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Both Inducible and Constitutive Activator Protein-1-Like Transcription Factors Are Used for Transcriptional Activation of the Galanin Gene by Different First and Second Messenger Pathways

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ABSTRACT

We investigated *trans*-acting factors mediating galanin (GAL) gene activation by protein kinase-dependent signal transduction pathways in chromaffin cells. GAL mRNA up-regulation via the protein kinase A (PKA) pathway (25 μM forskolin) required new protein synthesis. Stimulation via protein kinase C (0.1 μM phorbol myristate acetate) did not. The involvement of activator protein-1(AP-1) and cAMP response element-binding protein (CREB) in serine/threonine protein kinase activation of GAL gene transcription was assessed. Cotransfection of a GAL reporter gene along with expression plasmids encoding c-Jun plus c-Fos, or the catalytic subunit of PKA (PKAβ), resulted in a 4- to 8-fold enhancement of GAL reporter gene transcription. Transcriptional activation required the galanin 12-*O*-tetradecanoylphorbol-13-acetate (phorbol-12-myristate-13-acetate) response element (GTRE) octamer sequence (TGACGCGG) in the proximal enhancer of the GAL gene, previously shown to confer phorbol ester responsiveness in chromaffin cells. CREB coexpression did not stimulate GAL gene transcription or increase transcriptional activation by PKAβ. The GTRE preferentially bound in vitro synthesized Jun and Fos-Jun, compared with CREB, in electrophoretic mobility shift assays. The GTRE preference for binding AP-1-immunoreactive protein compared with CREB was even more pronounced in chromaffin cell nuclear extracts, in which the majority of GTRE-bound protein in electrophoretic mobility shift assays was supershifted with anti-Fos and anti-Jun antibodies. Thus, GAL gene regulation mediated by protein kinase activation appears to involve both constitutively expressed and inducible AP-1-related proteins. Elevated potassium stimulation of GAL mRNA was completely blocked, but pituitary adenylyl cyclase-activating polypeptide and histamine stimulations were only partially blocked, by cycloheximide. Both inducible and constitutive pathways are therefore used by physiologically relevant first messengers that stimulate GAL biosynthesis in vivo.

The neuropeptide galanin (GAL) is synthesized as a prohormone precursor, processed to a 29- to 30-amino-acid bioactive peptide, and secreted via the regulated pathway from neuroendocrine cells throughout the mammalian neuroendocrine axis (Bedecs et al., 1995). GAL hyperinnervation of cholinergic neurons of the nucleus basalis of Meynert may contribute to cholinergic hypofunction in Alzheimer's disease (Bowser et al., 1997) because GAL has been shown to inhibit stimulus-evoked release of acetylcholine from primate brain slices in vitro (Fisone et al., 1991). GAL is sharply up-regulated by peripheral axotomy in the rat, and its expression is associated with suppression of autotomy elicited by peripheral nerve injury (Ji et al., 1994). Pituitary GAL levels vary dramatically during the estrus cycle in rat and in response to treatment with steroid hormones (Kaplan et al., 1988). GAL plays a role in controlling lactotroph proliferation and differentiation in the anterior pituitary gland (Wynick et al., 1993). GAL released from sympathetic fibers innervating the endocrine pancreas is a negative modulator of insulin secretion (Sharp, 1996). GAL is likely to play a role in endocrine homeostasis at the level of the adrenal medulla: reflex stimulation of the splanchnic nerve elicited by insulin-induced hypoglycemia elicits a more than 70-fold increase in GAL content, which persists for several days after the cessation of

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ABBREVIATIONS: GAL, galanin; CRE, cAMP response element; PKA, protein kinase A; PKC, protein kinase C; GTRE, galanin 12-*O*-tetradecanoylphorbol-13-acetate (phorbol-12-myristate-13-acetate) response element; GTREM, mutated galanin 12-*O*-tetradecanoylphorbol-13-acetate (phorbol-12-myristate-13-acetate) response element; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; CREB, cAMP response element-binding protein; TRE, 12-*O*-tetradecanoylphorbol-13-acetate (phorbol-12-myristate-13-acetate) response element; PMA, phorbol-12-myristate-13-acetate; PACAP, pituitary adenylyl cyclase-activating polypeptide; DMEM, Dulbecco's modified Eagle's medium; AP-1, activator protein-1.

stimulation (Fischer-Colbrie et al., 1992). In general, the neuroendocrine actions of GAL involve rapid up-regulation of its biosynthesis in response to hormonal and other physiological stimuli.

The *trans*-synaptic stimulation of GAL biosynthesis in the adrenal medulla occurs via enhanced production of GAL mRNA (Anouar and Eiden, 1995). Transcriptional activation of the GAL gene in vivo presumably involves secretion of first messengers such as acetylcholine, pituitary adenylyl cyclase-activating polypeptide (PACAP), and vasoactive intestinal peptide from splanchnic nerve fibers, which increase intracellular cAMP, calcium, and other second messengers and subsequently activate intracellular protein kinases in chromaffin cells (Malhotra et al., 1989; Przywara et al., 1996).

The pathways linking protein kinase stimulation to GAL gene activation were investigated here using primary cultures of bovine chromaffin cells derived from the adrenal medulla. Chromaffin cells in primary culture have been used for more than a decade as a model system to study the molecular basis for stimulus-secretion-synthesis coupling, the process by which neuroendocrine cells establish, maintain, and regulate neuropeptide synthesis to compensate for cellular peptide loss during secretion (MacArthur and Eiden, 1996). The inducible immediate-early gene products c-Fos and c-Jun and constitutively expressed proteins related to them have been implicated as *trans*-activators of neuropeptide gene transcription in response to *trans*-synaptic signals in chromaffin cells (Mar et al., 1992; Bacher et al., 1996; MacArthur, 1996). Distinguishing between these two types of signaling pathways to the nucleus is critical for determining how neuroendocrine cells respond to various trophic and *trans*-synaptic stimuli to maintain or alter the levels of the neuropeptides they produce.

Here we report that up-regulation of the biosynthesis of GAL by protein kinase A (PKA) requires new protein synthesis, whereas GAL mRNA up-regulation by the protein kinase C (PKC) signaling pathway does not. Cotransfection of GAL reporter gene constructs with activator protein-1 (AP-1) and cAMP response element-binding protein (CREB), together with gel shift analysis, suggest that AP-1-related proteins function as both pre-existing and inducible regulators of GAL gene transcription in response to specific signal transduction pathways in chromaffin cells. Constitutive and inducible *trans*-acting factors are differentially used by various first messengers, including histamine, PACAP, and cell depolarization causing calcium influx, to regulate the GAL gene in chromaffin cells.

Materials and Methods

Cell Culture. Primary cultures of bovine chromaffin cells were obtained by perfusion of bovine adrenal glands with 0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ) and 30 U/ml DNase (Sigma Chemical Co., St. Louis, MO) as described previously. Cells were directly plated at a density of 0.2 to 1×10^6 in 24-well dishes or further purified by differential plating in T150 flasks to remove contaminating nonchromaffin cells followed by replating of nonadherent cells 24 h later in 24-well plates coated with poly(D-lysine). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Life Technologies/GIBCO, Grand Island, NY) containing 5% heat-inactivated FBS (Biowhitaker Bioproducts, Walkersville, MD) and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cytosine arabinofuranoside (50 μg/ml; Sigma) and nystatin (100 U/ml, Life Technologies, Inc., Gaithersburg, MD) were present in the medium until the final medium change before drug addition.

Drug Treatments. At 24 h after differential plating of chromaffin cells, medium was removed and replaced with fresh medium or medium containing 0.5 μg/ml cycloheximide. Thirty minutes later, medium was again replaced with fresh medium or medium containing either 0.1 μM PMA, 25 μM forskolin, 40 mM KCl (prepared by isotonic replacement of sodium chloride with potassium chloride in DMEM), 10 μM histamine, or 10 nM PACAP, each with or without 0.5 μg/ml cycloheximide. Cells were harvested for total RNA and Northern blotting as described below.

Transient Expression Assays. When prepared for transfection, differentially plated cells were plated onto poly(D-lysine)-coated 60 mm culture dishes at 3 to 5×10^6 cells/dish in 5 ml of medium and transfected by calcium phosphate/DNA coprecipitation using the Promega Profection Mammalian Transfection System-Calcium Phosphate according to the manufacturer's instructions (Promega Technical Manual TM012; Promega, Madison, WI) and as described by Anouar et al. (1994). Briefly, 5 to 20 μg of DNA was added to 27.75 μl of 2 M calcium chloride and adjusted to 0.225 ml with water. The DNA solution was added to 2× HEPES-buffered saline dropwise with vortexing. The final solution was incubated at room temperature for 30 min, revortexed, and added dropwise to each well. After overnight incubation at 37°C in 5% CO_2/air, the medium was removed, and the cells were washed once with PBS and incubated for 3 min at room temperature in 15% glycerol in DMEM prewarmed to 37°C. Cells were washed twice with PBS, and returned to 5% FBS in DMEM. Drugs or vehicle were added 24 h after this, and cells were harvested for measurement of luciferase activity after 1 day of drug or vehicle treatment. In cotransfection experiments, 5 μg of reporter plasmid (pGTRE or pGTREM) was cotransfected with 2 μg of each expression plasmid or with 2 μg of Bluescript DNA to maintain input DNA concentration constant in all transfections. The luciferase activity was determined by chemiluminescence in a Berthold Lumat 9501 luminometer using the luciferase assay system (Promega). This activity was corrected for transfection efficiency by a cotransfected β-galactosidase control plasmid whose activity was measured using the galactolight system (Tropix, Bedford, MA) as described previously (Anouar et al., 1994).

mRNA Measurements. RNA was harvested from individual cell culture wells after removal of medium, by extraction with Tris buffer containing SDS, EDTA, and proteinase K. RNA was electrophoresed on denaturing agarose gels, electroblotted onto nylon membranes, and hybridized with bovine galanin cRNA probes as previously described (Rökaeus et al., 1990; Anouar and Eiden, 1995).

Identity and Construction of Reporter and Expression Plasmids. The plasmids pGTRE or pGTREM functioned as the reporter genes in all transfection/expression assays. To construct both pGTRE and pGTREM, a double-stranded oligonucleotide containing GAL promoter sequences between −88 and −50 including the GTRE was inserted upstream of a GAL minimal promoter fused to the luciferase encoding gene. In pGTREM, three nucleotides of the GTRE were mutated from TGACGCGG to gGcaGCGG. The construction of pGTRE and pGTREM and their transcriptional activity in chromaffin cells in response to PMA and forskolin have been previously described (Anouar et al., 1994). A rat CREB transcription plasmid was constructed by subcloning a *Sac*I/*Bam*HI fragment obtained from the Rous sarcoma virus CREB plasmid (Gonzalez and Montminy, 1989) and containing the entire CREB coding sequence into Bluescript KS^+ (Stratagene). The expression plasmids cytomegalovirus (CMV) c-Fos and CMV c-Jun were constructed by subcloning an *Eco*RV/*Xba*I fragment from Bluescript KS^+ c-Fos (Bravo et al., 1987) or an *Eco*RI/*Sph*I fragment from pGEM2 c-Jun (Nakabeppu et al., 1988) containing the entire coding sequences for mouse c-Fos and c-Jun, respectively, into the corresponding sites of the pcdna I polylinker downstream of the CMV promoter (InVitrogen). The plas-

mids pGEM2 Jun-B (Ryder et al., 1988) and pPKAβ (Maurer, 1989) were also used without modification in this study.

In Vitro Transcription and Translation. CREB RNA was obtained after digestion of Bluescript CREB plasmid by *Bam*HI and subsequent transcription of the linearized plasmid using a T7 RNA polymerase transcription system (Ambion, Inc., Austin, TX). The c-Fos plasmid was linearized by *Xba*I and transcribed using T3 RNA polymerase. The c-Jun plasmid was linearized with *Hin*dIII and transcribed with SP6 RNA polymerase. pGEM2 Jun-B was transcribed with T7 RNA polymerase after linearization with *Bam*HI. RNAs were then translated in rabbit reticulocyte lysates (Promega). To verify the quality of the proteins made, ^{35}S-methionine was included in the translation reactions and the labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Electrophoretic Mobility Shift Assays. Chromaffin cell nuclear extracts were prepared from phorbol-12-myristate-13-acetate (PMA)-treated cells maintained in T150 flasks as previously described (Anouar et al., 1994). Nuclear extract (2 μg protein) or in vitro translated proteins (2 μl of the 50-μl translation reaction) were assayed for their binding to the DNA elements by gel mobility shift assay as described (Anouar et al., 1994). Double-stranded oligonucleotides containing the somatostatin cAMP response element (CRE) (Montminy and Bilezikjian, 1987), the collagenase 12-*O*-tetradecanoylphorbol-13-acetate (PMA) response element (TRE) (Angel et al., 1987), and the galanin 12-*O*-tetradecanoylphorbol-13-acetate (PMA) response element (GTRE) (Anouar et al., 1994) were used and were labeled by fill-in (Sambrook et al., 1989).

In the supershift EMSA, specific antibodies were incubated with the nuclear extracts or in vitro translated proteins for 30 min before the addition of the labeled oligonucleotide. The incubation was then continued for an additional 20 min. The DNA-protein complexes were resolved on 3.75% nondenaturing polyacrylamide gels in 0.25× TBE (12.5 mM Tris borate, pH 8.3, 1 mM EDTA). The antibodies used (1 μl/reaction) were affinity purified IgG fractions recognizing ATF-1 p35 and CREB-1 p43 in the case of the anti-ATF-1 antibody, all known Fos homologs in the case of the anti-Fos antibody, and all known Jun homologs in the case of the anti-Jun antibody (all antibodies obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specificity of the antibodies was checked in EMSAs using the in vitro translated proteins described above. As expected, each antibody interacted only with the corresponding bZIP protein.

Chemicals and Other Materials. Collagenase was obtained from Worthington Biochemical Corp. Nystatin was from Life Technologies, Inc. DNase, cytosine arabinofuranoside, histamine, and cycloheximide were from Sigma Chemical Co. PMA was from LC Services Corp. (Woburn, MA). Forskolin was from Calbiochem (San Diego, CA). PACAP-27 was from Phoenix Pharmaceuticals (Mountain View, CA).

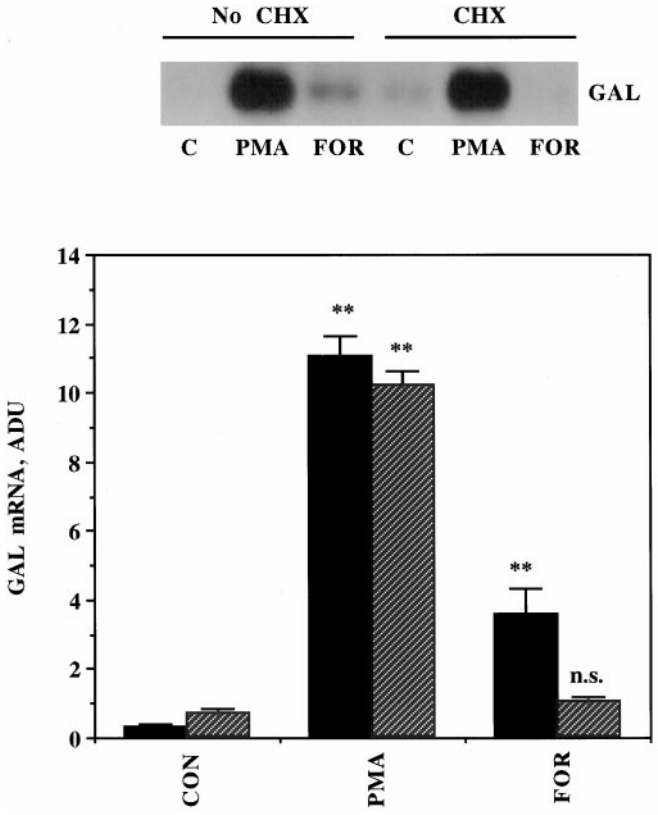


Fig. 1. Induction of GAL mRNA by forskolin (FOR) and PMA. Differential blockade by cycloheximide (CHX). Twenty-four hours after differential plating (2 days in vitro), medium was removed from chromaffin cells in 24-well Costar plates at a density of 500,000 cells/well and replaced with medium containing 0.5 μg/ml cycloheximide or vehicle (CON). Thirty minutes later, medium was again replaced with medium containing 25 μM forskolin or 0.1 μM PMA or vehicle, with or without cycloheximide. Cell were harvested for total RNA and Northern blotting 18 h later as described in *Materials and Methods*. Ethidium bromide-stained gels were photographed and negatives were scanned densitometrically before RNA transfer to Nylon membranes to ensure equal loading of RNA between treatment groups. Values represent arbitrary densitometric units (ADU) and are the mean ± S.E.M. of four separate determinations (wells) from a single experiment repeated at least once for each drug with qualitatively similar results. $^{**}p < .01$, Student's two-tailed t test; n.s., not significantly different from corresponding (cycloheximide-treated) control group.

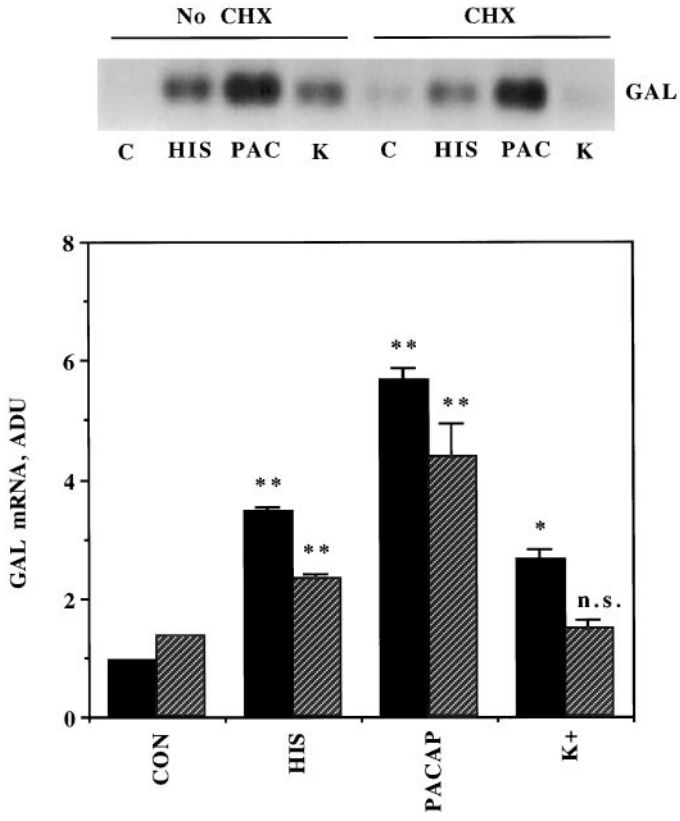
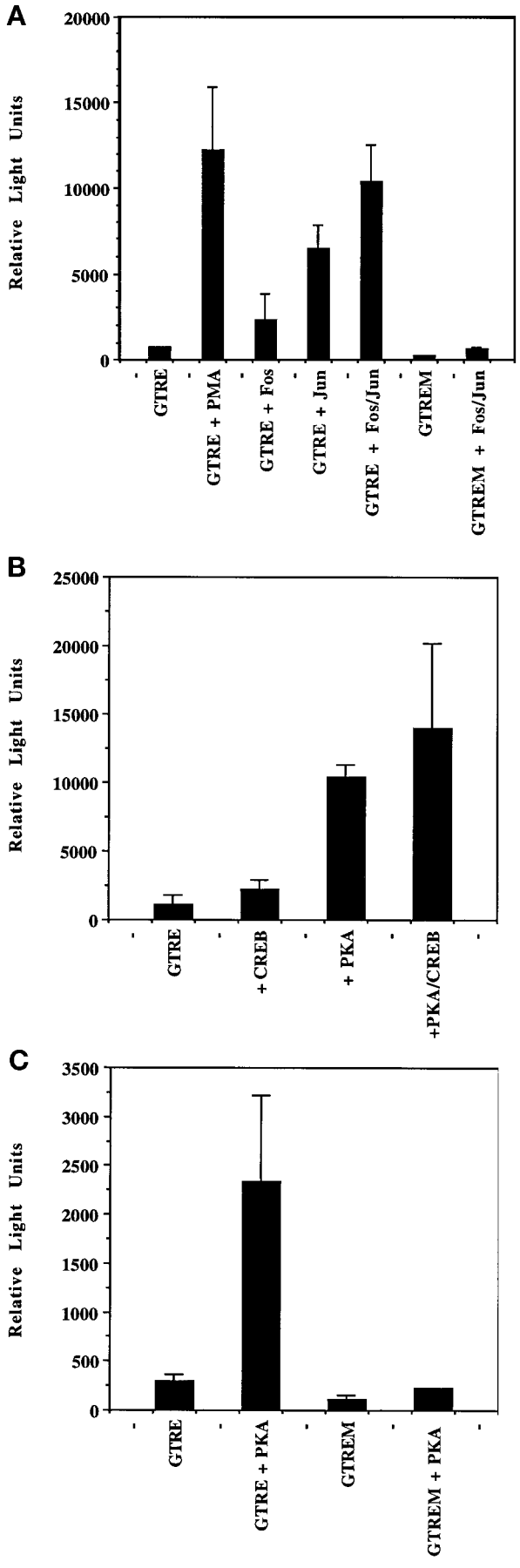


Fig. 2. Induction of GAL mRNA by PACAP and histamine, but not by elevated potassium, occurs in the absence of new protein synthesis. Experiments were performed as described in *Materials and Methods* and the legend to Fig. 1. K^+, 40 mM potassium; PACAP, 10 nM PACAP-27; HIS, 10 μM histamine. $^{*}p < .05$, $^{**}p < .001$, n.s. not significantly different from corresponding (cycloheximide-treated) control group, using Student's two-tailed t test.



Results

GAL mRNA levels were elevated 8- to 12-fold by 25 μM forskolin and more than 20-fold by 0.1 μM PMA, as reported previously (Rökaeus et al., 1990). Forskolin- and PMA-induced up-regulation of GAL mRNA were differentially affected by inhibition of new protein synthesis with cycloheximide. The increase in the level of GAL mRNA after forskolin treatment was completely blocked in the presence of cycloheximide, whereas PMA induction of GAL mRNA was unaffected by inhibition of protein synthesis with this drug (Fig. 1).

Several first messengers previously shown to stimulate neuropeptide biosynthesis in chromaffin cells were tested for their ability to up-regulate GAL mRNA, and sensitivity to inhibition by treatment with cycloheximide was assessed. Histamine and PACAP, at concentrations shown to up-regulate the biosynthesis of enkephalin, increased GAL mRNA severalfold (Fig. 2). Induction by both first messengers was only partially dependent on new protein synthesis (Fig. 2). Elevated potassium, similarly to activation of PKA, increased GAL mRNA levels via a signaling pathway that was completely dependent on induction of new protein synthesis (Fig. 2).

The requirement for new protein synthesis for calcium influx- and PKA-mediated GAL gene regulation suggests the involvement of immediate-early genes in this process. Little is known about the *cis*-active sequences required for induction of galanin gene transcription by various second messenger signaling pathways, except that PMA responsiveness can be localized to a region of the GAL promoter containing the sequence TGACGCGG, referred to as the GTRE (Anouar et al., 1994). To demonstrate the dual responsiveness of the GAL GTRE, chromaffin cells were cotransfected with a GAL reporter gene and expression plasmids expressing Fos and Jun proteins, or the catalytic subunit of PKA, which phosphorylates and activates CREB (Maurer, 1989). Transcription of the reporter gene was increased by coexpression of both PKA and Fos/Jun (Fig. 3). Coexpression of CREB, however, had no consistent effect on GAL reporter gene expression (Fig. 3). To test the possibility that endogenous PKA levels are too low to allow significant phosphorylation of exogenous CREB, both CREB and PKA expression plasmids were cotransfected with the GTRE reporter. These conditions produced no further transcriptional activation than expression of PKA alone (Fig. 3), suggesting that PKA signaling to the GAL gene does not require the participation of CREB.

Fig. 3. Effects of exogenously expressed components of protein kinase signaling pathways on GAL reporter gene transcription and dependence on the GTRE in chromaffin cells. A, transcription from the GTRE-containing luciferase reporter gene (pGTRE) or the identical GAL reporter gene mutated in the GTRE (pGTREM), as described in *Materials and Methods*, transfected into untreated chromaffin cells or chromaffin cells treated with 0.1 μM PMA or cotransfected with c-Fos and c-Jun expression plasmids (CMV c-Fos and CMV c-Jun, respectively) or a combination of c-Fos and c-Jun expression plasmids. B, transcription from the pGTRE luciferase reporter gene cotransfected with CREB or PKA expression plasmids separately or together. C, transcriptional activity of pGTREM cotransfected with the PKA expression plasmid. pGTRE transcription with and without PKA was included in the same experiment as a positive control. Data are presented as relative light units (luciferase activity per well, corrected for transfection efficiency by β-galactosidase activity of a cotransfected RSVβgal plasmid). Values represent the mean and range of duplicate determinations from a single experiment repeated at least once with qualitatively similar results.

Although we have previously shown that the GTRE mediates responsiveness to PMA in chromaffin cells, the possibility exists that Fos and Jun bind to regions of the gene other than the GTRE. To investigate this possibility, a reporter construct in which the GTRE is mutated by replacement of three nucleotides within the GTRE (GTREM; see Anouar et al., 1994) was cotransfected with Fos and Jun in chromaffin cells. This construct was not responsive to PMA, as previously reported, or to cotransfected Fos or Jun (Fig. 3A). Thus, the pattern of nuclear protein binding to the GTRE is likely to be an accurate indication of the binding pattern for functional transactivation of the GAL reporter gene, and of the endogenous GAL gene itself, in chromaffin cells.

EMSA analysis of the GTRE, in comparison to consensus CRE and TRE oligonucleotides, was carried out to determine the proteins that might be involved in mediating the new protein synthesis-independent induction of this gene by PMA. A more highly resolving gel system than that reported previously was used to show that several complexes with different mobilities form with the GTRE on incubation with chromaffin cell nuclear extracts (Fig. 4). Components of these complexes are also bound by the CRE or TRE based on their mobilities and the cross reactions between the three elements (Fig. 4). To assess the possibilities for binding to the GTRE, the proteins CREB, Fos, and Jun were synthesized in vitro (Fig. 5A) and allowed to interact with the GTRE. EMSA demonstrated that the GTRE is capable of high-affinity binding to Fos/Jun heterodimers and, to a lesser extent, to c-Jun (relative to the consensus TRE) and to CREB (relative to the consensus CRE; Fig. 5B), consistent with a unique dual TRE/CRE recognition capability of this element as suggested previously (Anouar et al., 1994). The ability of the GTRE to recognize members of the ATF/CREB and Fos/Jun transcription factor families was confirmed by supershift assays in the EMSA format with pan-specific ATF, Jun, and Fos antibodies (Fig. 6). The GTRE has a unique interaction profile with these proteins compared with either a canonical CRE or TRE.

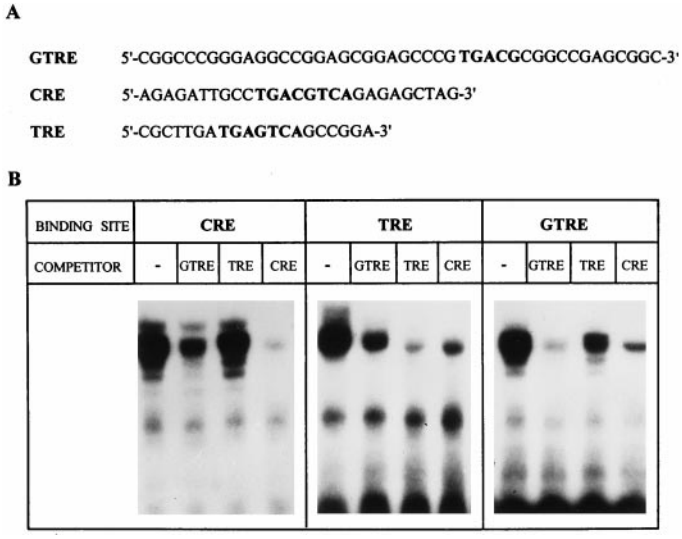


Fig. 4. EMSAs of CRE, TRE, and GTRE with chromaffin cell nuclear extract. A, oligonucleotides used in EMSAs with GTRE, CRE, and TRE sequences in bold. GTRE, bovine galanin gene fragment; CRE, consensus cyclic AMP response element; TRE, consensus phorbol ester response element. B, EMSA carried out as described in *Materials and Methods*. Unlabeled excess oligonucleotides were present at 100× the concentration of labeled GTRE, CRE, or TRE oligonucleotides when present.

Thus, the CRE binds both CREB and Fos/Jun, whereas the TRE binds AP-1, but not CREB. In contrast, the GTRE binds little or no immunoreactive CREB in chromaffin cell nuclear extracts as evidenced by the anti-CREB supershifting experiment shown in Fig. 6. Furthermore, the GTRE is capable of binding Fos/Jun-immunoreactive proteins found in chromaffin cell nuclear extracts with a slightly different profile than that for either the TRE or the CRE. Thus, anti-Fos supershifts GTRE complexes much more completely than CRE or TRE complexes, whereas anti-Jun supershifts the GTRE complexes from chromaffin cell nuclear extracts less than for the TRE. Because Fos homodimers do not bind AP-1 recognition sites (Hai and Curran, 1991), the GTRE AP-1 complexes formed in chromaffin cell nuclei may be composed of Fos-related antigen homodimers, or heterodimers composed of both Fos- and Jun-related proteins, which are distinct from those complexes that form with consensus TRE or CRE *cis*-active gene regulatory sequences.

Discussion

Various first messengers stimulate neuropeptide biosynthesis in chromaffin cells. Elevated potassium causes passive depolarization and calcium influx via voltage-sensitive calcium channels and has been reported to stimulate the biosynthesis of all chromaffin cell neuropeptides examined to date (MacArthur and Eiden, 1996, and references therein). PACAP increases inositol trisphosphate, intracellular calcium, and cAMP production and up-regulates enkephalin, vasoactive intestinal peptide, and atrial natriuretic peptide biosynthesis in chromaffin cells (Rius et al., 1994; Babinski et

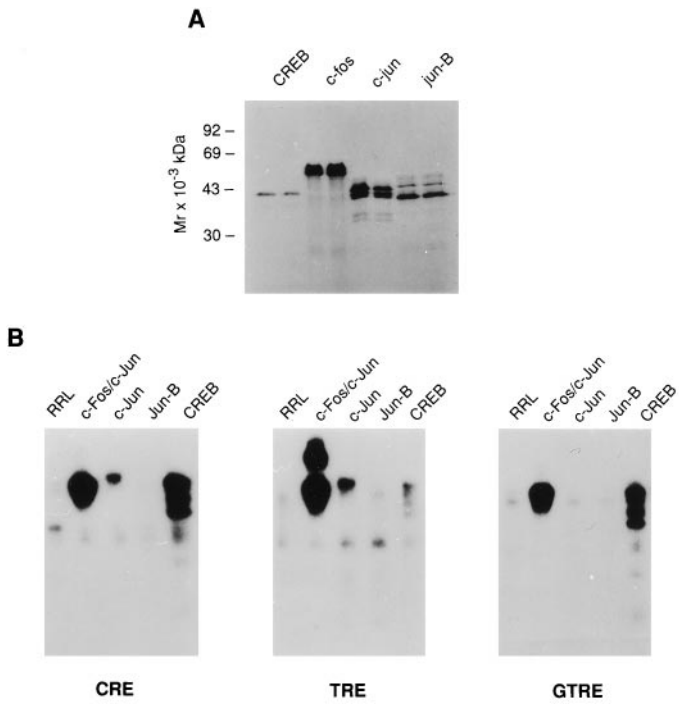


Fig. 5. Cell-free synthesis of Jun, Fos, and CREB; EMSAs with TRE, CRE, GTRE. A, polyacrylamide gel electrophoresis of in vitro translation products from CREB, c-Fos, c-Jun, and Jun-B transcripts carried out in the present of ^{35}S-methionine. B, EMSA of RRL (unprogramed reticulocyte lysate), c-Fos/c-Jun lysate mixture, and c-Jun, Jun-B, and CREB unlabeled in vitro translation products incubated with ^{32}P-labeled CRE, TRE, and GTRE oligonucleotide probes shown in Fig. 4A.

al., 1996; Hahm et al., 1998; Tanaka et al., 1998). Histamine increases intracellular calcium mobilization as well as calcium influx and has been shown to up-regulate enkephalin and secretogranin II biosynthesis (Kley et al., 1987; Bauer et al., 1993). Inositol trisphosphate, calcium, and cAMP are all second messengers that can directly or indirectly activate serine/threonine kinases such as PKA, PKC, and calmodulin kinase in chromaffin cells. These kinases, in turn, activate AP-1, CREB, and other *trans*-activators of neuropeptide gene transcription either by inducing their transcription and de novo biosynthesis, by direct phosphorylation of the pre-existing factors, or by stimulating phosphatases that in turn activate either pre-existing or induced transcription factors such as AP-1 by serine dephosphorylation (Goodman, 1990; Armstrong and Montminy, 1993; MacArthur and Eiden, 1996). Finally, transcriptional *trans*-activators, induced de novo on stimulation or post-translationally activated by phosphorylation/dephosphorylation, bind to response elements usually contained in the proximal promoter/enhancer of neuropeptide genes, to accelerate transcription and ultimately increase neuropeptide content within the secretory compartments of the cell.

Both inducible and constitutively expressed AP-1-like proteins and CREB have been implicated in *trans*-synaptic activation of neuropeptide gene transcription in both the central nervous system and chromaffin cells (Sonnenberg et al., 1989; Giraud et al., 1991; Mar et al., 1992; Konradi et al., 1993; Bacher et al., 1996; MacArthur, 1996). However, direct evidence for the involvement of a specific protein or proteins in vivo is still lacking. The critical test of which *trans*-acting factor(s) actually bind to the enhancer of a given neuropeptide gene to effect transcriptional up-regulation during *trans*-synaptic signaling, however, is not easily made in vivo. Thus, determining whether gene induction is blocked by inhibition of new protein synthesis, indicating immediate-early gene induction, or is mediated through post-translational modification of pre-existing cellular factors requires the study of purified populations of neuropeptide-synthesizing neuroendocrine cells. In primary cultures of chromaffin cells, these mechanisms can be distinguished by treatment of cells with cycloheximide before stimulation with inducing agents: processes occurring through the induction of immediate-early genes (Morgan and Curran, 1995) are abrogated by inhibition of new protein synthesis, and those occurring through action of pre-existing factors are not. Having made this determination, *trans*-acting factors that meet the criteria for mediating transcriptional regulation of a given gene can then be identified with gelshift and cotransfection experiments.

GAL gene activation through forskolin, which increases cAMP and activates PKA, and PMA, which activates PKC, exhibit differential requirements for inducible versus constitutive transcriptional factors or cofactors in chromaffin cells. Forskolin and PMA both stimulated GAL mRNA production in acutely cultured chromaffin cells, as previously reported for long-term chromaffin cell cultures (Rökaeus et al., 1990). Up-regulation of GAL mRNA levels by forskolin was sensitive to treatment with cycloheximide, but up-regulation by PMA was not. These data suggest that inducible *trans*-activating factors function in GAL gene transcriptional activation by the PKA pathway, while the PKC pathway increases GAL gene transcription by post-translational modification of a pre-existing factor or complex in chromaffin cells.

This pattern of differential utilization of apparently constitutive and inducible pathways for protein kinase regulation applies as well to first messenger stimulation with calcium, PACAP, and histamine. Elevated potassium levels require new protein synthesis for up-regulation of GAL mRNA, whereas histamine and PACAP can both induce GAL mRNA, albeit to a more limited extent than in untreated cells, in the presence of cycloheximide. These data suggest that PACAP and histamine signaling to the GAL gene involves a signaling pathway composed of pre-existing factors, similar to that initiated by activation of PKC, and that a second, inducible pathway similar to that initiated by activation of PKA is required for complete signaling to the GAL gene by these first messengers. Signaling to the GAL gene via calcium influx, on the other hand, involves exclusively a pathway dependent on inducible factors, similar to activation of GAL transcription by PKA. In fact, depolarization and calcium influx can increase cAMP levels in chromaffin cells, demonstrating the potential for activation of PKA by elevated potassium and other first messengers that increase calcium flux (Keogh and Marley, 1991).

PKA and PKC may be merely prototypical for various serine/threonine kinases, including calmodulin and MAP kinases, that link first messenger activation to GAL gene tran-

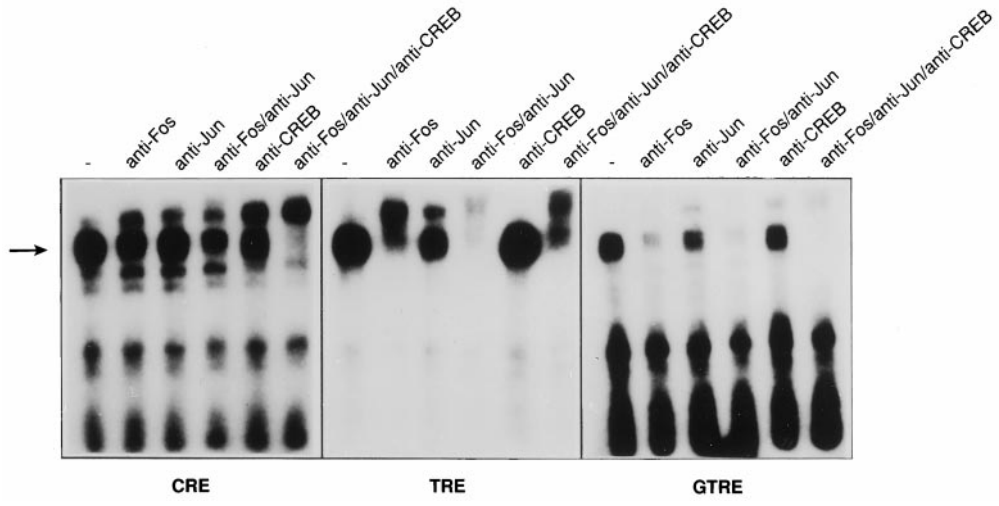


Fig. 6. Supershift EMSA of GTRE by Fos, Jun, and CREB antibodies: Comparison with consensus TRE and CRE. EMSA and supershift EMSAs using chromaffin cell nuclear extracts; GTRE, TRE, and CRE labeled oligonucleotide probes; and antibodies directed to Fos, Jun, and CREB singly and in the combinations indicated.

scription through inducible and constitutive *trans*-acting factors, respectively. The requirement for new protein synthesis for induction of GAL mRNA by PKA does, however, imply that GAL gene regulation by PACAP cannot occur exclusively via this cAMP-dependent pathway. Likewise, histamine is unlikely to act solely through calcium mobilization because histamine action appears to require both constitutive and inducible pathways for full activation of GAL transcription, whereas activation by potassium is completely dependent on new protein synthesis. Reports that histamine and PACAP may activate multiple second messenger pathways in chromaffin cells are consistent with convergent signaling pathway regulation of the GAL gene by these two first messengers (Kley et al., 1987; Tanaka et al., 1998).

Candidates for inducible factors mediating calcium and cAMP effects on neuropeptide gene transcription include members of the AP-1 complex family. Several groups have demonstrated prompt and dramatic up-regulation of c-Fos and c-Jun mRNA and protein on stimulation of chromaffin cells with elevated potassium and phorbol ester and increased intracellular cAMP (Mar et al., 1992; Bacher et al., 1996). As mentioned above, the GAL gene contains a phorbol ester response element, or GTRE (sequence TGACGCGG), at −59 to −52 within the proximal promoter/enhancer of the gene, that mediates cell-specific PMA up-regulation in chromaffin cells (Anouar et al., 1994). Jiang et al. (1998) confirmed that the GTRE functions as a PMA-response element in human neuroblastoma cells. The GTRE, as shown here, is also the element within the GAL gene required for responsiveness to stimulation of the PKA signaling pathway. Our previous inability to demonstrate up-regulation of a GAL transgene in chromaffin cells stimulated with forskolin alone may indicate that the GAL GTRE is a relatively weaker response element for cAMP than for PMA and may require more sustained activation of the PKA signaling pathway to elicit a response detectable by GAL reporter genes, generally less responsive than the endogenous gene to first and second messenger stimuli examined to date in chromaffin cells (Rökaeus et al., 1990; Anouar et al., 1994). Additional elements may be required to increase sensitivity of response to the PKA signaling pathway under conditions in which maximum elevation of cAMP, and therefore PKA activation, is transient, rather than prolonged, as is the case for constitutively activated PKA.

CREB and AP-1 have both been implicated in the induction of many neuropeptide genes by the PKA and calcium signaling pathways, and in fact, in vitro synthesized CREB and AP-1 proteins, as well as CREB- and AP-1-immunoreactive proteins in chromaffin cell nuclear extracts, bind specifically to the GTRE. Furthermore, cotransfection of a GAL reporter gene into chromaffin cells with expression vectors providing exogenous c-Fos and c-Jun confirmed that signal transduction pathways culminating in activation of AP-1 proteins can access the GAL gene. Cotransfection of CREB, however, did not result in activation of the GAL reporter containing the GTRE. Furthermore, PKA expression clearly enhanced GAL reporter transcription without a further effect of coexpressed CREB, demonstrating that the ability of the cAMP pathway to activate GAL transcription is essentially independent of CREB. The ability of the GTRE to bind purified Fos and Jun and Fos- and Jun-immunoreactive protein in chromaffin cell nuclear extracts supports the idea that up-regulation of the GAL gene by calcium and cAMP occurs via induction of AP-1 proteins acting as immediate-early gene products, binding of these AP-1 proteins to the GTRE, and consequent *trans*-activation of GAL transcription. Up-regulation of the GAL gene by PMA, on the other hand, must involve another AP-1-like protein or proteins, whose action does not depend on new protein synthesis. These data strongly suggest that two classes of AP-1-related proteins, one constitutively expressed and activated post-translationally by PKC, and one inducible by calcium and PKA, regulate galanin gene transcription in chromaffin cells. MacArthur (1996) argued that a novel Fos-like protein mediates up-regulation of the proenkephalin gene by elevated potassium, which is also insensitive to blockade by cycloheximide (H.-W.L., L.E.E., S. H. Hahm, manuscript in preparation). This protein would be a candidate for mediating PKC regulation of the GAL gene as well.

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