

Research article

Interaction of neuronal nitric oxide synthase with α_1 -adrenergic receptor subtypes in transfected HEK-293 cells

Andre S Pupo and Kenneth P Minneman*

Address: Department of Pharmacology, Emory University, Atlanta, GA 30322 USA

E-mail: Andre S Pupo - aspupo@ibb.unesp.br; Kenneth P Minneman* - kminneman@pharm.emory.edu

*Corresponding author

Published: 16 August 2002

Received: 26 June 2002

BMC Pharmacology 2002, 2:17

Accepted: 16 August 2002

This article is available from: <http://www.biomedcentral.com/1471-2210/2/17>

© 2002 Pupo and Minneman; licensee BioMed Central Ltd. This article is published in Open Access: verbatim copying and redistribution of this article are permitted in all media for any non-commercial purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: The C-terminal four amino acids (GEEV) of human α_{1A} -adrenergic receptors (ARs) have been reported to interact with the PDZ domain of neuronal nitric oxide synthase (nNOS) in a yeast two-hybrid system. The other two α_1 -AR subtypes have no sequence homology in this region, raising the possibility of subtype-specific protein-protein interactions.

Results: We used co-immunoprecipitation and functional approaches with epitope-tagged α_1 -ARs to examine this interaction and the importance of the C-terminal tail. Following co-transfection of HEK-293 cells with hexahistidine/Flag (HF)-tagged α_{1A} -ARs and nNOS, membranes were solubilized and immunoprecipitated with anti-FLAG affinity resin or anti-nNOS antibodies. Immunoprecipitation of HF α_{1A} -ARs resulted in co-immunoprecipitation of nNOS and vice versa, confirming that these proteins interact. However, nNOS also co-immunoprecipitated with HF α_{1B} - and HF α_{1D} -ARs, suggesting that the interaction is not specific to the α_{1A} subtype. In addition, nNOS co-immunoprecipitated with each of the three HF α_1 -AR subtypes which had been C-terminally truncated, suggesting that this interaction does not require the C-tails; and with Flag-tagged β_1 - and β_2 -ARs. Treatment of PC12 cells expressing HF α_{1A} -ARs with an inhibitor of nitric oxide formation did not alter norepinephrine-mediated activation of mitogen activated protein kinases, suggesting nNOS is not involved in this response.

Conclusions: These results show that nNOS does interact with full-length α_{1A} -ARs, but that this interaction is not subtype-specific and does not require the C-terminal tail, raising questions about its functional significance.

Background

α_1 -Adrenergic receptors (ARs) are G protein-coupled receptors that mediate some of the actions of norepinephrine and epinephrine. Three human α_1 -AR subtypes have been cloned and named α_{1A} , α_{1B} and α_{1D} -ARs[1]. These receptors regulate several important central and peripheral processes, such as neuronal excitability, vascular and nonvascular smooth muscle contraction, and cellular

growth and differentiation. The three α_1 -AR subtypes are structurally and pharmacologically distinct, but all couple through $G_{q/11}$ to cause activation of apparently similar intracellular signaling pathways.

The last four amino acids of the intracellular C-tail of the α_{1A} -AR, GEEV, matches the motif G(D/E)XV shown previously to interact with the class III PDZ domain of neuron-

al nitric oxide synthase (nNOS). Experiments using the yeast two-hybrid system showed previously that a protein corresponding to the last 114 amino acids of the rat α_{1A} -AR (previously referred to as α_{1C} -AR) interacted strongly with the PDZ domain of nNOS[2]. Since the corresponding amino acids at the C-terminus of α_{1B} (PGQF) and α_{1D} -ARs (ETDI) would not be predicted to interact with this PDZ domain, an interaction between α_{1A} -ARs and nNOS could represent an interaction unique to this subtype.

PDZ domains are protein-binding modules involved in assembly of signaling complexes and subcellular protein targeting[3]. For example, NMDA receptors in cultured cortical neurons associate with nNOS through PSD-95, a protein containing three PDZ domains[4]. Consequently, NMDA receptor activation increases nitric oxide production and neurotoxicity; while suppression of PSD-95 expression inhibits these responses. These results suggest that the PDZ domains of PSD-95 may facilitate the assembly of signaling complexes involving both NMDA receptors and nNOS, and the increases in intracellular Ca^{2+} caused by NMDA receptor activation may facilitate nNOS activation.

Since α_{1A} -AR activation also increases intracellular Ca^{2+} , we studied the interaction between this receptor and nNOS. We wanted to determine whether full-length α_{1A} -ARs interact with full-length nNOS, whether the interaction is subtype-specific, and whether it involves the GEEV motif in the C-terminal tail. We co-expressed epitope-tagged full length or C-terminally truncated α_1 -ARs with nNOS in HEK-293 cells and examined the ability of anti-Flag and anti-nNOS antibodies to immunoprecipitate both proteins. We found that nNOS does interact with full-length α_{1A} -ARs, but that it also interacts with other α_1 -AR subtypes and β -ARs. In addition, the interaction does not require the C-terminal tail, confirming that it is not specific to the GEEV motif.

Results

Co-immunoprecipitation of nNOS with HF α_{1A} -ARs

To study the interaction between α_{1A} -ARs and nNOS, HEK-293 cells were transfected with rat nNOS and selected with geneticin (400 μ g/ml). Western blots using an anti-nNOS antibody showed a strong immunoreactive band of \sim 170 kDa corresponding to nNOS in stably transfected cells as expected, but little or no signal in untransfected cells (data not shown). Expression of nNOS was similar to that observed with equal amounts of rat brain membrane protein run in parallel, suggesting similar expression levels. HEK-293 cells stably transfected with nNOS were co-transfected with the cDNA encoding HF α_{1A} -ARs. Expression levels of transiently transfected α_1 -ARs in these cells ranged from 100–500 fmol/mg pro-

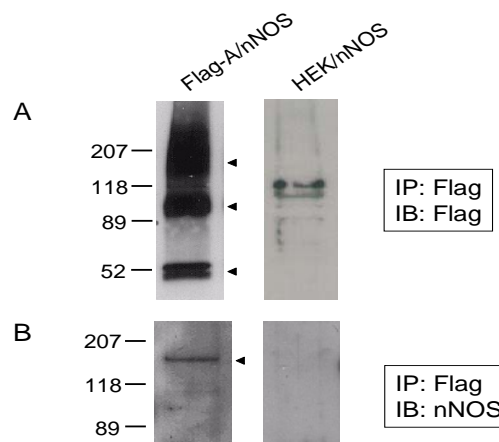


Figure 1

Immunoprecipitation with anti-Flag M2 affinity resin of solubilized HEK-293 cells co-expressing nNOS and HF-tagged α_{1A} -AR (Flag-A/nNOS) or HEK-293 expressing only nNOS (HEK/nNOS). Cells were solubilized, immunoprecipitated with anti-Flag M2 affinity resin, eluted with Flag peptide, run on SDS-PAGE, and transferred to nitrocellulose as described in Methods. A: Western blot with M2 anti-Flag antibody with arrows showing monomers, dimers and trimers; B: Western blot with anti-nNOS antibody. Blots are representative of at least two other experiments with similar results. IP = immunoprecipitation; IB = immunoblot.

tein, also similar to levels observed in rat brain. Cells were then solubilized, immunoprecipitated with anti-Flag M2 affinity resin, eluted, and blotted with anti-Flag (Fig. 1A) or anti-nNOS antibodies (Fig. 1B). Western blots of anti-Flag immunoprecipitates showed that HF α_{1A} -ARs migrated as monomers of \sim 50 kDa (Fig. 1), and also appeared as dimers and trimers, as reported previously[5]. Immunoprecipitation of HF α_{1A} -ARs with anti-Flag affinity resin resulted in co-immunoprecipitation of nNOS, as revealed by the 170 kDa band detected in immunoblots using anti-nNOS antibody (Fig. 1B). Note that nNOS immunoreactivity was not present in anti-Flag affinity resin immunoprecipitates from solubilized HEK-293 cells not transfected with HF α_{1A} -ARs (Fig. 1B), showing that co-immunoprecipitation of nNOS requires presence of the tagged receptor construct.

Co-immunoprecipitation of nNOS with HF α_{1B} - and HF α_{1D} -ARs

To determine whether the interaction between nNOS and α_{1A} -ARs was subtype-specific, cells stably expressing nNOS were co-transfected with HF α_{1B} or HF α_{1D} -ARs, solubilized, immunoprecipitated with anti-Flag affinity resin, eluted, and blotted with anti-Flag (Fig. 2A) or anti-nNOS antibodies (Fig. 2B). Monomers of the HF α_{1B} and

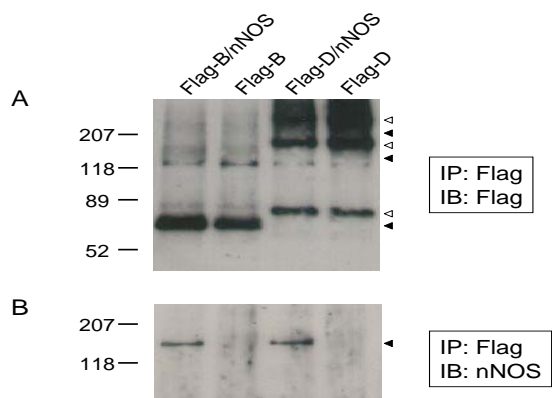


Figure 2
 Immunoprecipitation with anti-Flag M2 affinity resin of solubilized HEK-293 cells co-expressing nNOS and HF-tagged α_{1B} -ARs (Flag-B/nNOS) or α_{1D} -ARs (Flag-D/nNOS) or HEK-293 cells expressing only the HF-tagged α_1 -AR subtypes (Flag-B, Flag-D). A: Western blot with anti-Flag antibody; arrows indicate monomers, dimers and trimers of the HF-tagged α_{1B} -ARs (closed) and α_{1D} -ARs (open). B: Western blot with anti-nNOS antibody. Blots shown are representative of at least two other experiments with similar results. IP = immunoprecipitation; IB = immunoblot.

HF α_{1D} -ARs migrated as bands of ~65 and ~75 kDa (Fig. 2A), and dimers and trimers were also detected. Surprisingly, nNOS was also co-immunoprecipitated from cells co-expressing nNOS and HF α_{1B} or HF α_{1D} -ARs (Fig. 2B), although the GEEV motif predicted to interact with nNOS is not present in either of these subtypes.

Interaction of C-terminally truncated HF α_1 -ARs with nNOS

The role of the C-terminus of α_1 -ARs in the interaction with nNOS was examined by truncation. Stop codons were introduced approximately 20 amino acids after the predicted 7th transmembrane domain, at a conserved glutamine (α_{1A} , Gln 344; α_{1B} , Gln 366) or an adjacent arginine (α_{1D} , Arg 418). Truncated HF α_1 -ARs were transfected into HEK-293 cells stably expressing nNOS, and cells were solubilized, immunoprecipitated with anti-Flag M2 affinity resin, and blotted with anti-Flag (Fig. 3A) or anti-nNOS antibodies (Fig. 3B). The monomeric truncated receptors migrated with molecular masses ~25% lower than that of the full length receptors (Fig. 3A), and higher order oligomers were also apparent as observed with full-length receptors. Specific immunoreactivity to anti-nNOS antibody was also detected in these immunoprecipitates (Fig. 3B), showing that the C-terminal cytoplasmic tail of the HF α_1 -ARs is not required for interaction.

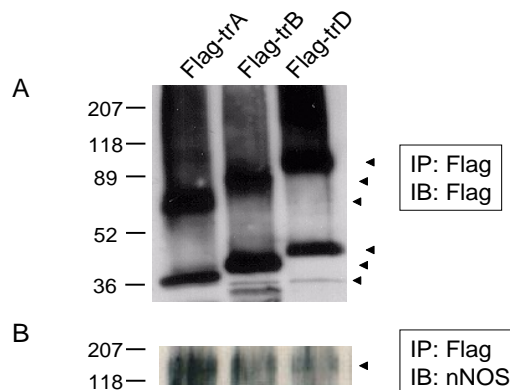


Figure 3
 Immunoprecipitation with anti-Flag M2 affinity resin of solubilized HEK-293 cells expressing C-terminally truncated HF-tagged α_1 -AR subtypes (Flag-trA, Flag-trB and Flag-trD) and nNOS. A: Western blot with anti-Flag M2 antibody; arrows indicate positions of monomers and dimers. B: Western blot with anti-nNOS antibody; arrow indicates nNOS. Blots shown are representative of at least two other experiments with similar results. IP = immunoprecipitation; IB = immunoblot.

Co-immunoprecipitation of HF-tagged α_1 -ARs with anti-nNOS antibody

We also examined the ability of anti-nNOS antibodies to co-immunoprecipitate full length and truncated HF α_1 -ARs. HEK-293 cells expressing nNOS were transfected with each receptor construct and harvested after 48–72 hr. Samples were solubilized, incubated with anti-nNOS antibody, immunoprecipitated with Protein A agarose, and blotted with anti-Flag antibody. This procedure resulted in a strong non-specific band migrating at ~50 kDa (approximately the size of the HF α_{1A} -AR), probably representing IgG heavy chains. For comparison, parallel samples were immunoprecipitated with anti-Flag affinity resin and loaded on the same gel. Fig. 4A shows that anti-nNOS antibodies caused co-immunoprecipitation of all three full length and truncated HF α_1 -ARs. Note that neither protein A agarose alone, nor anti-nNOS antibody plus Protein A agarose, caused immunoprecipitation of HF α_1 -ARs in cells not expressing nNOS (data not shown). This indicates that HF α_1 -ARs do not nonspecifically interact with anti-nNOS antibody and/or protein A agarose.

Co-immunoprecipitation of nNOS with Flag-tagged β_1 - and β_2 -ARs

To further examine the specificity of this interaction, HEK-293 cells stably expressing nNOS were co-transfected with Flag-tagged β_1 or β_2 -ARs, solubilized, immunoprecipitated

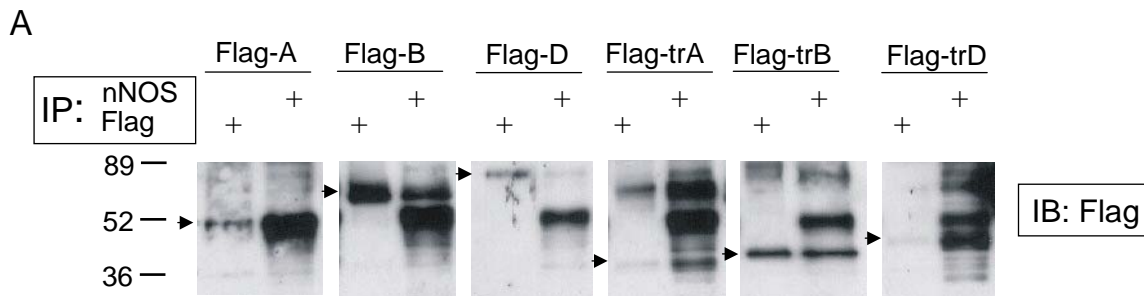


Figure 4

Immunoprecipitation with anti-nNOS antibody of solubilized HEK-293 cells co-expressing nNOS and HF-tagged full length (Flag-A, Flag-B, Flag-D) or C-terminally truncated (Flag-trA, Flag-trB, Flag-trD) α_1 -AR subtypes. For comparison, immunoprecipitation of HF-tagged receptors with anti-Flag M2 affinity resin is also shown. Monomers of each full-length and truncated subtype are indicated by arrows on the left of each blot. Blots shown are representative of at least two other experiments with similar results. IP = immunoprecipitation; IB = immunoblot.

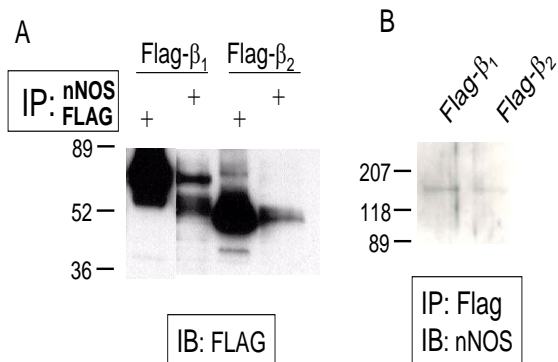


Figure 5

Immunoprecipitation with anti-nNOS antibody or anti-Flag M2 affinity resin of solubilized HEK-293 cells co-expressing nNOS and Flag-tagged β_1 - or β_2 -ARs. A: Western blot with anti-Flag antibody; B: Western blot with anti-nNOS antibody. Blots shown are representative of at least two other experiments with similar results. IP = immunoprecipitation; IB = immunoblot.

ed with anti-Flag affinity resin or anti-nNOS antibody plus Protein A agarose, and blotted with anti-Flag (Fig. 5A) or anti-nNOS antibodies (Fig. 5B). Fig. 5A shows that both Flag-tagged β_1 and β_2 -ARs migrated as monomers (β_1 at ~70 kDa and β_2 at ~50 kDa) as well as oligomers (data not shown), and that immunoprecipitation of nNOS caused co-immunoprecipitation of both Flag-tagged β_1 or β_2 -ARs. Fig. 5B shows that nNOS was also observed following immunoprecipitation with anti-Flag M2 affinity resin in cells transfected with either Flag-tagged β_1 and β_2 -ARs.

Effect of l-NAME on α_{1A} -AR-induced ERK activation in PC12 cells

It is known that nitric oxide produced by nNOS is required for PC12 cell differentiation induced by nerve growth factor (NGF) and that treatment of PC12 cells with NGF induces nNOS expression[6]. Since α_{1A} -AR stimulation also activates ERKS and induces differentiation in PC12 cells stably transfected with this subtype[7], we investigated the effects of l-NAME, an inhibitor of NOS, on norepinephrine-induced ERK phosphorylation in PC12 cells stably transfected with HF α_{1A} -ARs. Fig. 6 shows that treatment of HF α_{1A} -PC12 cells with high concentrations of l-NAME did not block ERK phosphorylation induced by norepinephrine, or by UTP, EGF or NGF, suggesting

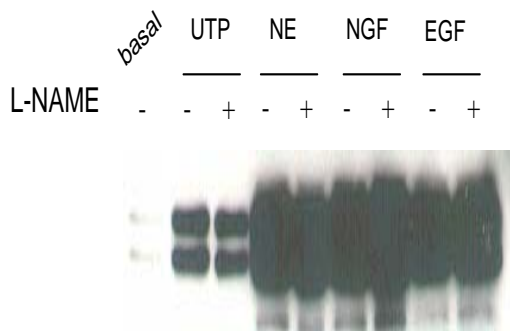


Figure 6

Effect of L-NAME (500 μM) on UTP (100 μM), norepinephrine (NE, 100 μM), NGF (100 ng/ml) and EGF (100 ng/ml) induced ERK phosphorylation in PC12 cells. HFα_{1A}-transfected PC12 cells were serum-starved for 2 hr, pretreated with L-NAME for 30 min, and exposed to agonist for 15 min before harvesting. ERK activation was determined by Western blotting with dual phospho-specific ERK antibodies as described in Methods. Although the blot shown is overexposed, similar strength of signals were seen with NE, NGF and EGF after shorter exposures.

that nitric oxide is not required for mitogenic signals in this cell line.

Discussion

We evaluated the specificity and functional importance of the reported interaction of the C-terminus of α_{1A}-ARs with the PDZ domain of nNOS. Previous work using a yeast two hybrid assay showed that the C-terminal 114 amino acids of rat α_{1A}-ARs (referred to by the previous name of α_{1C}-) strongly interacted with residues 1–111 of nNOS[2]. The bradykinin B2 receptor, also a G protein coupled receptor, has been shown to bind directly to the oxygenase domain of nNOS and form an inhibitory complex[8], and it has been proposed that nNOS is released and activated upon receptor stimulation. The domain of the bradykinin B2 receptor that interacts with nNOS is in the C-tail shortly after the predicted 7th transmembrane domain, and spatially similar but structurally dissimilar domains of the rat angiotensin AT1 receptor and human endothelin-1 ETB receptors have been proposed to block endothelial NOS (eNOS) activity, possibly through a similar mechanism[9]. Therefore we wanted to determine whether there was a specific interaction between full-length α_{1A}-ARs and nNOS in intact cells.

Co-immunoprecipitation experiments showed that epitope-tagged α_{1A}-ARs do interact with full-length nNOS when expressed together in HEK-293 cells. Immunoprecipitation of HFα_{1A}-ARs from cells stably expressing nNOS caused co-immunoprecipitation of nNOS. Similarly, immunoprecipitation of nNOS caused co-immunoprecipitation of HFα_{1A}-ARs. This interaction appeared to be specific, since nNOS was not immunoprecipitated by anti-Flag affinity resin in cells not transfected with tagged receptors, and tagged receptors were not immunoprecipitated by anti-nNOS antibody in cells not expressing nNOS.

These results support an interaction between nNOS and α_{1A}-ARs, which could be due to the previously reported interaction of the receptor C-terminus and the PDZ domain of nNOS[2]. However, this hypothesis is weakened by the unexpected observation that nNOS also co-immunoprecipitates with both α_{1B} and α_{1D}-ARs. There is little or no homology between the C-terminal sequences of α_{1A}, α_{1B} and α_{1D}-ARs, and neither α_{1B} nor α_{1D}-ARs contain the GEEV motif predicted to mediate the interaction between α_{1A}-ARs and nNOS. However, nNOS was found to co-immunoprecipitate with both HFα_{1B}- and HFα_{1D}-ARs after co-expression in HEK-293 cells. These interactions could be observed by blotting for the tagged receptors after immunoprecipitation with anti-nNOS antibody, or by blotting for nNOS after immunoprecipitation of the tagged receptors with anti-Flag antibody. Direct comparison of α_{1A}, α_{1B} and α_{1D}-ARs in the same experiment showed similar degrees of interactions of all three subtypes with nNOS (data not shown), further demonstrating that this interaction is not specific to the α_{1A} subtype.

We examined the role of the C-terminal tail in this interaction by constructing receptors in which the C-terminus was truncated. Studies with HFα₁-AR subtypes with short (~20 aa) C-terminal tails suggested that the C-terminal tails are not required for interaction with nNOS. Following transfection into nNOS-expressing cells, immunoprecipitation of all three C-terminally truncated HFα₁-AR constructs caused co-immunoprecipitation of nNOS similar to that observed with full-length receptors. Although PDZ domains of some proteins, such as PSD-95 and syn- trophin, can bind internal peptide sequences that fold as β-fingers and mimic canonical C-terminal peptides[10,11], there is very little homology between the intracellular loops of α₁-AR subtypes, making it unlikely that there is a common internal amino acid sequence involved in interaction with nNOS.

Since we found nNOS to co-immunoprecipitate with all three α₁-AR subtypes, we also determined whether it would directly associate with β-ARs. Flag-tagged β₁ and β₂-ARs were transfected into cells stably expressing nNOS, and after solubilization and immunoprecipitation with

anti-Flag M2 affinity resin we again found co-immunoprecipitation of nNOS. Since β_1 and β_2 -ARs show no sequence homology to α_1 -AR subtypes in their intracellular domains, this further supports the conclusion that interaction with nNOS is not localized to discrete intracellular domains.

Conclusions

Our data suggest that full-length α_{1A} -ARs do interact with nNOS; however this interaction is not subtype-specific since α_{1B} - and α_{1D} -ARs showed similar interactions. The interaction did not require the receptor C-terminus, and similar interactions were observed with β_1 and β_2 -ARs. This data does not support a proposed specific interaction between the α_{1A} -AR C-terminus and the nNOS PDZ domain suggested by studies with fusion proteins. Studies on α_{1A} -ARs in transfected PC12 cells showed no role for nitric oxide in mitogenic signaling, also raising questions about the functional significance of this interaction.

Methods

Materials

HEK-293 cells were purchased from ATCC. PC12 cells were obtained from Cindy Miranti and Michael Greenberg (Harvard Medical School, Boston, MA, USA). The cDNA encoding rat nNOS was from Dr. Thomas Michel (Harvard Medical School, Boston, MA), the human α_{1A} -AR cDNA [12] from Dr. Gozoh Tsujimoto (National Children's Hospital, Tokyo, Japan), the human α_{1B} -AR cDNA [13] from Dr. Dianne Perez (Cleveland Clinic), and the human α_{1D} -AR cDNA [14] was cloned in our lab. Materials were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM); L-NAME; norepinephrine ((-)-arterenol); streptomycin, penicillin, Flag peptide, anti-Flag M2 affinity resin, HRP-conjugated anti-Flag M2 antibody and goat anti-rabbit HRP-conjugated secondary antibodies (Sigma, St Louis, MO); geneticin; n-Dodecyl- β -D-maltoside (Calbiochem); anti-nNOS rabbit polyclonal antibody, Protein A-agarose resin (Santa Cruz); rabbit polyclonal dual phospho-specific anti-ERK antibody, PNGase F (New England Biolabs); and ECL reagent (Amersham).

Cell culture

HEK-293 cells were propagated in Dulbecco's Minimal Essential Medium with sodium pyruvate, 10% heat inactivated fetal bovine serum, 100 U/l streptomycin, and 100 U/l penicillin at 37°C in a humidified atmosphere with 5% CO₂. Confluent plates were subcultured at a 1:3 ratio. PC12 cells were propagated in Dulbecco's Minimal Essential Medium containing 4.5 g/l glucose, 1.4% glutamine, 20 mM Hepes, 100 U/l streptomycin, 100 U/l penicillin, 10% donor horse serum, and 5% fetal bovine serum. For measurement of ERK phosphorylation, 35 mm dishes of PC12 cells were seeded at a density of 600,000 cells/2 ml.

Transfections

Receptor coding sequences were generated by PCR, sequenced, and subcloned into the mammalian expression plasmid pDT containing sequential N-terminal hexahistidine and FLAG (HF) epitopes as previously described [5]. HEK-293 cells (150 mm plates) were transfected with 50 μ g cDNA encoding the rat isoform of nNOS by calcium phosphate precipitation, and stably transfected cells selected with geneticin (400 μ g/ml). cDNAs encoding each of the HF-human α_1 -ARs were transfected into parental HEK-293 cells or cells stably transfected with nNOS by calcium phosphate precipitation and cells harvested 48–72 h later. The density of HF α_1 -ARs was measured by specific binding of [¹²⁵I]-HEAT [7], and ranged from 100–500 fmol/mg protein.

Immunoprecipitation

HEK-293 cells expressing HF-tagged α_1 -ARs, nNOS, or both, were harvested by scraping and fractionated by repeated centrifugation and homogenization. Cell lysates (1–2 mg protein) were solubilized in 1X buffer (25 mM Hepes and 150 mM NaCl, pH 7.4) with 2% n-Dodecyl- β -D-maltoside for 90 min at 4°C in buffer A (25 mM HEPES, 150 mM NaCl, pH 7.4) supplemented with protease inhibitors (aprotinin 2 μ g/ml, leupeptin 2 μ g/ml, pepstatin 2 μ g/ml, benzamidine 2 μ g/ml, PMSF 2 mM, and EDTA 50 mM). Solubilized samples were centrifuged, the supernatant diluted 10-fold with buffer A containing protease inhibitors, and incubated with 100–200 μ l anti-Flag M2 affinity resin for 90 min at 4°C with gentle rotation [5]. Alternatively, the supernatant was incubated with 5 μ l of anti-nNOS rabbit polyclonal antibody (200 μ g/ml) for 90 min at 4°C and then incubated with 20 μ l Protein A-agarose overnight at 4°C. Immunoprecipitated material was recovered by centrifugation and washed at least 4 times with buffer A containing protease inhibitors. After washing, samples immunoprecipitated with anti-Flag affinity resin were eluted with 100 to 200 μ l buffer A containing 400 μ g/ml Flag peptide, while samples immunoprecipitated with anti-nNOS antibody were eluted with 40 μ l of 2X Laemmli loading buffer. All samples were deglycosylated after immunoprecipitation by treatment with 1 μ l PNGase F for 2 h at room temperature. Aliquots of 30 μ l were separated by 4–20% SDS-PAGE, transferred to nitrocellulose, and blotted with anti-Flag M2 antibodies conjugated to HRP (1:600) or anti-nNOS rabbit polyclonal antibodies (1:1,000) followed by goat anti-rabbit HRP-conjugated secondary antibodies (1:15,000). Proteins were visualized by ECL.

ERK phosphorylation in PC12 cells

Confluent PC12 cells stably transfected with HF- α_{1A} -ARs [15] (~1 pmol/mg of protein) were serum-starved for 2 h before use, and incubated with or without L-NAME (500 μ M/30 min). Cells were then incubated with nore-

pinephrine or other agonists for 15 min and lysed with Laemmli sample buffer. Cell lysates were centrifuged and proteins (10 µg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Phosphorylation of ERKs was detected by immunoblotting using a 1:1000 dilution of rabbit polyclonal dual phospho-specific ERK antibodies with HRP-conjugated goat anti-rabbit IgG as a secondary antibody and visualized by ECL.

List of abbreviations

AR, adrenergic receptor; nNOS, neuronal nitric oxide synthase; HF, hexahistidine/Flag tagged; ERK, extracellular signal regulated kinase; l-NAME, l-nitroarginine methyl ester; NGF, nerve growth factor

Authors' contributions

ASP carried out most of the biochemical work and performed the statistical analysis. The study was conceived and designed by ASP and KPM, who both participated in data analysis and writing. Both authors read and approved the final manuscript.

Acknowledgements

Supported by NIH and FAPESP. We thank Dr. Randy Hall for providing Flag-tagged β_1 - and β_2 -AR constructs.

References

1. H Zhong, KP Minneman: **Alpha1-adrenoceptor subtypes**. *Eur J Pharmacol* 1999, **375**:261-76
2. J Schepens, E Cuppen, B Wieringa, W Hendriks: **The neuronal nitric oxide synthase PDZ motif binds to -G(D,E)XV* carboxyterminal sequences**. *FEBS Lett* 1997, **409**:53-6
3. M Sheng, C Sala: **PDZ domains and the organization of supramolecular complexes**. *Annu Rev Neurosci* 2001, **24**:1-29
4. R Sattler, Z Xiong, WY Lu, M Hafner, JF MacDonald, M Tymianski: **Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein**. *Science* 1999, **284**:1845-8
5. A Vicentic, A Robeva, G Rogge, M Uberti, KP Minneman: **Biochemistry and Pharmacology of Epitope-Tagged alpha(1)-Adrenergic Receptor Subtypes**. *J Pharmacol Exp Ther* 2002, **302**:58-65
6. YT Phung, JM Bekker, OG Hallmark, SM Black: **Both neuronal NO synthase and nitric oxide are required for PC12 cell differentiation: a cGMP independent pathway**. *Brain Res Mol Brain Res* 1999, **64**:165-78
7. H Zhong, KP Minneman: **Differential activation of mitogen-activated protein kinase pathways in PC12 cells by closely related alpha1-adrenergic receptor subtypes**. *J Neurochem* 1999, **72**:2388-96
8. R Golser, AC Gorren, A Leber, P Andrew, HJ Habisch, ER Werner, K Schmidt, RC Venema, B Mayer: **Interaction of endothelial and neuronal nitric-oxide synthases with the bradykinin B2 receptor. Binding of an inhibitory peptide to the oxygenase domain blocks uncoupled NADPH oxidation**. *J Biol Chem* 2000, **275**:5291-6
9. MB Marrero, VJ Venema, H Ju, H He, H Liang, RB Caldwell, RC Venema: **Endothelial nitric oxide synthase interactions with G-protein-coupled receptors**. *Biochem J* 1999, **343**:335-40
10. JE Brenman, DS Chao, SH Gee, AW McGee, SE Craven, DR Santillano, Z Wu, F Huang, H Xia, MF Peters, et al: **Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains**. *Cell* 1996, **84**:757-67
11. BJ Hillier, KS Christopherson, KE Prehoda, DS Bredt, WA Lim: **Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex**. *Science* 1999, **284**:812-5
12. A Hirasawa, K Horie, T Tanaka, K Takagaki, M Murai, J Yano, G Tsujimoto: **Cloning, functional expression and tissue distribution of human cDNA for the alpha 1C-adrenergic receptor**. *Biochem Biophys Res Commun* 1993, **195**:902-9
13. CS Ramarao, JM Denker, DM Perez, RJ Gaivin, RP Riek, RM Graham: **Genomic organization and expression of the human alpha 1B-adrenergic receptor**. *J Biol Chem* 1992, **267**:21936-45
14. TA Esbenshade, A Hirasawa, G Tsujimoto, T Tanaka, J Yano, KP Minneman, TJ Murphy: **Cloning of the human alpha 1d-adrenergic receptor and inducible expression of three human subtypes in SK-N-MC cells**. *Mol Pharmacol* 1995, **47**:977-85
15. NG Williams, H Zhong, KP Minneman: **Differential coupling of alpha 1-, alpha 2-, and beta-adrenergic receptors to mitogen-activated protein kinase pathways and differentiation in transfected PC12 cells**. *J Biol Chem* 1998, **273**:24624-32

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMedcentral will be the most significant development for disseminating the results of biomedical research in our lifetime."

Paul Nurse, Director-General, Imperial Cancer Research Fund

Publish with **BMC** and your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours - you keep the copyright



BioMedcentral.com

Submit your manuscript here:

<http://www.biomedcentral.com/manuscript/>

editorial@biomedcentral.com