Glucocorticoid activation of the neuronal nicotinic acetylcholine receptor α7 subunit gene: involvement of transcription factor Egr-1

Carmen Carrasco-Serrano, Manuel Criado*

Department of Biochemistry and Molecular Biology and Instituto de Neurociencias, Universidad Miguel Hernández-C.S.I.C., 03550-San Juan, Alicante, Spain

Received 26 February 2004; revised 21 April 2004; accepted 21 April 2004

Available online 3 May 2004

Edited by Ned Mantei

Abstract The α7 subunit is a component of nicotinic acetylcholine receptors expressed in bovine chromaffin cells. The peculiar localization of these receptors at adrenomedullary areas adjacent to the adrenal cortex suggests that factors, probably glucocorticoids, arising from the cortex might diffuse and regulate α7 receptor expression. In reporter gene transfection experiments, dexamethasone increased α7 promoter activity by up to fivefold in a concentration- and time-dependent manner despite the absence of consensus glucocorticoid receptor elements at the α7 promoter. Transcriptional activation induced by glucocorticoids was abolished through simultaneous mutation of at least two of the three sites for the immediate early transcription factor Egr-1, present in the proximal promoter region of the α7 subunit gene. Therefore, glucocorticoids activate the α7 subunit gene through Egr-1 in an indirect way.

Keywords: Adrenal gland; Nicotinic receptor; α7 subunit; Gene promoter; Glucocorticoid; Egr-1

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric oligomers composed of related subunits which are commonly classified as agonist-binding (designated α2–α10) and structural (β2–β4) subunits (reviewed in [1,2]). Considerable effort has been dedicated to understanding how the regional and developmental expression of neuronal nAChRs is controlled at the transcriptional level (reviewed in [3]). Previously, we have characterized the promoters of the bovine α7 [4,5], α5 [6], and β4 [7] subunits which are components of nAChRs present in chromaffin cells of the bovine adrenal medulla. One of the subtypes, sensitive to α-bungarotoxin, is formed by α7 subunits and is differentially expressed in adrenergic cells adjacent to the adrenal cortex [4]. Since the latter may provide high levels of glucocorticoids to the contiguous adrenergic cells, we asked whether the α7 promoter would be regulated by glucocorticoids.

* Corresponding author. Fax: +34-965-919-561.
E-mail address: manuel.criado@umh.es (M. Criado).

Abbreviations: nAChR, nicotinic acetylcholine receptor; PNMT, phenylethanolamine *N*-methyl transferase

2. Materials and methods

2.1. Plasmid constructions

The isolation and characterization of the 5′ flanking sequence of the bovine α7 subunit gene has been previously described [4]. All α7 promoter-LUC gene fusions were made in the pGL2-Basic vector (Promega, Spain), introducing in its polylinker, upstream of the luciferase gene, the suitable α7 promoter fragments. The basic strategy for site-directed mutagenesis of the different elements at the −77 to −15 region of the α7 promoter (see Fig. 2) has been previously described [5].

2.2. Cell culture and reporter assays

Chromaffin cells were isolated from bovine adrenal glands as described [8] and cultured in 90% Dulbecco's modified Eagle's medium (Sigma, Spain), 10% fetal calf serum, and 10 μM cytosine arabinoside and 10 μM 5-fluoro-2′-deoxyuridine (Sigma) to prevent fibroblast proliferation. C2C12 cells were grown in 85% Dulbecco's modified Eagle's medium (Sigma), and 15% fetal calf serum. Serum was treated with charcoal to eliminate hormones [9] and medium contained no phenol red [10].

Plasmids were purified by Concert columns (Invitrogen). Both cell types were transfected by the calcium phosphate procedure [11]. Chromaffin cells on 48 wells-plates (5×10^5 cells/well) or C2C12 (10^4 cells/well) cells on 24 wells-plates were incubated with 0.75 μg of pGL2 vector or an equivalent amount (in molar terms) of the different constructs derived from this vector, and with 0.75 μg of β-galactosidase expression vector pCH110 (Amersham Pharmacia Biotech) as a control of transfection efficiency (amounts given per well). Cells were washed the next day and dexamethasone treatment started immediately. Cells were harvested after 48 h and β-galactosidase and luciferase were then determined in the lysates with the corresponding assay systems (Promega). Luciferase activity was normalized to values obtained with the p77α7LUC plasmid in the same cell type and conditions but without dexamethasone treatment. Data were analyzed with the Graphpad Prism program (Graphpad Software Inc.) applying the ANOVA-Bonferroni's multiple comparison test. Statistical significance was set to $P < 0.05$.

2.3. Electrophoretic mobility shift assay

Crude nuclear extracts were prepared from transfected C2C12 cells as described [12]. Extracts from cells treated or not with 100 nM dexamethasone for 48 h were compared. The DNA fragment corresponding to the region −77 to +43 of the α7 promoter was obtained and end-labeled by Klenow-filling as previously described [5]. The conditions of the DNA–protein binding reactions have also been described [5].

3. Results and discussion

In order to examine the possibility that glucocorticoids were activating the transcription of the α7 subunit gene, bovine chromaffin cells were cotransfected with a plasmid coding for the human glucocorticoid receptor (pRS-hGRα kindly

0014-5793/$22.00
doi:10.1016/j.febslet.2004.04.049

provided by Dr. R. Evans, San Diego, USA) and the α7 promoter-luciferase gene construct p2700α7LUC, which contains about 2700 bp of α7 promoter sequence plus 43 bp of 5′ non-coding region linked to the luciferase gene and yields the maximal promoter activity in chromaffin cells [4]. The effect of the synthetic glucocorticoid dexamethasone on α7 promoter activity was examined by measuring luciferase expression in cells lysed at different times of dexamethasone treatment. Although a slight enhancement (118% of non-treated control) of luciferase activity was already observed at 18 h after 100 nM dexamethasone treatment, maximal activation (712%) was obtained at 48 h.

To define the minimal promoter region that mediates glucocorticoid activation, chromaffin cells were also transfected with reporter constructs bearing nested 5′ deletions of the α7 promoter fused to luciferase and further treated with 100 nM dexamethasone during 48 h.Constructs bearing 1011, 665, 353, 128 and 77 bp of promoter sequence relative to the transcriptional start site mediated dexamethasone-inducible gene expression at levels comparable to the one observed with p2700α7LUC. Therefore, the smaller tested construct, p77α7LUC, contains the elements necessary for dexamethasone induction. We have previously shown that this construct defines the minimal α7 promoter region required for optimal gene expression in cultured chromaffin cells [5].

Although dexamethasone activation of the α7 promoter was always observed in chromaffin cells, results were highly variable when comparing different cell batches, ranging between two- and seven-fold activation. We think that this is a consequence of donor heterogeneity, perhaps produced by different living conditions, hormonal treatments, age, etc. For this reason, we decided to use the C2C12 cell line for a more detailed study. We have previously shown that C2C12 muscle cells express transcripts coding for the α7 subunit and that their differentiation produced an increase in the amount of α7 transcripts, which is accompanied by the activation of the α7 core promoter [13]. In C2C12 cells we first compared two different α7 promoter constructs, the ones containing 1011 and 77 bp of promoter sequence. They exhibited similar activation of their transcriptional activity (Fig. 1A), as it was previously observed in chromaffin cells. Dexamethasone stimulated reporter gene expression in a time-dependent manner (Fig. 1B). Whereas activation was not significant after 3 h, a fourfold enhancement of luciferase activity produced by construct p77α7LUC was observed at 24 h after 100 nM dexamethasone treatment. An increase of about fivefold was observed at 48 h (statistically non-significant when compared to the activation observed at 24 h). Dexamethasone also exhibited activation of reporter gene expression in a concentration-dependent manner (Fig. 1C). A significant stimulation of luciferase expression was observed at 5 nM dexamethasone. Maximum activation was observed at 5 μM, although saturation of the response was evident at 50 nM. In fact, values obtained at 50 nM and higher concentrations were not significantly different and we decided to use a concentration of 100 nM dexamethasone in further experiments. Cells non-transfected with the glucocorticoid receptor plasmid did not show dexamethasone activation of p77α7LUC, as they do not express endogenous glucocorticoid receptors, suggesting that the dexamethasone effect is specific.

No consensus binding sites for glucocorticoid receptors have been found at the α7 promoter, which is, however, activated by the immediate-early gene product Egr-1 through its binding to

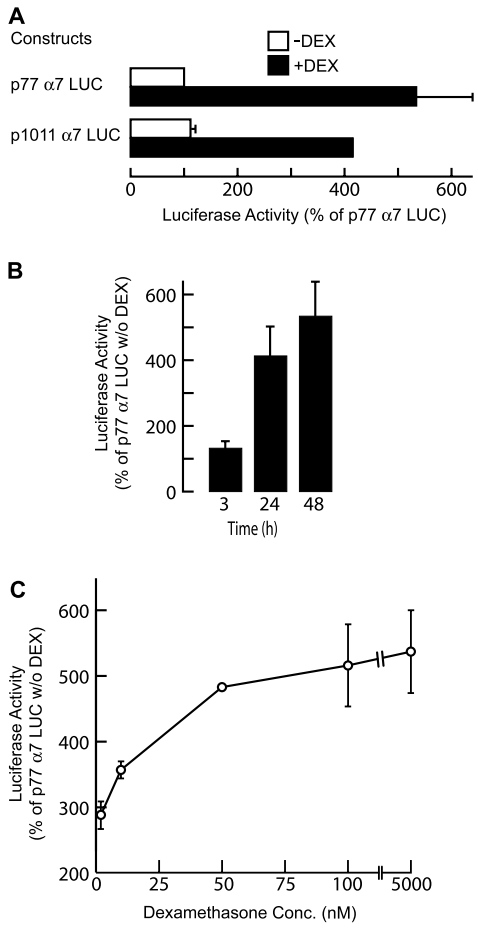

Fig. 1. Activation of the α7 subunit promoter by dexamethasone in transfected C2C12 cells. A: Cells were transfected with the α7 promoter-luciferase gene constructs p77α7LUC or p1011α7LUC plus the glucocorticoid receptor expression plasmid pRS-hGRα and treated with 100 nM dexamethasone for 48 h. Luciferase expression was determined as described in Section 2, and activity was expressed as a percentage with respect to values in cells transfected with p77α7LUC and pRS-hGRα in the same conditions, but not treated with the glucocorticoid. Data are means ± S.E. (bars) values for two individual experiments. All experimental points were performed in triplicate. Basal activities of p77α7LUC and p1011α7LUC were not significantly different. Dexamethasone activation was significant in both cases B: Activation of the α7 subunit promoter in C2C12 cells as a function of time. Cells were transfected with p77α7LUC and pRS-hGRα and treated with 100 nM dexamethasone for different intervals. Activity was expressed as a percentage with respect to values in cells cultured and transfected in the same conditions but not treated with dexamethasone. C: Activation of the α7 subunit promoter in C2C12 cells as a function of dexamethasone concentration. Cells were transfected with construct p77α7LUC and treated with different concentrations of dexamethasone for 48 h. Luciferase activity was determined and expressed as indicated in panel A. Data from panels B and C are from three or four experiments performed in triplicate.

three closely located sites in the proximal promoter region [5]. Egr-1 can be rapidly induced by various extracellular stimuli (reviewed in [14]), so we asked whether the dexamethasone induction of the α7 promoter occurs through a mechanism potentially dependent on Egr-1. Thus, we compared dexamethasone-induced luciferase activity expressed by the wild-type promoter (p77α7LUC) and by derivatives of this construct mutated at the Egr-1 binding sites and also at an USF site required for maximal transcriptional activity (see

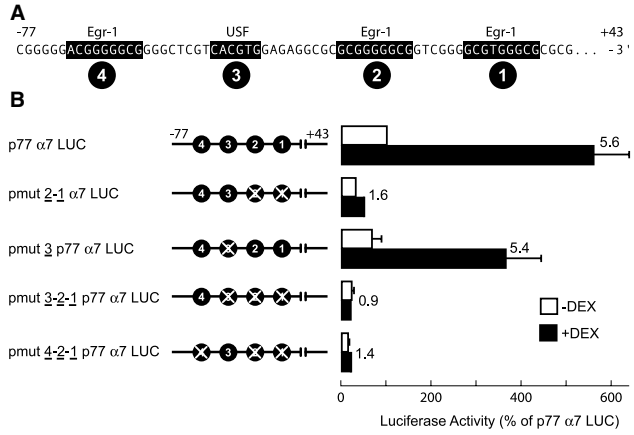

Fig. 2. (A) Egr-1 sites in the proximal promoter region of the α7 gene are involved in glucocorticoid activation. The nucleotide sequence (nucleotides −77 to −12) of the bovine α7 subunit gene promoter is shown at the top. Egr-1 and USF regulatory elements are indicated (filled boxes, numbered 1–4). Several nucleotides of each element were mutated, individually or simultaneously, in the parent construct p77α7LUC to yield mutant derivatives that were transfected in C2C12 cells together with pRS-hGRα. The name of each mutant construct indicates the element(s) that have been altered (also crossed out in the schematic diagram of panel B). Luciferase activity was determined as indicated in Fig. 1 and normalized to values obtained with the p77α7LUC construct. Open boxes indicate luciferase activity of the different constructs in untreated control cells, whereas solid boxes indicate activity in cells treated with 100 nM dexamethasone for 48 h. Numbers above the columns indicate the fold induction by dexamethasone relative to the activity of the same construct in untreated cells. Data are from two experiments performed in triplicate.

Fig. 2A for their locations). Single mutations of Egr-1 elements (boxes 1, 2 and 4 at Fig. 2A) did not have any significant effect on dexamethasone induction observed with the non-mutated construct (results not shown). However, simultaneous mutation of two of the three Egr-1 binding elements (sites 1 and 2) clearly reduced dexamethasone induction to statistically non-significant levels (Fig. 2B, pmut 2-1 α7LUC). The additional mutation of Egr-1 site 4 did not affect dexamethasone induction further (Fig. 2B, pmut 4-2-1 α7LUC). Suppression of the USF site alone did not affect dexamethasone activation (Fig. 2B, pmut 3 α7LUC), whereas the simultaneous mutation of this site and Egr-1 sites 1 and 2 totally abolished hormonal activation (Fig. 2B, pmut 3-2-1 α7LUC). These results indicate that Egr-1 sites are required for maximal induction of the α7 promoter in response to glucocorticoids.

The involvement of Egr-1 in the glucocorticoid activation of the α7 gene was further analyzed by gel mobility shift assays. Since the highest induction in the α7 promoter was obtained after 48 h of treatment, we prepared nuclear extracts from C2C12 cells transfected with the glucocorticoid receptor plasmid and treated or not with 100 nM dexamethasone for this time, and compared the pattern of complexes obtained with an α7 probe covering from −77 to +43, in the minimal promoter region. The probe showed the same pattern with nuclear extracts from treated and untreated cells (not shown). Moreover, the main complexes, due to the binding of USF and Egr-1 (see [13]), had the same intensity in both extracts, suggesting that the glucocorticoid activation of the α7 gene does not involve an increase in the amount of Egr-1 or the emergence of new complexes. A possibility would be that glucocorticoids could induce post-translational modifications of Egr-1 that might enhance its transactivation ability. For instance, phosphorylation [15], the redox state of Egr-1 [16] or the interaction with corepressors [17] might modify its DNA binding capacity. Whatever the mechanism, in the bovine adrenal gland a specific stimulation of Egr-1 function by glucocorticoids appears very plausible, since localization of adrenergic cells expressing Egr-1 and also the enzyme phenylethanolamine *N*-methyl transferase (PNMT) and α7 nAChRs is so peculiar: they are only in the region of the adrenal medulla close to the adrenal cortex and to the portal vein [4]. In this scenario, glucocorticoids arising from the cortex would diffuse into the nearby area of the adrenal medulla and then act directly on adrenergic chromaffin cells, especially because the glucocorticoid receptor has been localized only to the cytoplasm of adrenergic cells [18]. Egr-1, in turn, would activate the promoter of PNMT [19] and α7 [4,5] genes. Recently, two overlapping glucocorticoid response elements have been identified in the promoter of the rat PNMT gene, which are primarily responsible for its glucocorticoid sensitivity [20]. We could not detect such elements in a sequence of about 1500 bp of the α7 gene promoter. Moreover, the smallest construct p77α7LUC showed glucocorticoid activation, hence it can be concluded that in the case of the α7 gene, glucocorticoid activation is indirect, through the Egr-1 elements. This might also explain, at least partially, why it is necessary to transfect glucocorticoid receptor DNA into chromaffin cells to observe activation of the α7 promoter by dexamethasone: the concentration of endogenous hormone receptor may not be high enough for such an indirect effect to take place.

Finally, it is interesting to notice the central role that this transcription factor plays in the integration of signals that regulate the expression of the α7 gene. Thus, we have previously shown that the α7 promoter is activated by phorbol esters [21] and also during muscle differentiation [13]. In both cases, activation takes place through the Egr-1 sites previously mentioned. Therefore, it is reasonable to expect that other physiological stimuli that increase Egr-1 expression (see [22] for a review), such as the ones driving to neuronal stimulation [23,24], could also activate the nAChR α7 subunit gene.

Acknowledgements: This work was supported by Research Grants from the Ministry of Education (PM98-0104, DGICYT)and Science and Technology (BMC2002-00972) of Spain, and the Agencia Valenciana de Ciencia y Tecnología (CTIDIB/2002/138 and GRUPOS03/038). C.C.-S. was the recipient of a predoctoral fellowship from Generalitat Valenciana. The excellent technical assistance of Eva Martínez is also appreciated. We thank Dr. R. Evans (San Diego, USA) for kindly providing pRS-hGRα and Dr. F. Sala (Alicante, Spain) for his help in the statistical analysis.

References

[1] Lindstrom, J.M. (2003) Ann. N.Y. Acad. Sci. 998, 41–52.
[2] Hogg, R.C., Raggenbass, M. and Bertrand, D. (2003) Rev. Physiol. Biochem. Pharmacol. 147, 1–46.
[3] Fornasari, D., Battaglioli, E., Terzano, S. and Clementi, F. (1998) in: Neuronal Nicotinic Receptors: Pharmacology and Therapeutic Opportunities (Arneric, S.P. and Brioni, J.D., Eds.), pp. 25–42, Wiley-Liss, New York.
[4] Criado, M., Domínguez del Toro, E., Carrasco-Serrano, C., Smillie, F.I., Juíz, J.M., Viniegra, S. and Ballesta, J.J. (1997) J. Neurosci. 17, 6554–6564.
[5] Carrasco-Serrano, C., Campos-Caro, A., Viniegra, S., Ballesta, J.J. and Criado, M. (1998) J. Biol. Chem. 273, 20021–20028.

[6] Campos-Caro, A., Carrasco-Serrano, C., Valor, L.M., Viniegra, S., Ballesta, J.J. and Criado, M. (1999) J. Biol. Chem. 274, 4693–4701.
[7] Valor, L.M., Campos-Caro, A., Carrasco-Serrano, C., Ortiz, J.A., Ballesta, J.J. and Criado, M. (2002) J. Biol. Chem. 277, 8866–8876.
[8] Gandía, L., Casado, L.F., López, M.G. and García, A.G. (1991) Br. J. Pharmacol. 103, 1073–1078.
[9] Eberwine, J.H., Jonassen, J.A., Evinger, M.J. and Roberts, J.L. (1987) DNA 6, 483–492.
[10] Berthois, Y., Katzenellenbogen, J.A. and Katzenellenbogen, B.S. (1986) Proc. Natl. Acad. Sci. USA 83, 2496–2500.
[11] Graham, F.L. and van der Eb, A.J. (1973) Virology 52, 456–467.
[12] Schreiber, E., Matthias, P., Müller, M.M. and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419.
[13] Campos-Caro, A., Carrasco-Serrano, C., Valor, L.M., Ballesta, J.J. and Criado, M. (2001) DNA Cell Biol. 20, 657–666.
[14] Beckmann, A.M. and Wilce, P.A. (1997) Neurochem. Int. 31, 477–510.
[15] Cao, X., Mahendran, R., Guy, G.R. and Tan, Y.H. (1993) J. Biol. Chem. 268, 16949–16957.
[16] Huang, R.-P. and Adamson, E.D. (1993) DNA Cell Biol. 12, 265–273.
[17] Sevetson, B.R., Svaren, J. and Milbrandt, J. (2000) J. Biol. Chem. 275, 9749–9757.
[18] Ceccatelli, S., Dagerlind, A., Schalling, M., Wikstrom, A.-C., Okret, S., Gustafsson, J.A., Goldstein, M. and Hökfelt, T. (1989) Acta Physiol. Scand. 137, 559–560.
[19] Ebert, S.N., Balt, S.L., Hunter, J.P.B., Gashler, A., Sukhatme, V. and Wong, D.L. (1994) J. Biol. Chem. 269, 20885–20898.
[20] Tai, T.C., Claycomb, R., Her, S., Bloom, A.K. and Wong, D.L. (2002) Mol. Pharmacol. 61, 1385–1392.
[21] Carrasco-Serrano, C., Viniegra, S., Ballesta, J.J. and Criado, M. (2000) J. Neurochem. 74, 932–939.
[22] Thiel, G. and Cibelli, G.J. (2002) Cell Physiol. 193, 287–292.
[23] Al-Sarraj, A. and Thiel, G. (2002) Neurosci. Lett. 332, 111–114.
[24] Illing, R.B., Michler, S.A., Kraus, K.S. and Laszig, R. (2002) Exp. Neurol. 175, 226–244.