Differential Expression of α -Bungarotoxin-Sensitive Neuronal Nicotinic Receptors in Adrenergic Chromaffin Cells: A Role for Transcription Factor Egr-1

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Adrenomedullary chromaffin cells express at least two subtypes of acetylcholine nicotinic receptors, which differ in their sensitivity to the snake toxin α -bungarotoxin. One subtype is involved in the activation step of the catecholamine secretion process and is not blocked by the toxin. The other is α -bungarotoxin-sensitive, and its functional role has not yet been defined. The $\alpha 7$ subunit is a component of this subtype. Autoradiography of bovine adrenal gland slices with α -bungarotoxin indicates that these receptors are restricted to medullary areas adjacent to the adrenal cortex and colocalize with the enzyme phenylethanolamine *N*-methyl transferase (PNMT), which confers the adrenergic phenotype to chromaffin cells. Transcripts corresponding to the $\alpha 7$ subunit also are localized exclusively to adrenergic cells. To identify possible transcriptional regulatory elements of the $\alpha 7$ subunit gene involved in the restricted expression of nicotinic receptors, we iso-

lated and characterized its 5' flanking region, revealing putative binding sites for the immediate early gene transcription factor Egr-1, which is known to activate PNMT expression. In reporter gene transfection experiments, Egr-1 increased $\alpha 7$ promoter activity by up to sevenfold. Activation was abolished when the most promoter-proximal of the Egr-1 sites was mutated, whereas modification of a close upstream site produced a partial decrease of the Egr-1 response. Because Egr-1 was found to be expressed exclusively in adrenergic cells, we suggest that this transcription factor may be part of a common mechanism involved in the induction of the adrenergic phenotype and the differential expression of α -bungarotoxin-sensitive nicotinic receptors in the adrenal gland.

Key words: ACh receptors; α -bungarotoxin; adrenergic; chromaffin cell; $\alpha 7$ subunit; Egr-1

Neuronal nicotinic acetylcholine receptors (nAChR) sensitive to α -bungarotoxin (α -Bgt) are widely expressed in the central and peripheral nervous systems (for review, see Sargent, 1993; McGehee and Role, 1995). The molecular cloning of nAChR subunits that assemble into channels activated by cholinergic agonists and are blocked by α -Bgt (termed $\alpha 7$, $\alpha 8$, and $\alpha 9$; Couturier et al., 1990; Schoepfer et al., 1990; Elgoyhen et al., 1994) has allowed structural and functional studies of these nAChR subtypes. Although α -Bgt-sensitive nAChRs on neurons function as ligand-gated ion channels (Alkondon and Albuquerque, 1993; Zhang et al., 1994), their physiological role *in situ* have not yet been elucidated fully. It has been suggested that they could enhance fast excitatory transmission via a presynaptic mechanism (McGehee et al., 1995). In addition, they could have a growth or trophic

role, because there is evidence that these receptors are involved in the guidance of nerve fibers (Pugh and Berg, 1994), inhibition of neurite extension in cultured cells (Chan and Quik, 1993), regulation of neurotrophic growth factor expression in rat hippocampus (Freedman et al., 1993), motoneuronal death (Hory-Lee and Frank, 1995), and modulation of proliferative responses in lung tumor cells (Codignola et al., 1994; Quik et al., 1994). Recently, postsynaptic responses for α -Bgt-sensitive nAChRs on neurons have been detected (Zhang et al., 1996).

We have shown previously that the α -Bgt-sensitive nAChRs from the chromaffin cells of the adrenal medulla contain an $\alpha 7$ subunit, homologous to those previously cloned from other species (García-Guzmán et al., 1995). In the adrenal gland, acetylcholine released on stimulation of the splanchnic nerve activates nAChRs in chromaffin cells and triggers catecholamine secretion, but, interestingly, this functional response is not blocked by α -Bgt. This suggests the involvement of other nAChRs in the activation of exocytosis and would imply a segregation of functions for different nAChRs subtypes in the same cell. Alternatively, different nAChRs with distinct functions might be expressed in specific chromaffin cell subsets. Given any of these cases, complex molecular mechanisms may underlie nAChR subunit expression, which in turn may influence the physiological characteristics of a cellular group or a neuronal circuit. To investigate these mechanisms, we studied the expression of α -Bgt-sensitive nAChRs in the adrenal gland of several mammals, consistently finding that they were present only in adrenergic cells. This heterogeneous distribution is attributable, at the very least, to restricted transcription of the $\alpha 7$ subunit gene in this

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chromaffin cell subset. Furthermore, we have identified transcription factor Egr-1 (Cao et al., 1990), which also is restricted to adrenergic cells, as the candidate potentially involved in this differential expression.

MATERIALS AND METHODS

α -Bgt autoradiography. The procedure followed for [125 I] α -Bgt binding has been described previously (Clarke et al., 1985). Cryostat sections (15–20 μ m) were incubated with 5 nM [125 I] α -Bgt for 2 hr at room temperature. After nonbound toxin was washed, sections were dried and stored in cassettes for 48 hr at 4°C with Kodak X-OMAT x-ray film.

Immunohistochemistry. Rat tissue was perfused and processed as previously described (Dominguez del Toro et al., 1994). Cat and bovine adrenal glands were fixed by immersion in 4% paraformaldehyde in 75 mM sodium phosphate buffer, pH 7.35, and then processed in the same manner as the rat tissue. Cryostat sections (25–30 μ m) were free-floated in continuously agitated solutions at 4°C. Phenylethanolamine *N*-methyl transferase (PNMT; EC 2.1.1.28) was immunolabeled with a rabbit antiserum against bovine PNMT (Eugene Tech, Ridgefield Park, NJ), diluted 1:1000. Egr-1 was localized with an affinity-purified polyclonal antibody (588) raised in rabbit against a peptide that corresponds to the 14 C-terminal residues of mouse Egr-1 p82 (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody was used at a 1:1000 dilution.

In situ hybridization. Sense and antisense digoxigenin-labeled riboprobes were synthesized with SP6 polymerase from the corresponding linearized template (pSPT18) with a Boehringer Mannheim (Barcelona, Spain) RNA labeling kit. Probes were directed to a region of the M3–M4 cytoplasmic loop unique to the $\alpha 7$ subunit [amino acids 315–382; see García-Guzmán et al. (1995) for the bovine $\alpha 7$ sequence and Séguéla et al. (1993) for its rat counterpart]. Frozen adrenal gland tissue was cut in 10 μ m sections, mounted on silanated slides, fixed in 4% formaldehyde (5 min at room temperature), deproteinized (10 min in 0.2 M HCl at room temperature), and dehydrated in ethanol. Hybridization was for 16 hr at 45°C in a buffer containing 50% formamide, 5 $\times$ SSC, 1 $\times$ Denhardt's solution, 10% dextran sulfate, 0.2% SDS, 10 mM dithiothreitol, 250 μ g/ml herring sperm DNA, and 250 μ g/ml yeast tRNA. Digoxigenin-labeled probes were diluted (in the range from 1:25 to 1:400) with hybridization buffer to obtain a suitable and equivalent concentration of the different probes, as verified by dot blot assays. After hybridization, sections were washed for 2 $\times$ 30 min in 0.1 $\times$ SSC at 60°C. Visualization of the probe was performed with anti-digoxigenin antiserum, as indicated by the manufacturer (Boehringer Mannheim).

Reverse transcription-PCR (RT-PCR) analysis. Total RNA was extracted by guanidinium isothiocyanate and acid phenol extraction (Promega, Barcelona, Spain) from adrenomedullary tissue dissected from areas near to or far from the adrenal cortex. $\alpha 7$ subunit transcripts were detected by a combined RT-PCR assay (Singer-Sam et al., 1990). Briefly, samples of total RNA (1.2 μ g) in a final volume of 30 μ l were reverse-transcribed with 1.25 U avian myeloblastosis virus (AMV) reverse transcriptase (10 min at 50°C); then PCR was performed for 30 cycles (1 min at 94°C, 2 min at 58°C, and 2.5 min at 72°C). The following primers were chosen: sense, 5'-CCAGGGCTGGTTTCCGAGA-3'; antisense, 5'-TGTTCAAGTCATTTGTAGCCA-3'. The PCR fragment had 281 base pairs (bp) from the 5' noncoding region to amino acid 80; the amplified DNA fragment expanded over three introns to rule out the possibility of amplifying a potential contamination of genomic DNA. In fact, RT-PCR performed in the absence of AMV reverse transcriptase (see Fig. 2B, inset) did not yield any fragment.

Isolation and analysis of the 5' flanking sequence of the $\alpha 7$ subunit. A cDNA probe containing 87 bp of the 3' end of exon 1 and 105 bp of the 5' end of exon 2 was used to screen a bovine genomic library in EMBL-3 SP6/T7 (Clontech, Heidelberg, Germany), as previously described (García-Guzmán et al., 1995). Several overlapping bacteriophage clones were purified and characterized. Clone λ a7-11, which contained ~17 kb of bovine genomic sequence, including exons 1 and 2, and ~7 kb of 5' flanking sequence, was characterized further.

5' RACE analysis of 5' mRNA ends. The rapid amplification of cDNA ends (RACE) was performed as described (García-Guzmán et al., 1995). The first-strand cDNA, after reverse transcription of bovine adrenal medulla mRNA, was polyadenylated and amplified by two PCR rounds. An oligo dT was the sense primer, and the two antisense primers were 5'-TGTTCAAGTCATTTGTAGCCA-3' (first round) and 5'-ATGTTGGTGGTCAACAC CTG-3' (second round), coding for amino acids 74–80 and 67–73, respectively, of the $\alpha 7$ sequence. The resultant

DNA was cloned into pBluescript (Stratagene, Heidelberg, Germany), and the sequence of the inserts was determined and compared with genomic DNA to establish the 5' end of $\alpha 7$ mRNA.

RNAse protection. A 305 bp *SacII*–*BamHI* fragment of the $\alpha 7$ gene that included 126 bp 5' and 46 bp 3' to exon 1 was subcloned into pBluescript. After linearization of the plasmid with *EcoRI*, a probe of 397 nucleotides was synthesized with T7 polymerase and [α - 32 P]CTP. For calibration, several other RNAs of known size were synthesized also. RNAse protection experiments were performed with the RNAse Protection Kit (Boehringer Mannheim). Protected fragments were separated on a 6% acrylamide/urea gel.

Plasmid constructions. All $\alpha 7$ promoter–LUC gene fusions were made in the pGL2-Basic vector (Promega), introducing in its polylinker upstream of the luciferase gene the suitable $\alpha 7$ promoter fragments. These fragments were generated with restriction enzymes (as indicated in Fig. 4) and cloned directly into pGL2-Basic or subcloned first in pBluescript and then transferred to pGL2-Basic. The vector pGL2-Control, which express the luciferase gene under the regulation of the SV40 promoter and enhancer sequences, was used to check luciferase activity.

Deletion analysis of the most promoter-proximal region was performed by partially digesting an *ApaI*–*HindIII* fragment (from –339 to +41 in the $\alpha 7$ sequence) with *Bst*UI, which leaves blunt ends at GCGC sequences, and subcloning the partial digests into pBluescript vector cut with *HincII*–*HindIII*. Sequence analysis allowed selection of the appropriate fragments, which were cloned into pGL2-Basic and transfected further.

For site-directed mutagenesis of the putative Egr-1 sites at the –38 to –15 region of the $\alpha 7$ promoter (see Fig. 6), the following mutagenic primers were synthesized: mut1, 5'-CaaaaaCGGTTCGGGGCGTGGGCG-3'; mut2, 5'-CGGGGGCGGTaaaaaCGTGGGCGCGCGCTGGG-3'; mut3, 5'-CGGGGGCGGTTCGGGGaaaaaGCGCGCGCTGGGCTTTTAA-3' (the introduced mutations are indicated in lower case letters). PCR (25 cycles of 94°C for 10 sec, 62°C for 30 sec, and 68°C for 45 sec, using the Expand kit from Boehringer Mannheim) was performed with these oligonucleotides and an antisense primer (5'-CTTTATGTTTTTGGCGTCTTCC-3') that anneals to the pGL2-Basic vector downstream of the site of transcription initiation. PCR products were cloned into pBluescript, sequenced, and transferred further to pGL2-Basic.

Cell culture and reporter assays. Neuro-2a mouse neuroblastoma cells were cultured in 90% Eagle's minimal essential medium (EMEM), 10% fetal calf serum (FCS), and 2 mM glutamine. SH-SY5Y human neuroblastoma cells were grown in a 1:1 mixture of Ham's F12 medium and EMEM containing 1% nonessential amino acids and 10% FCS. Chromaffin cells were isolated from bovine adrenal glands as described (Gandia et al., 1991) and cultured in 90% DMEM, 10% FCS, and 10 μ M cytosine arabinoside to prevent fibroblast proliferation.

Plasmids were purified by Wizard maxipreps (Promega) and then banded in CsCl. All cell types were transfected by the calcium phosphate procedure (Graham and van der Eb, 1973). Cells ($1\text{--}3 \times 10^5$) on 3.5 cm plates were incubated with 3 μ g of pGL2 vector or an equivalent amount (in molar terms) of the different constructs derived from this vector and with 3 μ g of β -galactosidase expression vector pCH110 (Pharmacia, Uppsala, Sweden) as a control of transfection efficiency. Two micrograms of Egr-1 expression plasmid (pCMVEgr-1) (Gupta et al., 1991) or of its mutated inactive form (pCMVEgr-1 Δ 331–374) also were present in the transfection mixture when the effect of this transcription factor was studied. Cells were harvested after 48 hr and lysed with reporter lysis buffer (Promega). Then β -galactosidase and luciferase were determined in the lysates with the corresponding assay systems (Promega). Luciferase activity was normalized to values obtained with the pGL2-Control vector in the same cell type. Relative light units obtained with pGL2-Control in the different cell types are as follows (mean of three individual experiments and corrected for transfection efficiency): chromaffin, 273; neuro-2a, 371; SHSY-5Y, 801.

Electrophoretic mobility shift assay. Crude nuclear extracts were prepared from chromaffin cells, as described by Schreiber et al. (1989). DNA fragments corresponding to the region –38 to +41, mutated or not at the putative Egr-1 sites, were obtained by digesting pBluescript subclones with *EcoRI*–*HindIII* and end-labeled by Klenow filling with [α - 32 P]dATP. The DNA–protein binding reaction volumes were 20 μ l containing (in mM): 10 Tris, pH 7.5, 50 NaCl, 1 EDTA, and 1 dithiothreitol with 10% glycerol, 5 μ g of bovine serum albumin, 2 μ g of poly(dA-dT)–(dA-dT) (Pharmacia), 5 μ g of nuclear extract protein, and 20,000 cpm of 32 P-labeled probe. Reactions were incubated for 10 min at room

Figure 1. Localization of α -Bgt binding sites and PNMT in the adrenal gland. *A–F*, Autoradiography of [125 I] α -Bgt binding to adrenal gland slices. *A*, *B*, Bovine; *C*, *D*, rat; *E*, *F*, cat. Total binding is observed in *A*, *C*, and *E*, whereas nonspecific binding, determined by incubating in the presence of 0.1 mM nicotine, is observed in *B*, *D*, and *F*. *G–I*, Immunolocalization of PNMT in the bovine (*G*), rat (*H*), and cat (*I*) adrenal glands. Scale bars: 1000 μ m in *A–G*; 500 μ m in *H–I*. Note that sizes are not comparable between species, because sections were not obtained from analogous locations in each gland.

temperature, labeled probe was added, and the incubation was continued for an additional 20 min. For competition studies the nuclear extract was incubated with the competing probe before the labeled probe during 20 min. Competition was performed either with the same purified fragments or with double-stranded oligonucleotides containing consensus sites for Egr-1 (Santa Cruz Biotechnology), Sp1, or cAMP response element binding proteins (CREB; Promega). Supershift assays were performed by incubating with 2 μ l of anti-Egr-1 antibody (588X from Santa Cruz Biotechnology) or rabbit IgG (Sigma, Madrid, Spain) after probe addition, and the incubation continued for an additional 30 min at room temperature.

In some experiments purified Egr-1 protein fused to glutathione *S*-transferase (GST) was used. The expression vector for the Egr-1/GST fusion protein was kindly provided by Dr. X. Cao (National University of Singapore), and the protein was expressed in bacteria and purified as described by Jain et al. (1996). The mutated inactive form of Egr-1 (Egr-1 Δ 331–374) fused to GST also was cloned in the same expression

vector (pGEX-KG; Guan and Dixon, 1991) and used as a negative control.

RESULTS

α -Bgt binding sites and $\alpha 7$ subunit mRNA colocalize in chromaffin adrenergic cells

Autoradiography of adrenal gland slices labeled with [125 I] α -Bgt was performed to localize α -Bgt-sensitive nAChRs in chromaffin cells (Fig. 1). Three mammalian species were tested: cow, cat, and rat. The bovine adrenal medulla showed a characteristic labeling: the highest α -Bgt levels were found in the areas close to the adrenal cortex, at the periphery, and around the portal vein at the center (Fig. 1*A*). Labeling of the rat adrenal medulla was almost complete (Fig. 1*C*), whereas the cat showed a patchy distribution

Figure 2. Localization of $\alpha 7$ transcripts in the adrenal gland. *In situ* hybridization of $\alpha 7$ transcripts in the bovine (*A–C*) and rat (*D, E*) adrenal glands. *A*, Hybridization with bovine $\alpha 7$ antisense probe. *B*, Hybridization with bovine $\alpha 7$ sense probe. *C*, Detail of *A, B*. *Inset*, Reverse transcription and further PCR amplification of $\alpha 7$ subunit mRNA isolated from sites close (*Me*) or far (*Mi*) from the adrenal cortex (*Co*); + and – indicate reaction performed in the presence or absence of reverse transcriptase, respectively. *D*, Hybridization with rat $\alpha 7$ antisense probe; *Co*, adrenal cortex; *M*, adrenal medulla. *E*, Detail of *D*. Scale bars: 300 μ m in *A, B*; 100 μ m in *D*; 25 μ m in *C, E*.

of binding sites, which was difficult to categorize (Fig. 1*E*). In all cases labeling was abolished by preincubation with nicotine (Fig. 1*B, D, F*). The fact that the adrenergic cell-rich rat adrenal medulla was labeled almost totally by α -Bgt, whereas its bovine counterpart, in which only ~50% of cells are adrenergic, was partially labeled, suggested that α -Bgt-sensitive nAChRs were differentially expressed in adrenergic cells. To verify this, we performed immunolocalization of PNMT, the enzyme converting norepinephrine to epinephrine and, therefore, conferring the adrenergic phenotype. PNMT was present only in the bovine chromaffin cells close to the cortex or the portal vein (Fig. 1*G*), whereas most rat chromaffin cells were labeled (Fig. 1*H*). PNMT immunostaining in the cat gland had a patchy distribution (Fig. 1*I*). Hence, PNMT localization for all three mammals was shown to be very similar to the one observed for α -Bgt binding sites.

Because the $\alpha 7$ subunit present in bovine chromaffin cells is

probably a major component of α -Bgt-sensitive nAChRs (García-Guzmán et al., 1995), it seemed possible that the restricted expression of α -Bgt-sensitive nAChRs was regulated by the transcriptional activity of the $\alpha 7$ subunit gene. This hypothesis was supported by a qualitative assay, the RT-PCR analysis of $\alpha 7$ mRNA levels in bovine adrenomedullary tissue (Fig. 2*B, inset*) dissected from places near to (*Me*) or far (*Mi*) from the adrenal cortex, i.e., where α -Bgt-sensitive nAChRs were in high or low abundance, respectively. This experiment demonstrates that $\alpha 7$ mRNA was more abundant in the region of the medulla close to the cortex. This evidence was confirmed by *in situ* hybridization of bovine $\alpha 7$ transcripts: as shown by an antisense probe, they were present in the area adjacent to the adrenal cortex (Fig. 2*A, Me*; 2*C*), where adrenergic cells had been localized previously. The same probe in the sense orientation gave no labeling at all (Fig. 2*B*). On the other hand, most of the rat adrenal medulla was

-1489 GAATTCACCTA AAGAAGTTGT CAAGTCAAGA GGTTCCTTT GTTAGATTTT
 TGCTTACTGA TTCACTCTAG TTATGTGTCT ATTGAGGTTT TCTATTTCTT
 -1389 TATGATTATG TCCTGCTGTG TCAAGTTTCT AGCGACTTGT CCATTTCATG
 TAGGTTATCC AATTCACTGG CATACGGTCA CAGTACTCTC TTATGATCAT
 -1289 TTTTGTTCCT ACAGAAACAG TTCTACTGTC CCAACTTTCA TTTCTGATTT
 AATAATTTAA ATCTTCTCTC TTTTAAAGTA TTTCTGGCTA AAGGTTTCTC
 -1189 AATTTTCATTG GTCTTTGCAA TAAGCCAATT TTTCTCTATT GCTTTTCTAT
 TCTCTATTTA TTCCCACT AATCTTATTT TTTTCCTTCC GTTAGCTCTG
 -1089 GGTTCAGTTT GCGGTTTTTT TTCCTAGTTC ATTAAGTTGT ACAGTTGGGT
 TGTGTCTTGG AGATCCTAAC ATCTGGTGA GCTCAGATTC TCTGGGGCCT
 -989 AAGGAAGCTG AGCTGCAGAC CACCTAACTG AGTGCAGGGA CCCCTCGGCT
 CTGAGCCTTC TGAAACATGC TCGCTGAGGG CAAAAGGGAT CGAGTTTACC
 -889 CACCACCTTT GAAGGCTGTC CTCAGCATTC TCTCTTCCAT CCTCTCCACG
 AATCAGGCCCC CCTCTTCAAC ATCTGGGATA TGATTAAGCC CCTGACCTCT
 -789 TGATTGGTGT CTGCTCAGAA GCTTCCCTTG GCAATGGCCT TTTGTGGCT
 AAAAAAATAA AAAAAAATAA AGCCTAGATC ACACCAACAA CTTTTCCTCG
 -689 TCCTCTGGCA AGGAGCTGAG AGTTAACTTT TCCCCCAA CTTTTCGAAT
 TGCTTTTAAA AGAGAACTAA ACATAAAGAA GATATACACT GGAATTTCCA
 -589 TCACACACTT TTGCAGCGTC TCCTTGAATA ATGCTTCCCT GGCAGATTTC
 GGCTCTCCTC TGGACACCTC CAGAACCATC TGAGAATCAG GCCGTGATC
 -489 CCCAGCAAAAT GGTGCTGGA AGAATATCCA TCATGGCTCG CGGGGTAGG
 AGGGACCTGT CTGATGCTG TCTCTCTCC TTTGGGTGGA CTCGGAACCC
 -389 TGTGAGAGCC TGGTTCTGG GCTTTCGCTC CAGGAGCTGG CGTGGAGACA
 GGCCCTCGGG GAGCCAGATT CAGAAGGCTC CCACCGTCTC CCCAGCGACC
 -289 CGGCGTGGCT TCTGTCTAAG AGTGGCGGCC AGCGGACCGG GGACAAAGGA
 CAGAAGAGAC AGAGCCTGGC CATTGACAGC CTCGGGACAG CGGGCAGCTC
 -189 CGGCGGAGAC GAGCGCTGGG AGGTGGCGCG CCTCTCTGG ACTTCTGGGC
 TACTGTCTCC CCCGCGGCCG CCTACCGCTG GGGTCTGGGG GGGGCTGAG
 -89 GGGGAGGGGG GCGGGGGGAC GCGGGGGG GCGGGGGG GCGAGGCGCG
 GCGGGGGG TCGGGGGGCT GCGGGGGGCT GGGCTTTTAA AAGGCGCGCC
 +12 AGCCGAGCGG GGAGAGGCGG CTTTGC3GC CCAGGCGCAG GCCAGGCGCT
 GTTTCGAGA CGCGCAGCTC CGGCCCCCA ACATGCGGG CTCGTTGTGC
 M R G S L C
 +112 CTGGCGCTGG CAGCGTCGAT CTTGCACGCT AAAGCCGCGC ATCTCTCCAC
 L A L A A S I L H
 CCATCACTCT TGCTCCGTGG GATCCAGGCG GGCTCGGAGC GCCCGCGCG
 +212 GGGACGGGAT TGGGATTCG CGCCTGTGTC TAAGGGCAGC GGGAGGCGCT
 GCGGTGGAAG GCGAGCCCGG CACTTCTGGG CTGCGATGCT TGGGCGGCTG
 +312 GGGGTGCGGG CAGGGGGCGC ATTCCGCGCG GTTTCGCTGA GCAGCCGCGC
 CGCTCTCCTC TTCCTCTCCC CGAAGTGTCC CTGCAAGGCG AGTTCCAGAG
 V S L Q G E F Q R
 +412 GAAGCTCTAC AAAGACCTGG TGAAGAACTA CAACCCCTTG GAGAGGCGCG
 K L Y K D L V K N Y N P L B R P
 TGGCCACGGA CTCGCTGCGG CTCACAGTCT ACTTCTCCCT GAGCCTCCTG
 V A N D S L P L T V Y F S L S L L
 +512 CAG...3'
 Q

Figure 3. The 5' region of the bovine $\alpha 7$ subunit gene. A 2 kb *EcoRI*–*PstI* fragment of genomic clone $\lambda\alpha 7$ –11 carrying exons 1 and 2 (with the protein sequence indicated underneath in *italics*), the intron in between (within arrowheads, $\blacktriangledown$), and 1500 bp of 5' flanking sequence, was analyzed. The translation start codon is underlined, and the major transcription initiation site (+1) is denoted by the arrow. Perfect matches to transcription factors Sp1 (solid lines rectangle), Egr-1 (long dashed lines rectangle), Myc-Max (short dashed lines rectangle), and E-box (hatched lines rectangle) are indicated.

labeled with an antisense rat $\alpha 7$ probe (Fig. 2D,E), as in the case of PNMT immunolabeling. Thus transcriptional regulation of the $\alpha 7$ subunit gene seems to be involved in the differential expression of α -Bgt-sensitive nAChRs in adrenergic cells.

Functional analysis of the $\alpha 7$ subunit promoter

To examine the requirements for $\alpha 7$ subunit transcription, we isolated and analyzed its promoter region. A bovine genomic library was screened, and several overlapping clones were isolated. Their mapping showed the presence of the $\alpha 7$ 5' untranslated region (first exon) and further upstream sequences, as well as the second and third exons; the latter were separated by an intron of ~30 kb. Subcloning and sequencing of an isolated *EcoRI*–*PstI* fragment from one of the clones (Fig. 3) and further comparison of this sequence to a database of binding sequences of known transcription factors revealed the main features of the promoter/regulatory region of the $\alpha 7$ gene: lack of a TATA box and the presence of perfect matches to three potential Sp1 sites, one Egr-1 site, one Myc-Max site, and one E-box (Fig. 3), all of

Figure 4. Determination of the $\alpha 7$ subunit gene transcription initiation site. *A*, Sequence of the 5' end of the RACE product obtained by reverse transcription and PCR amplification of $\alpha 7$ subunit mRNA. *B*, Mapping 5' ends of $\alpha 7$ subunit mRNA by RNase protection by using an $\alpha 7$ probe that included exon 1 and its 5' and 3' flanking regions. *T*, Undigested probe, 397 bases; *N*, probe digested in the presence of 5 μ g of yeast tRNA; *P*, protected fragments using 5 μ g of bovine adrenal medulla poly(A⁺) mRNA. The size of several RNA fragments used for calibration of the gel is indicated at the left.

them concentrated into ~360 bases located 5' to transcription initiation site. Additional sites with one mismatch, regarding perfect consensus sequences, also were observed.

The 5' end of $\alpha 7$ mRNA was mapped by 5' RACE and RNase protection analyses (Fig. 4), because primer extension experiments produced inconsistent results. In 5' RACE only one DNA fragment was detected, the 5' end sequence of which is shown in Figure 4A. This would map transcription initiation to a stretch of 5Ts and 3As (see black arrow in Fig. 3). RNase protection analyses with a 397 residue antisense riboprobe complementary to +180 to –125 and poly(A⁺) RNA from bovine adrenal medulla yielded two main protected fragments of ~139 and 137 bases (Fig. 4B). The major one mapped to the first adenosine detected in the 5' RACE fragment and was located downstream of a GC-rich region. Therefore, this tentatively was considered the main transcription initiation site (position +1, black arrow in Fig. 3), although other initiation sites may exist, because this is a typical feature of promoters without TATA boxes.

Figure 5. Analysis of $\alpha 7$ gene promoter activity. The indicated cell types were transfected with each of the plasmids (named $p\alpha 7$ LUC, with the number of promoter base pairs included in the construct) containing the luciferase reporter under the control of the different fragments of the $\alpha 7$ subunit promoter and pCH110/ β -galactosidase as a transfection efficiency control. All experimental points were run in triplicate. The mean $\pm$ SE (error bars) are given for three individual experiments. The restriction enzymes used to clone the different fragments also are shown in the scheme.

The transcriptional activity of the $\alpha 7$ promoter was examined with a series of constructs made by fusing the $\alpha 7$ 5' flanking region to a luciferase reporter gene. The nested set of $\alpha 7$ expression plasmids, containing 5900–128 bp of $\alpha 7$ promoter sequence plus 41 bp of 5' noncoding region was transfected into a variety of cell types. Two of them, bovine chromaffin cells and the human neuroblastoma SH-SY5Y (which is of sympathetic adrenergic ganglion origin; see Biedler et al., 1973), endogenously express α -Bgt-sensitive nAChRs (Lukas et al., 1993), whereas the other, mouse neuroblastoma neuro-2a, does not (our unpublished results). All reporter constructs were found to be active in chromaffin and SH-SY5Y cells, showing much reduced activity in neuro-2a cells (Fig. 5). The smallest $\alpha 7$ promoter fragment tested, 128 bp, was sufficient to drive transcription, although larger fragments increased activity, especially in chromaffin cells. No luciferase expression was detected in any of the cell types tested when no promoter segments were present upstream of the luciferase gene (pGL2-Basic vector; data not shown).

The similar pattern of expression in the bovine adrenal gland of the enzyme PNMT and the $\alpha 7$ subunit suggested that they share common transcriptional regulatory elements. This idea was reinforced when the promoter regions were compared: both were

Figure 6. Egr-1 induction of luciferase expression from the $\alpha 7$ subunit promoter. Neuro-2a and bovine chromaffin cells were cotransfected with $p\alpha 7$ LUC plasmids and Egr-1 expression vectors encoding a nonfunctional (+Mut. Egr-1) or a functional (+Egr-1) Egr-1 protein. Numbers above the columns indicate the fold induction by Egr-1. Data are expressed as in Figure 5.

markedly G/C rich and contained several consensus sequences for transcription factors Sp1 and Egr-1. Moreover, it is known that these factors regulate PNMT expression in PC12 cells (Ebert et al., 1994; Ebert and Wong, 1995). To determine the possible regulation of $\alpha 7$ expression by Egr-1, we cotransfected different $\alpha 7$ -luciferase constructs into neuro-2a and chromaffin cells with either a construct allowing strong expression of Egr-1 (pCMVEgr-1) or a control yielding a truncated, nonfunctional Egr-1 protein (pCMVEgr-1 Δ 331–374) (Fig. 6). In neuro-2a cells the functional Egr-1 increased the luciferase activity up to fourfold with respect to that observed with the truncated Egr-1. In chromaffin cells activation was between three- and fivefold, depending on the construct used.

In both cell types activation already was observed with the smallest construct, which contains 128 bp of $\alpha 7$ promoter region. Several potential binding sites for Egr-1 are found in this region, although only one, located 31 bp 5' to the transcription initiation site (+1) of the $\alpha 7$ gene, comprises a perfect match to the Egr-1 consensus binding element (GCGGGGGCG) (Gashler and Sukhatme, 1995). To demonstrate a possible direct interaction of this transcription factor with the $\alpha 7$ gene, we performed several deletions, studying in each case the activation produced by Egr-1 expression on transfection into chromaffin cells (Fig. 7A). Thus, removal of nucleotides (nt) –128 to –78 did not affect Egr-1 activation (p77 $\alpha 7$ LUC, Fig. 7A), which was even larger than with previous constructs. The promoter basal activity was similar to that supported by p128 $\alpha 7$ LUC. Further removal of nt –77 to –39 (p38 $\alpha 7$ LUC, Fig. 7A) resulted in $\sim$ 30% decrease in promoter activity (relative to p77 $\alpha 7$ LUC). However, Egr-1 was able to activate this shortened construct to a similar extent. Additional removal of nt –38 to –16 (p15 $\alpha 7$ LUC, Fig. 7A) drastically reduced promoter activity ($\sim$ 20% of p77 $\alpha 7$ LUC), which was not modified by the presence of Egr-1. A construct lacking the site of transcription initiation (p+7 $\alpha 7$ LUC, Fig. 7) was used to demonstrate further that downstream sequences as well as vector sequences were not involved in the observed effects. Therefore, these experiments indicate that the region between nt –38 to

Figure 7. Two sites in the proximal promoter region of the $\alpha 7$ gene are involved in Egr-1 activation of transcription initiation. *A*, Deletion constructs (from p77 $\alpha 7$ LUC to p+7 $\alpha 7$ LUC) were obtained, using *Bst*UI restriction enzyme, as described in Materials and Methods. The $\alpha 7$ promoter sequences contained between the constructs p38 $\alpha 7$ LUC and p15 $\alpha 7$ LUC were mutated by PCR (*mut1*–*mut3*). Each construct was cotransfected with Egr-1 expression vectors into chromaffin cells, and its activity was measured. Numbers above the columns indicate the fold induction by Egr-1. Luciferase activity was normalized to values obtained with the p77 $\alpha 7$ LUC construct. Data are expressed as in Figure 5. *B*, The region between -38 and -15 contains several putative binding sites for Egr-1 (boxed in p38). Five nucleotides of each potential element were mutated as indicated in *mut1*, *mut2*, and *mut3* to yield constructs analyzed in transfection (p38 $\alpha 7$ LUC*mut1*–*mut3* in *A*) and bandshift (Figs. 8, 9) experiments.

-16, although probably lacking important elements for basal activity, can support Egr-1 activation. This region contains several potential sites for Egr-1 (boxed and termed 1, 2, and 3 in Fig. 7*B*), which were mutated in an attempt to identify the area of interaction between this factor and the $\alpha 7$ gene. When site 1, starting at the 5' end, was abolished (p38 $\alpha 7$ LUC*mut1*, Fig. 7*A*), the activation produced by Egr-1 decreased with respect to that observed with the wild construct but was still significant. This site also was modified in p128 $\alpha 7$ LUC by mutating the area at -37 to -39 from GCG to CAT, yielding a similar degree of Egr-1 activation to the one observed for p38 $\alpha 7$ LUC*mut1* (data not shown). Mutation of site 2 (p38 $\alpha 7$ LUC*mut2*, Fig. 7*A*) did not produce any decline on Egr-1 activation. Finally, mutation of site 3 (p38 $\alpha 7$ LUC*mut3*, Fig. 7*A*) totally abolished the effect of Egr-1. Therefore, two areas (at -38 to -31 and -24 to -16) seem to be responsible for the effect of Egr-1, emerging as more important the most proximal to the initiation start site (site 3, Fig. 7*B*). In addition, it is important to note that the three mutant constructs showed reduced basal promoter activity, indicating that the whole region is involved in providing essential elements for transcriptional activity.

To examine further the binding sites of Egr-1 detected in the

-38 to -16 region of the $\alpha 7$ promoter region, we performed gel mobility shift assays. DNA fragments carrying the wild-type -38/+41 promoter region and the corresponding mutants (*mut1*, *mut2*, and *mut3*, Fig. 7*B*) were labeled and used either with nuclear extracts from chromaffin cells or with purified Egr-1 protein. A prominent retarded band was observed when Egr-1 was used either with the wild-type DNA fragment or with *mut1* or *mut2* (Fig. 8*A*, lanes 2, 5, and 8, respectively, filled dot). The same band also was obtained with *mut3* but with much less intensity (Fig. 8*A*, lane 11). Because a mutant, inactive Egr-1 protein was unable to bind any of the DNA fragments (Fig. 8*A*, lanes 3, 6, 9, and 12), we deduced that Egr-1 was binding specifically to the DNA probes. Moreover, the fact that *mut3* showed very reduced capacity to bind Egr-1 confirms the significance, with respect to Egr-1 activation, of the Egr-1 binding site most proximal to the transcription initiation site (site 3). When nuclear extracts were used, several retarded bands were observed (Fig. 8*B*). One of them (indicated by a dot) was retarded further by an anti-Egr-1 antibody (arrowhead Fig. 8*B*, lane 3), but not by an unrelated IgG. The same band also was observed with *mut1*, *mut2*, and *mut3*, but, again, the latter fragment showed reduced binding capacity. Interestingly, *mut3* revealed the presence of a new band, only weakly observed with the other constructs, that suggests the existence of another protein, the binding of which is possible only when Egr-1 is not bound. Alternatively, the introduced mutation would create a new recognition site not present in the other fragments.

To evaluate further the specificity of Egr-1 binding to the proximal promoter region of the $\alpha 7$ gene, we performed competition experiments with oligonucleotides containing Egr-1, Sp1, or CREB consensus sequences or the same DNA fragments used in the bandshift assays (Fig. 8*C,D*). Preincubation of the nuclear extracts with increasing amounts (ranging from a 15- to a 300-fold excess) of the WT-38/41 fragment gradually decreased the intensity of the different complexes (Fig. 8*C*, lanes 2–5). Competition with *mut1* produced a similar decrease (Fig. 8*C*, lanes 6–9), although the band corresponding to Egr-1 appeared slightly more intense (compare, for instance, the corresponding lanes 2 and 6 in Fig. 8*C*), suggesting that the absence of the Egr-1 site 1 in this fragment caused a decrease in its competition ability. The *mut3* fragment was the less effective competitor, although it also induced a decrease in band intensity (Fig. 8*C*, lanes 10–13), suggesting that (1) the proximal Egr-1 site 3, absent in this fragment, has a higher affinity for the factor, and (2) the distant Egr-1 site, although weaker, can bind Egr-1 and induce competition. Finally, when competition was performed with a 400-fold molar excess of synthetic oligonucleotides (Fig. 8*D*, lanes 1–4), only the Egr-1 consensus fragment abolished the formation of the Egr-1 complex. Therefore, Egr-1 specifically interacts with the most promoter-proximal region of the $\alpha 7$ gene.

The above data also suggest that Egr-1 categorizes two non-equivalent sites in this area. Experiments with purified Egr-1 confirmed this assertion (Fig. 9). Thus, by raising the amount of protein used to shift the nonmutated DNA fragment WT -38/+41, it was possible to observe the formation of a second, larger complex (Fig. 9, lanes 2 and 3, double dot). This complex was not observed when any of the two Egr-1 sites (sites 1 and 3) were mutated (Fig. 9, lanes 5–6 and lanes 8–9), suggesting that it arises from the binding of Egr-1 polypeptides to both sites. The different intensity of the bands obtained with *mut1* and *mut3* DNA fragments (compare lanes 5 and 6 vs lanes 8 and 9) indicated, again, that the Egr-1 affinity of site 1, which is kept intact in *mut3*, is

Figure 8. Gel mobility shift assays. *A*, Labeled wild-type (*WT-38/+41*) and mutated (*Mut1*, *Mut2*, *Mut3*; see Fig. 7) DNA fragments were used as gel mobility shift probes in the presence of 0.3 μ g of purified Egr-1/GST fusion protein (*E*) or its mutated, inactive counterpart (Δ). *B*, The same DNA fragments were used with 5 μ g of crude chromaffin cell nuclear extracts (*N*). The results of a supershift assay with Egr-1 specific antibody (*E*) or control IgG (*I*) are shown in lanes 3 and 4, respectively, of this panel. *Dot*, Egr-1 bound probe; *arrowhead*, supershifted Egr-1 bound probe; $-$, probe run in the absence of protein extracts. *C*, *D*, The gel mobility assay was run, using the *WT-38/+41* DNA fragment as the labeled probe and nuclear extracts from chromaffin cells. The first lane in each series ($-$) is always without added competitor. *C*, Competitor DNA fragments *WT-38/+41*, *Mut1*, and *Mut3* were added in 15-, 45-, 150-, or 300-fold excess. *D*, Competitor oligonucleotides with consensus sites for Egr-1 (*E*), Sp1 (*Sp1*), or CREB (*CREB*) were added in 400-fold molar excess. *Dot*, Egr-1 bound probe.

much lower than the one of site 3. If this is the case, one could assume that Egr-1 binds first to site 3, and, subsequently, a second Egr-1 binds to site 1. Moreover, the binding of the second Egr-1 molecule might be facilitated when the first one is already bound, because the band intensity of the larger complex, which would result on binding of Egr-1 to site 1 when site 3 is already occupied (Fig. 9, lane 3, *double dot*), is clearly stronger than the one produced on binding of Egr-1 to site 1 when site 3 is not available for binding (Fig. 8, lane 9). Accordingly, the enhancing activity of Egr-1 may take place via a cooperative interaction with two close elements at the $\alpha 7$ gene promoter region.

Egr-1 transcription factor is expressed only in adrenergic cells

If Egr-1 activates $\alpha 7$ and PNMT transcription, it should be present in the cells that express these two proteins. Immunolo-

Figure 9. Purified Egr-1 binds two nonequivalent sites within the most promoter-proximal region of the $\alpha 7$ gene. Labeled wild-type (*WT-38/+41*) and mutated (*Mut1*, *Mut3*) DNA fragments were used as gel mobility shift probes in the presence of 0.7 and 4 μ g of purified Egr-1/GST fusion protein. *Dot*, Egr-1 bound to a single site; *double dot*, Egr-1 bound to two sites; $-$, probe run in the absence of protein.

calization of Egr-1 in the bovine adrenal gland (Fig. 10*A*) with a polyclonal antibody against this transcription factor shows its expression in only the areas previously demonstrated to express PNMT, α -Bgt-sensitive nAChRs, and $\alpha 7$ transcripts, i.e., the domains close to the cortex and the portal vein. In these cells Egr-1 immunostaining was concentrated in the cell nuclei (Fig. 10*B*), corresponding to a transcriptional factor. In contrast to this restricted localization, Egr-1 labeling of the rat adrenal medulla was almost complete in accordance with PNMT and α -Bgt binding localization. In this case, Egr-1 also was found in the cell nuclei (Fig. 10*C*).

DISCUSSION

At least two classes of nAChRs have been identified on chromaffin cells. One class, probably formed by $\alpha 3$ (Criado et al., 1992), $\alpha 5$, and $\beta 4$ subunits, is present in all chromaffin cells of the adrenal medulla (Campos-Caro et al., 1997) and seems to be responsible for mediating catecholamine secretion. The other binds α -Bgt and contains $\alpha 7$ subunits (García-Guzmán et al., 1995). It was demonstrated only recently that these receptor subtype functions as a nAChR (Alkondon and Albuquerque, 1993; Zhang et al., 1994, 1996). The first conclusion that can be drawn from the present study is that, in contrast with the other nAChR subtype, α -Bgt-sensitive nAChRs are localized heterogeneously in the adrenal medulla at sites where the enzyme PNMT also is present. Moreover, $\alpha 7$ subunit transcripts also are detected in the same place. Cell type-specific gene expression frequently depends on *cis*-acting DNA sequences that are primarily located within the 5' flanking region of the gene. Activatory or inhibitory *trans*-acting proteins can bind to these sequence elements and regulate transcription. Thus, the 5' flanking region of the $\alpha 7$ subunit was isolated and characterized. The 128 bp sequence immediately upstream from the transcription initiation

Figure 10. Egr-1 immunolocalization in bovine and rat adrenal gland sections. *A*, A general view of the bovine gland shows that immunoreaction is present in the areas previously demonstrated to express α -Bgt-sensitive nAChRs and PNMT. *B*, Detail of the bovine gland showing nuclear labeling. *C*, High magnification of immunostaining in the rat adrenal medulla also shows nuclear labeling of Egr-1. Scale bars: 1000 μ m in *A*; 50 μ m in *B*; 25 μ m in *C*.

site was able to drive transcription of reporter genes in cells that express endogenous levels of the $\alpha 7$ gene, such as chromaffin cells (García-Guzmán et al., 1995) and SH-SY5Y neuroblastoma cells (Peng et al., 1994). Thus it was deduced that this region seems to contain the essential sequence elements required to establish a core promoter, although other elements further upstream also may be important, as indicated by the fact that the larger $\alpha 7$ promoter constructs yielded increased activity. The core promoter region does not contain TATA and CAAT boxes, but it does coincide with a G+C-rich domain, characteristic of many promoters, including the chick $\alpha 7$ promoter (Matter-Sadzinski et al., 1992). When this domain was inspected for motifs that might be relevant for the regulation of transcription, several putative binding sites for transcription factors Sp1 and Egr-1 were detected. Because Sp1 generally is expressed constitutively, our interest was directed to Egr-1 (also termed NGF1-A, krox-24, and zif268), an immediate early gene transcription factor known to activate PNMT gene expression in PC12 cells (Ebert et al., 1994; Ebert and Wong, 1995). Egr-1 activated the different $\alpha 7$ gene constructs tested in chromaffin and neuro-2a cells, indicating the requirement of this transcription factor for $\alpha 7$ gene expression.

The minimal promoter region of the $\alpha 7$ gene, which shows activity enhanced by Egr-1 (−38 to −16), contains several sequences that are similar to Egr-1 binding sites. Egr-1 binds to this

region at two locations. Interestingly, the site located −24 to −16, which contains a 1 bp mismatch regarding the Egr-1 consensus sequence, seems to have the highest affinity in bandshift experiments; consequently, its modification abolishes Egr-1 activation in transfection experiments. By contrast, the more distal site, which comprises a perfect Egr-1 consensus sequence, seems to play a secondary role, because Egr-1 activation is not eliminated totally when this site is modified. Accordingly, bandshift experiments showed that the site is recognized by Egr-1, although with lower affinity. These facts suggest that other elements, perhaps additional factors and/or DNA sequences outside the proximal Egr-1 binding site, may favor the binding of Egr-1 to this site. On the other hand, it may worth mentioning that the sequence of the proximal site (GCGTGGGCG), although containing a 1 bp mismatch, has been shown previously to be able to bind with high affinity an Egr-1 zinc finger peptide with which it was cocrystallized (Pavletich and Pabo, 1991). In any case, we propose that Egr-1 exerts its enhancing action via a mechanism that involves direct binding to two very close nonequivalent sites. However, this fact does not preclude the involvement of additional factors on $\alpha 7$ gene transcription. Consider, for instance, that the maximal activity obtained with Egr-1 in neuro-2a cells is always lower than the one exhibited by chromaffin cells, suggesting that, in the latter case, additional factors, probably not available in neuro-2a cells, are needed for enhanced transcription. This fact also is suggested by previous studies on the expression of the chicken $\alpha 7$ subunit, which described the ubiquitous activity of its gene promoter in undifferentiated neural cells and mesoderm stem cells (Matter-Sadzinski et al., 1992), as well as the presence of $\alpha 7$ transcript and protein in embryonic muscle (Corriveau et al., 1995). Thus $\alpha 7$ regulatory elements would acquire their cell specificity in the course of development (Matter-Sadzinski et al., 1992), depending on the availability of determined factors.

The Egr-1 polypeptide was localized exclusively to the same chromaffin cell subset as PNMT and α -Bgt-sensitive nAChRs; thus it seems likely that Egr-1 regulation is a common enhancing mechanism of transcription for these proteins. The transcription factors Egr-1–Egr-4 constitute a family of DNA binding proteins termed “early growth response genes,” which are induced rapidly and transiently in a variety of cells by mitogenic stimulation and by signals involved in cell growth and differentiation (for review, see Gashler and Sukhatme, 1995). In the brain Egr-1 displays manifest basal expression (Mack et al., 1990), which is highly responsive to neuronal stimulation. The level of Egr-1 transcript expression increases when high-frequency electric stimulation is associated with the induction *in vivo* of long-term potentiation. This increase is inhibited by NMDA receptor antagonists (Cole et al., 1989; Wisden et al., 1990) and suggests the potential involvement of Egr-1 in synaptic plasticity. In the bovine adrenal gland a specific stimulation of Egr-1 expression seems very plausible, because localization of adrenergic cells expressing Egr-1 (and also PNMT and $\alpha 7$ nAChRs) is so peculiar: they are only in the region of the adrenal medulla close to the adrenal cortex and to the portal vein. This might indicate that factors arising from the cortex diffuse into the nearby area of the adrenal medulla and induce, directly or indirectly, the expression of Egr-1, which, in turn, regulates the expression of PNMT and nAChRs. If this scenario is correct, glucocorticoids are strong candidates for substances that are released from the cortex and then act directly on nearby chromaffin cells, especially because the glucocorticoid receptor has been localized only to the cytoplasm of adrenergic cells (Ceccatelli et al., 1989). Interestingly, a functional glucocor-

ticoid response element in the 5' flanking region of the enzyme PNMT has been described (Ross et al., 1990). Moreover, by using a PC12 cell line that was stably expressing a glucocorticoid receptor, Ebert et al. (1994) subsequently demonstrated a concerted activation of Egr-1 and glucocorticoids. Preliminary experiments indicate that $\alpha 7$ promoter activity increases substantially when $\alpha 7$ constructs and a glucocorticoid receptor vector are cotransfected into chromaffin cell cultures (M. Criado, C. Carrasco-Serrano, S. Viniegra, unpublished observations), reinforcing our hypothesis that a direct or indirect interaction via the glucocorticoid receptor regulates $\alpha 7$ expression.

A final important question highlighted by this study is the functional significance of α -Bgt-sensitive nAChRs in adrenergic cells. A potential role is indicated by the high Ca^{2+} permeability of this receptor subtype (Séguéla et al., 1993), allowing the capacity for substantial control of intracellular Ca^{2+} levels, which may modulate exocytosis. Alternatively, the expression of $\alpha 7$ nAChRs in adrenergic cells may provide them with an additional mechanism, perhaps faster and specifically directed to trigger epinephrine release. This hypothetical mechanism might have been obscured until now if we consider that (1) α -Bgt-sensitive receptors have very rapid rates of desensitization, and the method of agonist application when testing catecholamine release would have not been fast enough to detect responses caused by α -Bgt-nAChR (see Zhang et al., 1994), and (2) the existence of other nAChR subtypes that desensitize more slowly than does α -Bgt-nAChR (Campos-Caro et al., 1997) would have darkened responses from the latter. Whatever the case, many studies point out significant differences in the secretory process of adrenergic and noradrenergic cells (Choi et al., 1993; Langley and Grant, 1995; Lomax et al., 1997), and the restricted expression of α -Bgt-sensitive nAChRs may contribute to this heterogeneity.

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