Thapsigargin suppresses phorbol ester-dependent human involucrin promoter activity by suppressing CCAAT-enhancer-binding protein α (C/EBP α) DNA binding

Sivaprakasam BALASUBRAMANIAN*, Chapla AGARWAL*, Tatiana EFIMOVA*, George R. DUBYAK*, Eric BANKS*, Jean WELTER* and Richard L. ECKERT*†‡§¶¹

*Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland, OH 44106-4970, U.S.A., †Department of Biochemistry, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland, OH 44106-4970, U.S.A., ‡Department of Reproductive Biology, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland, OH 44106-4970, U.S.A., §Department of Dermatology, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland, OH 44106-4970, U.S.A., and ¶Department of Oncology, Case Western Reserve University School of Medicine, 2109 Adelbert Road, Cleveland, OH 44106-4970, U.S.A.

Human involucrin (hINV) is a keratinocyte differentiation marker expressed in the suprabasal epidermal layers. In cultured keratinocytes hINV mRNA levels are increased 10-fold by a 24-h treatment with 50 ng/ml PMA, an agent that promotes keratinocyte differentiation. Previous studies show that thapsigargin (TGN), an agent that depletes intracellular calcium stores, inhibits keratinocyte differentiation. In the present study we show that TGN inhibits the PMA-dependent, differentiation-associated, increase in hINV mRNA levels and hINV promoter activity. Inhibition is half-maximal at 10 nM and maximal at 100 nM TGN. Neither basal hINV promoter activity nor glyceraldehyde-3-phosphate dehydrogenase mRNA levels are

inhibited. Mutation of a functionally important CAATT-enhancer-binding protein (C/EBP) site within the hINV promoter proximal regulatory region eliminates the regulation, suggesting that TGN may effect C/EBP-dependent promoter activation. Consistent with this hypothesis, TGN inhibits C/EBP α -dependent promoter activation via a mechanism that involves inhibition of C/EBP α binding to DNA without changing C/EBP α protein levels. These results suggest that TGN interferes with hINV expression by interfering with C/EBP transcription-factor function.

Key words: calcium, gene expression, PMA.

INTRODUCTION

Involucrin is a component of the keratinocyte cornified envelope that is specifically expressed in the suprabasal epidermal layers [1,2]. In cultured keratinocytes, involucrin mRNA levels and promoter activity are increased by agents such as PMA that promote keratinocyte differentiation [3–6]. Mechanistic studies show that basal promoter activity is determined, in part, by the contribution of several transcription-factor binding sites [3,4, 7–12], including a CAATT-enhancer-binding protein (C/EBP) site [13]. The PMA-dependent increase in human involucrin (hINV) promoter activity requires a functional C/EBP site [13], suggesting that C/EBP is a target of agents that regulate the PMA-dependent regulation.

Thapsigargin (TGN), a sesquiterpene lactone isolated from extracts of the plant *Thapsia garganica* [14], is a known tumour promoter. Unlike PMA, TGN does not bind to or activate protein kinase C, and therefore has been classified as a non-PMA-type tumour promoter [15]. Mechanistic studies indicate that TGN inhibits the Ca²⁺-ATPase of the endoplasmic reticulum (ER) [15]. This causes a chronic depletion of intracellular stored calcium without the hydrolysis of phosphoinositides or activation of protein kinase C. TGN has proven to be a useful agent for studying the effects of depletion/mobilization of intracellular calcium on gene expression in keratinocytes [16,17]

In the present study we examine the effects of TGN on hINV gene expression and promoter activity. We show that TGN treatment inhibits PMA-dependent hINV mRNA accumulation. TGN also inhibits PMA- and C/EBP α -dependent hINV promoter activation. The TGN-dependent suppression requires an intact C/EBP DNA-binding site and stored intracellular calcium. TGN appears to inhibit PMA-dependent promoter activation by reducing C/EBP α binding to the hINV promoter C/EBP α -binding site.

MATERIALS AND METHODS

Chemicals and reagents

Phorbol ester (PMA), DMSO and TGN were obtained from Sigma. PMA and TGN were prepared as 1000-fold stocks in DMSO. Keratinocyte serum-free medium (KSFM), trypsin, Hanks balanced salt solution, gentamicin and Lipofectin were obtained from Life Technologies. The plasmids pGL2-basic (promoterless luciferase reporter construct) and the chemiluminescent luciferase assay systems were from Promega. Chemiluminescence was measured using a Berthold luminometer, and oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. Dispace was from Boehringer Mannheim and [α -³²P]ATP from DuPont NEN. Fura-2 was obtained from

Abbreviations used: hINV, human involucrin; C/EBP, CAATT-enhancer-binding protein; TGN, thapsigargin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ER, endoplasmic reticulum; KSFM, keratinocyte serum-free medium.

¹ To whom correspondence should be addressed, at the Department of Physiology and Biophysics (e-mail rle2@po.cwru.edu).

Molecular Probes. BSA was purchased from ICN Immunologicals.

Plasmids and cell culture

The hINV reporter plasmids contain various lengths of hINV upstream promoter sequence fused to the luciferase reporter gene in pGL2-basic. These plasmids have been described previously [3,4]. C/EBP α was provided by Dr Richard Hanson (Case Western Reserve University, Cleveland, OH, U.S.A.).

Human keratinocytes, derived from human foreskin, were cultured as described previously [4]. The cells were grown in KSFM and used at the third passage.

RNA transfer blot

Near-confluent cultures of keratinocytes were maintained in KSFM containing no additives, 50 ng/ml PMA or 100 nM TGN for 48 h. The cells were then harvested. mRNA was prepared, electrophoresed on a denaturing agarose gel, transferred to Biotodyne A membrane and hybridized with 32 P-labelled cDNAs encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18] or hINV [19] as described previously [4].

Transfection and luciferase assay

Cells, growing in 60-mm-diameter dishes, were passaged when 80% confluent at a 1:5 split ratio. After attachment and when 50% confluent, the cells were transfected with 16 μ g of Lipofectin reagent and 2.5 μ g of hINV promoter plasmid in 3 ml of KSFM for 5 h at 37 °C. Then 3 ml of additional medium was added and the incubation continued for 19 h. The cells were then fed with fresh medium in the presence or absence of PMA and/or TGN and harvested 24 h later. Extracts were prepared and assayed for luciferase activity using the Promega assay system [4]. For co-transfections (Lipofectin-mediated), 2.5 μ g of hINV reporter plasmid was transfected with 0–2.5 μ g of expression vector. In each case, the total plasmid concentration was adjusted to 5 μ g with empty expression vector. In some experiments cells were transfected with FuGENE6 (Boehringer Mannheim) by mixing 3 μ g of hINV reporter plasmid, 4.5 μ l of FuGENE6 and 100 μ l of culture medium. This mixture was then added to 50% confluent keratinocyte cultures growing in 35-mm-diameter dishes containing 2 ml of medium. After 24 h, the FuGENE6 mixture was removed. The cells were then treated for 24 h with the desired agent prior to harvest for luciferase assay. The results are expressed as luciferase activity/ μ g of protein. Each transfection was performed in triplicate and the values averaged. The triplicates routinely varied by less than 10%. As a control to assure comparable transfection efficiency, we utilized a green-fluorescent-protein expression plasmid and determined the percentage of cells transfected by visual inspection. TGN did not influence transfection efficiency.

Detection of C/EBP α by immunoblot and gel mobility-shift assay

Gel mobility-shift assays and immunoblot detection were performed exactly as previously outlined [4,13]. For gel mobility shift, we used a double-stranded, end-labelled oligonucleotide, 5'-GGTTTGCTGCTTAAGATGCCTG-3' (with the C/EBP site shown in bold), that encodes the hINV C/EBP binding-site region [13]. C/EBP α was detected by immunoblot using rabbit anti-human C/EBP α antibody at a dilution of 1:500 (sc-61X, Santa Cruz Biologicals).

RESULTS

TGN inhibits the PMA-dependent increase in hINV mRNA and promoter activity

To test for effects of TGN on hINV RNA level, cultured normal human keratinocytes were treated with TGN for 48 h, and the level of hINV-encoding RNA was assayed by Northern-blot hybridization. As shown in Figure 1, PMA produces a 10-fold increase in hINV RNA levels. Simultaneous treatment with TGN completely inhibits this PMA-dependent increase. In contrast, the level of mRNA encoding GAPDH is not regulated, suggesting that the TGN treatment is not causing a non-specific general inhibition of mRNA synthesis.

To examine the effects of PMA and TGN on hINV promoter activity, we tested the effects of each agent on the activity of pINV-2473, a plasmid that includes the full-length hINV upstream regulatory region and TATA box linked to the luciferase gene [4]. Keratinocytes were transfected with pINV-2473 and treated for 24 h in the presence or absence of PMA and/or TGN. In the experiment shown in Figure 2, 50 ng/ml PMA produces a 5-fold increase in pINV-2473 activity. Treatment of cells with 50 ng/ml PMA and increasing concentrations of TGN suppresses promoter activity in a concentration-dependent manner. In the presence of 100 nM TGN, hINV promoter activity equals basal promoter activity. Activity was not suppressed further by 1000 nM TGN and basal promoter activity was not TGN-responsive. Thus the TGN-dependent reduction in mRNA level appears to result from an inhibition of hINV transcription.

Figure 1 TGN inhibits the PMA-dependent increase in hINV mRNA level

Human epidermal keratinocytes were grown until 80% confluent in KSFM and then treated for 48 h with KSFM (control) or KSFM supplemented with 50 ng/ml PMA (TPA) or 50 ng/ml PMA + 100 nM TGN. RNA was prepared, fractionated on a denaturing agarose gel, transferred to Biotodyne A membrane and hybridized with 32 P-labelled cDNAs encoding involucrin [19] or GAPDH [18]. Treatment with TGN alone did not regulate hINV mRNA level (results not shown).

Figure 2 TGN inhibits PMA-dependent hINV promoter activity

Human epidermal keratinocytes were grown until 80% confluent in KSFM and then transfected with pINV-2473 according to the procedure outlined in the Materials and methods section. The cells were then treated for 24 h with KSFM (open bars) or KSFM supplemented with 50 ng/ml PMA (TPA; hatched bars) and the indicated concentration of TGN. After treatment, the cells were harvested, and extracts were prepared and assayed for luciferase activity. The results are expressed as activity/μg of protein. The data represent means $\pm$ S.E.M. from assays performed in triplicate and the values varied by less than 5%. Similar results were obtained in each of four independent experiments.

Figure 3 TGN regulation of PMA-responsive truncated promoters

Human epidermal keratinocytes were grown until 80% confluent in KSFM and then transfected with the indicated hINV promoter construct. The cells were then treated for 24 h with KSFM (open bars) or KSFM supplemented with 50 ng/ml PMA (hatched bars) or 50 ng/ml PMA (TPA) + 100 nM TGN (solid bars). After treatment, the cells were harvested, and extracts were prepared and assayed for luciferase activity. The results are means $\pm$ S.E.M. expressed as activity/μg of protein. Similar results were obtained in each of three independent experiments.

Figure 4 Elimination of the C/EBP site eliminates the TGN response

Human epidermal keratinocytes were grown, transfected with the indicated plasmids and then treated with PMA (TPA) and TGN as outlined in Figure 3. After treatment, the cells were harvested, and extracts were prepared and assayed for luciferase activity. The results shown here are representative of four other experiments. The C/EBP-binding site, and ets-1- and ets-2-factor-binding sites (EBS-1 and EBS-2) were mutated as follows: C/EBP, 5'-GCTGCTTAAG-3' to 5'-GCTGAGATCT-3'; EBS-1, 5'-AGGAAG-3' to 5'-AGATCT-3'; EBS-2, 5'-AGGAAG-3' to 5'-ACGCGT-3'. The altered bases are underlined in each case. The complete promoter sequence is given in [25].

The PMA and TGN responses are mediated via a C/EBP-binding site

To determine the site of action of TGN in inhibiting hINV promoter activity, we tested the response of a set of hINV promoter constructs in which the 5' end was progressively truncated. As shown in Figure 3, constructs pINV-2473, pINV-2216, pINV-2100, pINV-1336, pINV-159 all responded to PMA with a 2–4-fold increase in activity. In each case, TGN antagonizes the PMA-dependent increase and returns expression to basal levels. The shortest construct, pINV-41, which displays only basal activity, is not regulated by these agents. These results suggest that the responsive region is located in the –159/–41 segment.

To further determine the site of TGN action, several constructs containing point mutations were tested. pINV-2473 is included in Figure 4 to show the response of the full-length promoter. The activity of pINV-2473 is increased by PMA and the increase is inhibited by TGN. A similar induction and suppression is observed with pINV-241, pINV-241(EBS-1m) and pINV-241(EBS-2m). The latter plasmids contain mutations, respectively, in the EBS-1 and EBS-2 sites, where EBS stands for ets-factor-binding site. These putative ets-factor binding sites regulate basal activity, but are not involved in mediating response to PMA (J. Welter and R. L. Eckert, unpublished work). In contrast, mutation of the C/EBP site (C/EBPm) eliminates both PMA- and TGN-dependent regulation. However, since

Figure 5 TGN inhibits C/EBP α -dependent promoter activity

Keratinocytes were transfected with pINV-241 and then treated with 50 ng/ml PMA (TPA), or 50 ng/ml PMA + 100 nM TGN (hatched bars). In parallel experiments, cells were transfected with 2.5 μ g of pINV-241 reporter plasmid in the presence of 2.5 μ g of C/EBP α expression vector (solid bars). The cells were then treated for 24 h in the presence or absence of 100 nM TGN and harvested for luciferase assay.

both PMA- and TGN-dependent activities are eliminated by mutation of the C/EBP site, this result, although suggestive that the response is linked to the C/EBP site, does not provide proof that the TGN response is mediated via this site. Since C/EBP α is known to activate the promoter via interaction at the C/EBP-binding site [13], we measured the effects of TGN on C/EBP α -dependent promoter activation.

Figure 6 TGN does not alter C/EBP α level

Keratinocytes were grown for 24 h in the presence of 50 ng/ml PMA (TPA), 100 nM TGN or PMA + TGN. Nuclear extracts were prepared and assayed for expression of C/EBP α by immunoblot using a C/EBP α -specific antibody. The positive control (C) was a nuclear extract prepared from C/EBP α -transfected keratinocytes. The C/EBP α -immunoreactive band is indicated by the arrow.

Figure 7 TGN treatment reduces C/EBP α binding to the hINV C/EBP site

C/EBP α binding to the hINV C/EBP site was measured by gel mobility-shift assay using the oligonucleotide indicated in the Materials and methods section. Keratinocyte nuclear extracts were prepared from cells following a 24-h treatment with 50 ng/ml PMA, 100 nM TGN or both. The extract was then incubated with the radiolabelled probe and then electrophoresed on a non-denaturing gel as described previously [13]. Migration of the authentic C/EBP α -containing complex is indicated by an arrow. FP indicates the migration of the free probe. Lane C shows probe migration in the absence of nuclear extract.

TGN suppresses C/EBP α -dependent activation and reduces C/EBP DNA binding

To provide additional evidence of a role for C/EBP α , we transfected cells with C/EBP α expression vector, which is known to activate the hINV promoter [20], in the presence or absence of 100 nM TGN. As shown in Figure 5, TGN inhibits the C/EBP α -dependent promoter activation.

The TGN-dependent reduction in hINV promoter activity could be caused by inhibition of synthesis, DNA binding or activity of C/EBP α . Therefore, we measured the effects of TGN on C/EBP α protein level. As shown in Figure 6, neither PMA nor TGN treatment altered C/EBP α levels. To determine whether these agents alter C/EBP α binding, we performed gel mobility-shift experiments using an oligonucleotide encoding the hINV promoter C/EBP site. Extracts from keratinocyte cultures grown in the absence of TGN or PMA produce a shifted band that is due to C/EBP α binding [13] (Figure 7). PMA treatment increases the level of C/EBP α binding. TGN treatment, in contrast, inhibits this increase (Figure 7).

DISCUSSION

TGN regulation of hINV expression

TGN is a useful agent for studying the effects of depletion/mobilization of intracellular calcium on gene expression in keratinocytes [16,17,21]. In mouse keratinocytes, 5–20 nM TGN inhibits expression of keratins 1 and 10 [17], inhibits activity of

the keratin 1 promoter, prevents redistribution of E-cadherin, inhibits morphological differentiation [17] and alters cell proliferation rate [21]. These responses are consistent with TGN inhibiting the differentiation of mouse keratinocytes. In cells maintained in low-calcium medium, the responses are associated with depleted ER calcium stores, and the TGN effects are reversed by increasing extracellular calcium levels to partially replenish ER calcium [17]. In human keratinocytes grown in low-calcium medium, TGN promotes a transient increase in the cytosolic free calcium level [16], whereas in keratinocytes maintained in high-calcium medium (2 mM), the transient increase in the level of cytosolic free calcium is followed by a sustained calcium increase caused by influx of extracellular calcium [16].

The studies described in the present report were performed under non-differentiating, low-calcium conditions. Under these conditions, TGN inhibits the PMA-dependent increase in hINV mRNA level and promoter activity. Promoter mapping studies localize the response to a promoter segment, nucleotides -159/-41, within the proximal regulatory region (PRR), that contains a cluster of binding sites that are important for hINV promoter activity [4,10,13]. Mutation studies suggest that the response is mediated via a C/EBP site located within this region [13]. C/EBP α , acting via this site, is thought to participate as a mediator of PMA-dependent activation [20]. We show further that TGN inhibits the C/EBP α -dependent activation of the hINV promoter. Furthermore, our studies show that TGN inhibits this activation by inhibiting C/EBP α binding to the hINV promoter C/EBP site without changing total C/EBP α levels. These results, when considered in the context of a previous report [13] showing that PMA increases promoter activity via a mechanism that involves increased C/EBP α binding to the promoter, indicate that the quantity of C/EBP α bound to the hINV promoter is a major determinant of the level of activation.

The reduction in C/EBP α DNA binding could be explained by a direct effect of TGN on hINV C/EBP α or by effects on an associated factor that is required for DNA binding. In addition, TGN could increase the levels of a factor that inhibits binding. GADD153 is a candidate gene. GADD153, a member of the C/EBP family of transcription factors, is induced in HeLa cells following treatment with TGN and other agents that increase intracellular calcium [22]. We have shown that GADD153 suppress hINV promoter activity [13]. GADD153 works by inhibiting the activity of other C/EBP proteins [23,24]. Increased GADD153 expression could explain the inhibition of the C/EBP α -dependent activation. However, it is unlikely that GADD153 is playing a role in the present regulation, since, in contrast to HeLa cells [22], we could find no increase in GADD153 mRNA level following treatment of keratinocytes with TGN (results not shown).

One other report describes the effect of TGN on hINV gene expression. In this study TGN was not able to inhibit the increase in involucrin following stimulation with 2 mM extracellular calcium [16]. We suspect that TGN-dependent inhibition was not observed in these experiments because the extracellular calcium concentration (2 mM) was sufficient to partially refill ER calcium stores. Taken together with our present results, these results suggest that stored calcium is required for differentiation-associated involucrin expression and that at least part of the reason for the loss of hINV expression when intracellular stores are depleted is a reduction in C/EBP α transcription-factor binding to DNA. Finally, it should be noted that although treatment with TGN suppresses PMA-dependent hINV promoter activity, it does not suppress basal promoter function. This may suggest that, although TGN is inhibiting the C/EBP α -dependent promoter response, other factors that maintain basal

promoter activity are not effected by TGN treatment. Further study will be required to assess this possibility.

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