$  | $7.9 \pm 0.11$ | $7.8 \pm 0.05$    |
| **Antagonists**|                 |         |                   |
| Prazosin       | $8.9 \pm 0.03$  | $8.9 \pm 0.03$ | $9.1 \pm 0.02$    |
| 5-Methylurapidil| $8.5 \pm 0.16$  | $8.7 \pm 0.19$ | $8.6 \pm 0.32$    |

$^a$ Competition binding assays were performed as described under “Experimental Procedures.” Data are mean ± SEM; $n$ = three to nine independent experiments, each performed in triplicate.

$^b$ [3H]Inositol-labeled rat-1 fibroblasts expressing HA-$\alpha_{1a}$ or HA-T348 were treated for 20 min with varying concentrations of NE and total inositol phosphates were quantitated. Data are mean ± SEM; $n$ = three to six independent experiments each performed in triplicate.

**Fig. 2.** Time course of total IP accumulation in response to NE stimulation of HA-$\alpha_{1a}$ and HA-T348. Rat-1 fibroblasts expressing HA-$\alpha_{1a}$ or HA-T348, metabolically labeled with [3H]inositol for 20–22 h, were stimulated with $10^{-5}\text{ M}$ NE for different periods of time in the presence of LiCl. Resultant levels of total IPs were quantitated. Data are mean ± S.E., $n$ = four independent experiments, each performed in triplicate and normalized to maximum IP production at 30 min.
Although the intracellular carboxyl terminus has been implicated in regulation of several GPCRs including the \( \alpha_{1a} \)AR, these data suggest that the carboxyl terminus of the human \( \alpha_{1a} \)AR does not play a similar role in regulating agonist-induced desensitization.

**Potential PKC Involvement in Acute Agonist-induced Desensitization of \( \alpha_{1a} \)ARs**

Agonist stimulation of \( \alpha_{1a} \)ARs leads to activation of the second messenger-dependent kinase PKC, a kinase that could potentially feed back to phosphorylate and desensitize the receptors. To examine a possible role of PKC in \( \alpha_{1a} \)AR desensitization, we utilized the active phorbol ester PMA to stimulate PKC directly. PMA pretreatment of cells expressing \( \alpha_{1a} \)AR or \( \alpha_{1a} \)AR effectively reduces the IP response of both full-length and truncated receptors to a subsequent stimulation by NE, although \( \alpha_{1a} \)AR is desensitized to a greater extent than \( \alpha_{1a} \)AR at each time point (Fig. 3B, Table II). PMA-induced desensitization is dose-dependent, with maximal effects achieved with 10 Con neurotransmitters (data not shown). Similar to the time course of NE desensitization, PMA-induced desensitization is rapid, with maximal desensitization occurring within 2 min of PMA treatment. Thus PKC could potentially play a role in agonist-mediated desensitization of \( \alpha_{1a} \)ARs. It is worth noting, however, that while carboxyl-truncated \( \alpha_{1a} \)AR is less sensitive to PMA-mediated desensitization than the full-length receptor, the truncated receptors sensitivity to agonist-mediated desensitization is not different from the full-length \( \alpha_{1a} \)AR. This suggests that second messenger activation of PKC probably does not serve in an agonist-mediated desensitization feedback loop.

To explore this topic further, we utilized the specific PKC inhibitor bisindolylmaleimide I (GF 109203X) to block PKC activity. If PKC plays a substantial role in agonist desensitization, pretreatment of \( \alpha_{1a} \)AR expressing cells with the PKC inhibitor should reduce the desensitizing effects of NE pretreatment. Initial dose-response experiments determined that 1 \( \mu \)M bisindolylmaleimide I is sufficient to completely inhibit PMA-mediated effects on \( \alpha_{1a} \)AR signaling (data not shown). When \( [\text{H}] \)inositol-labeled cells expressing either full-length and truncated \( \alpha_{1a} \)ARs were treated with bisindolylmaleimide I complete inhibition of PMA-induced desensitization of both receptors was seen (Fig. 4). In contrast, treatment with the PKC inhibitor does not affect the degree to which either receptor is desensitized in response to NE stimulation. These data indicate that although direct PKC activation can lead to desensitization of \( \alpha_{1a} \)ARs, it does not play a significant feedback role in agonist-induced desensitization of \( \alpha_{1a} \)ARs.
GRK-mediated Desensitization of α₁ARs

After concluding that PKC does not function in a significant feedback capacity in agonist-mediated α₁AR desensitization, we examined the ability of members of the GRK family to affect α₁AR signaling. In these experiments, IP accumulation was assessed in rat-1 cells transiently expressing HA-α₁a, or HA-T348 with or without coexpressed GRK2 and GRK6 (Fig. 5). Coexpression with GRK2 desensitized both HA-α₁AR and HA-T348, resulting in 63.8 ± 2.6% of the activity of receptor alone, respectively (Fig. 5, A and B). In contrast, coexpression of GRK6 with HA-α₁AR resulted in no desensitization (Fig. 5C). The ability of GRK2 to desensitize HA-α₁AR was also confirmed in COS-7 cells where it was found to be similar to that observed for HA-α₁bAR (data not shown). Although perhaps coincidental, it is worth noting that GRK-mediated desensitization (Fig. 5) and NE-induced (compare with Table II and Fig. 3) desensitization were approximately the same. The identical desensitization observed for full-length and truncated α₁AR reinforces the evidence presented above indicating that the carboxyl terminus plays little if any role in desensitization of this receptor in rat-1 cells.

Potential Mechanisms Underlying NE- and PMA-induced α₁AR Desensitization

Phosphorylation of Full-length and Truncated α₁ARs in Cells—Discovery of rapid desensitization of human α₁ARs led us to explore possible mechanisms underlying this phenomenon. Rapid phosphorylation of GPCRs by GRKs and/or second messenger-activated kinases such as PKC is thought to lead to receptor desensitization; therefore, we desired to examine phosphorylation of full-length and truncated α₁ARs in intact cells. Experiments designed to identify α₁AR proteins showed that photoaffinity labeling of membranes expressing HA-α₁a with [125I]azidoprazosin yields a single diffuse band centered at ~60 kDa (Fig. 6, lane 1). This corresponds to the size of the glycosylated wild type α₁aAR reported previously (25). Labeling of membranes derived from cells expressing HA-T348 yields a diffuse band of ~48 kDa, corresponding to the expected gel mobility of the truncated receptor (Fig. 6, lane 2). Prazosin (1 μM) blocks labeling of both peptides, confirming specificity of the photoaffinity labeling reaction and identification of α₁ARs (Fig. 6, lanes 3 and 4).

Phosphorylation of α₁ARs was examined in rat-1 fibroblasts stably expressing HA-tagged full-length or truncated receptors that were equilibrated with inorganic 32P to label intracellular pools of ATP. Following drug treatment, cells were lysed and solubilized and the HA epitope-tagged receptors were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis. Treatment of cells expressing HA-α₁a or HA-T348 with either NE or PMA clearly resulted in increased phosphorylation of the receptors relative to untreated cells (Fig. 6). This phosphorylation response was both dose-dependent (data not shown) and time-dependent (see below). Less phosphorylation was observed for HA-T348 than for the HA-α₁a (Fig. 6). This
PMA-induced H9251/H9252 (10 M) phosphorylation from untreated cells normalized to basal levels of receptor shown in the lanes; data were quantitated and low arbitrary PhosphorImager units) shown net phosphorylation values (PUI in arbitrary S.E.; n = three to seven independent experiments.

These data demonstrate that α1a-ARs in rat-1 cells; furthermore, treatment with forskolin/isobutylmethylxanthine (10 M) does not change basal phosphorylation of α1aARs, ruling out a possible role for cAMP-dependent protein kinase A (PKA) in either basal or agonist-mediated α1aAR phosphorylation (data not shown). These data demonstrate that α1aARs are phosphorylated in response to both agonist stimulation and direct activation of PKC by PMA. In addition, stimulation with either NE or PMA causes receptor phosphorylation at sites outside of the carboxyl tail of the α1aAR.

To characterize the temporal correlation between α1aAR desensitization and receptor phosphorylation, we examined receptors from 32P-labeled rat-1 fibroblasts expressing either full-length or truncated receptors that were treated with NE or PMA for various periods of time (Fig. 7). In a manner that parallels time courses of both NE- and PMA-induced desensitization of α1aARs, the overall time frame of both NE and PMA-induced phosphorylation of α1aAR is approximately half that observed for full-length receptor. As for HA-α1a, phosphorylation of HA-T348 is maximal at about 2 min of receptor stimulation during which time 32P incorporation into the receptor rises 8.9-fold over basal levels (Fig. 7B). However, after 5 min receptor phosphorylation begins to decrease until a steady level of 4.2-fold over basal is achieved after 20 min of receptor stimulation. It is important to remember that HA-T348 remains maximally desensitized during this entire period (refer to Fig. 3A). In addition, quantification of Fig. 7 data (in arbitrary pixel units) suggests that NE-induced phosphorylation of the truncated receptor is approximately half that observed for full-length receptor. This observation could indicate that truncation has eliminated potential phosphorylation sites. However, other explanations are possible; for example, decreased solubilization or immunoprecipitation of HA-T348 compared with full-length receptor. Even if NE stimulation induces phosphorylation of sites in the carboxyl-terminal tail of α1a-AR, the absence of any apparent role for the carboxyl-terminal tail in desensitization (Figs. 2, 3, and 5) suggests the sites do not participate in controlling agonist-mediated desensitization. This does not preclude potential roles of other phosphorylation sites in other regulatory processes.

The time course of PMA effects on HA-T348 phosphorylation is similar to that of the full-length receptor. Maximal 32P incorporation was achieved between 2 and 5 min of treatment with PMA, continuing through 30 min (Fig. 7B). Quantitation indicates that PMA-induced phosphorylation of HA-T348 is about half that observed for the full-length receptor. In contrast to agonist-mediated desensitization, the carboxyl-terminal tail of α1a-AR does influence PMA-induced desensitization (see Fig. 3B above). This fact is consistent with phosphorylation of the tail playing a role is PKC mediated α1aAR desensitization. Nevertheless, PMA-induced phosphorylation and partial desensitization of HA-T348 suggests regions other than the carboxyl-terminal tail also participate in these events. As for full-length α1aARs, the overall time frame of both NE and PMA-induced phosphorylation of HA-T348 receptors is closely...
correlated with the temporal occurrence of acute receptor desensitization.

Agonist-induced Internalization of α₁₃ARs—Sequestration of receptors from the extracellular surface into various intracellular compartments may occur as a component of both agonist-dependent and agonist-independent desensitization and/or desensitization processes. To determine whether receptor sequestration plays a role in acute desensitization of α₁₃ARs, we performed radioligand binding on intact rat-1 fibroblasts expressing HA-α₁₃ or HA-T348 with the lipophilic α₁AR antagonist [³H]prazosin at 4 °C, as previously described. Ligand binding at this temperature has been shown to inhibit partitioning of lipophilic prazosin across cell membranes, thus preventing recognition of receptors located intracellularly and allowing quantitation of cell surface receptors; ligand binding at 37 °C, on the other hand, allows detection and quantitation of total cellular receptors (26). Effects of NE treatment were similar in cells expressing full-length α₁₃ARs and those expressing the truncated receptor. During at least 10 min of agonist stimulation, there is no measurable decrease in the number of cell surface α₁₃ARs (Fig. 8A). Between 10 and 30 min of agonist exposure, however, surface receptor numbers decrease by 25% and continue to slowly decline over the next 60 min. Total numbers of either full-length or truncated α₁₃ARs do not decline in response to NE or PMA stimulation over this same time period, indicating that loss of surface receptors following NE exposure is due to internalization and not degradation of receptors (data not shown). Thus, internalization of α₁₃ARs occurs at a much slower rate than does either phosphorylation or desensitization of the receptors and, therefore, cannot be responsible for rapid α₁₃AR desensitization that is seen within the first several min of agonist exposure.

Interestingly, PMA treatment has no effect on the number of full-length or truncated α₁₃ARs at the cell surface throughout 90 min of exposure (Fig. 8B). The inability of PMA-mediated stimulation of PKC to induce internalization of α₁₃ARs stands in stark contrast to its ability to induce phosphorylation and dampen signaling of these receptors, indicating that these are sharply distinct processes for the α₁₃AR. This also suggests that increased receptor phosphorylation alone does not induce α₁₃AR internalization, but that other factors are necessary to initiate this process. These α₁₃AR regulatory processes are clearly not induced by PKC activity. Furthermore, when cells stably expressing α₁₃ARs are treated with the PKC inhibitor bisindolylmaleimide I prior to agonist stimulation, there is no effect on the rate or extent of agonist-mediated internalization of HA-α₁₃ARs (data not shown). These data provide further evidence that, similar to agonist-mediated α₁₃AR desensitization, agonist-induced internalization of α₁₃ARs occurs through a PKC-independent pathway.

Discussion

One unique feature of α₁₃AR expression is the presence of at least 12 receptor isoforms in various species, as opposed to single isomorph expression of α₁₃B and α₁₃D family members. The four full-length functional human isoforms (WT α₁₃-1, α₁₃-2, α₁₃-3, and α₁₃-4) differ in amino acid sequence at their distal carboxyl termini. Although the physiological significance of the α₁₃AR isoforms remains unclear particularly since their pharmacologic binding properties and second messenger coupling are identical, differences between their carboxyl-terminal amino acid sequences suggest that each α₁₃AR isoform may be differentially regulated through this receptor region. For example, 4 potential PKC sites are found in the carboxyl terminus of the α₁₃-1, 3 each in isoforms α₁₃-2, α₁₃-3, and α₁₃-4 (15, 16). Studies of α₁₃AR desensitization in endogenous systems face several potential difficulties, however, since all four functional α₁₃ARs and numerous nonfunctional truncated receptor isoforms are found in all tissues studied thus far. To focus on the most abundant α₁₃AR isoform in human prostate and heart, we profiled expression of functional α₁₃AR isomorph mRNAs in these tissues. α₁₃-1 is clearly the predominant splice variant in human prostate and heart, as well as in SK-N-MC cells. Although Chang et al. (16) originally suggested that the α₁₃-1 mRNA predominates in human prostate (based on reverse transcriptase-PCR experiments), our direct RNAse protection assay results clearly demonstrate much higher expression of the α₁₃-1 isoform in both prostate and heart. Therefore, we stably transfected epitope-tagged human α₁₃-1 AR cDNA in rat-1 fibroblasts to create a system in which acute regulation of the α₁₃-1 AR isoform can be studied without confounding expression of other splice variants.

Our studies provide convincing evidence that human α₁₃ARs are subject to acute agonist-induced desensitization of IP signaling in response to NE stimulation. Desensitization of α₁₃ARs is rapid (occurring within 2 min) and is characterized by a rightward shift and lowering of the NE dose-response curve (see Table II). We demonstrate that α₁₃AR phosphorylation is significantly increased within 2 min of agonist stimulation and closely correlates with acute agonist-mediated desensitization of α₁₃ARs. This agonist-induced process is most likely mediated through actions of at least one GRK as overexpression of GRK2 is able to diminish α₁₃AR-mediated IP signaling. These data also suggest that receptor phosphorylation plays an important role in acute agonist regulation of α₁₃AR signaling, as has been proposed for GPCRs in general. It is possible that agonist-induced desensitization and phosphorylation of α₁₃ARs may be concurrent yet independent events; indeed, the causal
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effect of protein phosphorylation on receptor desensitization has not been definitively demonstrated for any GPCR. However, mutagenic elimination of protein phosphorylation sites in several GPCRs (including \( \alpha_{1a} \)-ARs) does provide compelling evidence that the elimination of receptor phosphorylation sites results in significantly reduced receptor desensitization (8, 11, 12).

Internalization of GPCRs from the cell surface may also serve to desensitize receptor populations by removal of receptors from agonist exposure and prevention of further signaling. Agonist stimulation does induce internalization of human \( \alpha_{1a} \)-ARs (as demonstrated by loss of cell surface receptor binding). However, since this does not occur until after at least 10 min of agonist exposure, it cannot be responsible for rapid agonist-mediated \( \alpha_{1a} \)-AR desensitization that is seen after only 2 min. This finding corresponds with observations that receptor internalization does not play a role in rapid desensitization of several other GPCRs, including the \( \beta_{2} \)-AR (27), m3-muscarinic receptor (28), and \( \alpha_{1b} \)-AR (8).

Agonist-mediated desensitization of \( \alpha_{1a} \)-ARs occurs independently of PKC effects, as demonstrated by the failure of PKC inhibition to affect this process. A similar lack of phospholipase C involvement in agonist-mediated desensitization of several other phospholipase C-coupled receptors including substance P (29), m3-muscarinic (30), thromboxane (31), and \( \alpha_{1b} \)-adrenergic receptors (8) has also been demonstrated. In our hands, human \( \alpha_{1a} \)-ARs are nevertheless subject to PKC-mediated desensitization as phorbol ester (PMA) pretreatment substantially decreased IP production resulting from subsequent NE stimulation of the \( \alpha_{1a} \)-AR. PMA treatment also leads to a rapid increase in \( \alpha_{1a} \)-AR phosphorylation that closely correlates with the onset of PMA-induced desensitization. In this context, it is noteworthy that PMA treatment does not induce internalization of \( \alpha_{1a} \)-ARs from the cell surface. Conversely, PKC inhibition does not affect agonist-mediated internalization of \( \alpha_{1a} \)-ARs from the cell surface. These data support the hypothesis that exposure to agonist and PMA induces distinct and possibly separate \( \alpha_{1a} \)-AR regulatory responses. Although our data do not support a specific role of PKC “feedback” in agonist-induced desensitization of \( \alpha_{1a} \)-ARs, this second messenger-dependent kinase may play a role in modulating the ability of \( \alpha_{1a} \)-ARs to signal following stimulation of other PLC-coupled receptors within the same cell. This hypothesis is supported by recent demonstrations that stimulation of endothelin ETA receptors or bradykinin B2 receptors in rat-1 fibroblasts leads to a marked increase in phosphorylation of stably expressed \( \alpha_{1a} \)-ARs (32, 33).

Perhaps our most significant finding is that mechanisms underlying acute agonist-dependent regulation of human \( \alpha_{1a} \)-AR signaling appear to function independently of the receptor carboxyl terminus. This is demonstrated by equal sensitivity of the truncated and full-length \( \alpha_{1a} \)-AR to NE-induced desensitization. In addition, GRK2 desensitizes signaling by the truncated and full-length receptor equally, suggesting that the agonist-mediated pathway of desensitization remains intact in the absence of the \( \alpha_{1a} \)-AR carboxyl terminus. On the other hand, sensitivity of the truncated receptor to PMA-induced desensitization is less than that of the full-length \( \alpha_{1a} \)-AR. These data provide further evidence for the hypothesis that agonist- and PMA-induced desensitization proceed through separate, distinguishable mechanisms, and indicate that although the \( \alpha_{1a} \)-AR carboxyl terminus plays a substantial role in PMA-mediated desensitization, it is not required in the agonist-mediated pathway. This highlights a significant regulatory difference between human \( \alpha_{1a} \)-ARs and the \( \alpha_{1b} \)-AR subtype as the carboxyl-terminal of the \( \alpha_{1b} \)-AR plays an indispensable role in mediating both agonist- and PMA-induced phosphorylation and desensitization (8, 11, 12). In addition, our results place the human \( \alpha_{1a} \)-AR as the only \( G_{q} \)-coupled member of a very small group of GPCRs whose mechanisms of desensitization are primarily independent of carboxyl terminus regulation (e.g., follicltropin receptor (34), \( \alpha_{2a} \)-ARs (35), and the angiotensin II receptor (36)).

As acute desensitization of truncated \( \alpha_{1a} \)-ARs predicts, NE and PMA treatment each lead to significantly increased phosphorylation of the truncated \( \alpha_{1a} \)-AR. However, HA-T348 is modestly less phosphorylated than the full-length receptor under all conditions. This is not an unexpected result, given that potential phosphorylation sites were eliminated with truncation of the \( \alpha_{1a} \)-AR after Arg348. For example, the human \( \alpha_{1a} \)-AR contains 4 putative PKC phosphorylation sites within the carboxyl terminus as well as several potential sites in other intracellular regions of the receptor, including 1 in the second intracellular loop and 4 in the third intracellular loop. Thus truncation eliminates half of the potential PKC phosphorylation sites, perhaps explaining why the truncated \( \alpha_{1a} \)-AR cannot be desensitized as completely as the full-length receptor. Of course the truncated receptor still displayed substantial PMA-mediated desensitization concurrent with rapid phosphorylation strongly suggesting involvement of sites not in the tail.

Possible sites of GRK-mediated \( \alpha_{1a} \)-AR phosphorylation are more difficult to predict, since consensus sequences for receptor recognition and phosphorylation by individual GRKs are not as clearly defined. Early studies suggested that GRK2 and GRK3 preferentially phosphorylate serines or threonines in proximity to acidic amino acids (37) and Fredericks et al. (38) has observed that several receptors that are phosphorylated by GRKs contain a pair of acidic residues on the amino-terminal side of the phosphorylated residue. The human \( \alpha_{1a} \)-AR does not contain acidic pairs of residues, however, several potential GRK phosphorylation sites (represented by serines and threonines in the vicinity of acidic residues) are found within the carboxyl terminus and, more interestingly, within intracellular loops of the human \( \alpha_{1a} \)-AR. Experiments presented here indicate that elimination of some potential GRK phosphorylation sites by truncation does not affect the ability of the \( \alpha_{1a} \)-AR to undergo acute agonist-mediated desensitization even if overall receptor phosphorylation is decreased. Furthermore, agonist-induced phosphorylation of the truncated \( \alpha_{1a} \)-AR occurs concomitant with acute desensitization. These data are consistent with the hypothesis that phosphorylation of only a discrete subset of amino acids (retained within the truncated human \( \alpha_{1a} \)-AR) regulates agonist-mediated desensitization of the \( \alpha_{1a} \)-AR, although other sites may be phosphorylated that do not affect receptor desensitization. It is worth noting that the agonist-induced phosphorylation increase (over basal) was higher for HA-T348 (8.9-fold) than for full-length HA-\( \alpha_{1a} \)-AR (4.5-fold). It could certainly be the case that fold increases in phosphorylation can influence desensitization as much as absolute phosphorylation levels. If so, the fact that decreasing HA-T348 phosphorylation remains at 4.2-fold over basal even at the longest NE exposure times (30 min), may explain continued maximal desensitization. On the other hand, at times far removed from acute desensitization it is probable that mechanisms directed at downstream elements of the IP cascade are functioning.

While our manuscript was in preparation, a study of phosphorylation of the bovine \( \alpha_{1a} \)-AR stably expressed in rat-1 fibroblasts was published by Vazquez-Prado and colleagues (14). Both studies observe that exposure of the \( \alpha_{1a} \)-AR to NE causes rapid receptor phosphorylation (1 to 2 min), generating a 5–10-fold increase in phosphorylation compared with basal. Vazquez-Prado and colleagues (14) also suggested that phosphorylation of \( \alpha_{1a} \)-AR occurred primarily in the carboxyl-termi-
nal tail; however, the evidence presented was indirect. While phosphorylation of the chimeric α1aAR (consisting of the unphosphorylatable transmembrane region of α1bAR (12) and carboxyl terminus of α1aAR) does imply phosphorylation of the terminus, it does not demonstrate this phosphorylation is dominant or functionally significant. Indeed, no functional assays were performed using the chimeric protein. On the other hand, data presented in our current study are clear and direct; we demonstrate that the truncated α1aAR (HA-T348) is phosphorylated in response to NE stimulation in the same rapid manner as full-length receptor and furthermore, has identical desensitization properties. Although, phosphorylation of the carboxyl terminus may occur, it is not involved in agonist-mediated desensitization as deletion of the tail did not change any characteristic of desensitization.

In conclusion, our findings demonstrate that α1aAR signaling is subject to acute desensitization in response to NE as well as PMA exposure. In agreement with current paradigms, acute desensitization appears to be mediated by receptor phosphorylation since both occur simultaneously within 2 min of NE exposure. The GRK family of kinases is probably responsible for agonist-mediated phosphorylation as PKC was not involved in GRK exposure. In agreement with current paradigms, acute desensitization and begin to unravel the relationship of acute desensitization.

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Acute Agonist-mediated Desensitization of the Human $\alpha_1a$-Adrenergic Receptor Is Primarily Independent of Carboxyl Terminus Regulation: IMPLICATIONS FOR REGULATION OF $\alpha_1a$AR SPLICE VARIANTS
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