Phosphorylation and desensitization of α_{1d}-adrenergic receptors

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In rat-1 fibroblasts stably expressing rat α_{1d}-adrenoceptors, noradrenaline and PMA markedly decreased α_{1d}-adrenoceptor function (noradrenaline-elicited increases in calcium in whole cells and [^{35}S]guanosine 5′-[γ-thio]triphosphate binding in membranes), suggesting homologous and heterologous desensitizations. Photoaffinity labelling, Western blotting and immunoprecipitation identified α_{1d}-adrenoceptors as a broad band of 70–80 kDa. α_{1d}-Adrenoceptors were phosphorylated in the basal state and noradrenaline and PMA increased it. The effect of noradrenaline was concentration-dependent (EC_{50} 75 nM), rapid (maximum at 1 min) and transient. Phorbol ester-induced phosphorylation was concentration-dependent (EC_{50} 25 nM), slightly slower (maximum at 5 min) and stable for at least 60 min. Inhibitors of protein kinase C decreased the effect of phorbol esters but not that of noradrenaline. Evidence of cross-talk of α_{1d}-adrenoceptors with receptors endogenously expressed in rat-1 fibroblasts was given by the ability of endothelin, lysophosphatidic acid and bradykinin to induce α_{1d}-adrenoceptor phosphorylation. In summary, it is shown for the first time here that α_{1d}-adrenoceptors are phosphoproteins and that receptor phosphorylation is increased by the natural ligand, noradrenaline, by direct activation of protein kinase C and via cross-talk with other receptors endogenously expressed in rat-1 fibroblasts. Receptor phosphorylation has functional repercussions.

Key words: adrenoceptor, calcium, cross-talk, noradrenaline, protein kinase C.

INTRODUCTION

The actions of the natural adrenergic amines, adrenaline and noradrenaline (NA), are mediated through G-protein-coupled receptors. The function of these receptors, as that of many other receptors of this type, is tightly regulated and phosphorylation seems to be one of the major molecular mechanisms through which this regulation is accomplished [1–3]. The phosphorylation state of these receptors seems to modulate receptor sensitivity and therefore cellular responsiveness. Phosphorylation is considered to be the initial step in the process of desensitization. Current evidence indicates that, in homologous desensitization, agonist-activated receptors interact with G-proteins, which leads to guanine nucleotide exchange and to the release of $G\beta\gamma$ complexes. Such complexes recruit soluble G-protein-coupled receptor kinases, which catalyse receptor phosphorylation. β-Arrestins bind phosphorylated receptors, stabilizing an uncoupled state of the receptors and act as bridges, which bind clathrin molecules that initiate receptor internalization [1–3]. Second-messenger-activated protein kinases, such as protein kinase A and protein kinase C (PKC), also promote receptor phosphorylation, eliciting heterologous desensitization [1–3].

Three families of adrenergic receptor (AR) exist, the α_1-, α_2- and β-ARs, and three receptor subtypes of each family have been cloned [4]. Within a family of receptors there are differences in regulation, which are associated frequently with susceptibility of the various receptors to be phosphorylated. It is known that subtypes of the α_2- and β-ARs are subjected to desensitization according to their suitability as kinase substrates [5,6]. The information on α_1-ARs is still very incomplete (reviewed in [3]).

Three α_1-ARs have been cloned, i.e. the α_{1a}, α_{1b} and α_{1d} subtypes [7–10]. There is evidence that these isoforms are differentially regulated by activation of PKC [11]. Thus activation of PKC with PMA markedly inhibited the actions mediated through the α_{1b} and α_{1d} subtypes, whereas those mediated through α_{1a}-ARs were only marginally altered in side-by-side experiments using cells with similar expression levels [11,12]. α_{1b}-AR phosphorylation has been studied extensively and a major role of PKC in heterologous desensitization is well documented [11,13–16]. It was shown very recently that α_{1a}-ARs are also subject to phosphorylation, although to a much lesser extent than α_{1b}-ARs, and that such phosphorylation has only minor functional repercussions [12]. No data have been published yet on α_{1d}-AR phosphorylation.

In the present article we show that rat α_{1d}-ARs, stably expressed in rat-1 fibroblasts, are phosphorylated in the basal state and that such phosphorylation is increased in response to NA and PMA. These receptor phosphorylations are associated with desensitization in both whole cells and membrane preparations. In addition, we show that there is cross-talk with other receptors endogenously expressed in rat-1 fibroblasts, i.e. activation of the receptors for endothelin, bradykinin and lysophosphatidic acid induces α_{1d}-AR phosphorylation.

EXPERIMENTAL

Materials

(−)-Noradrenaline, lysophosphatidic acid, endothelin I, bradykinin, PMA, staurosporine, wortmannin, guanosine 5′-[γ-thio]triphosphate (GTP[S]), GDP and protease inhibitors were obtained from Sigma. Ro 31-8220 {3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)malimide methane

Abbreviations used: AR, adrenergic receptor; PKC, protein kinase C; NA, noradrenaline; GST, glutathione S-transferase, GTP[S], guanosine 5′-[γ-thio]triphosphate; fura-2/AM, fura-2 acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$.

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sulphonate} and bisindolylmaleimide I were from Calbiochem. Phentolamine was a generous gift from Ciba Geigy. Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, antibiotics and other reagents used for cell culture were from Gibco Life Technologies. [^{3}H]Prazosin (74.4 Ci/mmol), [^{125}I]arylazidoprazosin (2200 Ci/mmol), [^{35}S]GTP[S] (1250 Ci/mmol), [^{32}P]P_i (8500–9120 Ci/mmol) and labelled nucleotides were from New England Nuclear. Sepharose-coupled Protein A was from Upstate Biotechnology. Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes. DNA purification kits were from Qiagen. Restriction and modification enzymes were from New England Biolabs and Boehringer Mannheim. Nylon membranes were from Amersham. Oligonucleotides and automatic sequencing were done in the Molecular Biology Unit, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México City, México. A commercial antibody against α_{1d}-ARs obtained from Santa Cruz Biotechnology was used where indicated.

Cell line and culture

Rat-1 fibroblasts transfected with the rat α_{1d}-AR [9], provided generously to us by Dr R. J. Lefkowitz, Dr M. G. Caron and Dr L. Allen (Duke University, Durham, NC, U.S.A.), were cultured in glutamine-containing high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 300 μg/ml of the neomycin analogue, G-418 sulphate, 100 μg/ml streptomycin, 100 units/ml penicillin and 0.25 μg/ml amphotericin B at 37 °C under a 95% air/5% CO_2 atmosphere as described previously [11]. The α_{1d}-AR density in these cells was in the range of 0.6–1 pmol/mg of membrane protein with a K_D for [^{3}H]prazosin of 0.2–0.3 nM in agreement with previous reports [11]. For all the experiments, confluent cells were serum-deprived overnight in unsupplemented Dulbecco's modified Eagle's medium. Non-transfected wild-type rat-1 fibroblasts were cultured under the conditions described above but in the absence of G-418; as anticipated, no α_1-ARs were detected in [^{3}H]prazosin-binding studies and no functional response to NA was detected (results not shown).

Intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) measurements

Confluent fibroblasts were incubated overnight in Dulbecco's modified Eagle's medium 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. Experiments were performed with $\approx 10^6$ cells suspended in 3 ml of the above-mentioned buffer supplemented with 1.2 mM $CaCl_2$. Fluorescence measurements were carried out as described previously [12,16] with an Aminco-Bowman Series 2 spectrometer with excitation monochromator set at 340 and 380 nm, with a chopper interval of 0.5 s and the emission monochromator set at 510 nm. [Ca^{2+}]$_i$ was calculated according to Grynkiewicz et al. [17] using the software provided by Aminco-Bowman; traces were exported directly to the graphs.

Membrane preparation and [^{35}S]GTP[S] binding

Cells were treated with the different agents to be tested. After stimulation, cells were washed and scraped with a rubber policeman in buffer containing 20 mM Hepes, pH 7.5, 5 mM EDTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM $MgCl_2$ and protease inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml PMSF, 500 μg/ml bacitracin and 50 μg/ml soya bean trypsin inhibitor). Membranes were prepared as described in [12,16] and resuspended in binding buffer (50 mM Tris/HCl, 0.1 M NaCl, 10 mM $MgCl_2$ and 1 mM EDTA, pH 7.4, containing 0.1% BSA, 1 mM dithiothreitol and 1 μM GDP). [^{35}S]GTP[S] binding was performed as described by Wieland and Jakobs [18] with minor modifications. The binding reaction was carried out in a volume of 250 μl for 30 min at 25 °C in binding buffer containing 0.2 nM [^{35}S]GTP[S]. The reaction was initiated by the addition of membranes (25 μg protein/tube) and terminated by the addition of 2 ml of ice-cold 50 mM Tris, pH 7.4, containing 10 mM $MgCl_2$ and filtration on Whatman GF/C filters. Filters were washed three times and dried; radioactivity was measured with a Beckman LS 6000 SC liquid scintillation counter. Non-specific binding was determined in the presence of 30 μM unlabelled GTP[S] and represented about 10% of total binding. Statistical analysis between comparable groups was performed using ANOVA with Newman–Keuls analysis with the software included in the GraphPad Prism 3 program.

Photoaffinity labelling of the α_{1d}-ARs

Membranes from rat-1 cells expressing rat α_{1d}-AR were obtained as described previously [12,16]. In the dark, membranes (25 μg protein) were incubated with 6 nM of [^{125}I]arylazidoprazosin essentially as described in [12,16]. After 1 h at room temperature, 1 ml of 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 5 mM EDTA and the previously mentioned protease inhibitors was added. Open tubes were exposed to UV light for 3 min. Membranes were centrifuged at 12700 ***g*** for 15 min, washed and subjected to SDS/PAGE (10% gel) under reducing conditions. Specificity of the labelling was determined using 10 μM phentolamine as a competitor.

Construction of a glutathione S-transferase (GST)-α_{1d}-AR fusion protein

The C-terminal domain of the rat α_{1d}-AR was amplified by PCR using plasmid pMT2 containing the rat α_{1d}-AR cDNA [10] (donated generously by Dr R. M. Graham, V. Chang Cardiac Research Unit, Sidney, Australia). The oligonucleotides 5′-AT-CTGCAGGGATCCCTCCGCCTCCGGGAGTGGAGA-3′ (sense), corresponding to bases 1928–1943 of the rat α_{1d} open reading frame [9,10,19], with an additional *Bam*HI site, and 5′-TACTGCAGGAATTCTCAAATGTCAGTCTCTCGGAG-3′ (antisense), corresponding to bases 2245–2260 of the gene [9,10,19] with an *Eco*RI site, were used. The amplified fragment (243 bp) was digested with *Bam*HI–*Eco*RI and ligated with pGEX2T also digested with the same enzymes. The construction was analysed by restriction analysis and automatic sequencing. The fusion protein was expressed in *Escherichia coli* (strain MGT-7) under induction conditions and a protein of $\approx$29 kDa was expressed and affinity-purified with glutathione-Sepharose. The anticipated molecular mass of the fusion protein was 35.9 kDa, which lead us to suspect that proteolysis was taking place. The fusion protein was partially sequenced (N-terminal) and it was found to be intact, indicating that proteolysis took place at the C-terminus. Therefore, the fusion protein contained a non-proteolysed GST fused to a receptor peptide of the rat α_{1d}-AR sequence [9,10,19]. The fusion protein was proteolysed with thrombin [20] and the released peptide purified and sequenced: GST–SLR-LREWRLLGPLQR (with SLR as the linker peptide and the following section as the receptor fragment). Antisera against the GST-α_{1d}-AR fusion protein were generated by immunizing rabbits. Animals were injected intradermally with 1 mg of protein in each dose (4 or 5 sites). They were injected 5–7

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times with 2 week intervals between injections; the first administration was with complete Freund's adjuvant and all the following were with incomplete adjuvant. The ability of antisera to immunoprecipate photolabelled receptor was tested.

Immunoprecipitation of α_{1d}-ARs

Photoaffinity-labelled membranes (25 μg) expressing α_{1d}-ARs were used to standardize immunoprecipitation of the receptor. The procedure was similar to that used for the other subtypes [12,16]. Rabbit antiserum against the described GST-α_{1d}-AR fusion protein was used. Membranes were solubilized in 0.5 ml of solubilization buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1.5 % Triton X-100, 0.1 % SDS and the previously mentioned protease inhibitors). After 1 h at 4 °C, the extracts were centrifuged at 12 700 ***g*** for 15 min at 4 °C and the supernatants were transferred to new tubes containing 10 μl of immune serum. Tubes were incubated overnight at 4 °C. Then, 40 μl of Sepharose-coupled Protein A as 50 % (v/v) slurry was added and incubated for 1–2 h. Beads were washed five times (1 ml/each) with 50 mM Hepes, 50 mM NaH_2PO_4 and 100 mM NaCl, pH 7.2, containing 1 % Triton X-100, 0.25 % SDS and 100 mM NaF. Washed beads were suspended in 50 μl of 10 % SDS and vortexed, then 50 μl of $2\times$Laemmli sample buffer containing 5 % 2-mercaptoethanol were added. Samples were incubated during 5 min in a boiling bath and electrophoresed in SDS/PAGE (10 %) minigels. Gels were fixed, dried and exposed to Kodak X-OMAT X-ray film at −80 °C with an intensifying screen. Analysis of immunoprecipitation was performed in a Molecular Dynamics PhosphorImager with the included ImageQuant software.

Phosphorylation of α_{1d}-ARs

Rat-1 fibroblasts were cultured in culture dishes (10 cm diameter). Cells reaching confluence were serum-starved for 24 h. On the day of the experiment fibroblasts were maintained in phosphate-free Dulbecco's modified Eagle's medium for 1 h, and then incubated in 3 ml of the same medium containing $[^{32}P]P_i$ (50 μCi/ml) for 3 h at 37 °C. Labelled cells were stimulated with the agents indicated, then they were washed with ice-cold PBS and solubilized with 1.0 ml of ice-cold solubilization buffer (as used for immunoprecipitation, see above) supplemented with 50 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM *p*-serine and 1 mM *p*-threonine. The plates were maintained for 1 h on ice; then the extracts were centrifuged at 12 700 ***g*** for 15 min at 4 °C, and the supernatants were immunoprecipitated as described above. Receptor phosphorylation was detected with a Molecular Dynamics PhosphorImager and quantified with its ImageQuant software. Data were within the linear range of detection of the apparatus and were plotted using Prism 3. In some experiments the immunoprecipitates were subjected to SDS/PAGE and the proteins transferred to nitrocellulose membranes. Western blotting was performed using the serum obtained against the GST-α_{1d}-AR fusion protein or the commercial antiserum, a conjugated secondary antibody and the enhanced chemiluminescence (ECL) system of Pierce.

RESULTS

The basal intracellular concentration of calcium was 162±18 nM (mean±S.E.M., $n = 16$) in rat-1 fibroblasts stably expressing α_{1d}-ARs. In agreement with previous results [12,16] NA induced an almost immediate increase in $[Ca^{2+}]_i$ (to 500–700 nM at 10 μM NA). The effect of NA was concentration-dependent with an EC_{50} of 95±10 nM ($n = 4$). PMA (1 μM) induced a small but consistent decrease in the basal level of calcium (decrease of 53±4 nM, $n = 10$; Figure 1). This decrease in basal $[Ca^{2+}]_i$ has also been observed with inverse agonists and it has been suggested that this effect of PMA is due to blockade of the constitutive activity of these receptors [21]. If the incubation was continued the level of calcium increased slowly towards the basal level observed before the addition of PMA. The effect of NA was decreased markedly after the addition of PMA, suggesting that activation of PKC blocked/desensitized α_{1d}-ARs (Figure 1). The response to NA in the presence of PMA was decreased markedly at all the concentrations tested and the concentration-response curve was clearly shifted to the right compared with that of control cells (Figure 1; no estimate of the EC_{50} was made because even at the highest concentration tested, 100 μM NA, no clear saturation was observed). Also using this parameter, it was observed that when the cells were treated with 10 μM NA a transient calcium response was observed but that subsequent additions of NA did not result in any additional response (results not shown, see [21]). These data are consistent with the hypothesis that α_{1d}-ARs are subjected to homologous and heterologous desensitizations.

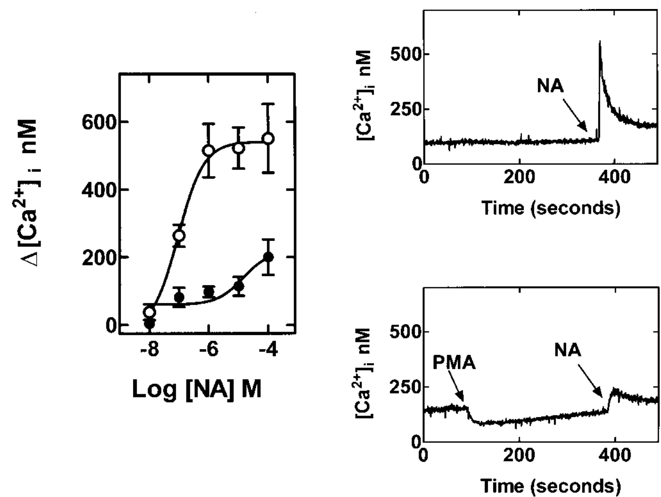

Figure 1 Effect of PMA on NA-induced $[Ca^{2+}]_i$ increase

Rat-1 fibroblasts expressing α_{1d}-ARs were loaded with fura-2/AM and $[Ca^{2+}]_i$ recorded. (Left-hand panel) Concentration-response curves for the effects of NA on $[Ca^{2+}]_i$ in cells incubated for 5 min in the absence (○) or presence (●) of 1 μM PMA. Plotted are the means±S.E.M. from four to six experiments using different cell cultures. (Right-hand panels) Representative traces of the effects of 10 μM NA and 1 μM PMA on $[Ca^{2+}]_i$.

The ability of NA to increase the binding of $[^{35}S]$GTP[S] to membranes was studied in order to substantiate these findings at a subcellular level. For this purpose, cells were incubated in the absence of any agent, 10 μM NA or 1 μM PMA, membranes were prepared and NA-stimulated $[^{35}S]$GTP[S] binding was studied. It can be observed in Figure 2 that in membranes from control cells NA induced an increase in guanine nucleotide binding of $\approx$30 %. This effect was decreased significantly in membranes obtained from cells treated with NA or PMA (Figure 2). Incubation with 1 μM lysophosphatidic acid also markedly decreased NA-induced $[^{35}S]$GTP[S] binding to membranes (Figure 2).

To study the possibility that the desensitization processes could be associated to receptor phosphorylation we proceeded to

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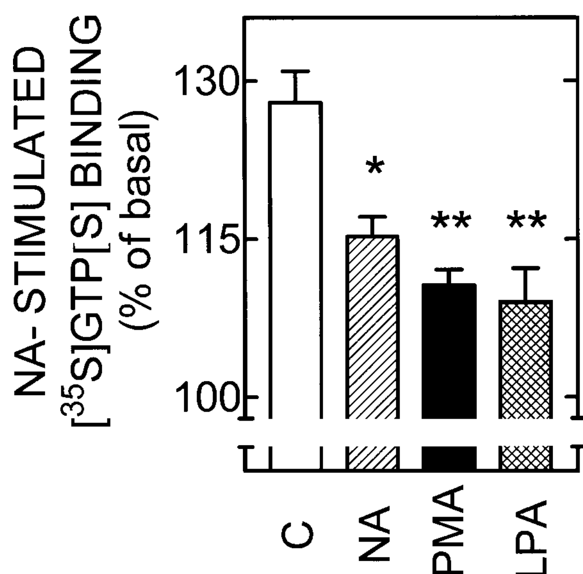

Figure 2 ***In vitro*** **NA-stimulated [^{35}S]GTP[S] binding to membranes**

Rat-1 fibroblasts expressing α_{1d}-ARs were incubated for 5 min in the absence of agonist (control, C), or in the presence of 10 μM NA, 1 μM PMA or 1 μM lysophosphatidic acid (LPA) and membranes were prepared. Plotted are the means ± S.E.M. from 15–18 determinations using membranes obtained from four different cultures. The mean vehicle-stimulated [^{35}S]GTP[S] binding was normalized to 100% for each group. *$P < 0.05$ versus control; **$P < 0.01$ versus control.

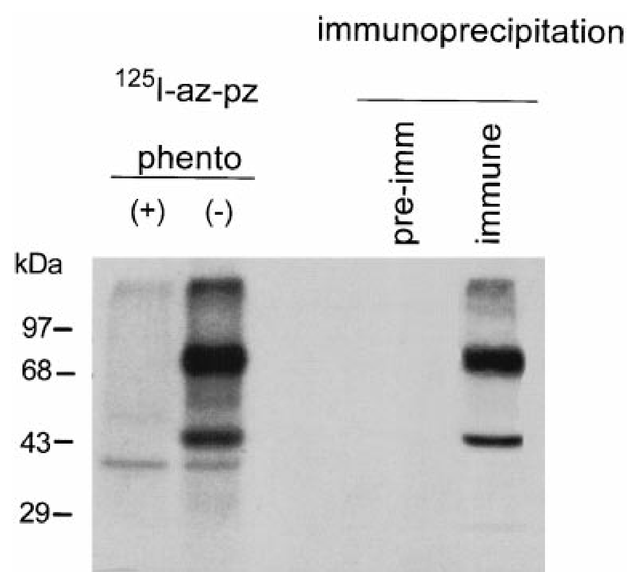

Figure 3 Photoaffinity labelling and immunoprecipitation of the α_{1d}-AR

Membranes from transfected rat-1 fibroblasts were labelled with [^{125}I]arylazidoprazosin (^{125}I-az-pz) in the absence (−) or presence (+) of 10 μM phentolamine (phento). [^{125}I]Arylazidoprazosin-labelled membranes were solubilized and the α_{1d}-AR immunoprecipitated as described in the Experimental procedures section; as a control, pre-immune serum was used (pre-imm). A representative autoradiograph of at least three independent experiments is shown.

perform receptor photoaffinity labelling and immunoprecipitation. Membranes that were incubated with [^{125}I]-arylazidoprazosin and subjected to UV irradiation were employed to define the conditions for immunoprecipitation. Photoaffinity labelling of membranes of rat-1 fibroblasts expressing α_{1d}-ARs resulted in the labelling of a major broad band of 70–80 kDa; the labelling of this band was competed for by phentolamine, indicating that it does correspond to the α_{1d}-ARs.

Three other bands were observed in our photoaffinity labelling studies. A band of ≈ 43 kDa was observed whose labelling was also blocked by phentolamine (Figure 3). This may represent a proteolytic fragment of the receptor either present in whole cells or generated during the experimental procedures, in spite of the presence of protease inhibitors. A faint band of ≈ 38 kDa, whose labelling was not blocked by phentolamine, was also detected. Similarly, a band of high molecular mass (150 kDa) was observed (Figure 3). High-molecular-mass bands have been observed with the other subtypes of this receptor [12,16]; it is possible that they may represent oligomeric forms [22] or aggregates with other proteins that are resistant to the conditions used for electrophoresis. No specific labelling was observed in membranes obtained from untransfected wild-type rat-1 fibroblasts (results not shown).

Photolabelled membranes were solubilized and the ability of the antiserum (generated with the GST-α_{1d}-AR fusion protein) to immunoprecipitate the receptor was tested. It can be observed in Figure 3 that the immune serum immunoprecipitated the receptor, the ≈ 43 kDa putative receptor fragment and the high-molecular-mass bands (Figure 3). Immune serum samples able to immunoprecipitate at least 80% of the photoaffinity-labelled receptor (as shown by PhosphorImager analysis) were used. Preimmune sera were unable to immunoprecipitate the receptor (Figure 3).

Next, we examined whether the receptor was phosphorylated under basal and stimulated conditions. Three major ^{32}P-labelled bands were observed (Figure 4): (i) a broad band of ≈ 70–80 kDa, (ii) a band of high molecular mass (150 kDa) and (iii) a band of ≈ 43 kDa. These are the same bands observed in the photoaffinity labelling experiments and that were immunoprecipitated by the α_{1d}-AR antiserum. No immunoprecipitation

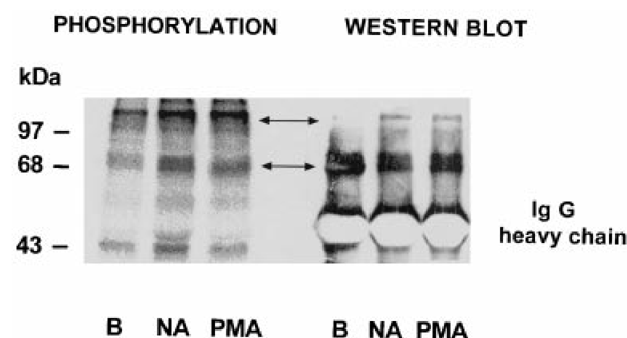

Figure 4 Effects of NA and PMA on α_{1d}-AR phosphorylation and Western blot of the immunoprecipitate

(Left-hand panel) Rat-1 fibroblasts expressing α_{1d}-ARs were metabolically labelled with [^{32}P]P_i and incubated for 5 min in the absence of any agent (B), or with 10 μM NA or 1 μM PMA. Positions of pre-stained molecular-mass markers are indicated. (Right-hand panel) Immunoprecipitates were subjected to SDS/PAGE, transferred to nitrocellulose and Western-blotted. A representative autoradiograph of at least 10 independent experiments is shown. The Western blot was repeated at least three times with identical results.

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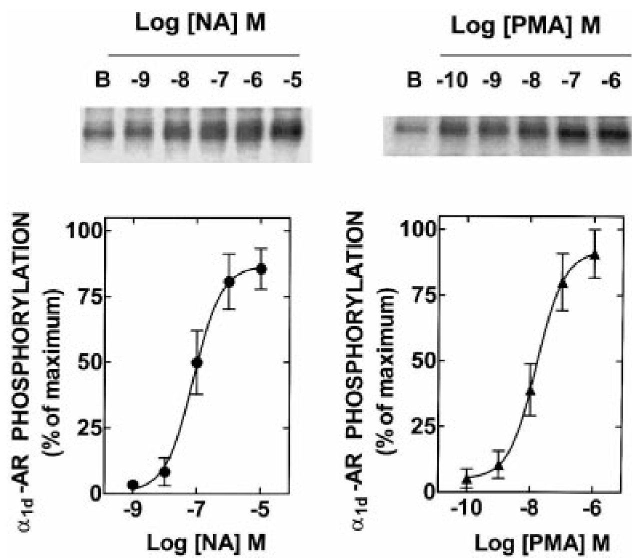

Figure 5 Concentration-response curves for the effects of NA and PMA on α_{1d}-AR phosphorylation

Rat-1 fibroblasts expressing α_{1d}-ARs were metabolically labelled with $[^{32}P]P_i$ and incubated with the indicated concentrations of NA or PMA for 5 min. Basal phosphorylation was subtracted and the highest value was considered as 100% in each experiment. Plotted are the means $\pm$ S.E.M. from four experiments; representative autoradiographs for the effects of NA and PMA are shown.

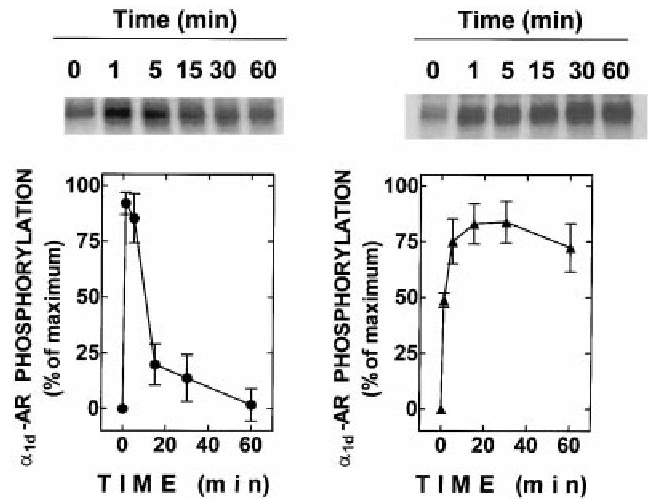

Figure 6 Time courses of the effects of NA and PMA on α_{1d}-AR phosphorylation

Rat-1 fibroblasts expressing α_{1d}-ARs were metabolically labelled with $[^{32}P]P_i$ and incubated for the times indicated with 10 μM NA (left-hand panel) or 1 μM PMA (right-hand panel). Plotted are the means $\pm$ S.E.M. from 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 NA and PMA are shown.

of these bands was observed when the receptor peptide was added. The bands corresponding to the receptor and the high-molecular-mass band (putative oligomers/aggregates) were also recognized in Western blots using the same antiserum. It was not possible to identify the $\approx$ 43 kDa band in the Western blots due to marked interference by the IgG heavy-chain signal. A commercial antibody generated against the α_{1d}-AR C-terminal decapeptide also identified the same bands (results not shown). Phosphorylation experiments were also carried out using untransfected wild-type rat-1 fibroblasts. In immunoprecipitates from these cells a few phosphorylated bands were observed that did not correspond to the receptor. The labelling of these bands was at least 10 times less than that observed in the α_{1d}-ARs in parallel experiments (results not shown).

When rat-1 fibroblasts stably expressing α_{1d}-ARs were stimulated with NA or PMA a marked stimulation of the labelling of the band corresponding to the receptor was observed (10 μM NA, 205$\pm$13% of basal labelling, $n = 11$, $P < 0.001$ versus basal; 1 μM PMA, 269$\pm$20% of basal labelling, $n = 10$, $P < 0.001$ versus basal). There were also increases in the labelling of the high-molecular-mass band and the $\approx$ 43 kDa band in cells stimulated with NA and PMA. In addition, in cells stimulated with these agents faint labelled bands in the region of 55–65 kDa were observed in some experiments (Figure 4). No bands were present when the immunoprecipitation was carried out using preimmune sera or when the α_{1d}-AR peptide (released from the fusion protein by thrombin-catalysed proteolysis) was added during the immunoprecipitation (results not shown). These data indicate that α_{1d}-ARs are phosphorylated in the basal (non-stimulated) state and that NA and PMA markedly increased such phosphorylation.

The concentration-response curves for NA and PMA and the time course of their effects are presented in Figures 5 and 6, respectively. The effect of NA was concentration-dependent (EC$_{50}$ 75$\pm$20 nM, $n = 5$). It was surprising that this concentration-response curve was shifted to the left by approx. one order of magnitude when compared with those observed with the

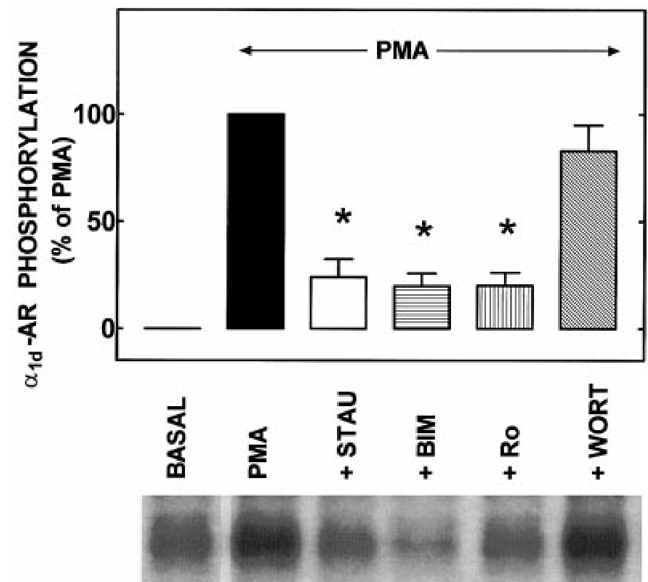

Figure 7 Effect of PKC inhibitors and wortmannin on PMA-induced α_{1d}-AR phosphorylation

Rat-1 fibroblasts expressing α_{1d}-ARs were metabolically labelled with $[^{32}P]P_i$, pre-incubated for 30 min with 300 nM staurosporine (STAU), 1 μM bisindolylmaleimide I (BIM), 300 nM Ro 31-8220 (Ro) or 100 nM wortmannin (WORT) and then incubated with 1 μM PMA for further 5 min. Basal phosphorylation was subtracted and the effect of 1 μM PMA alone was considered as 100% in each experiment. Plotted are the means $\pm$ S.E.M. from six independent experiments. A representative autoradiograph is shown. $^{*}P < 0.001$ versus PMA alone.

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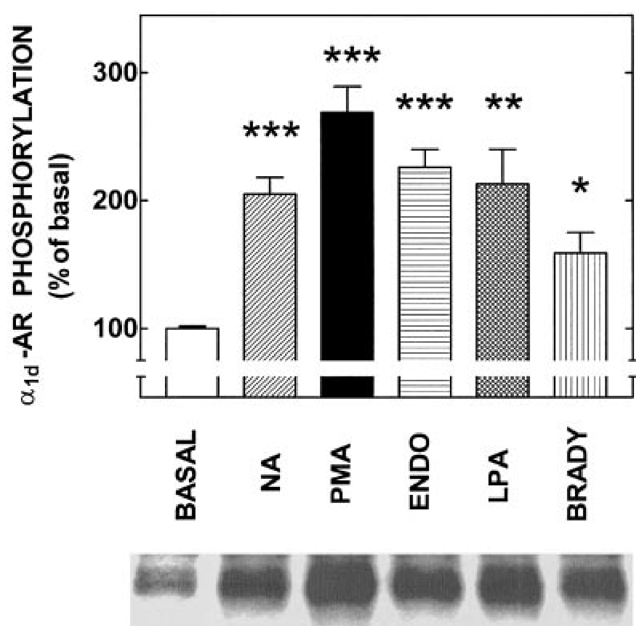

Figure 8 Effect of NA, PMA, endothelin, lysophosphatidic acid and bradykinin on α_{1d}-AR phosphorylation

Rat-1 fibroblasts expressing α_{1d}-ARs were metabolically labelled with [^{32}P]P_i and incubated for 5 min in the absence of any agent (BASAL) or with 10 μM NA, 1 μM PMA, 10 nM endothelin (ENDO), 1 μM lysophosphatidic acid (LPA) or 1 μM bradykinin (BRADY). Basal phosphorylation was considered as 100% in each experiment. Plotted are the means $\pm$ S.E.M. of four independent experiments. A representative autoradiograph is shown. $^{*}P < 0.05$ versus basal; $^{**}P < 0.005$ versus basal; $^{***}P < 0.001$ versus basal.

other subtypes [12,16]. Similarly, the effect of PMA was concentration-dependent (EC_{50} 25 $\pm$ 10 nM, $n = 4$). The effect of NA was rapid; it reached its maximum within the first minute, remained at this level for another 4 min and declined towards the basal level afterwards (Figure 6). In contrast, the effect of PMA was slower, reaching the maximum at 5 min, and this high level of receptor phosphorylation was maintained for 60 min.

As expected, the effect of PMA was markedly inhibited by 300 nM staurosporine, 300 nM Ro 31-8220 or 1 μM bisindolylmaleimide I, inhibitors of PKC, but not by 100 nM wortmannin, selective inhibitor of phosphoinositide 3-kinase (Figure 7). The α_{1d}-AR phosphorylation induced by NA was not altered by any of these kinase inhibitors (results not shown). The inhibitors were without effect on basal phosphorylation at the concentration tested (results not shown).

We have shown previously that activation of receptors endogenously expressed in rat-1 fibroblasts, such as endothelin ET_A receptors, lysophosphatidic acid receptors and bradykinin B2 receptors, results in α_{1b}-AR phosphorylation [11,23,24]. As shown in Figure 8, endothelin and lysophosphatidic acid induced marked phosphorylation of α_{1d}-ARs, and bradykinin induced a smaller but significant effect.

DISCUSSION

α_{1d}-ARs have been particularly intriguing and difficult to study. There were complications in defining them as a new receptor subtype [4,9,10] and detection by radioligand binding techniques has also been problematic [25]. Nevertheless, α_{1d}-ARs are functionally expressed in many arteries [26–28] and seem to be important in the regulation of vascular tone *in vivo* and associated with the pathogenesis/maintenance of hypertension [29].

Activation of α_{1d}-ARs induces a weak response in second-messenger generation [11,30]. This was confirmed in the present study where we observed that NA induced a rapid but relatively small increase in intracellular calcium, when compared with those observed in cells expressing similar densities of the other subtypes [11,12]. It is not currently known if this is an intrinsic property of these receptors or due to modulation of their activity.

In the present study, the ability of NA and PMA to block/desensitize α_{1d}-ARs in whole cells was confirmed and extended. NA-stimulated [^{35}S]GTP[S] binding reflects the ability of agonist-activated α_1-ARs to activate G-protein guanine nucleotide exchange and it is, therefore, an index of receptor–G-protein coupling. Thus our data with membranes confirmed what was observed in whole cells, i.e. that α_{1d}-ARs were subjected to homologous and heterologous desensitizations. Furthermore, the data indicate that the change(s) induced by the cell treatments were stable and persisted after cell disruption and membrane isolation.

The photoaffinity labelling studies identified the receptor as a major broad band of 70–80 kDa. The molecular mass of the peptide chain of the receptor is $\approx$ 60 kDa [9,10]. The broadness of the band and the difference in size suggest that α_{1d}-ARs are glycosylated extensively, as predicted by the sequence [9,10], and as this has been shown experimentally for other α_1-ARs [31]. To the best of our knowledge the only other α_{1d}-AR visualized by photoaffinity labelling has been the human isoform [32] and the reported data are consistent with our observations. α_{1d}-ARs were effectively immunoprecipitated and identified further by Western blotting using two different antibodies directed against different epitopes.

Our present data show that α_{1d}-ARs are phosphoproteins whose basal phosphorylation is increased by the agonist, NA, and by activation of PKC. The effects of these agents were concentration-dependent and their time courses were similar to those observed for the other subtypes [12,16]. As expected, PKC inhibitors blocked the effect of PMA. α_{1d}-AR phosphorylation induced by NA was not blocked by these agents, suggesting a major role of G-protein-coupled receptor kinases in this effect, as shown for the α_{1b}-AR subtype [14]. Nevertheless, the data do not exclude possible role(s) of PKC since the actions could be redundant and interplay between these kinases may exist.

α_{1d}-ARs were phosphorylated in response to activation of non-adrenergic receptors endogenously expressed in the cells. As shown, membranes from cells incubated in the presence of lysophosphatidic acid had a decreased NA-stimulated [^{35}S]GTP[S] binding, indicating clearly that the receptor phosphorylation induced by the lysophospholipid mediator had functional repercussions. This is consistent with what has been observed for the α_{1b}-AR [24]. The α_{1b}-AR phosphorylation induced by endothelin is mediated through pertussis toxin-insensitive G-proteins, putatively of the G_q family, and involves PKC and other, as-yet unidentified, kinases [3,16]. In contrast, the effect of lysophosphatidic acid is mediated through pertussis toxin-sensitive G-proteins, putatively of the G_i family, and is mediated through phosphoinositide 3-kinase and PKC [3,24]. It is likely that similar processes may participate in the effects here described for the α_{1d}-ARs; however, this needs to be shown experimentally.

It should be mentioned that there are marked structural differences between the hamster α_{1b}-AR and the rat α_{1d}-ARs. These receptors have only a 56.6% similarity (48.5% identity). As mentioned, the only α_1-AR in which phosphorylation sites have been defined experimentally is the hamster α_{1b}-AR [33].

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These sites have been located at the C-terminus [14] and correspond to Ser^{394} and Ser^{400} for PKC-mediated phosphorylation and to Ser^{404}, Ser^{408} and Ser^{410} for G-protein-coupled receptor kinase-mediated phosphorylation [33]. The C-terminus of the hamster α_{1b}-AR and that of the rat α_{1d}-AR differ in length, have only a 29.2% similarity (22.7% identity) and the phosphorylation sites detected in the hamster α_{1b}-AR are not conserved in the rat α_{1d}-AR [9,10,19]. In addition, phosphorylation sites are not present only in the C-termini of this family of receptors; G-protein-coupled receptor kinase-mediated phosphorylations have been detected in the middle portion of the third intracellular loop [34]. It is, therefore, clear that there are marked differences among these receptors and that the specific sites involved have to be defined experimentally.

Differential down-regulation of human α_1-AR subtypes, after long-term stimulation, has been observed using stably transfected fibroblasts [35]. Long-term incubation with phenylephrine induced receptor down-regulation in cells expressing human α_{1a}- and α_{1d}-ARs. However, in cells expressing the human α_{1d}-AR subtype an increase in receptor density was observed with this treatment [35]. Long-term treatment with PMA did not change receptor density and did not cause functional desensitization [35]. Receptor density is the result of the balance between synthesis and degradation and many factors participate in these events. In transfected systems factors such as the regulatory elements present in the plasmid construct and the site of insertion may play important roles in the regulation of receptor expression. Therefore, it is presently difficult to relate those long-term effects with our present findings. We have observed that human α_{1B}-ARs expressed in mouse fibroblasts are desensitized and phosphorylated in response to activation of PKC by PMA [36]. These actions were blocked by PKC inhibitors and by overnight treatment with PMA, which induces PKC down-regulation [36].

In summary, we show here for the first time that α_{1d}-ARs are phosphoproteins and that the phosphorylation state of these receptors is increased by NA, by direct activation of PKC, and via cross-talk with other receptors endogenously expressed in rat-1 fibroblasts. In addition, we showed that the phosphorylations induced by NA, PMA and lysophosphatidic acid had functional repercussions. These data complete an initial picture on α_1-AR phosphorylation, and indicate that the three receptor subtypes are subjected to this covalent modification. The α_{1b} and α_{1d} subtypes are efficiently phosphorylated and such phosphorylations have functional repercussions. In contrast, the α_{1a} subtype is phosphorylated to a much lesser extent and phosphorylation has only marginal functional significance. The role of these phosphorylations in a physiological context is as yet unknown. It has been shown that in cultured neonatal heart myocytes, which express the three α_1-AR subtypes, most of the longer-term actions are mediated through the α_{1a} subtype [37,38]. It is tempting to speculate that the susceptibility to phosphorylation may play a role in determining the intensity of acute and longer-term actions of adrenergic amines, the cell responsiveness and the cellular distribution of the receptors under basal, agonist-stimulated and cross-talk-regulated conditions. Further experiments will be required using cells that endogenously express one or more of these receptor subtypes to address these questions.

This research was supported in part by grants from Dirección General de Asuntos del Personal Académico (IN 205100) and Consejo Nacional de Ciencia y Tecnología (27569N). We thank Dr R. J. Lefkowitz, Dr M. G. Caron and Dr L. Allen for donation of the transfected rat-1 fibroblasts; Dr R. M. Graham for the plasmid containing the α_{1d}-AR cDNA; Dr Guillermo Mendoza of the School of Medicine of Universidad Nacional Autónoma de México for kindly sequencing the fusion protein and the receptor peptide; Dr Fernando López-Casillas for donation of the wild-type rat-1 fibroblasts; Jorge Ramirez for help with the programs for sequence analysis and the staff of the Vivarium, Computer Services Unit and Molecular Biology Unit of our institute for their help and advice.

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Received 23 August 2000/23 October 2000; accepted 9 November 2000

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