Thyrotropin-Releasing Hormone Regulation of Thyrotropin β-Subunit Gene Expression Involves Intracellular Calcium and Protein Kinase C†

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Received July 26, 1990; Revised Manuscript Received January 4, 1991

ABSTRACT: Our previous studies demonstrated TRH stimulation of TSHβ gene expression in rat pituitary cell cultures and GH_3 tumor cells in a transient expression assay. To begin to characterize the gene-proximal elements of the pathways involved in TRH stimulation of TSHβ gene transcription, we examined the effects of factors that increase intracellular calcium concentration, $[Ca^{2+}]_i$, or activate protein kinase C on TSHβ promoter activity in transfected GH_3 cells. TPA, a tumor-promoting phorbol ester, stimulated a dose-dependent increase in TSHβ promoter activity at 8 h similar to TRH (2–3-fold). TPA did stimulate protein kinase C activation without $[Ca^{2+}]$ mobilization. The calcium ionophore ionomycin increased cytoplasmic free $[Ca^{2+}]$ by stimulating both calcium influx and release from internal stores without affecting protein kinase C. Ionomycin also stimulated a dose-dependent increase (2-fold) in TSHβ promoter activity at 8 h. However, the voltage-dependent Ca^{2+} channel agonist Bay K 8644, which increased influx of extracellular calcium, had little or no effect on TSHβ gene expression until 48 h (5-fold). Similar effects on prolactin/mRNA levels were observed in these cells. Effects of these factors were not additive, suggesting a common pathway(s) to stimulate gene expression. Inhibition of intracellular calcium mobilization by treatment with 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) inhibited ionomycin effects on gene expression without affecting phorbol ester activity, and, conversely, inhibition of protein kinase C activity by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride (H-7) or TPA desensitization blocked TPA effects without affecting ionomycin activity. However, TRH stimulation of TSHβ gene expression was inhibited by decreasing both calcium mobilization and protein kinase C activity. These effects were mediated through the same 180 bp DNA sequence in the 5′-flanking region of the rat TSHβ subunit gene. These results strongly suggest that both protein kinase C and $[Ca^{2+}]$ mobilization are important factors in mediating TRH-induced TSHβ gene expression.

Understanding the molecular mechanisms by which polypeptide hormones regulate tissue-specific gene expression requires an integrated model of signal transduction, from initiating events at the plasma membrane through cellular pathways to rapid specific effects within the nucleus. The neuropeptide thyrotropin-releasing hormone (TRH)[1] is a major positive regulator of thyrotropin (TSH) and prolactin (PRL) secretion and synthesis in the rodent pituitary (Bowers et al., 1967; Gershengorn, 1986; Aizawa & Hinkle, 1985a,b; Kourides et al., 1984; Shupnik et al., 1986) and PRL synthesis and release from pituitary tumor cell lines (Murdoch et al., 1983, 1985; Camper et al., 1985; White et al., 1981; White & Bancroft, 1983; Laverriere et al., 1983, 1988). Both of the nonidentical, noncovalently associated subunits, α and β, of the glycoprotein hormone TSH are stimulated at the transcriptional level by TRH (Kourides et al., 1984; Shupnik et al., 1986). To understand further the physiological regulation of TSH synthesis at the level of gene expression, we have previously established a transient expression assay system and demonstrated that 5′-flanking DNA sequences direct TRH-responsive heterologous expression in the pituitary tumor GH_3 cells and primary pituitary cell cultures (Carr et al., 1989a,b). Nevertheless, the mechanism by which TRH induces changes in TSH gene activity is not well understood. Neither the identity of the predicted "second messengers" nor the putative DNA binding proteins that might mediate the transcriptional effects of TRH have been identified.

In the hormone-responsive rat pituitary tumor GH cells, the initial interaction of TRH with its receptor has been shown to influence several potential second-messenger systems including coupling of G-protein activation with phospholipase C stimulation (Aub et al., 1986, 1987; Masters et al., 1985; Martin et al., 1985; Hinkle & Kinsella, 1984). Recent studies of TRH stimulation of PRL synthesis indicated that extracellular calcium flux through voltage-sensitive calcium channels (VSCC) (Aizawa & Hinkle, 1985a,b; Enyeart et al., 1986, 1987; Hinkle et al., 1988), mobilization of intracellular calcium, $[Ca^{2+}]_i$ (Gershengorn et al., 1984; Gershengorn & Thaw, 1985; Ramsdell & Tashjian, 1985; Albert & Tashjian, 1985, 1986), and activation of protein kinase C may be involved (Murdoch et al., 1985; Ramsdell & Tashjian, 1985; Drust & Martin, 1984, 1985; Osborne & Tashjian, 1981). The relative

† A portion of these studies was performed under the auspices of Department of Clinical Investigation Protocol 1393-87 and under Walter Reed Army Institute of Research Work Unit WIAB.

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[1] Abbreviations: TRH, thyrotropin-releasing hormone; TSH, thyrotropin; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride; TMB-8, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

0006-2960/91/0430-3721$02.50/0

contribution of each of these factors is not clear, however. Extracellular calcium concentrations can markedly influence prolactin mRNA levels and transcription rates (White et al., 1981; Laverriere et al., 1988; Hinkle et al., 1988; Supowit et al., 1984; Gick & Bancroft, 1985) and more recently have been shown to stimulate PRL promoter activity in heterologous expression systems (Jackson & Bancroft, 1988). Finally, TRH has been shown to rapidly activate the phosphatidylinositol cycle (Sutton & Martin, 1982; Rebecchi et al., 1983; Martin, 1983; Rosenfeld et al., 1983; Rebecchi & Gershengorn, 1983; Martin & Kowalchyk, 1984; Imai & Gershengorn, 1986) to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). These intermediates, transiently produced by the cycling of the phosphatidylinositol pathway, initiate a cascade of cellular effects (Nishizuka, 1983) which may stimulate calcium release from intracellular stores and via protein kinase C activation, respectively.

To begin to characterize the gene-proximal elements of the path of TRH stimulation of TSHβ gene transcription, we investigated the effects of factors that increase $[Ca^{2+}]_i$ or activate protein kinase C on TSHβ promoter activity in a transient expression assay. We demonstrate that the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which mimics DAG and stimulates protein kinase C, and the calcium ionophore ionomycin rapidly stimulate TSHβ-directed synthesis similar to TRH. In contrast, Bay K 8644, a calcium channel agonist, results in a delayed but significant stimulation.

Experimental Procedures

Materials

Ionomycin and Bay K 8644 were obtained from Calbiochem; TMB-8 [8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate] was from Aldrich Chemical Co.; H-7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride] and Indo-1 were from Molecular Probes, Inc.; TRH was from Peninsula; TPA (12-*O*-tetradecanoylphorbol 13-acetate) was from Sigma. All enzymes were purchased from Boehringer-Mannheim. [γ-^{32}P]ATP (>6000 Ci/mmol) was obtained from New England Nuclear—Dupont.

Methods

Construction of TSHβ CAT Chimeric Plasmids. The chimeric plasmids contained various portions of the 5′-flanking sequences of the rat TSHβ subunit gene fused to the coding sequence of the reporter enzyme chloramphenicol acetyltransferase (CAT) (Figure 1). The DNA digest and subcloning procedures were performed as previously described (Carr et al., 1989a,b). Use of previously described transcriptional start sites was determined by the S1 nuclease protection assay as described (Carr et al., 1987; Carr & Chin, 1988). For comparison throughout these studies, pBLCAT8+ containing the heterologous herpes simplex virus thymidine kinase (TK) promoter was used.

Cell Culture. GH_3 cells, a clonal pituitary cell line responsive to TRH (Martin & Tashjian, 1977), were obtained from Dr. Barry Brown (University of Sheffield, Sheffield, U.K.). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 2 mM glutamine. Cells (1×10^6) were plated in 60-mm dishes 24 h before transfection. Unless otherwise indicated, media and other materials were from BRL-GIBCO. For calcium studies, the cells were grown in Corning 150 cm^2 tissue culture flasks.

Transient Expression of Fusion Genes and CAT Assays. The (diethylamino)ethyl- (DEAE-) dextran-mediated transfection technique (Lopata et al., 1984) was used to study transient expression of the TSHβ CAT chimeric plasmids as previously described (Carr et al., 1989a,b). After transfection (2 h), the cells were incubated in test media for 2 days (with or without various treatments added for 0–48 h of the 48-h incubation) before preparation of cell extracts for CAT assay. Extracts of GH_3 cells were assayed for CAT enzyme activity as described by Gorman et al. (1982). CAT activity is expressed as the percent conversion of chloramphenicol to the acetylated products per microgram of total cellular protein.

RNA Hybridization. RNA levels were examined by cytoplasmic dot hybridization (White & Bancroft, 1982) with a synthetic single-strand oligodeoxyribonucleotide probe, 36 nucleotides: −27 to −15 coding sequence region of the Sprague–Dawley rat PRL gene (Maurer et al., 1981), synthesized on a Pharmacia gene assembler. The 5′-end-labeled probe was hybridized as described (Carr & Chin, 1988), and quantitation of the autoradiograms was by video densitometry (Bio-Rad videodensitometer). The relative mRNA levels were presented as arbitrary densitometric units. Standardization of RNA was by comparison with hybridization results obtained with a random-primer-labeled (Boehringer Mannheim) actin and/or 18S RNA probe.

Protein Kinase C Activity. Cells were treated (TRH, 10^{-8} M; TPA, 10^{-8} M; ionomycin, 10^{-6} M) for 10 min, and total cytosolic protein was recovered by DEAE-cellulose chromatography. Phospholipid-dependent protein kinase C activity was determined by monitoring [γ-^{32}P]ATP incorporation into histone H1 substrate as described (Kikkawa et al., 1982). Protein kinase C when activated is translocated to the plasma membrane; a decrease in cytosolic activity thus reflects activation.

Cytosolic Free Calcium. GH_3 cells were incubated at 37 °C for 30 min with 5 μM Indo-1. Fluorescence was measured at an excitation wavelength of 350 nm and at emission wavelengths of 405 and 480 nm using a Deltascan spectrofluorometer (Photon Technology International, Inc., South Brunswick, NJ). Cytosolic free calcium concentration was determined by the method of Grynkiewicz et al. (1985). Basal calcium levels were approximately 150 nM.

Statistical Analysis. Simple comparison analysis was by Student's *t* test. Dose response experiments were assessed by logistic regression analysis, and multiple comparisons were assessed by analysis of variance and multiple *t* test (Duncan's) (Snedecor & Cochran, 1980).

Results

Time Course and Concentration Dependence of Calcium Ionophore and Phorbol Ester Stimulation of TSHβ-(−2900/+27−I1100)-CAT Promoter Activity. The transcriptional regulation of the rat TSHβ subunit gene was characterized by using plasmids constructed by fusing 5′-flanking DNA sequences containing the putative promoter elements to the coding sequences of the CAT reporter enzyme (Figure 1). The chimeric plasmid TSHβ-(−2900/+27−I1100)-CAT contains 2.9 kilobases (kb) of 5′-flanking DNA sequences, exon 1 [(+27), defined by transcriptional start site 2 (TSS2) (Carr et al., 1987)], and 1.1 kb of intron 1. This construct has been previously shown to yield the highest basal level of expression when transfected into GH_3 cells (Carr et al., 1989a,b) and was therefore chosen to assess potential regulatory effects of the various treatments unless otherwise indicated.

We have previously demonstrated that TRH stimulation of TSHβ promoter activity, as reflected by CAT synthesis, is detectable at 4 h and significant by 8 h (Carr et al., 1989a).

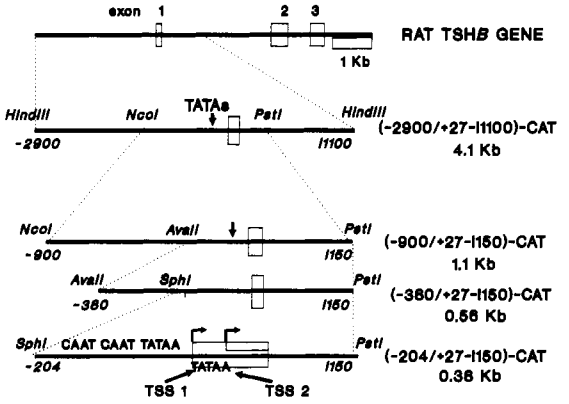

FIGURE 1: Construction of rat TSHβ subunit gene chimeric plasmids. DNA fragments encompassing various portions of the single rat TSHβ subunit gene were generated by restriction enzyme digestion as indicated. All gene fragments contained consensus promoter elements unique to the TSHβ gene. The gene fragments were ligated directly or after generating blunt-ends into the *Hin*dIII site of pBRCAT (Dr. W. Rutter) or *Hin*dIII or *Sph*I sites of pBLCAT 8+ (Dr. W. Wahli).

Table I: Calcium Ionophore and Phorbol Ester Stimulation of TSHβ Gene Promoter Activity[a]

	activity			
dose (M)	TRH	TPA	ionomycin	Bay K 8644
0	3.8 ± 0.2	3.0 ± 0.3	4.0 ± 0.5	4.8 ± 0.4
10^{-11}	4.9 ± 0.3	3.0 ± 0.6		
10^{-10}	7.1 ± 0.3[b]	4.8 ± 0.2[b]		
10^{-9}	10.1 ± 0.2[b]	5.5 ± 0.5[b]		5.7 ± 1.1
10^{-8}	11.2 ± 0.3[b]	6.4 ± 0.8[b]	7.0 ± 0.6	6.2 ± 0.9
10^{-7}	10.8 ± 0.7[b]	6.5 ± 0.7[b]	9.5 ± 0.7[b]	
10^{-6}			12.2 ± 1.1[b]	

[a] Transfected GH_3 cells were treated for the final 8 h of the incubation period. CAT activity is represented as percent total [^{14}C]chloramphenicol converted to acetylated products per microgram of total cell protein. Data are mean ± SEM, $n = 6$; three separate experiments for each of the treatments. [b] Statistical significance $p < 0.05$ compared with untreated control.

To assess the potential involvement of calcium flux and/or protein kinase C activation in this process, treatments were for the final 8 h of the total 48-h incubation. The dose-dependent effects of TRH, TPA, ionomycin, and Bay K 8644 on TSHβ-directed CAT synthesis are summarized in Table I. The CAT activity observed after the various treatments was increased relative to the activity with no hormone added. TRH stimulation of TSHβ promoter activity is noted at 10^{-10} M and maximal at 10^{-8} M with a half-maximal stimulatory effect at 0.5 nM. Ionomycin, which increases cytosolic [Ca^{2+}] both from intracellular mobilization and from extracellular flux, also stimulated a dose-dependent increase in TSHβ promoter activity.

To investigate the possible involvement of extracellular calcium flux through voltage-sensitive calcium channels (VSCC), the effects of Bay K 8644 on TSHβ promoter activity were investigated during an 8-h treatment (Table I). During this time period, a slight, but not significant increase in TSHβ promoter activity was detectable with Bay K 8644 at 10^{-8} M (100 ng/mL). Therefore, intracellular calcium mobilization is more important than Ca^{2+} influx in stimulating TSHβ promoter activity. Of interest, treatment of the transfected GH_3 cells with the phorbol ester TPA also stimulated a dose-dependent increase in TSHβ promoter activity with a maximal 2.2-fold stimulation observed at 10^{-7} M (Table I).

The time course of stimulation by ionomycin (10^{-6} M), TPA (10^{-8} M), Bay K 8644 (10^{-7} M), and TRH (10^{-8} M) was determined by addition of each factor to the incubation media at the indicated time (1–48 h) from the final hour of the total

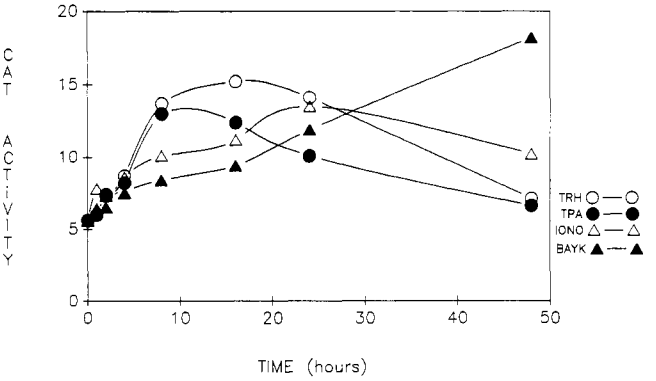

FIGURE 2: Time course of stimulation of TSHβ gene expression. Cells were transfected with 1 μg of plasmid DNA. TRH (10^{-8} M), TPA (10^{-8} M), ionomycin (IONO, 10^{-6} M), or Bay K 8644 (10^{-7} M) was included in the media for varying amounts of time (1–48 h as indicated) for the total 48-h incubation time. Results are pooled data from two separate experiments, $n = 4$. CAT activity is represented as percent total [^{14}C]chloramphenicol converted per microgram of total cell protein.

posttransfection period through the 48 h of incubation. Thus, the time actually reflects the hours of exposure of the transfected GH_3 cells to the factors during the 48-h posttransfection (Figure 2). As with TRH, ionomycin and TPA rapidly stimulated TSHβ promoter activity. While ionomycin and TPA stimulated maximal 2.5-fold increases at 8 h, similar to the 2.5-fold stimulation observed with TRH, maximal TRH effects occurred at 16–20 h with a greater cumulated stimulation than observed with ionomycin and TPA alone. Of interest, however, Bay K 8644 stimulated a 3–4-fold increase in TSHβ-directed CAT synthesis but not until after 24 h. The effects of these treatments were not additive. Incubation of TRH with any of the other agents at maximal doses at 8 h did not yield a significantly greater response than with TRH treatment alone (data not shown).

As with TRH, calcium ionophore and phorbol ester stimulation of TSHβ gene expression appears to be specific. No effect on heterologous non-TRH-responsive promoters such as herpes simplex virus thymidine kinase promoter was noted. During the time period studied, none of the factors altered total cellular protein content. Nevertheless, to observe early responses rather than maximal stimulation and to minimize potential cytotoxicity, all subsequent treatments involving transient expression assays were for the final 8 h of the total 48-h incubation period.

Effect of Inhibition of Mobilization of Intracellular Calcium on TRH Stimulation of TSHβ Gene Transcription. To examine the role of the mobilization of intracellular calcium on TRH stimulation of TSHβ gene transcription, the effect of an inhibitor of mobilization of calcium from intracellular stores, TMB-8 [8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate], was studied. TMB-8 has been shown to inhibit calcium release from intracellular stores in smooth muscle cells as well as fibroblasts, reticulocytes, and FRTL-5 thyroid cells (Malagodi & Chiou, 1974; Chiou & Malagodi, 1975; Rittenhouse-Simmons & Deykin, 1978; Villereal et al., 1986; Smallridge et al., 1991).

The response of cytosolic free calcium to treatment with TRH (10^{-8} M), ionomycin (10^{-6} M), TPA (10^{-8} M), and Bay K 8644 (10^{-8} M) was measured in the GH_3 cells to determine the specificity of the factors in inducing cellular effects. Ionomycin, TRH, and Bay K 8644, but not TPA, stimulated significant ($p < 0.05$) increases (105 ± 5, 42 ± 0.5, 63 ± 3, and 0% increase, respectively) in cytosolic free calcium levels in the presence of extracellular calcium. However, only ion-

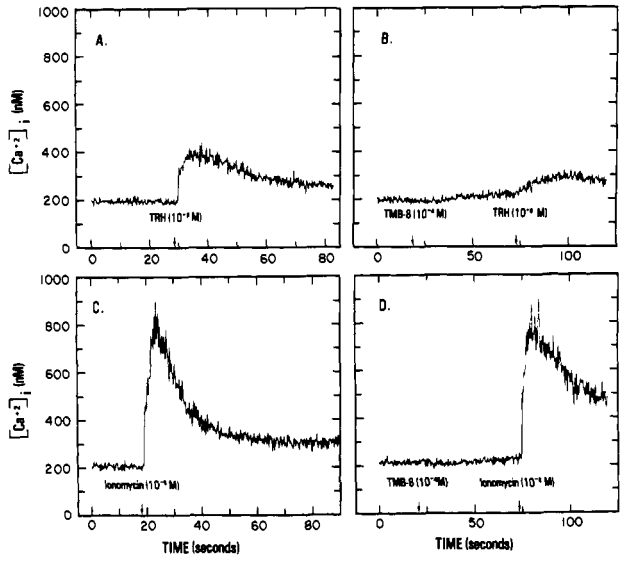

FIGURE 3: Cytosolic free calcium concentration in GH_3 cells. Representative tracings of single studies of cellular calcium in response to TRH (10^{-8} M) (A, B) or ionomycin (10^{-6} M) (C, D). The effect of TMB-8 (10^{-4} M) on TRH-induced (B) or on ionomycin-induced changes (D) is also illustrated. Cytosolic calcium concentration is expressed in nanomolar. TMB-8 was included 30 min prior to test treatment.

Table II: Inhibition of Intracellular Calcium Mobilization Affects Stimulation of TSHβ Gene Promoter Activity[a]

	activity		
TMB-8	control	TRH (10^{-8} M)	ionomycin (10^{-6} M)
0	5.6 ± 0.6	13.1 ± 0.5[b]	11.7 ± 2.1[b]
10^{-6}	6.6 ± 0.8	11.5 ± 1.5[b]	10.4 ± 1.2[b]
10^{-5}	7.8 ± 1.3	10.5 ± 0.8[b,c]	8.2 ± 1.1[b,c]
10^{-4}	6.4 ± 0.4	7.1 ± 1.2[c]	7.0 ± 0.6[c]

[a] TMB-8 (10^{-6}–10^{-4} M) was added to the transfected GH_3 cells 30 min prior to the addition of TRH or ionomycin. Cells were maintained in media containing TMB-8 alone or with TRH or ionomycin for 8 h. CAT activity data are mean Ω SEM; $n = 6$ per group. [b] $p < 0.05$ compared to untreated control. [c] $p < 0.05$ for effect of TMB-8 (10^{-6}–10^{-4} M) compared to base-line stimulation (TRH, ionomycin).

omycin and TRH stimulated changes in calcium levels in the absence of extracellular calcium (not illustrated). This confirmed the ability of ionomycin and TRH to release calcium from internal stores. In neither case did TPA at the doses used in this study induce changes in cytosolic free calcium concentration in this system. TMB-8 treatment prior to TRH completely blocked the initial phase of TRH-stimulated mobilization of intracellular Ca^{2+} without affecting extracellular Ca^{2+} influx (Figure 3). However, the reponse to ionomycin, which mobilizes calcium from multiple sites, was unaffected by TMB-8.

Treatment of transfected GH_3 cells with TMB-8 30 min prior to addition of ionomycin (10^{-6} M) blocked the stimulation of TSHβ promoter activity in a dose-dependent manner with maximal inhibition at 10^{-4} M (Table II). Higher doses of TMB-8 did not further decrease TSHβ promoter activity. TRH at 10^{-8} M stimulated a 2-fold increase in TSHβ promoter activity in the 8-h treatment. TMB-8 caused a dose-dependent decrease in TRH-stimulated TSHβ promoter activity when added to the incubation 30 min prior to addition of TRH. An 80% inhibition was noted at 10^{-4} M TMB-8. TMB-8 inhibition of TRH stimulation of TSHβ promoter activity appears to be in part through inhibition of intracellular Ca^{2+} mobilization.

Treatment of transfected GH_3 cells with TMB-8 over the dose range of 10^{-6}–10^{-4} M for 8 h had no significant effect

Table III: H-7 Inhibition of TPA and TRH Stimulation of TSHβ Gene Promoter Activity

	activity		
H-7 (M)	control	TRH (10^{-8} M)	TPA (10^{-8} M)
0	4.3 ± 0.5	7.6 ± 0.4[b]	6.6 ± 0.3[b]
2×10^{-5}	4.6 ± 0.7	5.2 ± 1.0	4.1 ± 0.7

[a] Transfected GH_3 cells were treated with 2×10^{-5} M H-7 for 30 min prior to the addition of either TRH or TPA. The combined treatments were continued for 8 h. CAT activity data are mean ± SEM, $n = 8$ per group. [b] $p < 0.05$ compared to untreated control.

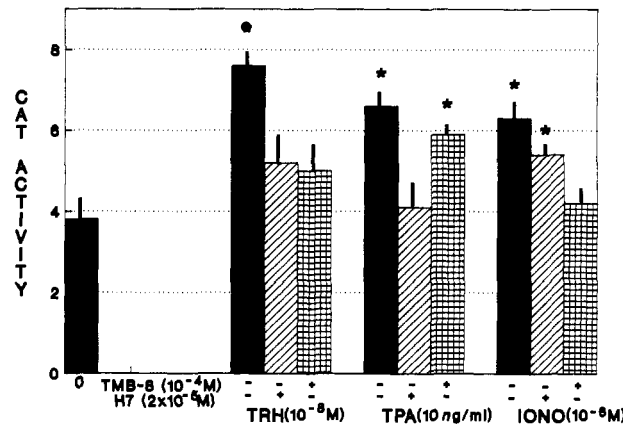

FIGURE 4: Effect of inhibition of calcium flux on protein kinase C activities and effect of protein kinase C inhibition on intracellular calcium activities. Cells transfected with −2900/+27–I1100–TSHβ–CAT were treated with either 2×10^{-5} M H-7 or 10^{-4} M TMB-8 30 min prior to and subsequent to addition of TRH, TPA, or ionomycin for the final 8 hr of the incubation period. Zero indicates the basal level of TSHβ gene promoter activity in parallel samples. The asterisk, indicates $p < 0.05$ compared to untreated control (0), multiple t test (Duncan's), $n = 6$ per group. CAT activity is percent [^{14}C]chloramphenicol converted per microgram of total cell protein.

on basal TSHβ promoter activity or pBLCAT8+ (TKCAT) activity, nor on cellular protein content (not illustrated). Therefore, there appears to be a selective inhibition by the drug of TRH- and ionomycin-stimulated TSHβ gene expression.

Effect of Inhibition of Protein Kinase C Activity on TRH Stimulation of TSHβ Promoter Activity. Since TRH also stimulates protein kinase C activity and TPA rapidly stimulates TSHβ promoter activity, the effects of inhibition of protein kinase C on TRH activity in the transient expression assay were also studied. In our studies, TPA (10^{-8} M) and TRH (10^{-8} M) increased protein kinase C activity 2-fold ($p < 0.05$) while ionomycin (10^{-6} M) caused no significant changes. H-7 at 2×10^{-5} M has been shown in a variety of cell systems to specifically inhibit protein kinase C activity (Imbra, 1987). Activation of protein kinase C and TSHβ promoter activity with either TRH or TPA at 10^{-8} M was inhibited by a 30-min pretreatment of the transfected cells with H-7 (Table III). Treatment with H-7 completely blocked TPA stimulation of TSHβ promoter activity and decreased TRH-induced effects by approximately 80%.

Specificity of TMB-8 and H-7 Inhibition. To determine the specificity of TMB-8 and H-7 inhibition of calcium ionophore and phorbol ester activities, the effect of TMB-8 treatment on TPA stimulation of TSHβ promoter activity and conversely the effect of H-7 on ionomycin activity were also investigated (Figure 4). While TMB-8 treatment blocked ionomycin-induced changes in TSHβ-directed CAT synthesis and inhibited TRH effects, TMB-8 decreased phorbol ester effects by only 10%. Although consistent, the effect was not significant. Inhibition of protein kinase C activity did block TPA effects and inhibit TRH stimulation of TSHβ promoter

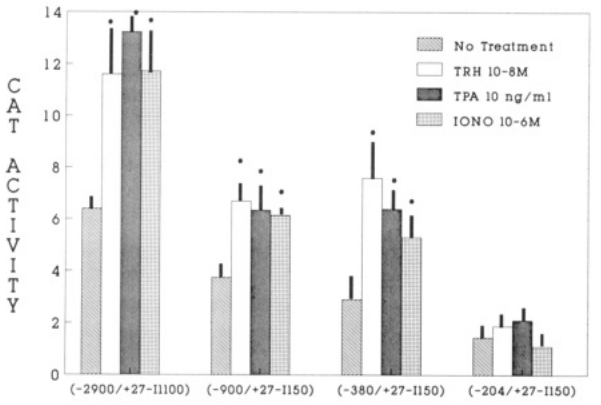

FIGURE 5: Deletion analysis of the TSHβ gene chimeras. Basal expression of the indicated plasmid constructs is depicted by the hatched bars. TRH, TPA, or ionomycin was added for the final 8 h of the 48-h incubation. Data are mean ± SEM, $n = 4$. Asterisks indicate $p < 0.05$ compared with the respective untreated control; logistic regression analysis. CAT activity is summarized as percent [^{14}C]chloramphenicol coverted per microgram of total cell protein.

activity while decreasing ionomycin activities by approximately 10–15%. Not illustrated, prolonged pretreatment of the cells with TPA to desensitize protein kinase C activity confirmed the results obtained with the protein kinase C inhibitor, H-7. However, neither TMB-8 nor inhibition of protein kinase C completely abolished TRH activity. Moreover, addition of both TMB-8 and H-7 together failed to completely block TRH activity.

Regulation of PRL mRNA in GH_3 Cells. Since GH_3 cell lines have been used to study the effects of various calcium ionophores, phorbol esters, and TRH on PRL secretion and synthesis, the calcium agents used in this study as well as TPA and TRH were tested for effects on steady-state PRL mRNA levels. Following an 8-h treatment, TRH (10^{-8} M), TPA (10^{-8} M), and ionomycin (10^{-6} M) stimulated a 1.8-, 1.7-, and 1.6-fold significant ($p < 0.05$) increase in PRL mRNA levels, respectively. Treatment of GH_3 cells with Bay K 8644 (10^{-8} M) in calcium-containing media caused no significant change in PRL mRNA levels at 8 h; however, at 48 h, it stimulated a 6-fold increase in PRL mRNA levels similar to previous reports (Laverriere et al., 1988; Hinkle et al., 1988; Gick & Bancroft, 1985) (data not illustrated). Treatment of the cells with TMB-8 (10^{-4} M) to block mobilization of intracellular calcium inhibited ($p < 0.05$) both ionomycin- and TRH-stimulated increases in PRL mRNA. Similarly, TPA desensitization of the GH_3 cells inhibited phorbol ester as well as TRH stimulation of PRL mRNA.

Deletion Mapping of the TSHβ Promoter with Regard to Calcium Ionophore, Phorbol Ester, and TRH Regulation of Its Activity. Basal expression of the various constructs is illustrated in Figure 5. The effects of treatment with TRH (10^{-8} M), TPA (10^{-8} M), and ionomycin (10^{-6} M) are illustrated in parallel. As demonstrated previously, TSHβ–(–2900/+27–I1100)–CAT includes a 4.1-kb gene fragment which directs CAT synthesis and contains sequences that confer sensitivity to TRH, TPA, and ionomycin stimulation of promoter activity. Deletion of 5′-flanking sequences to –900 and intronic sequences to I150 has been previously shown to result in a decrease in basal expression (Carr et al., 1989a,b). Further deletion of the 5′-flanking sequence to –380 results in a DNA fragment with decreased basal expression compared with 900/+27–I150 but similar to –204/+27–I150, suggesting the removal of an enhancer element or activation of a repressor located either in 5′-flanking DNA sequences –900 to –380 or in intronic sequences +I150 to I1100. Nevertheless, deletion of 5′-flanking sequences to –380 results in a TSHβ gene fragment which confers TRH, TPA, and ionomycin sensitivity. Further deletion of a 5′-flanking DNA sequence to –204 results in a loss of TRH sensitivity. Of interest, regulation by either TPA or ionomycin is also eliminated. Thus, the CIS elements responsible for conferring phorbol ester and calcium ionophore sensitivities to the TSHβ gene must reside, at least in part, within the same 180 bp region that is necessary for TRH responsivity. Transfer of this 180 bp DNA fragment 5′ to the thymidine kinase promoter in the *Hin*dIII site of pBLCAT8+ confers TRH, ionomycin, and TPA stimulatory effects to this heterologous promoter (not illustrated).

Discussion

We have demonstrated that activation of protein kinase C and mobilization of intracellular calcium rapidly stimulate TSHβ promoter activity in a time- and dose-dependent manner similar to the effects of the neuropeptide TRH. While at early time points (less than 12 h) either TPA or ionomycin may substitute for TRH in the transient expression assay, over the entire incubation time neither alone can completely mimic the effects of TRH; ionomycin stimulation is greater than TRH while TPA stimulation is less than TRH at different times. Yet, activation of voltage-sensitive calcium channels (Bay K 8644) to increase the intracellular free calcium concentration to levels similar to ionomycin does not produce a comparable increase in TSHβ promoter activity at 4 or 8 h, but does significantly contribute to increased TSHβ-directed CAT synthesis after 24 h. The effects of TRH, TPA, and ionomycin at maximal doses are not additive at 8 h, suggesting a final common pathway in the stimulation of TSHβ promoter activity.

The specificity of effects was determined by monitoring both intracellular calcium levels and directly measuring protein kinase C activity. Ionomycin clearly stimulated an increase in cytosolic free [Ca^{2+}] without stimulating protein kinase C, and TPA increased protein kinase C activity without affecting cytosolic free calcium concentration. As anticipated, TRH stimulated both systems. Ionomycin stimulates $^{45}Ca^{2+}$ transport from intracellular stores and acts as an ionophore to release TRH-regulated [Ca^{2+}] stores in GH4C1 cells (Albert & Tashjian, 1985, 1986). Although Albert and Tashjian (1985) found that TPA stimulated $[Ca^{2+}]_i$ release in the GH4C1 cells, we found no effect of TPA on cytosolic free calcium concentration in the GH_3 cells. Therefore, TPA effects on TSHβ promoter activity are due to activation of protein kinase C and not due to alterations in intracellular calcium concentration.

Since production of PRL in GH_3 cells has been extensively studied as a model of TRH action, we examined the effects of these agents on PRL mRNA levels after an 8-h treatment. As demonstrated with the TSHβ gene promoter activity, we found that TRH, TPA, and ionomycin stimulated an increase in steady-state PRL mRNA levels with little or no effect of the dihydropyridine Bay K 8644. No effect of these agents was detected on the non-TRH-responsive promoter herpes simplex virus thymidine kinase.

Our data thus indicate that mobilization of intracellular calcium and activation of protein kinase C, like TRH, rapidly stimulate TSHβ gene promoter activity and accumulation of PRL mRNA in GH_3 cells. In contrast, extracellular calcium does not rapidly stimulate TSHβ promoter activity and thus further emphasizes the specificity of calcium stimulation of PRL. Murdoch et al. (1983, 1985) and Supowit et al. (1984) implicate TRH stimulation of phosphatidylinositol turnover and subsequent activation of protein kinase C as important

early events in TRH stimulation of PRL synthesis. No effect of extracellular calcium was observed (Murdoch et al., 1985). However, mobilization of internal calcium as well as activation of protein kinase C was implicated by Ramsdell and Tashjian (1985). Specific protein phosphorylation coordinately linked with PRL synthesis further suggested involvement of a kinase (Sobel & Tashjian, 1983). In contrast, Laverriere et al. (1988) noted a predominant effect of extracellular calcium on PRL synthesis with little effect of either phorbol esters or ionomycin in this system. While our data and those of Murdoch et al. (1985) and Supowit et al. (1984) are in general agreement regarding the limited effect of external calcium in stimulating either TSHβ gene promoter activity or PRL synthesis as a rapid event, it should be emphasized that in our study extracellular calcium flux, as reflected by Bay K 8644, caused a significant increase in TSHβ gene promoter activity at 48 h, a time at which phorbol ester action and intracellular calcium flux are less effective. Perhaps extracellular calcium is an important factor in maintaining long-term effects of TRH for both TSH and PRL synthesis, as indicated by the studies of Laverriere et al. (1988). Events that are involved in the initiation of increased TSHβ promoter activity may well differ from the cellular processes required for the maintenance of the initial signal. The time course of stimulation by the various factors supports the suggestion that intracellular calcium mobilization and activation of protein kinase C are important early events while increased cellular calcium concentration, including from extracellular pools, is important for long-term stimulation of TSHβ promoter activity. Nevertheless, a key role for extracellular calcium as an important regulator of PRL synthesis probably distinct from TRH effects has been clearly established (White et al., 1981; White & Bancroft, 1983, 1987; Laverriere et al., 1988; Enyeart et al., 1987; Hinkle et al., 1988; Supowit et al., 1984; Gick & Bancroft, 1985; Jackson & Bancroft, 1988; White, 1985; Preston & White, 1987) and may distinguish regulation of TSH and PRL synthesis.

To further elucidate the potential role of the mobilization of intracellular calcium and the activation of protein kinase C on TRH stimulation of TSHβ gene transcription, we investigated the effects of an inhibitor of the mobilization of calcium from intracellular stores, TMB-8, and inhibitors of protein kinase C (H-7 and desensitization by pretreatment of the cells for 24 h with TPA). TMB-8, in a dose-dependent manner, inhibited TRH stimulation of TSHβ promoter activity. TRH stimulates a biphasic increase in intracellular $[Ca^{2+}]$ levels with the first peak reflecting release from intracellular stores and the second peak from influx of calcium from extracellular sources. Pretreatment of GH_3 cells with TMB-8 blocked the appearance of TRH-induced increases in $[Ca^{2+}]_i$ from intracellular stores without affecting the extracellular component. Therefore, TRH stimulation of TSHβ gene expression occurs, at least in part, through mobilization of intracellular calcium. TMB-8 also inhibited ionomycin-induced increases in TSHβ promoter activity. However, no significant effect of TMB-8 on ionomycin stimulation of $[Ca^{2+}]_i$ was observed. Ionomycin stimulation of calcium mobilization may be from numerous intracellular stores, resulting in a large increase in $[Ca^{2+}]_i$ compared with the small change in $[Ca^{2+}]_i$ in response to TRH. At the doses used in this study in calcium-containing media, TMB-8 inhibition of ionomycin effects on $[Ca^{2+}]_i$ might not be possible to detect if TMB-8 blocks $[Ca^{2+}]$ mobilization only from a small but critical pool. TMB-8 may also inhibit ionomycin-stimulated increases in TSHβ gene expression through a mechanism subsequent to calcium mobilization. This would require coordinate multiple intracellular signalling effects from ionomycin and TMB-8 in the GH_3 cells. Nevertheless, TMB-8 clearly inhibits TRH-induced changes in $[Ca^{2+}]_i$, and both TRH and ionomycin stimulated increases in TSHβ promoter activity. TMB-8 also blocked both TRH- and ionomycin-stimulated increases in PRL mRNA levels. TMB-8 inhibition of calcium-induced increases in PRL synthesis (Preston & White, 1987) suggests that the shift of $[Ca^{2+}]$ to a sequestered form is unfavorable for PRL gene expression (White & Bancroft, 1987). TMB-8 has been shown to block intracellular calcium mobilization in several muscle cell types (Malagodi & Chiou, 1974; Chiou & Malagodi, 1975) and growth factor induced calcium mobilization (Villereal et al., 1986) between 10^{-6} and 10^{-4} M. This effect is not specific, however, as TMB-8 not only inhibited receptor-mediated intracellular calcium mobilization but also increased Ca^{2+} influx in FRTL-5 cells (Smallridge et al., 1991). Nevertheless, TMB-8 has recently been shown to inhibit PRL gene expression after calcium induction in GH_3 cells (Preston & White, 1987) without affecting ^{45}Ca uptake into these cells (White & Bancroft, 1987).

Inhibition of protein kinase C by treatment with H-7 clearly blocked phorbol ester stimulation of TSHβ promoter activity and also inhibited TRH-induced changes in TSHβ-directed CAT synthesis. Although H-7 inhibits protein kinase C activity in a variety of cell types (Imbra, 1987), desensitization of protein kinase C activity by pretreatment with phorbol ester for 24 h (Hoeffler et al., 1989) was also tested to confirm the specificity of protein kinase C inhibition. In both cases, inhibition of protein kinase C decreased both phorbol ester and TRH stimulation of TSHβ promoter activity. Although not significant, both TMB-8 and H-7 caused consistent decreases in phorbol ester and calcium ionophore activities, respectively, suggesting either a linkage in the two pathways and/or a lack of specificity of the agents tested. Neither TMB-8 nor H-7 completely blocked TRH stimulation of TSHβ-directed CAT synthesis, further suggesting a calcium–protein kinase C linked system and/or an additional nonintracellular calcium, non-protein kinase C mechanism of TRH action on TSHβ gene promoter activity. Calmodulin inhibition by W-7 and trifluoperazine does decrease TRH-stimulated TSHβ-directed CAT synthesis (F. Carr, unpublished observation). However, the lack of specificity of these agents limits the interpretation of the possible involvement of calmodulin kinases in this system.

Finally, deletion analysis indicates that DNA sequences between −380 and −204 are required for TRH regulation of TSHβ promoter activity. Of importance, this sequence also confers phorbol ester and calcium ionophore sensitivity to the TSHβ gene. The CIS elements responsible for conferring TPA and ionomycin sensitivity must reside at least in part within the same 180 bp region of the rat TSHβ gene that is necessary for TRH responsivity. Recent studies of calcium regulation of PRL-directed CAT synthesis (Jackson & Bancroft, 1988) have identified a calcium response element in a −174 5′-flanking deletion fragment. This fragment also contains pituitary-specific transcription factor sites (Nelson et al., 1988; Castrillo et al., 1989) that are important elements in maintaining high basal levels of PRL gene promoter activity. While there are several regions of homology between the −380 to −204 gene fragment and the −174 PRL fragment, including a consensus PIT-1 factor, a TRH consensus element remains to be defined.

These studies suggest that TRH stimulation of TSHβ gene expression occurs in part through a common pathway involving

mobilization of intracellular calcium as reflected by ionomycin and activation of the calcium-dependent protein kinase C reflected by the phorbol ester TPA. This effect may be linked through the activation of the phosphatidylinositol cycle generating DAG and IP_3. DAG stimulation of protein kinase C activity and IP_3 mobilization of intracellular calcium may then participate in a cooperative, sequential, or hierarchial organization to facilitate intracellular signalling to a final common pathway regulating gene expression. Future studies directed toward the identification of a consensus region mediating all or part of these effects will facilitate the identification of the gene-proximal elements involved in the TRH pathway.

ACKNOWLEDGMENTS

We thank Ms. Sabita Lahiri for her careful preparation of the synthetic DNA primers and Mrs. Estelle Coleman-Fraser for expert editorial assistance in the preparation of the manuscript. Plasmids pBRCAT and pBLCAT8$^+$ were generously supplied by Dr. William Rutter (University of California, San Francisco, CA) and Dr. Walter Wahli (University of Lausanne, Lausanne, Switzerland).

Registry No. TRH, 24305-27-9; Ca, 7440-70-2; thyrotropin, 9002-71-5; prolactin, 9002-62-4; protein kinase, 9026-43-1.

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Subcellular Localization and Nucleosome Specificity of Yeast Histone Acetyltransferases†

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Received September 28, 1990; Revised Manuscript Received December 6, 1990

ABSTRACT: We have previously reported [López-Rodas et al. (1989) *J. Biol. Chem. 264*, 19028-19033] that the yeast *Saccharomyces cerevisiae* contains four histone acetyltransferases, which can be resolved by ion-exchange chromatography, and their specificity toward yeast free histones was studied. In the present contribution we show that three of the enzymes are nuclear, type A histone acetyltransferases and they are able to acetylate nucleosome-bound histones. They differ in their histone specificity. Enzyme A1 acetylates H2A in chicken nucleosomes, although it is specific for yeast free H2B; histone acetyltransferase A2 is highly specific for H3, and histone acetyltransferase A3 preparations acetylate both H3 and H4 in nucleosomes. The fourth enzyme, which is located in the cytoplasm, does not accept nucleosomes as substrate, and it represents a canonical type B, H4-specific histone acetyltransferase. Finally, histone deacetylase activity is preferentially found in the nucleus.

A variety of roles have been proposed for reversible acetylation of specific lysines in the N-terminal tails of the core histones, and it has been suggested that acetylation may be a specific signal for (i) histone removal during DNA replication, (ii) histone replacement in some differentiation processes, or (iii) H2A-H2B removal during transcription (Loidl, 1988). In addition to these nuclear acetylation events, a cytoplasmic H4 acetylation, related to histone deposition of newly synthesized histones, has been described [for a review, see Vidali et al. (1988)], and a relationship between the acetylation–deacetylation of newly assembled core histones and H1 deposition has recently been established (Perry & Annunziato, 1989).

It has been determined both by sequencing (Pesis & Matthews, 1986; Chicoine et al., 1986, 1987; Couppez et al., 1987; Richman et al., 1988) and by immunological methods (Turner & Fellows, 1989; Lin et al., 1989; Turner, 1989) that the usage of different lysyl residues of histones for reversible acetylation is not a random process. For instance, it has been proposed that acetylation of lysine 5 of H4 histone (lysines 4 and 11 in *Tetrahymena*) is related to histone deposition, whereas the turnover of acetyl groups at other lysines is connected with other functions (Pesis & Matthews, 1986; Richman et al., 1988). At any rate, the differential usage of sites for histone acetylation may be associated with the diversity of roles played by this histone modification (Loidl, 1988).

The turnover of acetyl groups depends on the activity of two kinds of enzymes, the histone acetyltransferases and the histone deacetylases. The knowledge of the regulation of these enzymatic activities may be a decisive stage in the way to elucidate how the different roles of histone acetylation are performed.

We have recently reported that the yeast *Saccharomyces cerevisiae* contains four histone acetyltransferases that can be resolved by DEAE-Sepharose chromatography (López-Rodas et al., 1989). Because of this multiplicity of enzymes, yeast may be the organism of choice to address the regulation of histone acetylation. When assayed with yeast free histones, two of the *Saccharomyces* histone acetyltransferases are specific for H4, one is specific for H2B, and the fourth one is H3-specific. However, the specificity of histone acetyltransferases may change when nucleosomes are used as substrate (Belikoff et al., 1980; Garcea & Alberts, 1980; Kelner & McCarty, 1984; Sendra et al., 1986), and, therefore, it is first interesting to know the histone specificity in chromatin.

†This work has been supported in part by Grant PB88-352 from DGICYT (Spain).

0006-2960/91/0430-3728$02.50/0