Dexamethasone represses cAMP rapid upregulation of TRH gene transcription: identification of a composite glucocorticoid response element and a cAMP response element in TRH promoter

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Abstract

Hypothalamic proTRH mRNA levels are rapidly increased (at 1 h) *in vivo* by cold exposure or suckling, and *in vitro* by 8Br-cAMP or glucocorticoids. The aim of this work was to study whether these effects occurred at the transcriptional level. Hypothalamic cells transfected with rat TRH promoter (–776/+85) linked to the luciferase reporter showed increased transcription by protein kinase (PK) A and PKC activators, or by dexamethasone (dex), but co-incubation with dex and 8Br-cAMP decreased their stimulatory effect (as observed for proTRH mRNA levels). These effects were also observed in NIH-3T3-transfected cells supporting a characteristic of TRH promoter and not of hypothalamic cells. Transcriptional regulation by 8Br-cAMP was mimicked by noradrenaline which increased proTRH mRNA levels, but not in the presence of dex. PKA inhibition by H89 avoided 8Br-cAMP or noradrenaline stimulation. TRH promoter sequences, cAMP response element (CRE)-like (–101/–94 and –59/–52) and glucocorticoid response element (GRE) half-site (–210/–205), were analyzed by electrophoretic mobility shift assays with nuclear extracts from hypothalamic or neuroblastoma cultures. PKA stimulation increased binding to CRE (–101/–94) but not to CRE (–59/–52); dex or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increased binding to GRE, a composite site flanked by a perfect and an imperfect activator protein (AP-1) site in the complementary strand. Interference was observed in the binding of CRE or GRE with nuclear extracts from cells co-incubated for 3 h with 8Br-cAMP and dex; from cells incubated for 1 h, only the binding to GRE showed interference. Rapid cross-talk of glucocorticoids with PKA signaling pathways regulating TRH transcription constitutes another example of neuroendocrine integration.

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Introduction

Thyrotropin-releasing hormone (TRH), synthesized in the paraventricular nucleus of the hypothalamus (PVN) and released from the median eminence, regulates the pituitary–thyroid axis; other endocrine functions include its effects on the synthesis and release of prolactin (de Greef *et al.* 1987, Segerson *et al.* 1987a, Haisenleder *et al.* 1992, Lechan & Toni 1992, Fink *et al.* 1993). TRH gene expression in the PVN is strictly controlled by the feedback action of thyroid hormones (Koller *et al.* 1987, Segerson *et al.* 1987b, Rondel *et al.* 1988, Hollenberg *et al.* 1995, Satoh *et al.* 1999) and is regulated in multiple physiological conditions including: circadian and estrous cycles (Covarrubias *et al.* 1988, Uribe *et al.* 1991), suckling and weaning (Uribe *et al.* 1993, 1995b, Sánchez *et al.* 2001), cold stress (Zoeller *et al.* 1990, Uribe *et al.* 1993, Sánchez *et al.* 2001) and fasting (Blake *et al.* 1991, Legradi *et al.* 1997a, Fekete *et al.* 2002). In addition, a

fast and transient increase in proTRH mRNA levels in the PVN (maximal at 30–60 min) is produced by cold exposure or suckling (Uribe *et al.* 1993, Sánchez *et al.* 2001), conditions that activate TRH release from the median eminence (Arancibia *et al.* 1983, de Greef *et al.* 1987, Rondel *et al.* 1988).

At the transcriptional level, the negative feedback of thyroid hormones is well characterized (Segerson *et al.* 1987b, Hollenberg *et al.* 1995, Satoh *et al.* 1996, Guissouma *et al.* 2000, Abel *et al.* 2001) and the consensus sequences for thyroid hormone receptor (TR) binding identified in the human (h) (Yamada *et al.* 1990), rat (r) (Lee 1988) and mouse (m) (Abel *et al.* 2001) TRH genes. The sequence between –547 to +84 of rTRH promoter confers almost full basal transcriptional activity in transfected cells (Balkan *et al.* 1998); within this sequence, response elements of the transcriptional factors: activator protein 1 (AP-1), cAMP response element (CRE)-binding protein (CREB), and glucocorticoid

receptors (GR) are present in the three species. Two DNA elements similar to consensus CRE are localized in the rTRH gene (−101/−94 (CRE-2) and −59/−52 (CRE-1)). CRE-1 (TGACCTCA) (also called site 4) binds CREB (Hollenberg *et al.* 1995, Wilber & Xu 1998, Harris *et al.* 2001) and overlaps a thyroid hormone response element (THRE (AGGTCA)), recognized as an important site for thyroid hormone negative feedback in the three species (Hollenberg *et al.* 1995, Satoh *et al.* 1996, 1999). The stimulatory role of CREB on TRH gene transcription has been evidenced in cells transfected with constructs including human or murine TRH promoters (Wilber & Xu 1998, Harris *et al.* 2001); 8-h incubation with α -melanocyte stimulating hormone (α MSH; which increases phosphorylated CREB (P-CREB) (Sakar *et al.* 2002)) enhances TRH transcription (Harris *et al.* 2001). The stimulatory effect of α MSH is reduced in the presence of 3,5,3'-triiodothyronine (T_3) and transfected thyroid hormone receptor TR β 2 (Harris *et al.* 2001).

The effects of glucocorticoids on TRH gene expression are less well understood. A stimulatory effect of long-term treatment (days) with dexamethasone (dex) on proTRH mRNA levels was first described in CA77 cells (Tavianini *et al.* 1989), and later in rat anterior pituitary or in diencephalic primary cultures (Bruhn *et al.* 1994, Luo *et al.* 1995). Primary cultures of hypothalamic cells have a time- and dose-dependent response to dex: a rapid increase of proTRH mRNA levels (1–3 h) occurs at 10^{-6} to 10^{-8} M while levels are diminished at 10^{-10} M or after 24-h incubation at 10^{-8} M (Joseph-Bravo *et al.* 1998, Pérez-Martínez *et al.* 1998). *In vivo*, glucocorticoids have an inhibitory influence (Kakucska *et al.* 1995) suggested to be due to their inhibitory effect on CREB phosphorylation in the PVN (Legradi *et al.* 1997a); upregulation of P-CREB levels follows glucocorticoid depletion (Whitehead & Carter 1997a). Rat TRH promoter lacks a canonical glucocorticoid response element (GRE) but has several copies of the strongest half-site (TGTTCT) (Dahlman-Wright *et al.* 1991) at: −735, −560 or −210, and a near-consensus inverted site at −284 (GGTCCAcacTCT TGT), as well as two other inverted half-sites at −275 and −310 (Lee 1988). Site −210/−205 binds GR (Lee *et al.* 1996). Hela cells transfected with the plasmid containing the −242/+84 sequence of rTRH promoter, or with deletions of a cis-acting element located between −242/−200, proved that this region is important for basal transcription and glucocorticoid response (Lee *et al.* 1996). Pancreatic cells transfected with rTRH promoter fragments −554/+84 or −242/+84, treated for 48 h with dex, showed increased transcriptional activity (Fragner *et al.* 2001). (See Fig. 1 for the position of putative response elements in the rTRH promoter).

Since most of the *in vivo* or *in vitro* studies on TRH gene regulation have dealt with long-term treatments (at

least 6 h) in various cell types, the role of the regulatory elements involved in a fast neuronal response has yet to be characterized. We have previously shown that proTRH mRNA levels are rapidly increased (1–3 h) in rat fetal hypothalamic primary cell cultures by PKA activators or by 10^{-8} M dex; 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increases proTRH mRNA levels only after 2 h (Uribe *et al.* 1995a). However, co-incubation of 8Br-cAMP with either dex or TPA reduces their stimulatory effect while the effect of dex with TPA is additive (Pérez-Martínez *et al.* 1998). The aim of this study was to elucidate if the cross-talk between glucocorticoids and the PKA pathway occurred at the transcriptional level and to determine the possible involvement of the postulated CRE and GRE elements of the proTRH promoter. We report that PKA and GR signaling pathways rapidly regulated TRH gene transcription and that noradrenaline (NA), a mediator of cold stimulus (Arancibia *et al.* 1989), mimicked cAMP effects. Characterization of the elements that could mediate these effects demonstrated that CRE-2 (−101/−94) could be an important target for TRH transcriptional regulation; this is also the case for GRE (−210/−205), which binds GR and has the characteristics of a composite GRE (Miner & Yamamoto 1992, Harrison *et al.* 1995, Malkoski & Dorin 1999).

Materials and methods

Animals

Wistar rats raised in the local vivarium were kept in a ratio of 12 h light:12 h darkness and were allowed to feed *ad libitum*. Care was taken to preserve adequate conditions following the FRAME guidelines and as approved by the institute's ethical commission.

Reagents

Poly-D-lysine, dex, 8Br-cAMP, TPA, the PKA inhibitor H89 and its inactive form H85, dithiothreitol, glucose, glutamine, cytosine arabinofuranoside and DNase were from Sigma. Poly (dI-dC), dNTPs and luciferase assay kit were from Roche; thyroxine (T_4) polynucleotide kinase was from Promega. Dulbecco's modified Eagle's medium (DMEM), Hank's medium, trypsin, fetal bovine serum, vitamins and antibiotic-antimycotic were from GIBCO; guanidinium thiocyanate was from Fluka Steinheim (Sigma) and [32 P]ATP from NEN Life Science Products (Boston, MA, USA). Expression vectors for CREB (SV-CREB) (González *et al.* 1989), AP-1 (c-Jun (RSV-cJun), c-Fos (RSV-cFos)) (Hirai *et al.* 1990, Schontal *et al.* 1988) and GR (CEO-GR) (Meyer *et al.* 1989) were a kind gift from Dr Gustavo Pedraza-Alva, IBT-UNAM; TRH-Luc plasmid was a gift from Dr Wayne Balkan, University of Miami School

of Medicine, Miami, FL, USA. Oligonucleotides with the consensus and mutant CRE and AP-1 sequences and antibodies used in supershift analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other oligonucleotides described in this work were synthesized at the institute.

Cell culture

Primary cultures of hypothalamic cells were performed as described previously (Joseph-Bravo *et al.* 2002) using embryos from anesthetized pregnant Wistar rats on the 17th day of gestation. Cells were plated on 1.5 µg/ml poly-D-lysine pre-coated 35 mm plates (2.7×10^6 cells for TRH release studies, electrophoretic mobility shift assays (EMSAs) and transient transfection assays); for proTRH mRNA quantification, 0.6×10^6 cells were plated in 16 mm plates. DMEM was supplemented with 10% fetal calf serum (FCS), 0.25% glucose, 2 mM glutamine, 3.3 µg/ml insulin, 1% antibiotic-antimycotic and 1% vitamin solution (S-DMEM). Cultures were maintained at 37 °C with 7% CO₂, 93% air atmosphere and 90% humidity. On the fourth day *in vitro* (DIV), cytosine arabinofuranoside (10^{-5} M) was added and maintained for 48 h to inhibit cell proliferation; afterwards half the incubation medium was replaced every second day with fresh S-DMEM (Charli *et al.* 1995). When drugs were dissolved in dimethyl sulfoxide (DMSO) (dex or TPA), an equivalent DMSO concentration was added to controls.

NIH-3T3 cells were maintained in DMEM supplemented with 10% FCS at 37 °C with 7% CO₂, 93% air atmosphere, and 90% humidity.

The SH-SY5Y (ATCC CRL-2266) neuroblastoma cell line was kindly donated by Dr Rosa Ma Pardós (Instituto de Fisiología, Benemérita Universidad Autónoma de Puebla, México). Cells were grown in 125 ml flasks to confluency in S-DMEM, then rinsed with Hank's medium, trypsinized (4 min, with 5 ml of trypsin), centrifuged (5 min) and the pellet was resuspended in S-DMEM. For experiments with undifferentiated cells, 7×10^6 cells were seeded in 60 mm plates and incubated for 24 h. Differentiated cells were obtained by seeding 5×10^6 cells in 60 mm plates, incubating them for 24 h and replacing the medium with S-DMEM containing 5% FCS and 1.6×10^{-9} M TPA; half the medium was replaced every 2 days for 8 days (Renauld & Spengler 2002).

Transient transfection and luciferase assays

Hypothalamic cultures from 12 DIV were transiently transfected using polyethylenimine (Guerra-Crespo *et al.* 2003). Transfections were carried out in 35 mm dishes with 2.7×10^6 hypothalamic cells, 5 µg of a reporter

plasmid containing rat TRH promoter (−776/+85) linked to the luciferase reporter (TRH-Luc) and 5 µg RSV-β-gal (Gynheung *et al.* 1982) used as internal control of transfection efficiency. Cells were cultured for 48 h in S-DMEM and then harvested for luciferase activity (following kit instructions) and β-galactosidase activity (Lucibello *et al.* 1990). Luciferase values were normalized to values obtained for β-galactosidase activity and protein content; data are expressed relative to luciferase activity.

NIH-3T3 cells were plated in 35 mm dishes and grown for 20–24 h to a confluency of around 60%. The cells were then transfected with polyethylenimine. Cotransfection was performed with 1 µg transcription factor expression vector(s) together with 5 µg reporter plasmid (TRH-Luc), 3 µg pRSV-β-gal and PUC18 to a total of 10 µg plasmid DNA per dish. Cells were cultured for 48 h in DMEM with 10% FCS; then treated for 3 h with 8Br-cAMP, TPA or dex and harvested for luciferase activity. Data are expressed as relative luciferase activity with respect to cells transfected only with TRH-Luc. For EMSAs, NIH-3T3 cells were transfected with 1 µg of the expression vector (CREB, AP-1 or GR) together with 3 µg pRSV-β-gal and PUC18 to a total of 10 µg plasmid DNA per dish.

Determination of TRH content and release by RIA

Hypothalamic primary cells (18 DIV) were incubated with drugs diluted in DMEM at concentrations and times stated in the figures. The medium was collected in a tube and immediately frozen; cells were stored at −20 °C. TRH cell content was extracted as described previously (Joseph-Bravo *et al.* 2002). It was not possible to measure TRH directly in the culture medium due to interference in the RIA; therefore, the medium was thawed and extracted with C-18 cartridges (Sep-pak, Millipore) activated according to the manufacturer's specifications; TRH was eluted with 60% methanol after washing with 2 ml water. The methanol extract was evaporated and the residue was resuspended in 250 µl 0.05 M phosphate buffer, pH 7.5, 0.25% BSA and immunoreactive TRH measured in duplicate by a specific RIA (Charli *et al.* 1995); Sep-pak-extracted medium included in the standard curve did not interfere with the RIA; recovery of 100 pg TRH (Peninsula, Belmont, CA, USA) added before extraction was >90% (not shown).

mRNA purification

Total RNA was extracted from frozen cells scraped with 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol as described previously (Pérez-Martínez *et al.* 1998).

Figure 1 Regulatory elements of the rat TRH promoter region and oligonucleotides used in the EMSA. (A) Nucleotide positions are relative to transcription start site; potential regulatory elements are shown in boxes. Two putative overlapping AP-1 and cAMP response elements (CRE) are located from nucleotide position -59 to -52 (CRE-1) and from -101 to -94 (CRE-2). A half-palindrome sequence for THRE overlaps the CRE/AP-1 element at position -59/-52. The GRE half-palindrome is located from -210 to -205. (B) Sequences utilized as probes for EMSA analysis. CRE-L, CRE-1 and CRE-2 with CRE sites in bold. GRE site (shown in bold) and in the complementary strand, two AP-1 sites (bold and underlined).

ProTRH mRNA semi-quantification by RT-PCR

Reaction conditions for proTRH mRNA quantification were as previously reported (Perez-Martinez *et al.* 1998) except that 11 pmol proTRH and 25 pmol glyceraldehyde-3-phosphate dehydrogenase (G3 PDH) primers were used for co-amplification (30 cycles). Gels were analysed by laser densitometry to calculate the relative amounts of proTRH vs G3 PDH cDNAs.

Nuclear extract preparation

Hypothalamic cells (14 DIV), transfected NIH-3T3 cells or SH-SY5Y neuroblastoma cells were washed with PBS before obtaining nuclear extracts, as described previously (Schreiber *et al.* 1989) but with the following modifications: cells were lysed by 5 min incubation at 4 °C in hypotonic buffer (10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 1 µg/ml leupeptin, 0.5 mM phenylmeth-

ylsulfonyl fluoride and 0.6% NP-40). Intact nuclei were washed with lysis buffer and nuclear extracts were obtained by incubating the nuclei in extraction buffer (20 mM Tris-HCl, pH 8.0, 450 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 1 µg/ml leupeptin, 5 mM spermidine and 25% glycerol) for 45 min under constant mild agitation at 4 °C. DNA was eliminated by centrifugation for 15 min at 13 000 *g*. Protein content was determined by the Bradford assay (Bradford 1976).

Electrophoretic mobility shift assay (EMSA)

The double-stranded rat TRH promoter oligonucleotides used in EMSAs were as follows. CRE-L, containing the two potential CRE elements of the TRH promoter (-101 to -94 and -59 to -52); CRE-1, -59/-52; CRE-2, -101/-94. For GRE: GRE-L, -220/-193; GRE-A, -215/-193. The sequences of double-stranded oligonucleotides used in EMSAs are

depicted in Fig. 1. Other probes used were: CRE consensus, 5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3'; CRE mutant, 5'-AGA GAT TGC CTG **TGG** TCA GAG AGC TAG-3'; GR consensus, 5'-GAC CCT AGA GGA TCT GTA CAG GAT GTT CTA GAT-3'; GR mutant, 5'- GAC CCT AGA GGA TCT **CAA** CAG GAT GTT CTA GAT-3'; STAT, 5'-CTC CTA TTG GCT TGA-3' (mutated bases in bold). EMSAs were conducted as described previously (Pedraza-Alva *et al.* 1994). Briefly, oligonucleotides were end-labeled with T4 polynucleotide kinase using 30 μ Ci [γ - 32 P]ATP/100 ng of oligonucleotide. Nuclear extracts (10 μ g) were incubated for 20 min at room temperature with the labeled oligonucleotide (1×10^5 c.p.m.) in band shift buffer (25 mM Hepes, pH 7.9, 40 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol and 10% glycerol) containing 1 μ g poly(dI-dC) as a non-specific competitor. DNA-protein complexes were resolved by electrophoresis on non-denaturing 6% polyacrylamide gels for 2–3 h at 150 V (50 mM Tris-HCl, 45 mM boric acid, 0.5 mM EDTA). They were analyzed either directly with a phosphor-imager (Molecular Dynamics, Piscataway, NJ, USA) or by film autoradiography (Fluor-S MultiImager; BioRad, Hercules, CA, USA).

For competition experiments, 10- to 100-fold molar excess of unlabeled oligonucleotide was added 5 min before adding the labeled probe. For immune band shift assays, 1 μ g antibody was incubated with nuclear extracts (5 h at 4 °C) and then with labeled oligonucleotide. The same amount of an irrelevant antibody (rabbit IgG) or normal rabbit serum (NRS) was used as control. DNA-protein complexes were resolved by electrophoresis on 5% polyacrylamide gels.

Statistical analysis

Results were calculated as percentages of controls of each culture; data were then calculated as the means $\pm$ S.E.M. Data were analyzed by ANOVA, considered significant at $P < 0.001$, followed by Fisher's PLSD test (P values stated in each figure).

Results

Involvement of PKA in regulating proTRH mRNA levels

To verify if the upregulation of proTRH mRNA levels by 8Br-cAMP (Pérez-Martínez *et al.* 1998) was due to PKA activation, we studied the effect of inhibiting PKA by preincubating (30 min) hypothalamic cells with 50 nM H89. As shown in Fig. 2, 1 mM 8Br-cAMP increased proTRH mRNA levels and this effect was avoided with H89; the presence of H89 revealed a dex stimulatory effect. As previously shown (Pérez-Martínez

Figure 2 PKA mediated 8Br-cAMP upregulation of proTRH mRNA levels in hypothalamic cells. Primary cultures of fetal hypothalami were preincubated at 14 DIV with 50 nM H89 for 30 min, followed by 1 h incubation with 1 mM 8Br-cAMP (A), with or without 10 nM dex (D). Total RNA was extracted and the levels of proTRH mRNA semi-quantified by RT-PCR. Values are expressed as the ratio of TRH/G3 PDH cDNA signal as a percentage of controls (100%). $n = 15$; * $P < 0.001$ vs control; &, $P < 0.001$ between groups, by Fisher's PLSD).

et al. 1998), 10 nM dex reduced the stimulation caused by PKA activation (Fig. 2).

Rapid modulation of TRH transcription

Hypothalamic cells from E17 rat embryos were transiently transfected with TRH-Luc (Balkan *et al.* 1998) to study whether the upregulation of proTRH mRNA levels by 8Br-cAMP, TPA or dex (Pérez-Martínez *et al.* 1998) occurred at the transcriptional level. Luciferase activity was increased after treatment with 8Br-cAMP when compared with untreated cells but the increase by dex was not significant; co-treatment with 8Br-cAMP and dex resulted in a reduction of the 8Br-cAMP-induced luciferase activity (Fig. 3A). These stimulatory and interference effects coincided with the reported variations in proTRH mRNA levels indicative of transcriptional effects. In contrast, although TPA increases proTRH mRNA levels after 2–3 h incubation and co-incubation with dex has additive effects (Uribe *et al.* 1995a, Pérez-Martínez *et al.* 1998), this was not significant in luciferase activity (Fig. 3A). The short time after which luciferase activity was assayed (3 h) could be insufficient for adequate c-Fos expression.

Figure 3 TRH promoter activity was regulated by different signaling pathways. (A) Cultured hypothalamic cells (12 DIV) were transiently transfected with 5 μ g reporter plasmid (TRH-Luc) and RSV- β -gal as described in Materials and methods; 48 h after transfection, cells were stimulated with 1 mM 8Br-cAMP, 100 nM TPA, 10 nM dex, alone or in combination. Total cell extracts were prepared 3 h after treatment and luciferase activity determined. Luciferase values were normalized to those obtained for β -galactosidase activity and protein content; results are plotted as a percentage of control cultures. Data are from six independent experiments with duplicate plates in each ($n=6$). * $P<0.01$ vs control; &, $P<0.01$ between groups. (B) NIH-3T3 cells transfected with TRH-Luc, RSV- β -gal (control) and with plasmids expressing CREB, GR, or c-Fos and c-Jun, alone or combined; 48 h after transfection cells were stimulated with 8Br-cAMP (1 mM), TPA (100 nM), or dex (10 nM), alone or in combination according to the cotransfected plasmid. Total cell extracts were prepared and luciferase activity determined as in panel A. Results are the mean of luciferase values normalized against β -galactosidase activity and calculated as a percentage of controls of three independent experiments ($n=6$; * $P<0.0001$ vs control; &, $P<0.0001$ between groups; &&, $P<0.05$ between groups).

To determine whether transcription factors activated by PKA, PKC or glucocorticoid pathways can regulate the TRH promoter (−776/+85), a heterologous system was used. NIH-3T3 cells were cotransfected with TRH-Luc and expression vectors for either CREB, AP-1 (c-Fos and c-Jun), or GR and later incubated for 3 h with 8Br-cAMP, TPA or dex respectively. Some cultures were cotransfected combining CREB and AP-1 or GR vectors and stimulated with 8Br-cAMP+TPA, 8Br-cAMP+dex, or TPA+dex, according to the combinations of vectors transfected. CREB expression and 3 h of

stimulation with 8Br-cAMP increased luciferase activity compared with unstimulated cultures transfected only with TRH-Luc (Fig. 3B). Luciferase activity was reduced when CREB was co-expressed with either GR or AP-1 and incubated with 8Br-cAMP+dex or 8Br-cAMP+TPA respectively (Fig. 3B). Therefore, the interference between CREB and GR or AP-1 pathways occurred at the transcriptional level, independent of the cellular context. Expression of the GR or AP-1 and 3 h stimulation with either dex or TPA augmented luciferase activity only slightly compared with controls (Fig. 3B).

TRH content and release in hypothalamic cells

Figure 4 Effect of dex and PKA or PKC activators on TRH content and release from hypothalamic cells. Hypothalamic cells (18 DIV) were incubated for 1 or 2 h with 8Br-cAMP (1 mM) or dex (10 nM), alone or in combination. Cells were extracted with 0.1 M acetic acid-methanol 60%, centrifuged and the supernatant evaporated and resuspended in RIA buffer for TRH quantification. The medium was extracted through Sep-pak cartridges and TRH quantified by RIA. Results are expressed as a percentage of controls of each culture. TRH cell content of controls from a representative culture: 257 ± 19 pg; TRH in medium of controls: 31 ± 3 pg ($n=16$; $*P < 0.05$ vs controls).

These effects were inhibited when GR and AP-1 were co-expressed in NIH-3T3 cells and stimulated simultaneously, in contrast to the effects of dex+TPA observed in hypothalamic cell cultures. Interference between GR and AP-1 appeared to be cell specific as previously reported, in particular for NIH-3T3 cells (Maroder *et al.* 1993).

Regulation of TRH release and content in hypothalamic cultures

Rapid effects of glucocorticoids can occur at the membrane level affecting intracellular calcium or protein kinases that can alter, for example, vasopressin release (Makara & Haller 2001, Chen & Qiu 1999). We studied if the release of TRH was regulated in hypothalamic cells treated for 1–2 h with dex or 8Br-cAMP, alone or combined. Increased release was detected after 1 h incubation with 8Br-cAMP and was still evident at 2 h but TRH cell content was augmented only after 2 h incubation (Fig. 4). 8Br-cAMP produced a higher response than dex in TRH release and content, coincident with the faster and higher increase in proTRH mRNA levels (Uribe *et al.* 1995a, Perez-Martinez *et al.* 1998). Longer times were not studied since it would be difficult to discern a higher basal release due to higher peptide cell content, from a direct effect on regulated exocytosis (Luo *et al.* 1995, Nillni *et al.* 2000). TRH content was increased by 8Br-cAMP and dex but not with combined treatment (Fig. 4B), as pre-

viously shown for proTRH mRNA levels (Pérez-Martínez *et al.* 1998). These results indicate a role for cAMP in modifying TRH release, besides its effect on biosynthesis. Changes in cell content reflected the effects found at transcription level: dex increased TRH cell content at 2 h and impeded the stimulatory effect of 8Br-cAMP. The lack of decreased content supported the assumption that, at the concentrations used, no cytotoxicity occurred.

NA mimicked cAMP effects

Fast modulation of TRH gene expression in the PVN has been observed *in vivo* when rats were exposed to cold ambient temperature (Uribe *et al.* 1993, Sánchez *et al.* 2001), a condition in which NA is a neurotransmitter candidate in the afferent pathway (Arancibia *et al.* 1989, Lechan & Toni 1992). Since PKA activation is one of the main intracellular pathways activated by catecholamines, we tested the effect of NA on TRH content and release, and proTRH mRNA levels in hypothalamic cell cultures. A 1-h incubation with 10 nM NA increased TRH release without affecting cell content (Fig. 5A). A fast and transient increase in proTRH mRNA levels was observed from 30 min, was highest at 60 min and decreased after 120 min incubation with 10 nM NA (Fig. 5B). NA at 1 or 100 nM increased proTRH mRNA levels to 180 ± 15 or $191 \pm 17\%$ respectively (controls = $100 \pm 5\%$; $n=6$, $P < 0.01$) after 1 h incubation. Pretreatment with H89 abrogated the stimulatory effect of NA while staurosporine did not (Fig. 5C). As observed for a cAMP analog (Pérez-Martínez *et al.* 1998), the NA-induced increase in proTRH mRNA levels was higher than that caused by dex and co-incubation of NA with dex diminished the response to NA (Fig. 5D and E). These results show that NA, a known physiological modulator, regulated TRH expression in a similar way to that observed with 8Br-cAMP in primary cultures of fetal hypothalamus.

Binding of nuclear extracts from hypothalamic cells to DNA sequences containing CRE and GRE sites

Binding to CRE

To study if the interference of CREB with GR or AP-1 elements was due to their binding to DNA cognate sequences, we first analyzed if the proposed elements in the TRH promoter, involved in CREB or GR recognition, would bind nuclear proteins extracted from cells of fetal primary hypothalamic cultures incubated with dex or drugs that activate PKA or PKC. Cultures were treated at 14 DIV since at this time they have maximal TRH mRNA levels (Pérez-Martínez *et al.* 2001). Putative DNA binding sites for CREB, GR, and AP-1 in the rTRH promoter region are shown in

Figure 5 Effect of NA on TRH metabolism. (A) Hypothalamic cells (14 DIV) were incubated with, or without, 10 nM NA (and 10 μ M ascorbic acid, present also in controls) for 1 h and TRH measured in the medium or cell content (as in Fig. 4); data are expressed as a percentage of control ($n=6$), $*P<0.01$. (B) Cells were incubated with 10 nM NA for 30, 60 or 120 min; they were rinsed, total RNA extracted, and proTRH mRNA levels measured by semi-quantitative RT-PCR. Values are expressed as a percentage of controls ($n=8$), $*P<0.01$. (C) Cells were preincubated for 30 min with H89 (50 nM) followed by 1 h with 10 nM NA or 10 nM staurosporine (Stau); proTRH mRNA levels were measured as in panel B ($n=6$), $*P<0.05$. (D) Representative gel of RT-PCR products (of samples from panel E). (E) Cells were treated for 1 h with 10 nM NA or 1 nM dex, alone or combined; proTRH mRNA levels were measured as in panel B ($n=15$), $*P<0.01$ vs control.

Fig. 1. Two sites have been described as possible CREs: CRE-2 at $-101/-94$ and CRE-1 at $-59/-52$; as mentioned, CRE-1 (site 4) has received exclusive attention as a target for CREB, AP-1 and TR. Since CREB phosphorylation increases DNA binding in canonical sequences and particularly in non-canonical sites (Benbrook & Jones 1994, Bullock & Habener 1998), we analyzed by EMSA whether nuclear extracts from hypothalamic cells treated with 8Br-cAMP had enhanced DNA-binding activity to the putative CRE elements located on the TRH promoter. Using CRE-1, two faint bands were observed with nuclear extracts from hypothalamic cells; they did not differ in intensity if

the nuclear extracts were obtained from cells incubated (1–3 h) with 8Br-cAMP, TPA or dex (representative gel of some groups, in Fig. 7A).

To avoid the possibility of unidentified transcription factors binding to sequences adjacent to CRE elements (Dan *et al.* 1999), the formation of DNA–protein complexes was studied with an oligonucleotide comprising the regions including CRE-2 and CRE-1 (CRE-L) (Fig. 1). Increased binding was observed with CRE-L and nuclear extracts from hypothalamic cells incubated for 1–3 h with 8Br-cAMP (Fig. 6A). Binding was prevented if cells were preincubated for 30 min with H89 while preincubation with H85 (inactive form of

Figure 6 Binding activity to CRE-L induced by 8Br-cAMP involved CREB protein. (A) Nuclear extracts from hypothalamic cells (14 DIV) treated with 1 mM 8Br-cAMP for 1–3 h were incubated with 32 P-labeled CRE-L (see Fig. 1). Some cells were preincubated with H89 or H85 for 30 min followed by 3 h with 8Br-cAMP. (B) As in panel A; DNA competition experiments were performed with: 10- or 100-fold of non-radioactive CRE-L; 100-, 10- or 1-fold of consensus CRE (cs); and mutated CRE (mt) (for sequences see Materials and methods). (C) AP-1 and CREB are able to bind independently to CRE-L. NIH-3T3 cells were transfected with either expression vector for β -galactosidase (β -gal), CREB, or c-Fos and c-Jun; 48 h after transfection nuclear extracts were prepared and tested by EMSA using the CRE-L element. Arrows indicate the position of the major DNA–protein complexes. (D) TPA and dex signaling pathways reduce 8Br-cAMP-induced binding to CRE-L. Nuclear extracts from 14-DIV hypothalamic cells treated for 3 h with: T, TPA; A, 8Br-cAMP; or D, dex; alone or combined; C, unstimulated cells. Data shown are representative of at least three independent experiments. (E) Protein–CRE-L complexes induced by 8Br-cAMP contain phospho-CREB. Nuclear extracts from 8Br-cAMP-treated hypothalamic cultures (3 h) were incubated with anti P-CREB before (a) or after (b, 1 μ g; c, 0.5 μ g) adding CRE-L, or with an irrelevant antibody (IgG) added after nuclear extracts were incubated with CRE-L. SS, the supershifted complex.

H89; Oki *et al.* 2000) had no effect (Fig. 6A), coincident with various reports that, in particular for non-canonical consensus sequences, phosphorylation induced by PKA stimulation increases binding to CRE (Bullock & Habener 1998). Binding to CRE-L was abolished by competing with a 100 $\times$ molar excess of cold CRE-L or consensus CRE; 100 $\times$ molar excess of mutated CRE had no effect on binding to CRE-L (Fig. 6B). To determine if AP-1 and CREB were able to bind independently to CRE-L, NIH-3T3 cells were trans-

fected with either CREB or c-Fos and c-Jun expression vectors; binding to CRE-L was observed with nuclear extracts from cells transfected with vectors for CREB, c-Fos and c-Jun while not with nuclear extracts from cells transfected with an expression vector for β -galactosidase (Fig. 6C). Primary cultures of hypothalamic cells were incubated for 3 h with dex, 8Br-cAMP or TPA, alone or combined, and their nuclei extracted to determine their ability to bind CRE-L. Binding was increased with nuclear extracts from dex- or

Figure 7 Binding to CRE elements. (A) Nuclear extracts from hypothalamic cells (14 DIV) incubated with: A, 1 mM 8Br-cAMP for 1 or 2 h; and A+D, 8Br-cAMP+10 nM dex for 1 h. They were tested for their ability to bind CRE-1; the two right-hand lanes contain nuclear extracts from controls (indicated by letter C) and 1 h incubation with 8Br-cAMP bound to CRE-2 (letter A). (B) Cells (14 DIV) were incubated for 1 or 3 h with 8Br-cAMP (1 mM), dex (10 nM), alone or combined; nuclear extracts were incubated with CRE-2 and the intensity of bands measured by densitometric analysis. Values are expressed as a percentage of control and are the mean of four independent experiments performed in duplicate ($n=8$ for 1-h incubation; $n=6$ for 3-h incubation; * $P<0.01$ vs control; & $P<0.01$ between groups). (C) Representative gel of nuclear extracts bound to CRE-2 (3-h incubations): C, control; A, 8Br-cAMP; D, dex. (D) As in panel C but 1-h incubation; the two right-hand lanes correspond to nuclear extracts from differentiated SH-SY5Y cells incubated with 8Br-cAMP (as described in Fig. 10). (E) Nuclear extracts of 14-DIV hypothalamic cells incubated for 1 h with 10 nM NA or 10 nM dex, alone or combined, were tested for their ability to bind CRE-2; D+St, nuclear extracts from cells preincubated with 10 nM staurosporine and then for 1 h with 10 nM dex.

TPA-incubated cells (albeit in less magnitude to that seen with 8Br-cAMP) (Fig. 6D). Compared with the intensity observed with nuclear extracts from 8Br-cAMP-stimulated cells, co-incubation of 8Br-cAMP with dex or TPA produced a less intense signal (Fig. 6D). Addition of P-CREB antibody to nuclear extracts from 8Br-cAMP-treated cells resulted in a supershifted complex (Fig. 6E), while the addition of an irrelevant antibody had no effect in the DNA-protein complex.

Thus, in hypothalamic cells, 8Br-cAMP induced P-CREB binding to an oligonucleotide containing both CRE-like elements, and binding to CRE-L was increased mainly with nuclear extracts from 8Br-cAMP and, to a lesser extent, with nuclear extracts from TPA-

or dex-treated cells. Combined treatments interfered with the 8Br-cAMP signal. To verify if the second CRE site -101/-94 was responsible for the increased binding, we used an oligonucleotide comprising the sequence -106/-89 (CRE-2). Hypothalamic cells were incubated for 1-3 h with 8Br-cAMP or dex, alone or combined. Densitometric analysis was performed in each gel and cultures were repeated at least three times with each condition in duplicate plates. Controls were taken as 100% for each culture and the mean of at least six values (two plates $\times$ three cultures) calculated. In contrast to the lack of increased binding to CRE-1 with nuclear extracts of 8Br-cAMP-incubated cells compared with non-treated cells, the same nuclear extracts showed

Figure 8 Binding to GRE-L in response to dex. (A) EMSA was performed using 32 P-labeled double-stranded GRE-L (see Fig. 2) and nuclear extracts from 14-DIV hypothalamic cells: C, untreated; D, treated for 3 h with 10 nM dex; p, probe with no nuclear extracts. DNA competition experiments were performed with 1-, 10- and 100-fold excess of non-radioactive GRE-L oligonucleotide: consensus GRE (cs), mutated GRE (mt) or STAT. (B) GR binds to GRE-L. NIH-3T3 cells were transfected with the expression vector for β -galactosidase (β -gal) or GR; 48 h after transfection nuclear extracts were prepared and tested by EMSA using GRE-L. (C) Interference between 8Br-cAMP and TPA signaling pathways with dex-induced binding to GRE-L. Hypothalamic cells (14 DIV) were treated with dex for 1–3 h (D) and TPA (T) or 8Br-cAMP (A) ($\pm$ dex) for 3 h. Nuclear extracts were incubated with 32 P-labeled GRE-L and subjected to EMSA; C, extracts from unstimulated cells indicate the basal binding activity.

higher DNA binding activity with CRE-2 (Fig. 7A and C). Two distinctive bands were observed, both increased in nuclear extracts from 3-h incubates with 8Br-cAMP, and only the lower mobility band (band a) was increased in nuclear extracts of cells treated with dex; co-incubation of dex+8Br-cAMP decreased intensity of band a (Fig. 7B and C). Nuclear extracts from cells incubated for 1 h either with 8Br-cAMP or dex, alone or combined, showed band b more intense than that observed in nuclear extracts from 3-h incubates. Both bands showed similar increments upon treatment but the analysis of several cultures showed no significant difference between them (Fig. 7A and D). As observed for CRE-L, preincubation with H89 avoided the effect of 8Br-cAMP (densitometric analysis of band a as a percentage of control ($100 \pm 12\%$): 8Br-cAMP, $279 \pm 54\%^*$; H89+8Br-cAMP, $112 \pm 9\%$; $*P < 0.01$ vs controls, $n=6$). Nuclear extracts from cells incubated with NA for 1 h gave similar complexes to those observed with 8 Br-cAMP and co-incubation with dex diminished binding; the increased binding to CRE-2 by dex was not altered by preincubation with staurosporine (Fig. 7E).

Binding to GRE

As mentioned, the GRE half-site located at $-210/-205$ confers dex response in transfected cells (Lee *et al.* 1996). However, adjacent to this site in the complementary strand are two sequences similar to the AP-1 response element (Fig. 1); this arrangement has been observed for corticotropin-releasing hormone (CRH) and other genes, and is referred to as composite GRE (Miner & Yamamoto 1992, Harrison *et al.* 1995, Malkoski & Dorin 1999). We tested the ability of nuclear extracts from dex-treated hypothalamic cells (3 h) to bind to the oligonucleotide sequence $-220/-193$ (GRE-L); two bands were observed with the upper one (lower mobility shown by letter a in Fig. 8A) increasing upon dex treatment (Fig. 8A). Competition assays with up to 100-fold excess of GRE-L or a consensus GRE completely abolished DNA binding while no competition was observed when the same amount of mutated GRE or STAT oligonucleotide was added (Fig. 8A). GR expressed in NIH-3T3 cells bound to GRE-L; no binding was detected with nuclear extracts

Binding to GRE-A Hypothalamic cells

Figure 9 Binding to GRE-A. (A) Hypothalamic cells (14 DIV) were incubated for 1 or 3 h with 1 mM 8Br-cAMP and 10 nM dex, alone or combined; nuclear extracts were incubated with GRE-A and the intensity of bands was measured by densitometric analysis. Values are expressed as a percentage of control and are the mean of four independent experiments performed in duplicate ($n=8$ for 1-h incubation; $n=6$ for 3-h incubation; $*P<0.01$ vs controls). (B) Representative gel of nuclear extracts bound to GRE-A (3-h incubations): C, control; A, 8Br-cAMP; D, dex; T, TPA. (C) As in panel B but for a 1-h incubation. (D) Nuclear extracts of 14-DIV hypothalamic cells incubated for 1 h with 10 nM NA and 10 nM dex, alone or combined, were tested for their ability to bind GRE-A; D+St, nuclear extracts from cells preincubated with 10 nM staurosporine and then for 1 h with 10 nM dex.

from cells transfected with RSV- β -gal (Fig. 8B). Binding was increased with nuclear extracts from hypothalamic cells incubated with dex (1–3 h); TPA stimulation (3 h) increased the intensity of the faster mobility complex (indicated by letter b; Fig. 8C). Co-incubation of dex with 8Br-cAMP diminished binding compared with nuclear extracts from cells incubated only with dex; a slight decrease was observed in the signal of nuclear extracts from cells co-incubated with dex+TPA (Fig. 8C).

To further characterize the TRH composite GRE (cGRE), another oligonucleotide for GRE was synthesized excluding the first four bases of GRE-L to avoid the second AP-1-like sequence; this shorter oligonucleotide was named GRE-A (Fig. 1). Binding to GRE-A was studied in the same nuclear extracts used

for CRE-2. Only one band was observed with GRE-A whether cells were incubated for 1 or 3 h with the drugs and the highest increase was upon dex treatment (Fig. 9); this band had a slower mobility than band b of gels with CRE-2 (not shown). Highest binding was observed with nuclear extracts of cells treated for 3 h with dex (alone or combined with TPA) followed by the signal of 8Br-cAMP-treated cells (Fig. 9A and B); co-incubation with dex and 8Br-cAMP diminished the signal observed after dex treatment. Nuclear extracts from cells stimulated with dex for 1 h also showed increased binding to GRE-A which was avoided when cells were co-incubated with 8Br-cAMP (Fig. 9A and C). The PKA inhibitor H89 diminished (although not statistically significantly) the effect of dex on GRE-A binding (controls, $100 \pm 17\%$; dex, $321 \pm 67\%*$;

SH-SY5Y cells

Figure 10 Binding of nuclear extracts of SH-SY5Y cells to CRE-2 and GRE-A. (A) SH-SY5Y cells (TPA-differentiated for 8 days) were incubated for 1 h with 1 mM 8Br-cAMP and 10 nM dex, alone or combined; nuclear extracts were incubated with CRE-2 and GRE-A and the intensity of bands measured by densitometric analysis. Values are expressed as a percentage of control and are the mean of seven independent experiments performed in duplicate; * $P < 0.01$ vs control. (B) CRE-2 bound with nuclear extracts from undifferentiated cells incubated with: A, 1 mM 8Br-cAMP for 0.5–3 h; or 10 nM NA for 0.5–1 h. (C) Representative gel of protein complexes bound to CRE-2 with nuclear extracts of differentiated cells treated with: A, 1 mM 8Br-cAMP; D, 10 nM dex; or P, 10 nM progesterone. (D) GRE-A bound to nuclear extracts as described in panel C.

H89+dex, $197 \pm 73\%$; * $P < 0.01$, $n = 4$). Nuclear extracts from NA-stimulated cells increased binding to GRE-A which was strongly diminished when cells were co-incubated with NA and dex; staurosporine pretreatment had no effect on the increased binding due to dex (Fig. 9D).

The increased binding of nuclear extracts treated with TPA or 8Br-cAMP with GRE-L or GRE-A, compared with controls, supports this region as a composite GRE

Neuroblastoma SH-SY5Y cells

Primary cultures of hypothalamic cells constitute a heterogeneous population of neurons and glia from different hypothalamic nuclei expressing several neuro-modulators. EMSA results are therefore due to the mean response of these cells. A neuronal cell line was used to

determine if events identified in the mixed background are likely to operate in a neuronal population; also, to verify if the interferences observed are due to transcription factors, exclusive of hypothalamic cells. We studied the binding of nuclear proteins extracted from neuroblastoma SH-SY5Y cells since they respond to adrenergic signals, kinases activation and glucocorticoids (Tank & Weiner 1992, Watters *et al.* 1997, Glick *et al.* 2000). Comparison was made between undifferentiated and differentiated cells. Nuclear extracts from undifferentiated cells treated with 8Br-cAMP or dex showed increased binding to CRE-2 and GRE-A but to a lesser extent than that observed from differentiated cells (not shown). We therefore used differentiated cells and results are summarized in Fig. 10A. As with hypothalamic cells, a faint band was detected with CRE-1 that did not differ between cell treatments (not shown). Increased binding to CRE-2 was observed after 30 min with nuclear extracts from cells incubated with 8Br-cAMP or NA

Figure 11 Competition for binding to short oligonucleotides. Nuclear extracts from 14-DIV hypothalamic cells treated for 1 h with 1 mM 8Br-cAMP or 10 nM NA were tested for binding to CRE-2 (A) or GRE-A (B). (A) Nuclear extracts preincubated without competitor or with 100 ng CRE-2, consensus CRE (CRE cs), CRE-1 or GRE-A, and then with labeled CRE-2. (B) Nuclear extracts preincubated with 100-fold cold GRE-A, consensus GRE (GRE cs), CRE-2 or CRE-1, and then incubated with labeled GRE-A. (C) Nuclear extracts from differentiated SH-SY5Y cells treated with 1 mM 8Br-cAMP (A), 10 nM dex (D), alone or combined (AD), were preincubated with 100-fold of either CRE-2, mutated CRE (CRE mt), CRE-1, CRE cs or GRE-A and then incubated with labeled CRE-2. (D) As in panel C, but incubated with labeled GRE-A and with GRE-A, consensus GRE, CRE-2 or CRE-1 as competitors.

(Fig. 10B). If gels ran for longer times, two bands were detected using CRE-2, with band b being stronger (Fig. 10C); the mobility of this band differed from that observed in nuclear extracts of hypothalamic cells, see Fig. 7D). Band b could correspond to AP-1 induced by TPA used for differentiation. The intensity of both bands was increased in nuclear extracts from 8Br-cAMP-treated cells and only slightly with dex-treated cells; co-incubation of dex+8Br-cAMP however, diminished the intensity of band a compared with that observed with 8Br-cAMP alone (Fig. 10C).

Binding to GRE-A was increased upon 8Br-cAMP incubation even further than with dex incubation; co-incubation considerably diminished the binding compared with that observed with 8Br-cAMP alone and also, to a lesser but still significant extent, compared with dex-treated cells (Fig. 10A and D). The GRE half-site is also recognized by progesterone receptors (PRs) (Tank & Weiner 1992) and, upon PKA activation, PR can bind to DNA without bound ligand (Lieberman *et al.* 1993, Cenni & Picard 1999). Incubation of differentiated

SH-SY5Y cells with progesterone caused increased binding to GRE-A (Fig. 10D) in contrast to a very light band observed with hypothalamic cells (not shown). No interference either to GRE-A or CRE-2 binding was observed if progesterone was co-incubated with 8Br-cAMP (Fig. 10C and D).

Competition and supershift analysis

Binding to CRE-2 with nuclear extracts from hypothalamic cells treated with 8Br-cAMP or NA was abolished with an excess of CRE-2 or consensus CRE; even though no increased binding to CRE-1 had been detected, this oligonucleotide was also able to displace binding of both complexes. GRE-A, which contains the perfect AP-1, displaced both bands but to a lesser extent, band a (Fig. 11A). The same extracts tested against GRE-A, with consensus GRE, GRE-A, CRE-2 and CRE-1 as competitors, showed complete displacement of band b. Mutual displacement of CRE-2 for GRE-A binding, and vice versa, suggests recognition of the AP-1

site present in GRE-A by some of the protein complexes bound to CRE-2 (Habener 1990, Rutberg *et al.* 1999). One band of lower mobility (band a) was observed with nuclear extracts from NA-treated cells and GRE-A; the band was displaced with GRE-A and CRE-1 but only slightly with CRE-2 or consensus GRE (Fig. 11B).

Displacement of CRE-2 binding to nuclear extracts from SH-SY5Y-differentiated cells incubated with 8Br-cAMP or dex was assayed. Gels were run for 3 h to separate the complexes efficiently. Only band a was displaced with cold CRE-1 or CRE-2 and slightly with GRE-A; in contrast, band b was preferentially displaced by GRE-A. Consensus CRE caused complete displacement of both bands (Fig. 11C). Binding to GRE-A was analyzed in gels run for 3 h. Band a was displaced by GRE-A, CRE-2 and CRE-1 but not consensus GRE, while band b was displaced by either consensus GRE or GRE-A and only partially by CRE-2 (Fig. 11D). These results suggest that both bands were formed by different protein complexes.

In an attempt to characterize the nature of the proteins binding to CRE-2 or GRE-A we performed supershift analyses using antibodies recognizing P-CREB, c-Jun or GR. We were unable to detect a shifted band whether the antibody was incubated before or after DNA–protein complex formation; this could be due to instability of protein complexes with the antibody due to the short length of the oligonucleotides used (16 bp). However, binding to CRE-2 with nuclear extracts from hypothalamic 8Br-cAMP-treated cells diminished with P-CREB antibody (46% vs 100% (signal in stimulated cells)) and with c-Jun antibody (52%); this also occurred in dex-treated cells but to a lesser magnitude (74%) (Fig. 12A). In contrast, binding to GRE-A with nuclear extracts from 8Br-cAMP-treated cells decreased only with c-Jun antibody (24%), while from dex-treated cells binding decreased with c-Jun and GR antibodies (54%) (Fig. 12B). The last lane of both gels corresponds to an equivalent amount of normal rabbit serum (NRS) and shows no decreased signal.

These results thus suggest that AP-1 and GR can bind to GRE-A and, also, to GR–c-Jun heterodimers (Miner & Yamamoto 1992, Maroder *et al.* 1993).

Discussion

The homeostatic control of the thyroid axis requires adjustment to long-term changes occurring in conditions that cause metabolic alterations, such as during fasting (Blake *et al.* 1991, Legradi *et al.* 1997a). However, a rapid response to environmental stimuli is also a characteristic of the neuroendocrine systems. We have shown that the rapid increase in TRH mRNA levels observed after cold exposure or suckling stimulation – and mimicked in primary cultures of fetal hypothalamic cells incubated

Figure 12 Supershift analysis. Hypothalamic cells (14 DIV) were incubated for 1 h with 1 mM 8Br-cAMP or 10 nM dex; nuclear extracts were extracted and incubated for 10 h at 4 °C with 1 µg of antibody against P-CREB, c-Jun or GR, or an equivalent amount of normal rabbit serum (NRS). ³²P-labeled CRE-2 or GRE-A was added to the mixture for 30 min. Electrophoresis ran for 8 h at 100 V.

with PKA activators – could be due, at least in part, to regulation of transcription, as is the case for the interference caused by glucocorticoids on PKA signaling. Regulation of transcription can occur at different levels: at the binding of transcription factors; interactions between these factors affecting their binding to DNA; by appropriate recruitment of co-activators or co-repressors, etc. (McKeena *et al.* 1999). We studied the possibility that the transcriptional interference observed between dex and 8Br-cAMP in neuronal cells could be at binding on their cognate sequences. The CRE-like sequence that recognized 8Br-cAMP-activated nuclear proteins was the one located at –101/–94. The GRE –219/–197, corresponding to a composite GRE with AP-1 response elements flanking in the opposite strand, responded to dex and cAMP stimulation. Interference on binding was more strongly observed with the GRE.

The hypothalamic cell culture optimized in our laboratory (Charli *et al.* 1995, Pérez-Martínez *et al.* 2001, Joseph-Bravo *et al.* 2002) has proven to respond not only to 8Br-cAMP but also to NA stimulation by causing an increase in variables that act as indicators for the activation of TRH neurons: increase in peptide release, and its mRNA (Joseph-Bravo *et al.* 1998).

Release of glucocorticoids occurs in almost any stressful condition and even in events such as suckling (Herman *et al.* 1992, Uribe *et al.* 1993, Sánchez *et al.* 2001). The rise is immediate and therefore it is difficult to envisage situations that are free of variations in the levels of this hormone. How these variations modulate further responses is currently being investigated by many research groups. Positive or negative effects of glucocorticoids depend on the gene and the cellular make-up; their actions are not exclusive to an interaction with the mineralocorticoid or glucocorticoid receptors (with or without DNA binding) but also affect the transcriptional activity of other factors (Newton 2000, de Bosscher *et al.* 2001, Schaaf & Cidlowski 2003). It is now recognized that glucocorticoids can act at the membrane level activating second messenger pathways and release of various neuromodulators and hormones (Chen & Qiu 1999). We found no effect of dex on TRH release after 1 h incubation but the possibility remains that some of its effects on proTRH mRNA levels were initiated at the plasma membrane. We have previously reported that dex-mediated upregulation of proTRH mRNA levels (1 h) occurs independently of protein synthesis (Pérez-Martínez *et al.* 1998). The stimulatory effect of PKA activation or glucocorticoids on proTRH mRNA levels in hypothalamic cell cultures can occur at the level of gene transcription since in transfected hypothalamic cells, 8Br-cAMP or dex increased luciferase activity under the control of TRH promoter. The degree of transcriptional activation varied between these pathways depending on the cell system. Compared with the extent of stimulation obtained by PKA activation or dex treatment in primary cultures on proTRH mRNA levels (4.5-fold by 8Br-cAMP vs 3-fold by dex (Pérez-Martínez *et al.* 1998)), 8Br-cAMP-induced luciferase activity was higher than that induced by AP-1 or GR, despite the fact that there were no major differences in the expression levels of the transcription factors as determined by DNA-binding activity (transfected homologous system: primary hypothalamic cell cultures, 7- vs 2-fold for 8Br-cAMP vs dex stimulation respectively; heterologous system: 32- vs 2.7-fold respectively). This discrepancy could be explained by the difference in GR activity on a stably integrated template (and its effect on chromatin remodeling and recruitment of coactivators) compared with a naked transiently transfected promoter (McKeena *et al.* 1999).

The interference caused by dex (or GR) on 8Br-cAMP (or CREB) activation on proTRH mRNA levels also

occurred at the transcriptional level as observed for CRH (Guardiola-Díaz *et al.* 1996, Malkoski *et al.* 1997, King *et al.* 2002), vasopressin (Kuwara *et al.* 2003) or the α -subunit gene of glycoprotein hormone (Stauber *et al.* 1992); while in some of these cases dex alone can cause inhibition or no effect, TRH regulation coincides with that of annexin A1 that is stimulated by dex or cAMP but when combined, inhibition is observed (Antonicelli *et al.* 2001).

Interaction between transcription factors occurs at different steps, some of which are independent of DNA binding, at least for one of them (Newton 2000, Schaaf & Cidlowski 2003). We studied whether the inhibitory effect of dex on 8Br-cAMP activation was related to altered DNA binding to either CRE or GRE sites. Activation of the PKA-dependent signal pathway led to the binding of P-CREB to a synthetic oligonucleotide containing in tandem both putative TRH CRE non-canonical sequences. The specific nature of P-CREB binding to the TRH CRE-L element was demonstrated by competition studies, by supershift assays using an antibody specific for P-CREB, and binding of transfected CREB. TPA-mediated TRH promoter activity in cell cultures correlated with the induction of protein complexes binding to the TRH CRE; these complexes probably included AP-1 since over-expression of c-Jun and c-Fos in NIH-3T3 fibroblasts produced a complex able to bind CRE-L. P-CREB and AP-1 could thus bind to overlapping regulatory elements of the rat TRH promoter. When both CRE elements were independently studied with short oligonucleotides (16 bp), CRE-2 showed increased binding with nuclear extracts from 8Br-cAMP-treated cells but not CRE-1, which presented a very faint non-inducible band. These two elements differ from the consensus CRE (TGACGTCA): CRE-2 (TGCCGTCA) and CRE-1 (TGACCTCA). Variations from the consensus sequence cause differences in binding affinity (Benbrook & Jones 1994) and phosphorylation of CREB influences DNA-binding affinities on sequences deviating from the canonical symmetrical site, and also that of the consensus sequence (Bullock & Habener 1998). CRE-1, within a longer oligonucleotide (−83/−36), is recognized by transfected CREB and only slightly by the endogenous form present in 293T cells (Harris *et al.* 2001). Although the sequence present in CRE-1 has a similar binding affinity to consensus CRE (Bullock & Habener 1998), it remains to be studied whether the adjacent contextual sequences are required for CRE-1 to function properly in the TRH promoter (Deutch *et al.* 1988). The rat serine dehydratase gene possesses two CRE sites: a CRE-1 identical to TRH CRE-1, and another CRE-like sequence that has the conserved critical internal CpG dinucleotide (TGCCGCAA) supposed to have an important role in adequate binding (Deutch *et al.* 1988, Haas & Pitot 1999); the second site was found to be the preferred binding site to CREB

(Haas & Pitot 1999). CRE-2 in the TRH promoter also contains this central CpG dinucleotide; whether this explains the difference in binding of hypothalamic nuclear extracts with CRE-2 vs CRE-1 will have to be confirmed by foot-printing and deletion analysis (in future studies). It cannot be overlooked, however, that when the CRE-1 site is mutated to TAAAACT, basal and α MSH-stimulated transcription of $-150/+5$ hTRH transfected promoter is lost (Harris *et al.* 2001). This site is also the preferred binding site of TRs and cells transfected with $-177/+83$ TRH promoter have increased transcriptional activity if cotransfected with TRs in the absence of T_3 , and repressed by addition of the hormone (Feng *et al.* 1994, Satoh *et al.* 1996, 1999). This site is close to the initiation site and the negative effect of T_3 has been proposed to alter TR conformation such that it interferes, through protein-protein interactions, with basal transcriptional machinery (Feng *et al.* 1994, Satoh *et al.* 1996). Whether mutation of this site affects not only CREB binding, but also TR or other factors, remains to be studied. Furthermore, since these studies have been performed in non-neuronal cells, the possibility remains that in other cell types CREB-like proteins (or particular heterodimers) different from those of neurons are able to recognize CRE-1.

Two DNA-protein complexes are observed with CRE-2: band a is only displaced by CREs but not by GRE-A; in contrast, band b could contain AP-1-related factors, as judged by preferential displacement by GRE-A. When binding to CRE-2 with nuclear extracts from hypothalamic cells was compared with that of SH-SY5Y cells, band a coincided in both cultures while band b differed from the most intense band, highly increased in TPA-differentiated cells. The nature of these complexes remains to be elucidated since CRE is recognized by several protein complexes with different affinities: various CREBs, CRE modulators (CREMs), AP-1, CREB-activating transcription factor-1 (ATF1), c-Jun homodimers and heterodimers with ATF2, to name a few (Habener 1990, Masquillier & Sassone-Corsi 1992, Rutberg *et al.* 1999). Partial characterization of complexes by supershift assays suggests that CREB and Jun antibodies inhibit binding to CRE-2; the relative levels of these factors may thus influence transcriptional activity and affect the levels of proTRH mRNA without evident changes in the intensity of bound complexes. The increased binding to CRE-2 in nuclear extracts from hypothalamic cells stimulated after dex incubation for 1 h points to a membrane-mediated effect through kinase pathways; although this is partially supported by the small inhibitory effect of H89, staurosporine had no effect; other pathways remain to be studied.

Many of the effects of dex on gene expression are mediated by binding of the glucocorticoid receptor to its cognate DNA sequences on the target promoters (Newton 2000, Schaaf & Cidlowski 2003). Our results

show that dex stimulation of hypothalamic cells induced GR binding to a TRH GRE. This binding activity was competed with an oligonucleotide containing a consensus GRE and not by a mutated GRE; transfected GR bound to GRE-L with similar mobility. Since TPA stimulation produced a second band, this oligonucleotide was shortened to avoid the second AP-1 site. Dex-treated hypothalamic cells showed higher binding to GRE-A than cells treated with 8Br-cAMP for 1 or 3 h; in contrast, neuroblastoma cells presented an equivalent or higher binding after 8Br-cAMP than after dex stimulation. PKA activation increases GR binding to GRE (Rangarajan *et al.* 1992); however, the presence in GRE-A of AP-1 consensus sequences that recognize nuclear proteins activated by TPA stimulation and even by CREB (Deutch *et al.* 1988, Habener 1990, Masquillier & Sassone-Corsi 1992, Hill & Treisman 1995, Malkoski & Dorin 1999, Rutberg *et al.* 1999) stresses the need for characterization of these complexes.

It has been recognized that there is no general competition model to explain interaction between transcription factors; effects depend on the cell type or the promoter, and can occur at different levels (Harrison *et al.* 1995, McKeena *et al.* 1999, Yamada *et al.* 1999, de Bosscher *et al.* 2001). A clear antagonism was observed between dex and 8Br-cAMP stimulation – whether in mRNA levels, transcription activity or, in certain conditions, DNA binding. Several forms of interaction have been proposed between PKA and glucocorticoids. Those related to DNA binding include: CREB-GR direct interactions avoiding DNA binding (Imai *et al.* 1993, Yamada *et al.* 1999) and sequestration of PKA signaling (Doucas *et al.* 2000). However, with these mechanisms, a mutual inhibition would be expected either on CRE or GRE sites but we observed an inhibition of protein binding to CRE only after 3 h incubation. The decreased binding to CRE (either CRE-L or CRE-2) could be due to glucocorticoids inhibiting CREB phosphorylation (Legradi *et al.* 1997b, Whitehead & Carter 1997). In contrast, binding to the composite GRE (GRE-A) was diminished at 1 and 3 h in dex+8Br-cAMP hypothalamic 14 DIV cells and in differentiated neuroblastoma cells. Since GR half-life is diminished to 3 h after hormone binding but is increased by cAMP to 10 h (Dong *et al.* 1999), decreased binding to GRE-A cannot be explained by shortening GR half-life after 1-h stimulation. In the CRH promoter, a consensus CRE sequence and a composite GRE are involved in its transcriptional regulation; the stimulatory effect of PKA activation is mediated by CRE (Guardiola-Díaz *et al.* 1996) but cGRE participates in glucocorticoid-dependent repression of PKA-induced transcription (Malkoski *et al.* 1997, Malkoski & Dorin 1999). Furthermore, the CRH gene can be induced by glucocorticoids in certain conditions through a different region in the promoter (King *et al.* 2002). The nature of

the different nucleoproteins able to bind to a composite GRE determines the composition of coactivators or corepressors that ultimately define the positive or negative effects. In the case of the proTRH gene, a heterodimer GR-Jun (suggested to bind to composite GREs (Miner & Yamamoto 1992, Maroder *et al.* 1993)) may be formed, and the response could depend on the type of complex bound (c-Jun homodimer, CREB, GR-Jun, etc). Differential effects of c-Jun forming particular dimers could affect CREB or GR binding, and/or their transcriptional activity; thus, a working hypothesis of c-Jun as a central regulator of proTRH expression seems worth considering.

In conclusion, the ensemble of these results demonstrates rapid regulation of TRH gene transcription by 8Br-cAMP which can be repressed by dex in hypothalamic or in NIH-3T3 cells. In hypothalamic cells, NA reproduced the stimulatory effect of 8Br-cAMP on proTRH mRNA levels and interacted similarly to 8Br-cAMP co-stimulation with dex, supporting a physiological relevance of this interaction. We identified a CRE site located at $-101/-94$, and a composite GRE ($-210/-205$), that may regulate the transcriptional effects of various information pathways in neuronal cells. We have restricted the analysis to the role of PKA but other pathways activated by cAMP remain to be studied; additional work such as DNA foot-printing and deletion analysis, as well as further characterization of the protein complexes observed in EMSA studies, is thus warranted.

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