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Short communication

Protein kinase C-α_{1b}-adrenoceptor coimmunoprecipitation: effect of hormones and phorbol myristate acetate

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Abstract

α_{1b}-Adrenoceptors immunoprecipitated with protein kinase C α, δ, and ε isoforms under basal conditions and such coimmunoprecipitations were increased in cells treated with phorbol myristate acetate. The increased coimmunoprecipitations induced by phorbol myristate acetate were concentration-dependent and reached their maxima 1 to 2 min after the addition of the tumor promoter. No coimmunoprecipitation of protein kinase C ζ and α_{1b}-adrenoceptors was detected. Norepinephrine, endothelin-1, lysophosphatidic acid and epidermal growth factor were also able to increase the coimmunoprecipitation of protein kinase C isoenzymes and α_{1b}-adrenoceptors. These data support the idea that protein kinase-receptor complexes might form and could be relevant in receptor desensitization. © 2001 Published by Elsevier Science B.V.

Keywords: α_1-Adrenoceptor; Protein kinase C; Desensitization

1. Introduction

Receptor phosphorylation seems to be a cardinal initial event in desensitization of G protein-coupled receptor signaling. Two major types of desensitizations exist: homologous and heterologous desensitizations. Homologous desensitization, in which receptors occupied by agonists reduce their responsiveness, mainly involves receptor phosphorylation by G protein-coupled receptor kinases. Heterologous desensitization, in which the stimulation by an agent attenuates the response to an unrelated agonist, mainly involves phosphorylation of receptors and other signaling entities by second messenger-activated kinases, such as protein kinase A and protein kinase C (García-Sáinz et al., 2000). Current ideas indicate that protein kinases and phosphatases form dynamic complexes with substrates and anchoring proteins; such dynamic complexes are organized and coordinated through specific protein–protein and protein–phospholipid interactions and it is through these signaling networks that cellular responses are regulated (Pawson and Scott, 1997).

α_{1b}-Adrenoceptors are members of the superfamily of the seven transmembrane domains G-protein-coupled receptors. Agonist-induced phosphorylation of α_{1b}-adrenoceptors seems to be mediated by G protein-coupled receptor kinase 2 and 3 isoenzymes (Diviani et al., 1996; Iacovelli et al., 1999), whereas the phosphorylation of these adrenoceptors by protein kinase C mainly participates in heterologous desensitization (Diviani et al., 1997; Vázquez-Prado et al., 1997; Casas-González et al., 2000; García-Sáinz et al., 2000; Medina et al., 2000).

Protein kinase C is a family of enzymes that have been divided into three structurally related groups: conventional (α, βI, BII and γ), novel (δ, ε, η, θ and μ) and atypical (ζ and λ) isoenzymes, with different requirements for calcium and diacylglycerol (Newton, 1995). Although great progress has been achieved, it is yet far from clear what role(s) each isoenzyme plays and to what extent their functions overlap.

In the case of α_{1b}-adrenoceptors, there is no information on the protein kinase C isoenzymes that participate in receptor phosphorylation and desensitization. We took advantage of the availability of antibodies that effectively immunoprecipitate α_{1b}-adrenoceptors (Vázquez-Prado et al., 1997) to define what protein kinase C isoenzymes coimmunoprecipitate with the adrenoceptor, under basal and stimulated conditions. Our data indicate that in rat-1 fibroblasts stably expressing α_{1b}-adrenoceptors, protein kinase C α, δ and ε isoforms coimmunoprecipitate with

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these adrenoceptors under basal conditions and that such a coimmunoprecipitation is increased by agents that induce receptor phosphorylation.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium, EGF (epidermal growth factor), fetal bovine serum, protein kinase C isoenzyme-selective antibodies and others reagents used for cell culture were obtained from Gibco BRL. (−)-Noradrenaline, lysophosphatidic acid, endothelin-1, phorbol myristate acetate (PMA), phospho-amino acids and protease inhibitors were from Sigma. Sepharose-coupled protein A was from Upstate Biotechnology. Nitrocellulose membranes were from Bio-Rad. Secondary antibodies were from Santa Cruz Biotechnology and the chemiluminescence kits were obtained from Pierce.

2.2. Cell line and culture

Rat-1 fibroblasts transfected with the hamster α_{1b}-adrenoceptor, generously provided to us by Drs. R.J. Lefkowitz, M.G. Caron and L. Allen (Duke University), 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 analog, G-418 sulfate, 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B at 37°C under a 95% air/5% CO_2 atmosphere as described previously (Vázquez-Prado et al., 1997, 2000). In all the experiments, confluent cells were serum-deprived overnight in unsupplemented Dulbecco's modified Eagle's medium.

2.3. α_{1b}-Adrenoceptor-protein kinase C coimmunoprecipitation studies

α_{1b}-Adrenoceptors were immunoprecipitated as described previously (Vázquez-Prado et al., 1997, 2000), with minor modifications. In brief, following treatment with inhibitors and/or agonists, cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h on ice in buffer containing 10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% sodium dodecyl sulfate, 50 mM NaF, 100 μM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM phosphoserine, 1 mM phosphothreonine and protease inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, 500 μg/ml bacitracin, 50 μg/ml soybean trypsin inhibitor). Cell lysates were centrifuged at 12,700 × *g* for 15 min and the supernatants were incubated overnight at 4°C with the anti-α_{1b}-

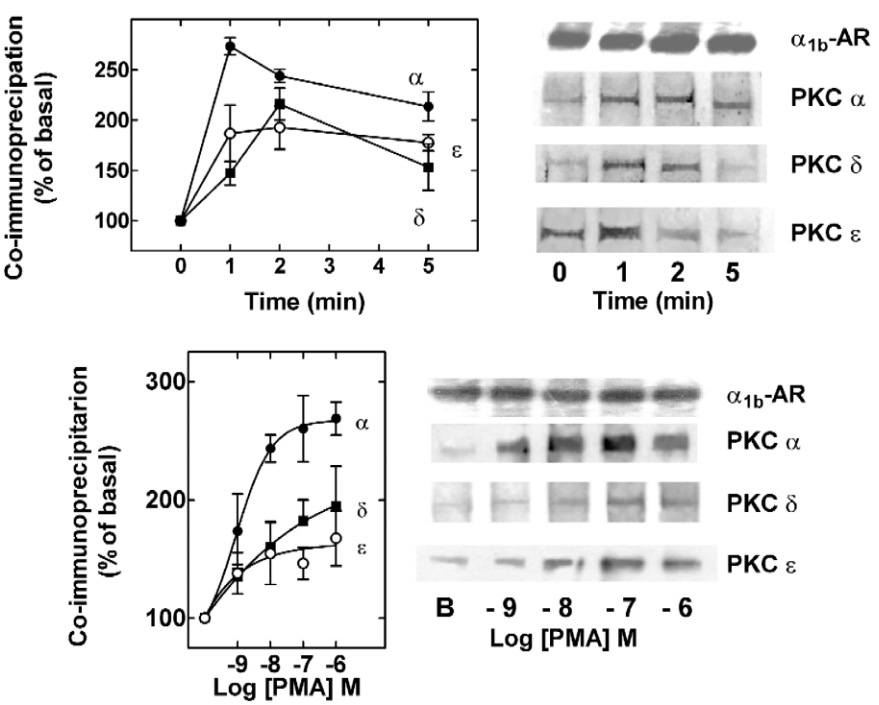

Fig. 1. Time course and concentration–response curves for the effect of PMA on the coimmunoprecipitation of α_{1b}-adrenoceptors and protein kinase C isoforms. Upper panels: Cells were incubated for the times indicated in the presence of 1 μM PMA. Lower panels: Cells were incubated with the indicated concentration of PMA for 2 min. Data were normalized to the coimmunoprecipitation observed in the absence of stimulus (100%). Plotted are the means and vertical lines represent the S.E.M. of four to five determinations using different cell cultures. α_{1b}-AR, α_{1b}-adrenoceptors. Representative immunoblots are presented.

adrenoceptor antiserum (Vázquez-Prado et al., 1997) and protein A-Sepharose. After four washes with 50 mM HEPES, 50 mM NaH_2PO_4, 100 mM NaCl, pH 7.4, 0. 1% Nonident P40, the immune complexes were denatured by boiling in sodium dodecyl sulfate-sample buffer containing 5% β-mercaptoethanol, and subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose membranes and immunoblotting was performed. Incubation with the protein kinase C isoenzyme-selective antibody was for 12 h at 4°C and with the secondary antibody for 3 h at room temperature. Super signal enhanced chemiluminescence kits from Pierce were used exposing the membranes to X-Omat X-ray films. The level of receptor immunoprecipitated under these conditions was consistent as evidenced by immunoblotting (see figures) and represented 80–85% of total α_{1b}-adrenoceptors as evidenced by immunoprecipitation of photoaffinity-labeled receptor (Vázquez-Prado et al., 1997, 2000); protein kinase C coimmunoprecipitation was assessed by densitometric analysis.

3. Results

As shown in Fig. 1 (upper panels), we were able to detect coimmunoprecipitation of α_{1b}-adrenoceptors with the α, δ, and ε isoforms of protein kinase C under basal conditions and such coimmunoprecipitations were increased in cells treated with PMA. The increased α_{1b}-adrenoceptor-protein kinase C coimmunoprecipitations induced by PMA reached their maxima 1 to 2 min after the addition of the tumor promoter and remained at high level for at least 5 min. Thirty minutes after the stimulation with the phorbol ester the coprecipitation of the different protein kinase C isoforms with α_{1b}-adrenoceptors was still above basal, but only ≈30–40% (data not shown). Attempts to detect coimmunoprecipitation of protein kinase C ζ and α_{1b}-adrenoceptors were unsuccessful, although this isoform was easily detected in cell extracts (data nor shown, see García-Sáinz and Alcántara-Hernández, 1998). These isoforms were observed as discrete bands of protein kinase C α (≈80 kDa; in some experiments (see Figs. 1 and 2) as a doublet ≈80–82 kDa and the bands were quantitated together), protein kinase C δ (≈78 kDa) and protein kinase C ε (≈95 kDa), in agreement with previous studies (García-Sáinz and Alcántara-Hernández, 1998). The concentration–response curves to PMA for the different protein kinase C isoforms are presented also in Fig. 1 (lower panels). It can be observed that all the isoforms increased their coprecipitation with α_{1b}-adrenoceptors in concentration-dependent fashions, but that the magnitude varied from almost threefold (protein kinase C α) to less than twofold (protein kinase C δ and ε isoforms).

We next studied if other agents known to induce α_{1b}-adrenoceptor phosphorylation were able to increase coimmunoprecipitation of these receptors and protein kinase C isoforms. Concentrations of the agents to maximally stimulate phosphorylation and a incubation time of 2 min were used. As shown in Fig. 2, norepinephrine, endothelin-1, lysophosphatidic acid and EGF were able to increase the coimmunoprecipitation of protein kinase C isoenzymes

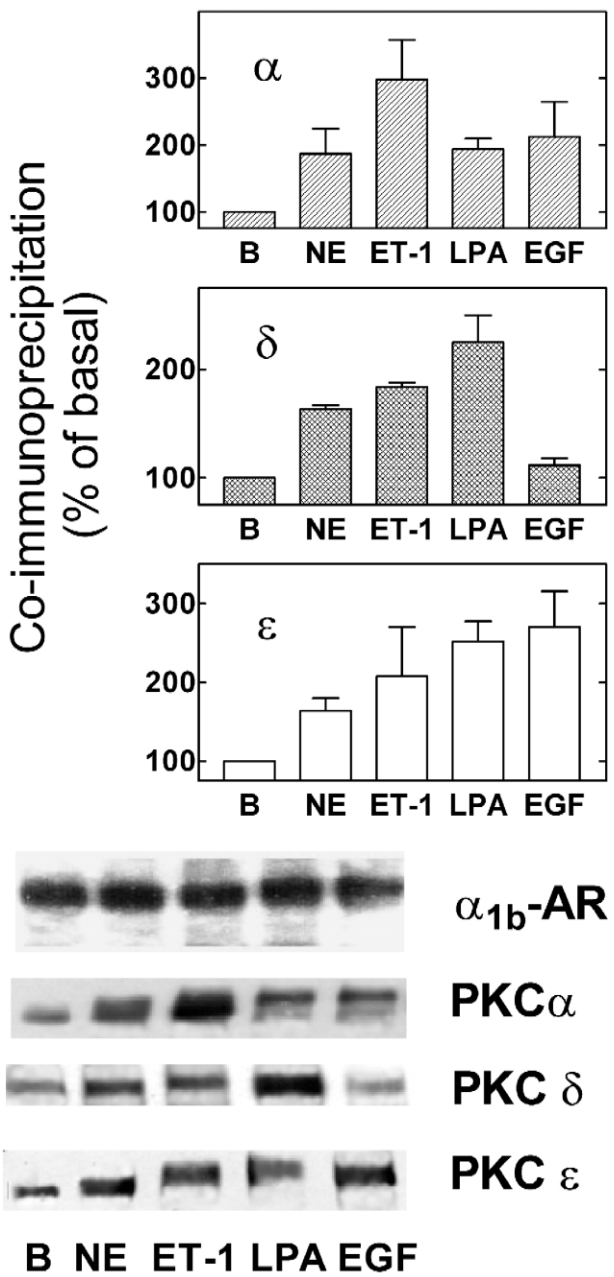

Fig. 2. Effect of hormones on the coimmunoprecipitation of α_{1b}-adrenoceptors and protein kinase C isoforms. Cells were incubated for 2 min in the absence of any agent (B) or presence of 10 μM norepinephrine (NE), 10 nM endothelin-1 (ET-1), 1 μM lysophosphatidic acid (LPA) or 100 ng/ml EGF (EGF). Data were normalized to the coimmunoprecipitation observed in the absence of stimulus (100%). Plotted are the means and vertical lines represent the S.E.M. of four to five determinations using different cell cultures. α_{1b}-AR, α_{1b}-adrenoceptors. Representative immunoblots are presented.

and α_{1b}-adrenoceptors. There was some variation in the ability of these agents to induce coprecipitation of the different kinase isoforms.

4. Discussion

It is known that rat-1 fibroblasts express protein kinase C α, δ, ε and ζ isoforms (Berti et al., 1994; García-Sáinz and Alcántara-Hernández, 1998). In the present work, we showed that protein kinase C isoforms immunoprecipitate with α_{1b}-adrenoceptors in the basal state and that agents known to increase α_{1b}-adrenoceptor phosphorylation promoted such coimmunoprecipitation (Diviani et al., 1997; Vázquez-Prado et al., 1997; Casas-González et al., 2000; García-Sáinz et al., 2000; Medina et al., 2000).

Activation of protein kinase C is well known to block α_{1b}-adrenoceptor actions and to induce α_{1b}-adrenoceptor phosphorylation (Diviani et al., 1997; Vázquez-Prado et al., 1997; Casas-González et al., 2000; García-Sáinz et al., 2000; Medina et al., 2000). The protein kinase C-catalyzed receptor phosphorylation sites have been located in the carboxyl tail (Ser^{394} and Ser^{400}) (Diviani et al., 1997). The present results indicate that several isoforms immunoprecipitate with the receptor. This suggests redundancy in their function or that they may achieve different functions. Protein kinase C α was the isoform that more strongly immunoprecipitate with the receptor in response to the phorbol ester.

Norepinephrine is well known to induce homologous desensitization of α_{1b}-adrenoceptors and receptor phosphorylation (Diviani et al., 1997; Vázquez-Prado et al., 1997; Casas-González et al., 2000; García-Sáinz et al., 2000; Medina et al., 2000). G protein-coupled receptor kinases seem to play the major role (Diviani et al., 1996; Iacovelli et al., 1999). However, our data suggest that protein kinase C isoforms may participate in the desensitization/phosphorylation processes. It is well known that there is a close interplay between G protein-coupled receptor kinases and protein kinase C (Chuang et al., 1995; Winstel et al., 1996).

Activation of receptors for endothelin, lysophosphatidic acid and EGF induce desensitization of α_{1b}-adrenoceptors associated to receptor phosphorylation (Vázquez-Prado et al., 1997; Casas-González et al., 2000; Medina et al., 2000) and in such processes protein kinase C plays a key role. Our present data are consistent with those findings. The differences observed in the immunoprecipitation of the protein kinase C isoforms with the adrenoceptors may reflect the various processes involved in such actions (García-Sáinz et al., 2000).

Recently, it was reported that α_{1b}-adrenoceptor stimulation activated the Jak (Janus kinase)/STAT (signal transducer and activator transcription factor) pathway and that Jak and STAT also interact with these receptors as suggested by coimmunoprecipitation studies (Sasaguri et al., 1999). Also recently, Shih et al. (1999) reported that protein kinase A and protein phosphatases associate with β_2-adrenoceptors forming dynamic complexes with participation of the anchoring protein gravin. Our current data further support the idea that protein kinase-receptor complexes could be relevant in receptor desensitization. Further experiments will be required to define if other molecular entities are involved and the relevant site(s) for such interactions.

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References

Berti, L., Mosthaf, L., Kroder, G., Kellerer, M., Tippmer, S., Mushack, J., Seffer, E., Seedorf, K., Häring, H., 1994. Glucose-induced translocation of protein kinase C in rat-1 fibroblast is paralleled by inhibition of the insulin receptor tyrosine kinase. J. Biol. Chem. 269, 3381–3386.

Casas-González, P., Vázquez-Prado, J., García-Sáinz, J.A., 2000. Lysophosphatidic acid modulates α_{1b}-adrenoceptor phosphorylation and function. Roles of Gi and phosphoinositide 3-kinase. Mol. Pharmacol. 57, 1027–1033.

Chuang, T.T., Le Vine, H., De Blasi, A., 1995. Phosphorylation and activation of β-adrenergic receptor kinase by protein kinase C. J. Biol. Chem. 270, 18660–18665.

Diviani, D., Lattion, A.L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J.L., Cotecchia, S., 1996. Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the α_{1B}-adrenergic receptor. J. Biol. Chem. 271, 5049–5058.

Diviani, D., Lattion, A.-L., Cotecchia, S., 1997. Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitization of the α_{1B}-adrenergic receptor. J. Biol. Chem. 272, 28712–28719.

García-Sáinz, J.A., Alcántara-Hernández, R., 1998. Hormonal modulation of membrane association of protein kinase C isoforms in rat-1 fibroblasts expressing α_{1b}-adrenoceptors. Pharmacol. Rev. Commun. 10, 119–124.

García-Sáinz, J.A., Vázquez-Prado, J., Medina, L.C., 2000. α_1-Adrenoceptors: function and phosphorylation. Eur. J. Pharmacol. 389, 1–12.

Iacovelli, L., Franchetti, R., Grisolia, D., De Blasi, A., 1999. Selective regulation of G protein-coupled receptor-mediated signaling by G protein-coupled receptor kinase 2 in FRTL-5 cells: analysis of thyrotropin, α_{1B}-adrenergic and A1 adenosine receptor-mediated responses. Mol. Pharmacol. 56, 316–324.

Medina, L.C., Vázquez-Prado, J., García-Sáinz, J.A., 2000. Cross-talk between receptors with intrinsic tyrosine kinase activity and α_{1b}-adrenoceptors. Biochem. J. 350, 413–419.

Newton, A.C., 1995. Protein kinase C: structure, function and regulation. J. Biol. Chem. 270, 28495–28498.

Pawson, T., Scott, J.D., 1997. Signaling through scaffold, anchoring and adaptor proteins. Science 278, 2075–2080.

Sasaguri, T., Teruya, H., Ishida, A., Abumiya, T., Ogata, J., 1999. Linkage between α_1-adrenergic receptor and the Jak/STAT signalling pathway in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 258, 25–30.

Shih, M., Lin, F., Scott, J.D., Wang, H.-Y., Malbon, C.C., 1999. Dynamic complexes of β_2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. J. Biol. Chem. 274, 1588–1595.

Vázquez-Prado, J., Medina, L.C., García-Sáinz, J.A., 1997. Activation of endothelin ET_A receptors induce phosphorylation of α_{1b}-adrenoceptors in rat-1 fibroblasts. J. Biol. Chem. 272, 27330–27337.

Vázquez-Prado, J., Medina, L.C., Romero, M.T., González-Espinosa, C., García-Sáinz, J.A., 2000. Norepinephrine and phorbol ester-induced phosphorylation of α_{1a}-adrenergic receptors. Functional aspects. J. Biol. Chem. 275, 6553–6559.

Winstel, R., Freund, S., Krasel, C., Hoppe, E., Lohse, M.J., 1996. Protein kinase cross-talk: membrane targeting of the β-adrenergic receptor kinase by protein kinase C. Proc. Natl. Acad. Sci. U. S. A. 93, 2105–2109.