Published in final edited form as:

Naunyn Schmiedebergs Arch Pharmacol. 2016 August ; 389(8): 911–920. doi:10.1007/s00210-016-1254-2.

Carboxyl terminus-truncated α_{1D}-adrenoceptors inhibit the ERK pathway

Marco A. Alfonzo-Méndez[1], **Jean A. Castillo-Badillo**[1], **M. Teresa Romero-Ávila**[1], **Richard Rivera**[2], **Jerold Chun**[2], and **J. Adolfo García-Sáinz**[1]

[1]Instituto de Fisiología Celular, UNAM, Universidad Nacional Autónoma de México, Apartado Postal 70-248, 04510 Mexico D. F, Mexico

[2]Department of Molecular and Cellular Neuroscience, Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, CA 92037, USA

Abstract

Human α_{1D}-adrenoceptors are G protein-coupled receptors that mediate adrenaline/noradrenaline actions. There is a growing interest in identifying regulatory domains in these receptors and determining how they function. In this work, we show that the absence of the human α_{1D}-adrenoceptor carboxyl tail results in altered ERK (extracellular signal-regulated kinase) and p38 phosphorylation states. Amino terminus-truncated and both amino and carboxyl termini-truncated α_{1D}-adrenoceptors were transfected into Rat-1, HEK293, and B103 cells, and changes in the phosphorylation state of extracellular signal-regulated kinase was assessed using biochemical and biophysical approaches. The phosphorylation state of other protein kinases (p38, MEK1, and Raf-1) was also studied. Noradrenaline-induced ERK phosphorylation in Rat-1 fibroblasts expressing amino termini-truncated α_{1D}-adrenoceptors. However, in cells expressing receptors with both amino and carboxyl termini truncations, noradrenaline-induced activation was abrogated. Interestingly, ERK phosphorylation that normally occurs through activation of endogenous G protein-coupled receptors, EGF receptors, and protein kinase C, was also decreased, suggesting that downstream steps in the mitogen-activated protein kinase pathway were affected. A similar effect was observed in B103 cells but not in HEK 293 cells. Phosphorylation of Raf-1 and MEK1 was also diminished in Rat-1 fibroblasts expressing amino- and carboxyl-truncated α_{1D}-adrenoceptors. Our data indicate that expression of carboxyl terminus-truncated α_{1D}-adrenoceptors alters ERK and p38 phosphorylation state.

Keywords

α1-adrenoceptors; Desensitization; GPCR; Carboxyl tail; ERK phosphorylation

Correspondence to: J. Adolfo García-Sáinz.

Jean A. Castillo-Badillo is currently affiliated at Research Support Network, Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán”, Universidad Nacional Autónoma deMéxico (CICUNAM), Vasco de Quiroga 15, 14000 México, D.F, Mexico

Electronic supplementary material The online version of this article (doi:10.1007/s00210-016-1254-2) contains supplementary material, which is available to authorized users.

Introduction

G protein-coupled receptors (GPCRs) are targets of a large proportion of the therapeutic agents currently in use and constitute the most abundant type of membrane-bound receptors in mammals (Fredriksson and Schioth, 2005, Lagerstrom and Schioth, 2008, Lefkowitz, 2013). α_1-Adrenoceptors (α_1-ARs) belong to this group of receptors and comprise a subfamily that mediates some of the actions of adrenaline and noradrenaline. These receptors participate in the maintenance of homeostasis and play roles in the physiopathology of some diseases, such as hypertension and benign prostatic hyperplasia (García-Sáinz et al., 1999).

The α_{1D}-adrenoceptor is a member of the α_1-AR subfamily (composed of α_{1A}, α_{1B}, and α_{1D} subtypes) (Hieble et al., 1995) and, like all GPCRs, is structurally characterized by seven transmembrane domains joined by three extracellular and three intracellular loops, with an extracellular amino terminus domain and an intracellular carboxyl tail (Hieble et al., 1995, Lefkowitz, 2013). Agonist binding to α_{1D}-ARs triggers calcium mobilization through Gαq/11, phosphoinositidase (phospholipase C) activation which generates diacylglycerol and inositol tris-phosphate (García-Sáinz and Villalobos-Molina, 2004). In addition, it has been shown that α_{1D}-AR activation stimulates the mitogen-activated protein (MAP) kinase pathway (Keffel et al., 2000, McCune et al., 2000, Waldrop et al., 2002).

Receptor phosphorylation is a very early event in desensitization of GPCRs. This allows β-arrestin binding and receptor internalization and triggers a second round of signaling (Lefkowitz, 2013). Receptor phosphorylation is mainly mediated by G protein-coupled receptor kinases (GRKs) and second messenger-activated protein kinases, such as protein kinases A and C (PKC) (Pitcher et al., 1998, Vázquez-Prado et al., 2003, Rajagopal et al., 2010) at regulatory domains, such as the third intracellular loop and the carboxyl tail (Lefkowitz, 2013). Therefore, there has been a growing interest in elucidating the function of these receptor domains.

Cottechia and coworkers showed that serine residues located specifically at the α_{1B}-AR carboxyl tail are targets of GRK and PKC and that substitution of these amino acids for non phosphorylatable residues, or the truncation of the receptor carboxyl tail, markedly decreases receptor phosphorylation and desensitization (Lattion et al., 1994, Diviani et al., 1997). This is in contrast with what has been observed with the α_{1D}-AR subtype, i. e., α_{1D}-AR-mediated calcium/phosphoinositide signaling, desensitization, and phosphorylation can take place in a carboxyl tail-truncated mutant (Rodríguez-Pérez et al., 2009). However, other pathways modulated by α_{1D}-ARs have not been studied.

Hence, the aim of this paper was to study the role of the human α_{1D}-AR carboxyl tail in MAP kinase pathway activation. We found that in Rat-1 fibroblast expressing the amino and carboxyl termini-truncated (ΔNΔC) α_{1D}-AR mutant, the adrenergic activation of ERK (extracellular signal-regulated kinase) was greatly reduced. In addition, expression of this mutant also markedly decreased ERK phosphorylation induced by unrelated receptors, such as those for lysophosphatidic acid and epidermal growth factor. Phosphorylation of Raf-1 and MEK1 was also diminished in Rat-1 cells expressing the amino and carboxyl termini-

truncated (ΔNΔC) α_{1D}-AR mutant. Data indicate that this mutant receptor alters downstream step(s) in this pathway.

Material and methods

Reagents

(−)-Noradrenaline, phorbol myristate acetate, lysophosphatidic acid, bisindolylmaleimide I, prazosin, propranolol, and yohimbine were obtained from Sigma-Aldrich Chemical (St. Louis, MO), Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, G418, Lipofectamine 2000, streptomycin, penicillin, amphotericin B, and Fura-2AM were purchased from Invitrogen Life Technologies (Carlsbad, CA). Epidermal growth factor was from Millipore Corporation (Billerica, MA). Polyvinylidene difluoride membranes were from BioRad (Hercules, CA) and SuperSignal West Pico Chemiluminescence kits were from Thermo Fisher Scientific (Waltham, MA). Anti-phospho-p42/44 ERK (extracellular signal-regulated kinases) 1/2 (Thre202/Tyr204), anti-total ERK (p42/44), anti-phospho c-Raf-1 (Ser338), anti-total c-Raf-1, anti-phospho MEK1 (Ser217/Ser221), and anti-total MEK-1 antibodies were obtained from Cell Signaling Technology (Danvers, MA); antitotal ERK2 p42 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids

The cytoplasmic ERK activity reporter EKAR (EGFP (enhanced green fluorescent protein)-mRFP (monomeric Red Fluorescent Protein)) (Harvey et al., 2008), was purchased from Addgene (Cambridge, MA).

Plasmids coding for HA-tagged human α_{1D}-adrenoceptor (Δ1–79, amino terminus-truncated, ΔN) and (Δ1–79 and 441–572, amino and carboxyl termini-truncated, ΔNΔC) were previously reported (Rodríguez-Pérez et al., 2009). Plasmid coding for carboxyl terminus GFP-tagged proteins of the mutants described above (ΔN-GFP and ΔNΔC-GFP) were synthesized commercially by Mutagenex Inc.

Cell lines

Rat-1 (rat fibroblasts), HEK 293 (human embryonic kindney cells), and B103 cells (rat neuroblastoma cells) were obtained from the American Tissue Culture Collection. Generation of Rat-1 cell lines expressing the α_{1D}-adrenoceptor deletion mutants was previously reported (receptor density ~ 1500 fmol/mg membrane protein) (Rodríguez-Pérez et al., 2009). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, G418 300 μg/ml, streptomycin 100 μg/ml, penicillin 100 U/ml and amphotericin B 0.25 μg/ml. Cells were cultured at 37 °C, under a 95% air/5% CO_2 atmosphere. HEK 293 and B103 cells were transfected with the GFP-tagged human α_{1D}-adrenoceptor (ΔN-GFP and ΔNΔC-GFP) indicated above. In the case of HEK 293 cells, transient transfection was used because expression was very robust, as evidenced by fluorescent confocal microscopy and calcium responsiveness to noradrenaline plus propranolol (the β-adrenergic antagonist was used in all experiments using these cells, due to the endogenous expression of these receptors). Cells were transfected with 1 μg of plasmid DNA per 2.5-cm dish using Lipofectamine 2000 and harvested 48 h later. B103

cells were transfected in an identical manner; however, because receptor expression was not as robust as that observed in HEK 293 cells, selection was performed using G418 (600 μg/ml initially (approximately for 1 month, 4–5 cell passages) and reduced afterwards to 300 μg/ml).

Intracellular calcium determinations

Intracellular calcium concentration was assessed as previously described (Rodríguez-Pérez et al., 2009). In brief, cells were serum-starved overnight, then loaded with 2.5 μM of the fluorescent Ca^{2+} indicator, Fura-2/AM, in Krebs-Ringer-HEPES containing 0.05% bovine serum albumin, pH 7.4 for 1 h at 37°C, and then washed three times to eliminate unincorporated dye. Fluorescence measurements were carried out at 340- and 380-nm excitation wavelengths and at a 510-nm emission wavelength, with a chopper interval set at 0.5 s, utilizing an Aminco-Bowman Series 2 Luminescence Spectrometer (Rochester, NY). Intracellular calcium levels were calculated according to Grynkiewicz et al. (Grynkiewicz et al., 1985).

Western blot assays

Cells were serum-starved overnight; after stimulation with the indicated agents, they were washed twice with ice-cold phosphate-buffered saline and lysed with Laemmli sample buffer (Laemmli, 1970). Lysates were centrifuged at 12, 700 × *g* for 5 min and proteins in supernatants were separated by electrophoresis on 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis. Proteins were electrotransferred onto polyvinylidene difluoride membranes and immunoblottings were performed. Duplicate samples were run in parallel to determine total- and phospho-kinases. Incubation with primary selective antibodies was conducted for 12 h at 4 °C and with the secondary antibody for 1 h at room temperature. SuperSignal West Pico chemiluminescence kits were employed, exposing the membranes to X-Omat X-ray films. Signals were quantified by densitometric analysis. Cells expressing the ΔN or the ΔNΔC α_{1D}-adrenoceptor were cultured in parallel and were subjected to the same treatments in all the experiments; afterwards, samples were subjected to electrophoresis, electrotransference, and immunoblotting in the same gels and membranes; representative Western blots are presented in the figures. For data normalization, the treatment that resulted in a consistent bigger response was considered as 100% (indicated in the figures). Anti-total ERK or anti-total ERK 2 antibodies were used in the different experiments obtaining identical results.

FRET-based ERK activity reporter assays

Rat-1 cells expressing the ΔN or ΔNΔC α_{1D}-adrenoceptors were transfected with a cytoplasmic extracellular signal-regulated kinase activity reporter plasmid (EKAR, EGFP-mRFP) (Harvey et al., 2008) using Lipofectamine 2000. EKAR is a genetically encoded, fluorescence resonance energy transfer-based sensor of ERK activity. This reporter contains an EKR substrate peptide, an ERK docking domain, a phospho-binding domain, and, at the amino and carboxyl termini, mRFP and EGFP, respectively (Harvey et al., 2008). ERK substrate peptide phosphorylation, by the endogenous protein kinase, triggers a reporter conformational change, which allows proximity of the fluorescent proteins and fluorescence resonance energy transfer (FRET) (Harvey et al., 2008). Confocal images were obtained 48

h post transfection using an Olympus Fluoview FV 100 laser confocal system attached/interfaced to an Olympus IX81 inverted light microscope with a ×60 water immersion objective; EGFP was excited at 488 nm and emission was simultaneously recorded at the green (500–530 nm) and red (555–655 nm) channels. Confocal images were viewed and processed employing FV10-ASW 1.6 software (Olympus).

Statistical analysis

Statistical analysis between groups was performed using analysis of variance with Bonferroni's post-test and was carried out with the software included in the GraphPad Prism program. In all statistical comparisons, $p < 0.05$ was considered significant.

Results

Amino terminus (Δ1–79) (ΔN)-truncated mutant was used to improve receptor plasma membrane expression as it has been described (Pupo et al., 2003, Hague et al., 2004, García-Sáinz et al., 2010). In order to determine the role of the α_{1D}-adrenergic carboxyl terminus in signaling, a mutant with this deletion of the amino terminus (Δ1–79) (ΔN) and another with an additional carboxyl terminus deletion (Δ 441–572) (ΔNΔC) were expressed in Rat-1 cells (Rodríguez-Pérez et al., 2009) and changes in ERK phosphorylation and intracellular calcium were assessed. In cells expressing the ΔN mutant, we observed a subtle basal level of ERK phosphorylation that was markedly increased after 1 and 5 min of noradrenaline stimulation, and returned to near baseline after 30 min (Fig. 1a). In contrast, in cells expressing the ΔNΔC receptor ERK phosphorylation in response to noradrenaline was either very weak or absent (Fig. 1a). Despite this differential response, cells expressing the ΔN and the ΔNΔC mutant were able to show similar robust increases in intracellular calcium concentration upon noradrenaline stimulation and preincubation with phorbol myristate acetate, to activate PKC, markedly reduced the adrenergic effect in both mutants (Fig. 1b), confirming our previous observations (Rodríguez-Pérez et al., 2009).

No ERK phosphorylation was observed in non-transfected Rat-1 control fibroblast in response to noradrenaline alone or in the presence of α_1-(prazosin), α_2-(yohimbine), or β-adrenergic (propranolol) blockers (Supplementary Fig. S1); however, these cells exhibited a strong ERK phosphorylation in response to lysophosphatidic acid (Supplementary Fig. S1). Similarly, no response to noradrenaline in calcium signaling was observed (data not shown).

Puzzled by the inhibition of ERK signaling in cells expressing the ΔNΔC receptor mutant, we explored whether such effect was selective for the α_{1D}-adrenergic action or a more general phenomenon, affecting the action of other receptors. We studied the effect of agents known to have endogenous receptors expressed in Rat-1 fibroblasts such as lysophosphatidic acid (Fig. 2a), whose signaling is mediated through GPCRs, and epidermal growth factor, that signals through receptor tyrosine kinases (ErbB receptors) (Fig. 2b). In both cases, a classic time-dependent ERK phosphorylation was observed in cells expressing the ΔN mutant, but it was markedly diminished, and most frequently absent, in cells expressing ΔNΔC α_{1D}-adrenoceptors (Fig. 2a, b). It goes by default that α_{1D}-adrenoceptor activation was not necessary to observe these carboxyl terminus-truncated mutant effects.

ERK phosphorylation has complex space temporal dynamics. For this reason and in order to confirm the previous findings, we employed a genetically encoded FRET-based ERK activity reporter. Endogenously expressed LPA receptors were stimulated in cells expressing ΔN or ΔNΔC α_{1D}-adrenoceptor mutants. An increase in FRET signal was observed upon stimulation with 1 μM lysophosphatidic acid in cells expressing the ΔN mutant receptor (Fig 3a); in contrast, no FRET signal was detected in cells expressing ΔNΔC α_{1D}-adrenoceptors, under the same conditions (Fig 3b). These results are consistent with our former findings and provide additional proof that the α_{1D}-adrenoceptor controls activation of the ERK pathway even if activation of that pathway occurs through non-related receptor-ligand interactions.

To determine if the effect of ΔNΔC α_{1D}-adrenoceptor expression, on ERK phosphorylation, was cell-specific, ΔN and ΔNΔC α_{1D}-adrenoceptor constructs were transiently expressed in HEK293 cells. Surprisingly, in these cells, 10 μM noradrenaline (plus propranolol 1 μM, to block endogenous β-adrenoceptors) and 1 μM lysophosphatidic acid clearly increased ERK phosphorylation (Fig 4a). Similar experiments were performed in B103 cells except that stably transfections were used, and the truncated receptors, fused to GFP at their respective carboxyl termini, were expressed to rapidly and easily detect receptor expression by fluorescent microscopy (expression was also checked by noradrenaline-induced intracellular calcium increases; data not shown). In these cells, very robust noradrenaline- and EGF-mediated ERK phosphorylations were observed in cells expressing the ΔN mutant receptor (Fig. 4a); interestingly, similar to the effects observed in Rat-1 fibroblasts, the activity of these agents was markedly and consistently attenuated in cells expressing the ΔNΔC mutant receptor (Fig. 4b).

To investigate the mechanism through which ERK signaling is inhibited in ΔNΔC-expressing Rat-1 fibroblasts, we tried to identify a pathway element that blocks transduction. Our first candidate was PKC for the following reasons: (a) both (ΔN and ΔNΔC) mutant receptors respond to agonist stimulation by increasing intracellular calcium, and both are phosphorylated and desensitized by PKC activation (Rodríguez-Pérez et al., 2009) (see also Fig. 1); (b) in addition, it has been shown that PKC is able to directly phosphorylate and activate Raf-1 (MAP Kinase Kinase Kinase) in vitro and in vivo (in NIH 3T3 fibroblasts) (Kolch et al., 1993). Therefore, we tested whether PKC could be involved in ERK activation in our model. To do this, Rat-1 fibroblasts expressing ΔN mutant receptors were incubated for 30 min in the absence or presence of the general PKC inhibitor, bisindolylmaleimide (1 μM). Then, we assessed ERK phosphorylation after noradrenaline stimulation during 1, 5, and 30 min. We observed a marked increase of ERK phosphorylation in cells incubated in the absence of the inhibitor (positive control), but no signal in cells pretreated with bisindolylmaleimide I (Fig. 5a). The inhibitor alone did not alter either basal or EGF-mediated ERK activation (Fig. 5, panel B). These data indicated that noradrenaline-induced (but not EGF-induced) ERK activation is PKC dependent (Supplementary Fig. S2).

To provide additional evidence that PKC plays a role in ERK activation in ΔN-expressing cells, we pharmacologically activated this enzyme, using phorbol myristate acetate, in both ΔN- and ΔNΔC-expressing Rat-1 fibroblasts. ERK phosphorylation was markedly increased in cells expressing the ΔN mutant receptors activated with this phorbol ester, but not in those

expressing ΔNΔC α_{1D}-adrenoceptors (Fig 6a, Supplementary Fig. S2). Therefore, in these ΔNΔC α_{1D}-adrenoceptor-expressing cells, direct activation of PKC was not able to turn on ERK signaling, but was capable of desensitizing and inducing α_{1D}-adrenoceptors phosphorylation (Rodríguez-Pérez et al., 2009). Taken together these data indicate that the pathway is inhibited downstream of PKC. Therefore, we evaluated the phosphorylation state of kinases Raf-1 and MEK-1. We observed that phorbol myristate acetate increased phosphorylation of both Raf-1 and MEK1 in cells expressing the ΔN receptor mutant but not in cells expressing the ΔNΔC receptor mutant (Fig. 6b). This means that, in cells expressing ΔNΔC α_{1D}-adrenoceptors, phorbol esters cannot activate ERK signaling from the Raf-1 kinase on, despite being able to activate PKC (as shown by α_{1D}-adrenoceptor desensitization and phosphorylation (Fig. 1 and (Rodríguez-Pérez et al., 2009)) (Supplementary Fig. S2).

Finally, we explored whether other MAP kinase pathway branches were similarly affected, i. e., p38. In Rat-1 fibroblasts expressing the ΔN α_{1D}-adrenoceptor mutant, baseline p38 phosphorylation showed some variability but its phosphorylation was clearly increased by noradrenaline or phorbol myristate acetate (Fig. 7). In contrast, in cells expressing ΔNΔC mutant receptors, p38 was markedly phosphorylated under baseline conditions (Fig. 7). No further effect was produced by noradrenaline and only a marginal increase was observed in response to the active phorbol ester (Fig 7).

Discussion

The major finding of the present work is that the amino- and carboxyl-termini-truncated mutant α_{1D}-adrenoceptors (ΔNΔC) was able to signal through the phospholipase C/IP_3-calcium mobilization pathway (Rodríguez-Pérez et al., 2009) but that signaling through the MAP kinase pathway was markedly altered. The data indicate that the actions of this receptor are biased. On one side, the sole expression of this receptor mutant markedly diminished signaling of one of the MAP kinase pathway branches, ERK 1/2, and that this happens not only through the receptor itself but also through other unrelated GPCRs, receptor tyrosine kinases, such as the EGF receptor, and even through pharmacological activation of PKC; in other words, the ΔNΔC receptor behaves as a dominant negative mutant. On the other side, the sole expression of the ΔNΔC α_{1D}-adrenoceptors was sufficient to increase p38 phosphorylation to near maximal levels, and the natural adrenoceptor agonist, noradrenaline, or pharmacological PKC activation, were unable to further increase it, i. e., ΔNΔC α_{1D}-adrenoceptors behave as a constitutively active mutant for this MAP kinase branch.

It should be kept in mind that an amino truncation of the receptor was utilized to increase its membrane expression, and that signaling was similar to what has been reported for other GPCRs ((Pupo et al., 2003, Rodríguez-Pérez et al., 2009) and present work). Therefore, the changes observed with the carboxyl-truncated receptor should be attributed to the absence of this domain.

Wild type α_{1D}-adrenoceptor-induced ERK phosphorylation has been studied by another group (Pérez-Aso et al., 2013) and there are some similarities with our data with the ΔN

receptor-expressing cells. However, those experiments were performed using HEK 293 cells and ERK phosphorylation was not altered by inhibition of protein kinase C (Pérez-Aso et al., 2013). It is possible that the difference in the cell types employed could explain the distinct roles of PKC in the adrenergic action and that this might be related to the fact that expression of ΔNΔC α_{1D}-adrenoceptor in such cells did not alter ERK phosphorylation.

To the best of our knowledge, no similar effects have been reported for other GPCR mutants. However, there is evidence that mutants of signaling proteins and also of scaffolding proteins exert marked effects on the MAP kinase pathway, for example, β-arrestin 3 and glutathione S-transferase P1 (Breitman et al., 2012, Okamura et al., 2015). It is known that β-arrestins bind to GPCRs during their desensitization/signaling switch and that these proteins are involved in regulating the MAP kinase pathway (Breitman et al., 2012, Lefkowitz, 2013). In this regard, a study using different β-arrestin mutants has shown that GPCR binding, interaction with the MAP kinase JNK3 (c-Jun N-terminal Kinase 3), and the ability to modulate this kinase are three distinct functions of this scaffold protein (Breitman et al., 2012). Among the mutants, one containing 12 alanine substitutions of the key receptor-binding residues was unable to bind β_2-adrenoceptors but was able to inhibit JNK3 activation by wild type β-arrestin (Breitman et al., 2012). Similarly, it was recently reported that tyrosine phosphorylation of glutathione S-transferase P1, by the EGF receptor, favors the transferase interaction with JNK and inhibits downstream signaling of this MAP kinase pathway (Okamura et al., 2015). In addition, it has been observed that β_2-adrenoceptors bind to membrane-associated guanylate kinase inverted-3 (MAGI-3) and that such association substantially retards ERK activation by the adrenoceptor (Yang et al., 2010).

It has been previously shown that α_{1D}-adrenoceptors are phosphorylated and desensitized by phorbol ester-mediated activation of PKC (García-Sáinz et al., 2001, García-Sáinz et al., 2004, Rodríguez-Pérez et al., 2009). These effects were observed with wild-type receptors and also when the ΔN and ΔNΔC α_{1D}-adrenergic mutant receptors were utilized ((García-Sáinz et al., 2001, García-Sáinz et al., 2004, Rodríguez-Pérez et al., 2009) and present work). Therefore, it is clear that PKC is functional in cells expressing the carboxyltail truncated receptors. α_{1D}-Adrenergic-stimulated ERK phosphorylation is PKC mediated, as evidenced by the blockade induced by the inhibitor, bisindolylmaleimide I. Investigation on the phosphorylation state of the intermediate kinases, Raf-1 and MEK indicated that signaling is blocked after PKC.

Interestingly Raf-1 is modulated through phosphorylation by PKC (Kolch et al., 1993) and also by growth factor receptors containing endogenous tyrosine kinase activity, such as the EGF receptor, with the key involvement of Ras GTPase activity (Kolch et al., 1993, Kolch, 2000). Activation and inhibition of Raf-1 activity via GPCRs has also been reported with the involvement of G protein βγ subunits, phosphoinositide 3 kinase and Akt/protein kinase B, among other signaling entities (Slupsky et al., 1999, Kolch, 2000, Merighi et al., 2006).

Raf is a proto-oncogene that participates in regulating cell proliferation and differentiation (Kolch, 2000). The Ras/Raf/MEK/ERK pathway constitutes a master regulator of growth control of great importance in health and disease, including malignant transformation, and it is a target for therapeutic intervention (Knight and Irving, 2014). Interestingly, Raf-1 is

known to interact with a large variety of proteins including, receptors, G proteins, scaffolds/adaptors, cytoskeleton components, chaperones, kinases, and phosphatases, among others (Kolch, 2000). Raf-1 is localized within a large protein complex in its inactive state; activated Ras binds with high affinity Raf-1 and participates in its activation and localization within the cells (Kolch, 2000).

We did not observe a decrease in Raf-1 abundance in extracts from cells expressing ΔNΔC α_{1D}-adrenergic mutant receptors as compared to those expressing the ΔN mutant. Hence, a dysfunctional change rather than an alteration in the amount seems to be responsible of the observed actions.

It is worth noting that α_{1D}-adrenoceptors carboxyl terminus is particularly long (167 amino acids in the human orthologue; http://www.uniprot.org/uniprot/P25100). It is possible that its absence could leave intracellular domains exposed, which might directly or indirectly (i. e., through other molecular entities) interact with members of the MAP kinase pathway. At present, the α_{1D}-adrenoceptor crystal structure has not been resolved, and there is hardly any information on that of the carboxyl tails structures of GPCRs. Our current working hypothesis is that the absence of the α_{1D}-adrenoceptor carboxyl tail could lead to exposure of site(s) that might bind Raf-1 or associated proteins, preventing its phosphorylation and activation. By default, a role of the long α_{1D}-adrenoceptor tail could be to avoid exposure of such sites. Obviously, at the present time, this is just a hypothesis and experimental proof remains to be obtained. Analysis of in cellulo and in vitro interaction of MAP kinases and related scaffolding proteins with ΔN and ΔNΔC α_{1D}-adrenoceptors might help to support or discard this hypothesis. Similarly, analysis of pull-down proteins using receptor fragments as baits and yeast two-hybrid test system might give some clues. It is clear that more structural work is required to fully understand GPCR action.

It seems likely that the roles of the α_{1D}-adrenoceptor carboxyl tail extend beyond signaling and desensitization. It has been observed that a PDZ-interacting domain present in this tail associates with syntrophin (Chen et al., 2006); knockout of syntrophin isoforms results in the loss of α_{1D}-adrenergic action in aortic smooth muscle (Lyssand et al., 2008). This adrenoceptor subtype plays major roles in the regulation of blood pressure and in the pathogenesis of hypertension (Villalobos-Molina and Ibarra, 1996, Villalobos-Molina et al., 1999) a disease estimated to cause 4.5% of the total human disease burden (Whitworth, 2003). Knowledge on the functional domains of this receptor might help to better understand this disease and to design more effective therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was partially supported by grants from CONACyT [177556-253156] and DGAPA-UNAM [200812-200915]. JC was supported by the NIH (NS084398). Marco Antonio Alfonzo is a student of the Programa de Maestría y Doctorado en Ciencias Bioquímicas-UNAM and the recipient of a fellowship from CONACyT; the present work constitutes a major part of his thesis. We express our gratitude to Dr. Rocío Alcántara-Hernández, Dr.

Claudia Rivera, Dr. Héctor Malagón, Juan Barbosa, Aurey Galván, and Manuel Ortínez for technical help and advice.

References

Breitman M, Kook S, Gimenez LE, Lizama BN, Palazzo MC, Gurevich EV, Gurevich VV. Silent scaffolds: inhibition of c-Jun N-terminal kinase 3 activity in cell by dominant-negative arrestin-3 mutant. J Biol Chem. 2012; 287:19653–19664. [PubMed: 22523077]

Chen Z, Hague C, Hall RA, Minneman KP. Syntrophins regulate alpha1D-adrenergic receptors through a PDZ domain-mediated interaction. J Biol Chem. 2006; 281:12414–12420. [PubMed: 16533813]

Diviani D, Lattion AL, Cotecchia S. Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitization of the alpha1B-adrenergic receptor. J Biol Chem. 1997; 272:28712–28719. [PubMed: 9353340]

Fredriksson R, Schioth HB. The repertoire of G-protein-coupled receptors in fully sequenced genomes. Mol Pharmacol. 2005; 67:1414–1425. [PubMed: 15687224]

García-Sáinz JA, Rodríguez-Pérez CE, Romero-Ávila MT. Human alpha-1D adrenoceptor phosphorylation and desensitization. Biochem Pharmacol. 2004; 67:1853–1858. [PubMed: 15130762]

García-Sáinz JA, Romero-Ávila MT, Medina LC. Alpha(1D)-adrenergic receptors constitutive activity and reduced expression at the plasma membrane. Methods Enzymol. 2010; 484:109–125. [PubMed: 21036229]

García-Sáinz JA, Vázquez-Cuevas FG, Romero-Ávila MT. Phosphorylation and desensitization of alpha1d-adrenergic receptors. Biochem J. 2001; 353:603–610. [PubMed: 11171057]

García-Sáinz JA, Vázquez-Prado J, Villalobos-Molina R. Alpha 1-adrenoceptors: subtypes, signaling, and roles in health and disease. Arch Med Res. 1999; 30:449–458. [PubMed: 10714357]

García-Sáinz JA, Villalobos-Molina R. The elusive alpha(1D)-adrenoceptor: molecular and cellular characteristics and integrative roles. Eur J Pharmacol. 2004; 500:113–120. [PubMed: 15464025]

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem. 1985; 260:3440–3450. [PubMed: 3838314]

Hague C, Chen Z, Pupo AS, Schulte NA, Toews ML, Minneman KP. The N terminus of the human {alpha}1D-adrenergic receptor prevents cell surface expression. J Pharmacol Exp Ther. 2004; 309:388–397. [PubMed: 14718583]

Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, Svoboda K. A genetically encoded fluorescent sensor of ERK activity. Proc Natl Acad Sci U S A. 2008; 105:19264–19269. [PubMed: 19033456]

Hieble JP, Bylund DB, Clarke DE, Eikenburg DC, Langer SZ, Lefkowitz RJ, Minneman KP, Ruffolo RR Jr. International Union of Pharmacology. X. Recommendation for nomenclature of alpha 1-adrenoceptors: consensus update. Pharmacol Rev. 1995; 47:267–270. [PubMed: 7568329]

Keffel S, Alexandrov A, Goepel M, Michel MC. Alpha(1)-adrenoceptor subtypes differentially couple to growth promotion and inhibition in Chinese hamster ovary cells. Biochem Biophys Res Commun. 2000; 272:906–911. [PubMed: 10860850]

Knight T, Irving JA. Ras/Raf/MEK/ERK pathway activation in childhood acute lymphoblastic leukemia and its therapeutic targeting. Front Oncol. 2014; 4:160. [PubMed: 25009801]

Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J. 2000; 351(Pt 2):289–305. [PubMed: 11023813]

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature. 1993; 364:249–252. [PubMed: 8321321]

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680–685. [PubMed: 5432063]

Lagerstrom MC, Schioth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov. 2008; 7:339–357. [PubMed: 18382464]

Lattion AL, Diviani D, Cotecchia S. Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the alpha 1B-adrenergic receptor. J Biol Chem. 1994; 269:22887–22893. [PubMed: 8077240]

Lefkowitz RJ. A brief history of G-protein coupled receptors (Nobel lecture). Angew Chem Int Ed Engl. 2013; 52:6366–6378. [PubMed: 23650015]

Lyssand JS, Defino MC, Tang XB, Hertz AL, Feller DB, Wacker JL, Adams ME, Hague C. Blood pressure is regulated by an {alpha}1D-adrenergic receptor/Dystrophin Signalosome. J Biol Chem. 2008; 283:18792–18800. [PubMed: 18468998]

McCune DF, Edelmann SE, Olges JR, Post GR, Waldrop BA, Waugh DJ, Perez DM, Piascik MT. Regulation of the cellular localization and signaling properties of the alpha(1B)- and alpha(1D)-adrenoceptors by agonists and inverse agonists. Mol Pharmacol. 2000; 57:659–666. [PubMed: 10727510]

Merighi S, Benini A, Mirandola P, Gessi S, Varani K, Leung E, Maclennan S, Baraldi PG, Borea PA. Modulation of the Akt/Ras/Raf/MEK/ERK pathway by a(3) adenosine receptor. Purinergic Signal. 2006; 2:627–632. [PubMed: 18404465]

Okamura T, Antoun G, Keir ST, Friedman H, Bigner DD, Ali-Osman F. Phosphorylation of glutathione S-transferase P1 (GSTP1) by epidermal growth factor receptor (EGFR) promotes formation of the GSTP1-c-Jun N-terminal kinase (JNK) complex and suppresses JNK downstream signaling and apoptosis in brain tumor cells. J Biol Chem. 2015; 290:30866–30878. [PubMed: 26429914]

Pérez-Aso M, Segura V, Monto F, Barettino D, Noguera MA, Milligan G, D'Ocon P. The three alpha1-adrenoceptor subtypes show different spatio-temporal mechanisms of internalization and ERK1/2 phosphorylation. Biochim Biophys Acta. 2013; 1833:2322–2333. [PubMed: 23797059]

Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem. 1998; 67:653–692. [PubMed: 9759500]

Pupo AS, Uberti MA, Minneman KP. N-terminal truncation of human alpha1D-adrenoceptors increases expression of binding sites but not protein. Eur J Pharmacol. 2003; 462:1–8. [PubMed: 12591089]

Rajagopal S, Rajagopal K, Lefkowitz RJ. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov. 2010; 9:373–386. [PubMed: 20431569]

Rodríguez-Pérez CE, Romero-ÁvilaMT, Reyes-Cruz G, García-Sáinz JA. Signaling properties of human alpha(1D)-adrenoceptors lacking the carboxyl terminus: intrinsic activity, agonist-mediated activation, and desensitization. Naunyn Schmiedeberg's Arch Pharmacol. 2009; 380:99–107. [PubMed: 19458937]

Slupsky JR, Quitterer U, Weber CK, Gierschik P, Lohse MJ, Rapp UR. Binding of Gbetagamma subunits to cRaf1 downregulates G-protein-coupled receptor signalling. Curr Biol. 1999; 9:971–974. [PubMed: 10508586]

Vázquez-Prado J, Casas-González P, García-Sáinz JA. G protein-coupled receptor cross-talk: pivotal roles of protein phosphorylation and protein-protein interactions. Cell Signal. 2003; 15:549–557. [PubMed: 12681442]

Villalobos-Molina R, Ibarra M. Alpha 1-adrenoceptors mediating contraction in arteries of normotensive and spontaneously hypertensive rats are of the alpha 1D or alpha 1A subtypes. Eur J Pharmacol. 1996; 298:257–263. [PubMed: 8846824]

Villalobos-Molina R, López-Guerrero JJ, Ibarra M. Functional evidence of alpha1D-adrenoceptors in the vasculature of young and adult spontaneously hypertensive rats. Br J Pharmacol. 1999; 126:1534–1536. [PubMed: 10323583]

Waldrop BA, Mastalerz D, Piascik MT, Post GR. Alpha(1B)- and alpha(1D)-adrenergic receptors exhibit different requirements for agonist and mitogen-activated protein kinase activation to regulate growth responses in rat 1 fibroblasts. J Pharmacol Exp Ther. 2002; 300:83–90. [PubMed: 11752101]

Whitworth JA. 2003 World Health Organization (WHO)/International Society of Hypertension (ISH) statement on management of hypertension. J Hypertens. 2003; 21:1983–1992. [PubMed: 14597836]

Yang X, Zheng J, Xiong Y, Shen H, Sun L, Huang Y, Sun C, Li Y, He J. Beta-2 adrenergic receptor mediated ERK activation is regulated by interaction with MAGI-3. FEBS Lett. 2010; 584:2207–2212. [PubMed: 20353789]

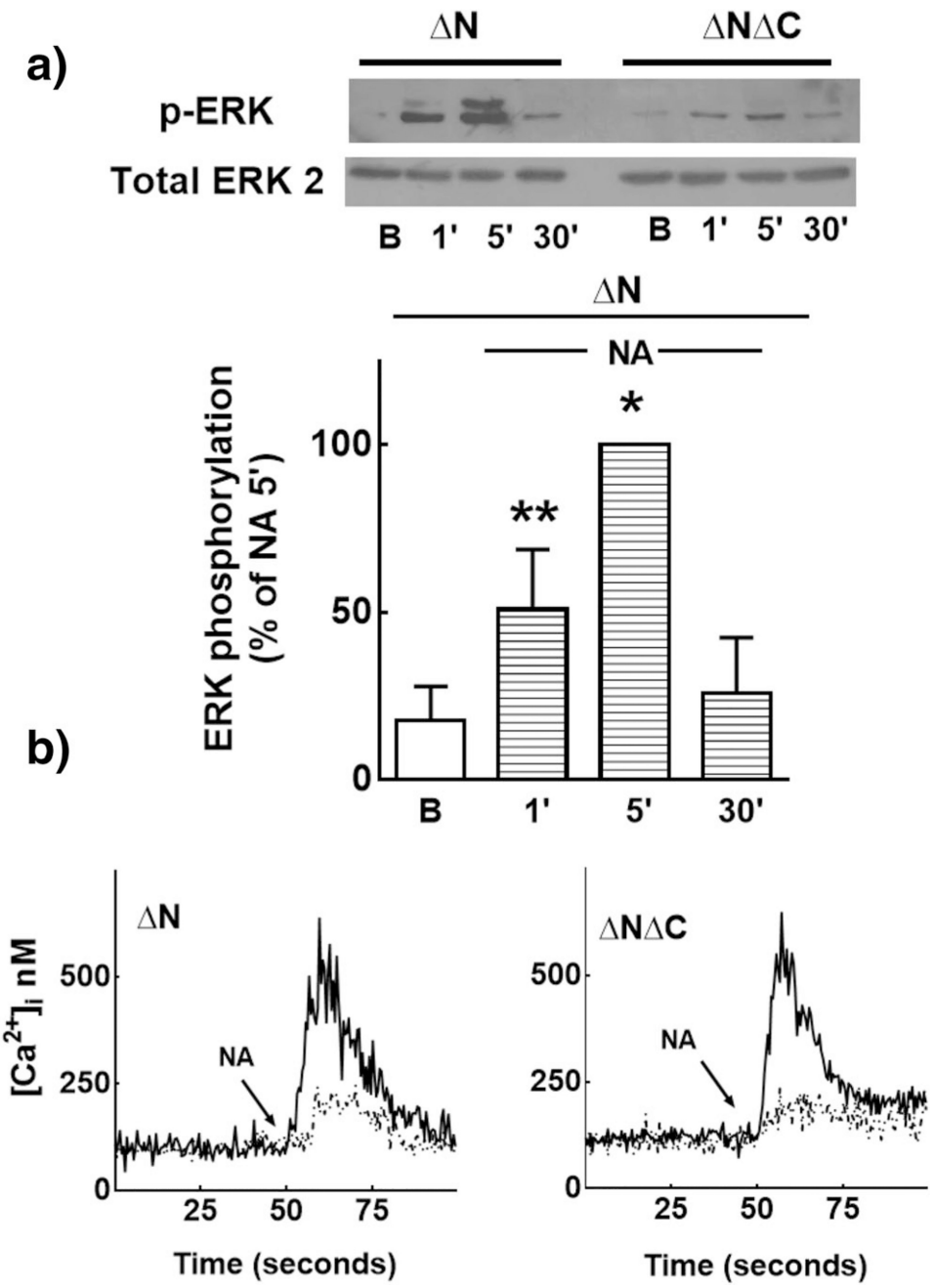

Fig. 1.

Effect of noradrenaline on ERK phosphorylation and intracellular calcium concentration. **a** Rat-1 fibroblasts expressing ΔN or ΔNΔC α_{1D}-adrenoceptors were stimulated with 10 μM noradrenaline (NA) for 1, 5 and 30 min. The response of cells expressing ΔN α_{1D}-adrenoceptors was normalized to the effect of 10 μM noradrenaline (NA) (5 min, maximum observed) considered as 100 %. No quantitative data of cells expressing ΔNΔC α_{1D}-adrenoceptors are presented because these were very small and in most cases absent. Plotted are the means and vertical lines representing the S.D. of 3 experiments using different cell preparations. *$p < 0.001$ vs. baseline (*B*); **$p < 0.05$ vs. baseline (*B*). Representative

Western blots for phospho-ERK 1/2 (p-ERK) and total ERK 2 are presented. **b** Intracellular calcium determinations using Rat-1 fibroblasts expressing amino terminus-truncated (ΔN) (*left graph*) or amino and carboxyl termini-truncated (ΔNΔC) (*right graph*) α_{1D}-adrenoceptors; cells were stimulated with 10 μM noradrenaline (NA) (*arrow*); cells were preincubated for 5 min in the absence (*solid line*) or presence (*dotted line*) of 1 μM phorbol myristate acetate

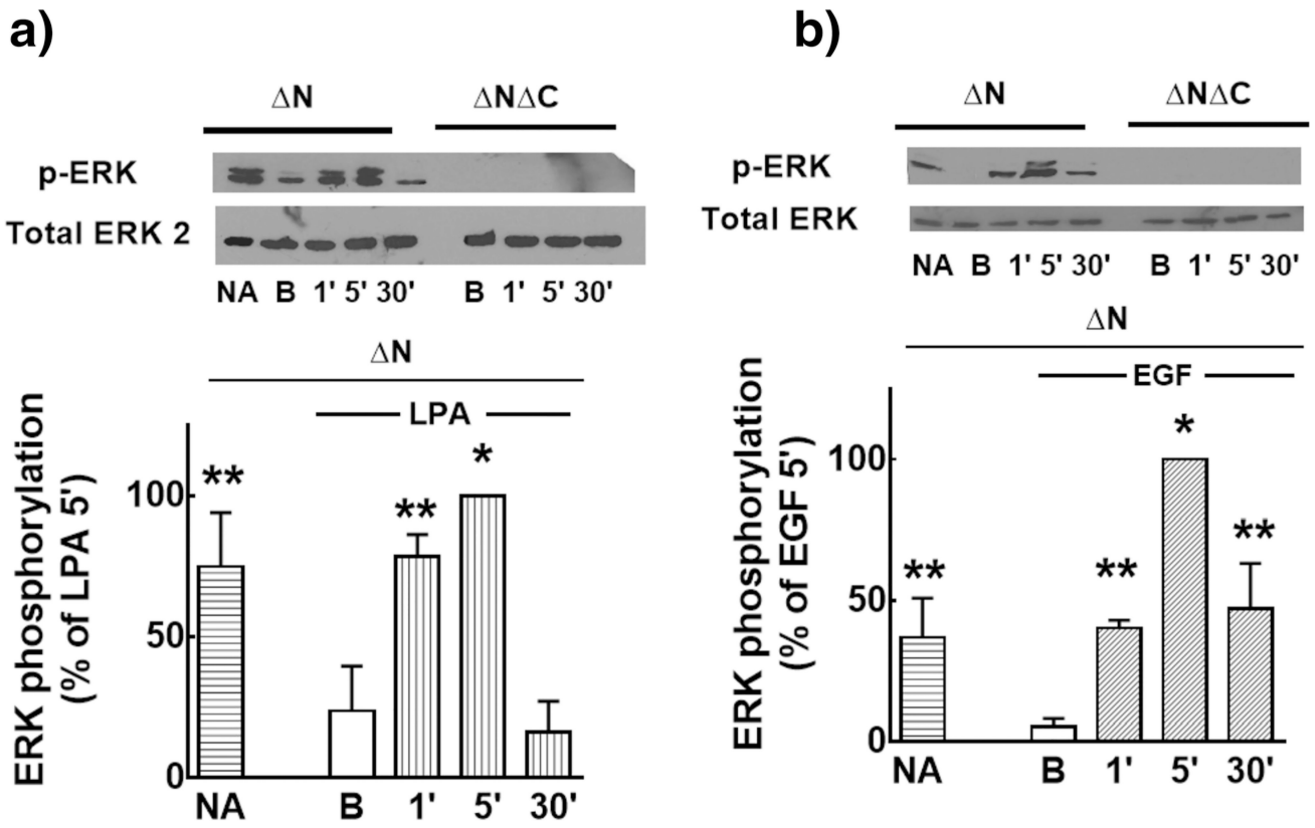

Fig. 2.

Effects of lysophosphatidic acid and EGF on ERK phosphorylation. **a** Representative Western blots of phospho ERK 1/2 (42–44 kDa) and total ERK 2. Rat-1 fibroblasts expressing ΔN or ΔNΔC α_{1D}-adrenoceptors were stimulated with 10 μM noradrenaline (NA)(positive control) for 5 min or with 1 μM lysophosphatidic acid (LPA) for 1, 5 and 30 min. The response of cells expressing ΔN α_{1D}-adrenoceptors was normalized to the maximal effect (1 μM lysophosphatidic acid (5 min)) as 100 %. *B* indicates baseline levels. **b** Experiments were performed as indicated for **a** except that 100 ng/ml EGF was used in the time course and the effects were normalized to the effect of EGF (5 min) considered as 100 %. Quantitative data of cells expressing ΔNΔC α_{1D}-adrenoceptors are not presented because they were very small or absent. In both panels, plotted are the means and vertical lines representing the S.D. of 3–4 experiments using different cell preparations. *$p < 0.001$ vs. baseline (*B*); **$p < 0.01$ vs. baseline (*B*)

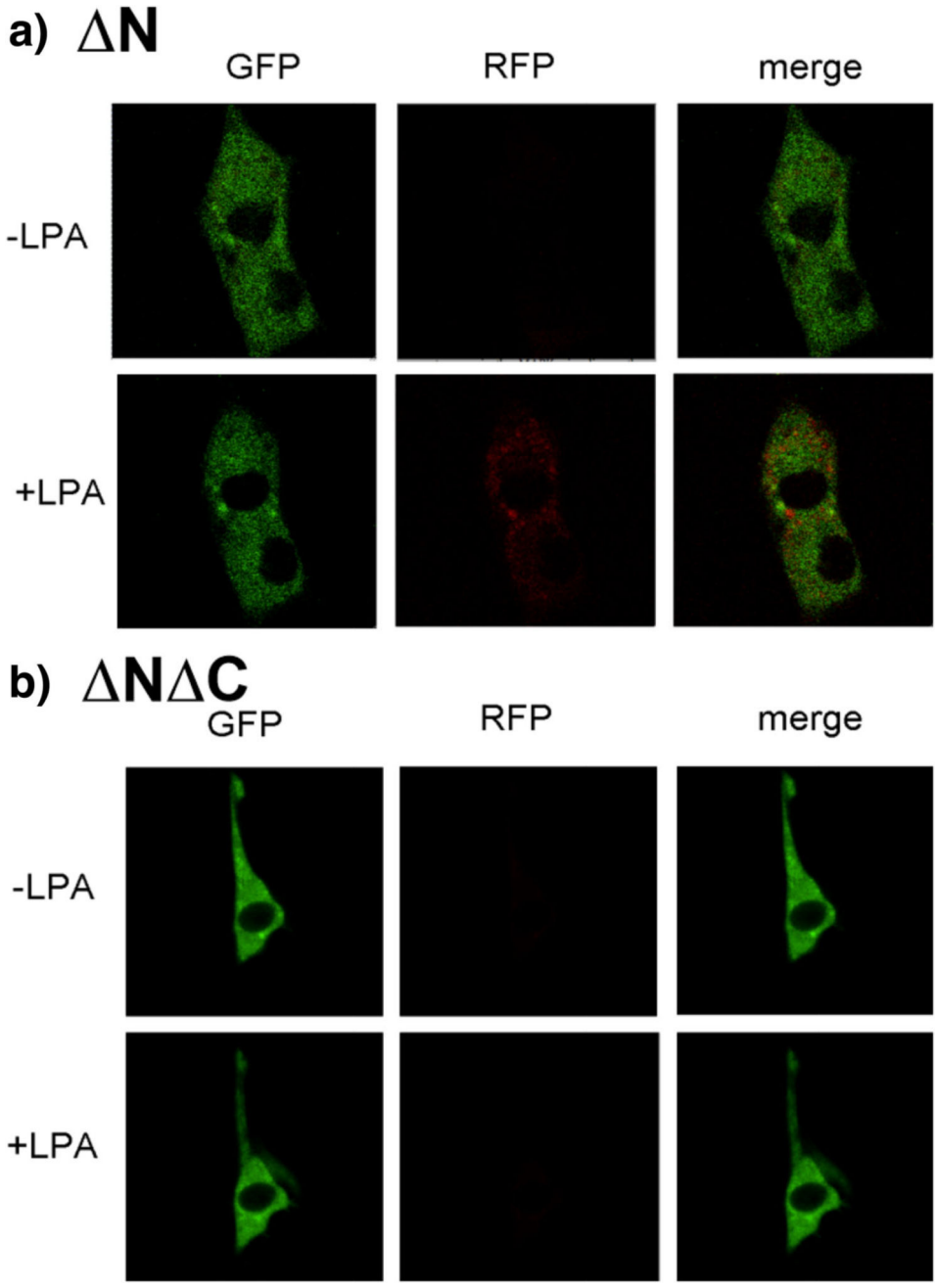

Fig. 3.

Effect of lysophosphatidic acid on ERK phosphorylation detected using the FRET-based activity sensor, EKAR. Rat-1 fibroblasts expressing ΔN or ΔNΔC α_{1D}-adrenoceptors and transfected with the cytoplasmic ERK Activity Reporter (EKAR, EGFP-mRFP) were stimulated with 1 μM lysophosphatidic acid for 5 min and observed with a confocal microscope. Excitation was at 488 nm and emission was simultaneously recorded in the *green* (GFP) and *red channels* (RFP); *merged images* are also shown. In these experiments mRFP was not directly excited at any time

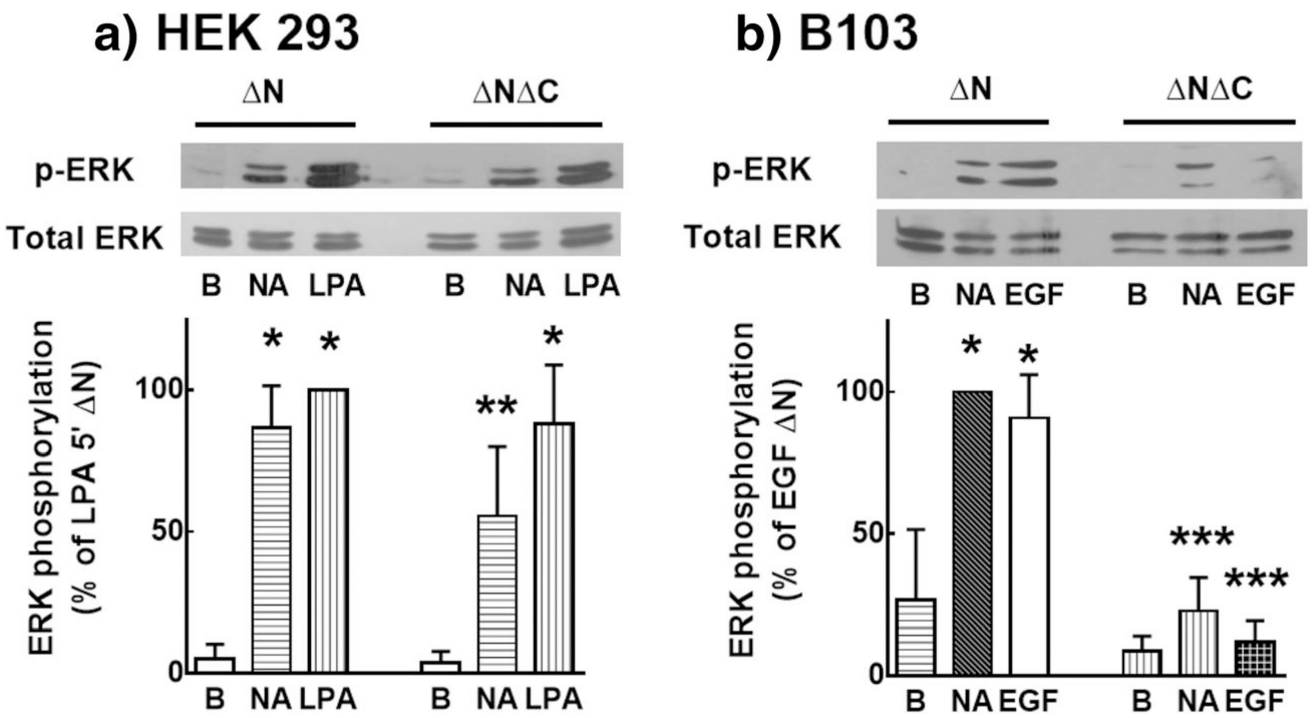

Fig. 4.

Expression of ΔN or ΔNΔC α_{1D}-adrenoceptors in HEK 293 and B103 cells show that the ERK phosphorylation effects are cell-type specific. HEK 293 (**a**) and B103 (**b**) cells transfected to express ΔN or ΔNΔC α_{1D}-adrenoceptors were incubated for 5 min in the absence (*B*, baseline) or presence or 10 μM noradrenaline plus 1 μM propranolol (NA), 1 μM lysophosphatidic acid (LPA) or 100 ng/ml EGF and ERK phosphorylation was determined by Western blotting. The response was normalized to the maximal effect observed (lysophosphatidic acid (**a**) or EGF (**b**) in ΔN α_{1D}-adrenoceptor-expressing cells) considered as 100 %. In both panels, the means are plotted and vertical lines represent the S.D. of 4–6 experiments using different cell preparations. *$p < 0.005$ vs. its respective baseline (*B*); **$p < 0.01$ vs. its respective baseline (*B*); ***$p < 0.005$ vs. same treatment in cells expressing ΔN α_{1D}-adrenoceptors. Representative Western blots are shown

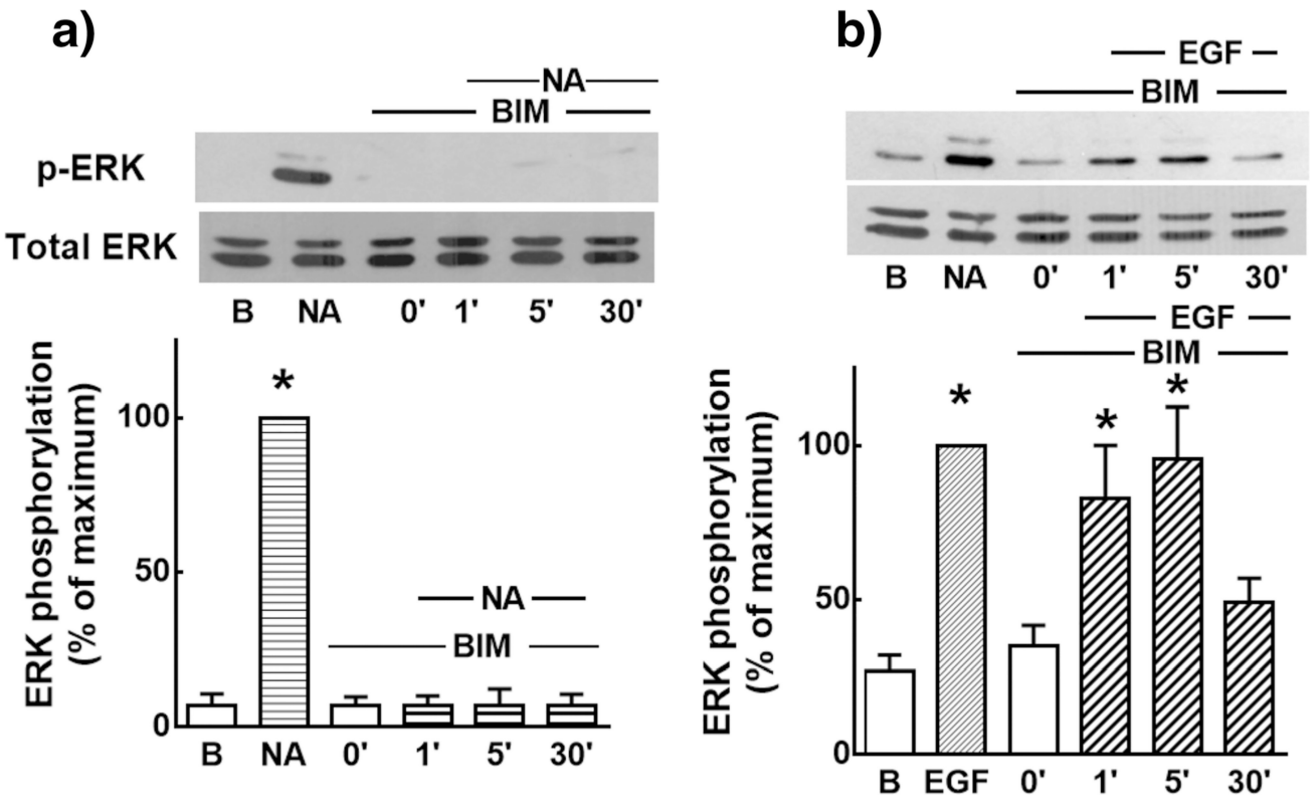

Fig. 5.

Roles of protein kinase C in noradrenaline- and EGF-induced ERK phosphorylation. Rat-1 fibroblast expressing ΔN α_{1D}-adrenoceptors were pre-incubated for 30 min with 1 μM bisindolylmaleimide I (BIM) and then challenged for the times indicated with 10 μM noradrenaline (NA) (**a**) or 100 ng/ml EGF (**b**) and ERK phosphorylation was determined by Western blotting. Internal controls included preincubation for 30 min with vehicle and incubation for 5 min in the absence (*B*, baseline) or presence of 10 μM noradrenaline (NA) (**a**) or 100 ng/ml EGF (**b**). Data were normalized to the maximal observed effect (noradrenaline (**a**), EGF (**b**)) considered as 100 %. In both panels, the means are plotted and vertical lines represent the S.D. of 4–6 experiments using different cell preparations. *$p <$ 0.005 vs. its respective baseline (*B*). Representative Western blots for phospho-ERK (p-ERK) and total ERK are shown

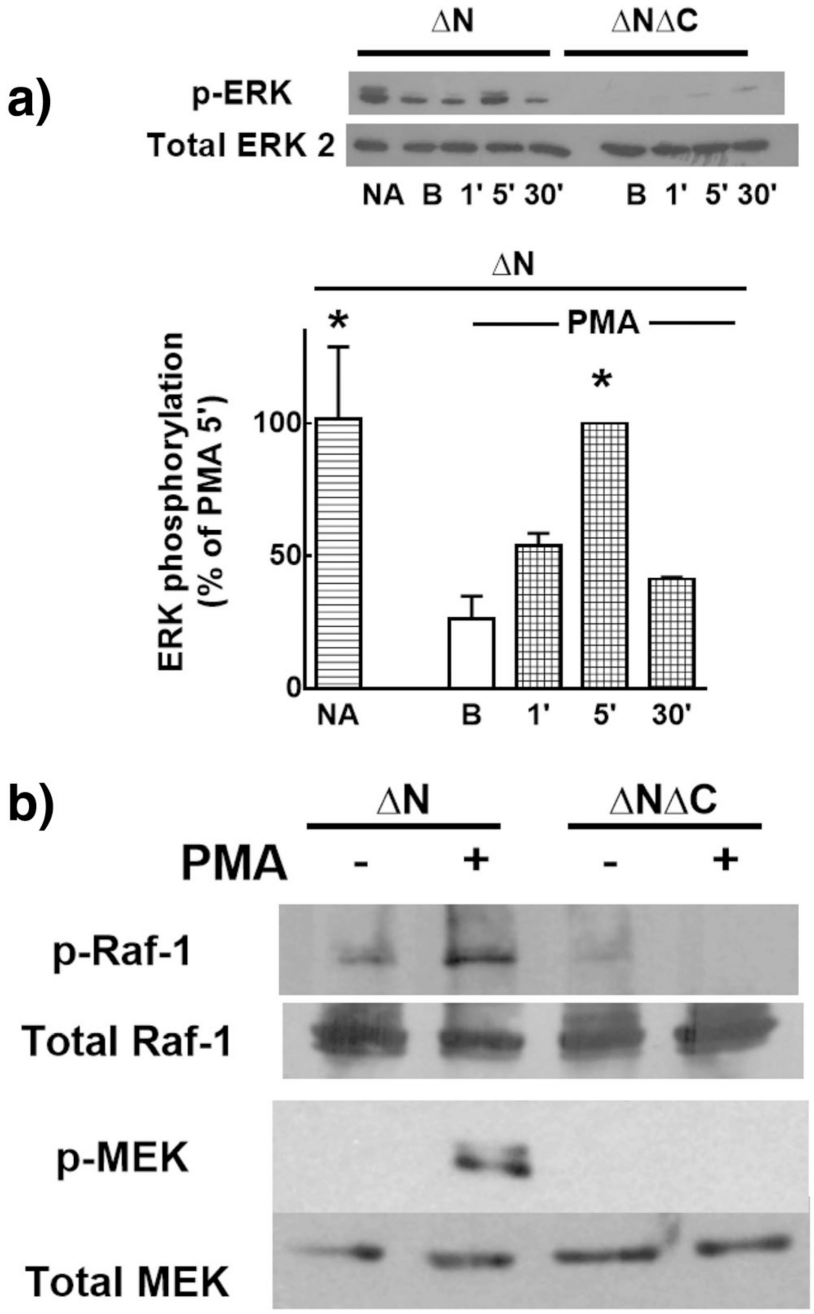

Fig. 6.

Effect of phorbol myristate acetate on the phosphorylation of the MAP kinases: ERK, Raf-1 and MEK. Rat-1 fibroblast expressing ΔN or ΔNΔC α_{1D}-adrenoceptors were incubated with 1 μM phorbol myristate acetate (PMA) and ERK, Raf-1 and MEK phosphorylation was determined by Western blotting. In **a**, cells were incubated for the indicated times in the presence of 1 μM PMA; 10 μM noradrenaline (NA) for 5 min was used as a positive control. Data were normalized to the maximal observed effect (phorbol myristate acetate, 5 min) considered as 100 %. No quantitative data of cells expressing ΔNΔC α_{1D}-adrenoceptors are presented because these were very small and in most cases absent. The means are plotted

and vertical lines represent the S.D. of 4–6 experiments using different cell preparations. *$p < 0.005$ vs. its respective baseline (*B*). Representative Western blots for phospho-ERK (p-ERK) and total ERK 2 are shown. In **b**, representative Western blots showing total and phosphorylated Raf-1 and MEK are presented. These experiments were repeated four times for each kinase using different cell preparations, obtaining the same results

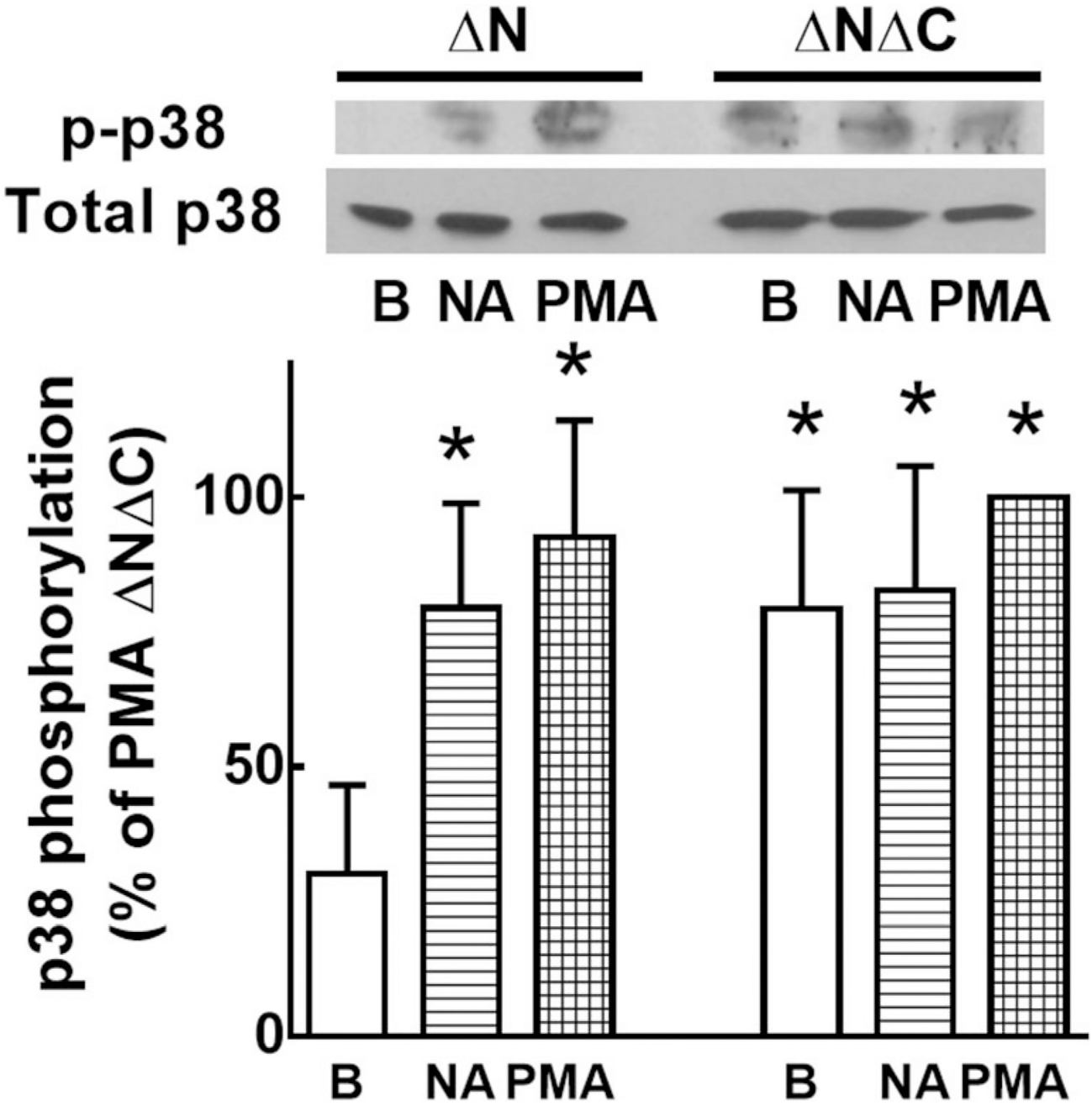

Fig. 7.

Effect of noradrenaline and phorbol myristate acetate on p38 phosphorylation. Rat-1 fibroblast expressing ΔN or ΔNΔC α_{1D}-adrenoceptors were incubated with 10 μM noradrenaline (NA) or 1 μM phorbol myristate acetate (PMA) for 5 min and p38 phosphorylation was determined by Western blotting. The response was normalized to the maximal effect observed (phorbol myristate acetate in ΔNΔC α_{1D}-adrenoceptor-expressing cells) considered as 100 %. Plotted are the means and vertical lines representing the S.D. of

4–6 experiments using different cell preparations. *$p < 0.005$ vs. ΔN baseline (*B*). Representative Western blots for phospho-p38 (p-p38) and total p38 are shown