Signal-transduction-pathway-specific desensitization of expression of orphan nuclear receptor TIS1

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Induction of many primary-response genes by a variety of stimuli occurs in a transient manner. The precise mechanism responsible for these transient kinetics is not completely understood. We report here that the orphan nuclear receptor, TIS1, which is a potent sequence-specific transcription factor, was transiently induced by the adrenergic agonist isoprenaline in the C2C12 skeletal-muscle cell line. Moreover, we showed that the rapid decline in mRNA level after peak induction was due in part to a specific desensitization of the isoprenaline-mediated induction pathway. Desensitization of the induction response presumably

occurred at the level of the receptor, as agents that either bypass the adrenergic receptor or activate alternative signalling pathways were able to induce TIS1 expression in the desensitized cells. However, stimulation by agents that directly activate intracellular enzymes also resulted in the signal-transduction-pathway-specific desensitization of TIS1 inducibility. Our results suggest that the pathway-specific nature of the desensitization process may be important for directing an integrated response to multiple physiological stimuli.

INTRODUCTION

One of the earliest responses to growth factors and many other agonists is the induction of a group of primary-response genes which are induced by the activation of pre-existing transcription factors (reviewed by Herschman [1]). The induction of these genes can occur without protein synthesis and is generally transient in nature. This is reflected in a rapid rise in the mRNA level which peaks within 30 min to 1–2 h and quickly returns to the basal level within 3–5 h [2–4]. The behaviour of this response implies that the mRNA of the early primary-response genes may be intrinsically unstable. More importantly, it also indicates that the transcription of these genes must be terminated rather abruptly, shortly after the initial presentation of the stimulus. Furthermore, as the transcription of primary early-response genes often ceases while the inducing agent continues to be present outside the cell, some alterations in the intracellular signalling process must occur to prevent the inducing signal from reaching the nucleus. Exactly how the cell manages to terminate and desensitize its transcriptional response to the incoming signal is not well understood. In particular, whether the desensitization process reflects a general suppression of the transcriptional activity of the primary-response gene or affects an individual specific signal-transduction pathway has not been established.

We are interested in the potential role of various primary-response genes in the regulation of the proliferation and differentiation of skeletal-muscle cells. We have reported previously that treatment with growth factors and other agents induces a number of primary-response genes in a variety of cell lines [4–6]. One of these genes, TIS1 (also known as nur77, N10), encodes an orphan receptor-like transcription factor [7–9]. The rat homologue of TIS1, NGFIB, has been found recently to regulate the expression of the gene encoding the steroidogenic enzyme 21-hydroxylase [10], and may also recognize a sequence element in the 5' flanking region of the acetylcholine receptor γ -subunit gene [11]. The human homologue of TIS1, NAK-1, has been isolated from a fetal muscle cDNA library [12], and the expression level of TIS1 appeared to increase in skeletal-muscle

tissue during murine postnatal development [13]. Moreover, a recent study showed that TIS1 expression *in vivo* in the mouse adrenal gland may be under neuroendocrine regulation [14]. Thus, although TIS1 was originally identified as a mitogen-inducible gene, these findings suggest that hormone-regulated expression of TIS1 may play a role in mediating the transcriptional responses of skeletal-muscle tissue to changes in the physiological environment. In agreement with such an hypothesis, we report here that the TIS1 mRNA was induced by the adrenergic agonist isoprenaline in the C2C12 mouse skeletal-muscle cell line. We showed that the induction of TIS1 occurred in a transient manner, as might be expected from the results of previous studies. Interestingly, termination of the transcriptional response to isoprenaline was accompanied by the desensitization of the isoprenaline receptor, as TIS1 expression was no longer induced by re-addition of the agonist, but remained inducible by agents that either bypass the membrane receptor or activate alternative signalling pathways. Moreover, pretreatment with pharmacological agents that stimulate TIS1 expression by activating intracellular enzymes also resulted in an attenuation of the induction response in a signal-transduction-pathway-specific manner. Taken together, the results from this study suggest that multiple mechanisms are involved in terminating the TIS1-induction signal in a pathway-specific manner at both receptor and post-receptor levels. The pathway-specific attenuation of the TIS1 induction would enable cells to fine-tune the expression of TIS1 in response to multiple stimuli, thus allowing an integrated response to complex physiological changes.

MATERIALS AND METHODS

Cell culture

C2C12 mouse myoblasts (obtained from ATCC) were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum on gelatinized plastic tissue culture dishes in a 37 °C, 5 % CO₂ humidified incubator. Recom-

Abbreviations used: bFGF, basic fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; CRE, cyclic AMP-responsive element; CREB, cyclic AMP-responsive element-binding protein.

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binant human basic fibroblast growth factor (bFGF) used in the experiments was generously given by Dr. Stephen Hauschka and Dr. Bradley Olwin. Phorbol 12-myristate 13-acetate (PMA), isoprenaline and forskolin were obtained from Sigma (St. Louis, MO, U.S.A.).

Plasmids

The derivation and properties of the TIS1 recombinant cDNA plasmids have been described previously [4]. The plasmid pCHO_b which recognizes a constitutively expressed mRNA species [15] was used as a probe to normalize for RNA loading and transfer as described earlier [4].

RNA isolation and analysis

Total RNA was isolated by the guanidinium isothiocyanate/acid/phenol extraction procedures of Chomczynski and Sacchi [16] with slight modifications. Two sequential phenol-extraction steps were performed instead of the single extraction step used in the original protocol. The second propan-2-ol precipitation step was eliminated. RNA samples were analysed by electrophoresis on a 1.1% agarose gel containing 2.2 M formaldehyde, blotted on to nitrocellulose by capillary action and hybridized to radioactive cDNA probes generated by random-primed labelling of the respective plasmids by standard protocols. Hybridization and washing conditions were as previously described [4]. The intensity of the TIS1 hybridization signals was quantified by densitometry scanning using a laser densitometer from LKB and normalized to the signals obtained with the pCHO_b plasmid.

RESULTS

Rapid and transient TIS1 induction by isoprenaline

C2C12 cells were stimulated with isoprenaline (100 nM), and RNA was harvested at different times after stimulation. Expression of TIS1 was monitored by Northern-blot hybridization. As expected from the behaviour of many primary-response genes under a variety of experimental conditions [2–4], the induction of TIS1 occurred rapidly, reaching a peak by 0.5–1 h, but returned to basal level by 4 h after the addition of the agonist (Figure 1).

Figure 1 Time-dependent isoprenaline-induced TIS1 expression

Proliferating C2C12 cells were treated with 100 nM isoprenaline for the indicated times from 0.5 to 4 h. Total cellular RNA was harvested and analysed by Northern-blot hybridization with a full-length radioactive TIS1 cDNA insert. Plasmid pCHO_b which recognizes a constitutively expressed mRNA was used as a control to normalize for RNA loading and transfer. Similar results were obtained from two other experiments.

Figure 2 Desensitization of TIS1 inducibility by pretreatment with isoprenaline

C2C12 cells were pretreated with 100 nM isoprenaline for 4 h or left untreated for the same time period. Various compounds were then added and the cells were incubated for an additional hour. Total cellular RNA was harvested and analysed by Northern-blot hybridization. N, no addition; I, cells rechallenged with isoprenaline (100 nM); P, cells stimulated with PMA (100 nM); F, cells stimulated with forskolin (10 μ M). Similar results were obtained from two other experiments.

Figure 3 Signal-transduction-pathway-specific desensitization of TIS1 induction by pretreatment with forskolin or PMA

Proliferating cells were either pretreated with 10 μ M forskolin or 100 nM PMA for 4 h or left untreated and then challenged with forskolin (10 μ M) or PMA (100 nM). Incubation was continued for a further hour. Total cellular RNA was then isolated and analysed by Northern-blot hybridization. N, control cultures that were left untreated; F, cultures treated with forskolin; P, cultures treated with PMA. Similar results were obtained from two experiments.

Loss of TIS1 induction response as a result of prolonged treatment with isoprenaline

The rapid decline in TIS1 mRNA level suggests that in addition to the mRNA having a relatively short half-life, the transcriptional activation of the TIS1 gene must also be rather transient. To understand the rapid cessation of the induction response, we tested whether reapplication of isoprenaline could cause restimulation of TIS1 expression. Cells were treated with the agonist for 4 h and then challenged with additional isoprenaline or a number of other agents for another hour before RNA isolation. As shown in Figure 2, TIS1 could no longer be induced by isoprenaline, indicating that the cells had indeed become refractory to stimulation by the same inducing agent. Interestingly, TIS1 was still inducible by PMA; in fact, inducibility was even slightly enhanced. (The PMA-induced TIS1 mRNA level was increased by 76% after normalization for

Figure 4 Rapid desensitization caused by forskolin and slow recovery after forskolin removal

(a) To determine how rapidly cells become desensitized to the effect of forskolin, cells were exposed to 10 μ M forskolin for 5 min. The incubation medium was removed, the cultures were rinsed twice with Hank's balanced-salt solution and fed serum-free Dulbecco's modified Eagle's medium. After 4 h, various compounds were added as indicated. Total cellular RNA was harvested after another hour and analysed for TIS1 expression. NA, no addition; FK, stimulated with forskolin (10 μ M); PMA, stimulated with PMA (100 nM); FGF, stimulated with bFGF (2 ng/ml). Parallel cultures not subjected to forskolin pretreatment were stimulated in an identical manner to serve as positive controls for TIS1 inducibility (no pretreatment lanes). (b) To determine if cells would remain desensitized in the continuous presence of forskolin, they were treated with 20 μ M forskolin for various periods of time from 4 to 30 h as indicated, and then challenged with the same concentration of forskolin. Total cellular RNA was extracted 1 h after the second addition of forskolin and subjected to Northern-blot analysis. +, forskolin readdition; —, no forskolin readdition; P, cells without pretreatment were stimulated with forskolin for 1 h to serve as a positive control; C, cells were treated with DMSO (used to dissolve forskolin) and harvested 30 h later without further stimulation to serve as negative control. (c) Slow recovery from the desensitized state. Cells treated for 4 h with 20 μ M forskolin were rinsed and re-fed with growth medium containing no forskolin. At various times indicated (4–45 h) after forskolin removal, cells were rechallenged with 20 μ M forskolin for 1 h. Total

RNA loading in the isoprenaline-pretreated cells in comparison with cells that had not undergone the pretreatment.) Furthermore, forskolin, which is believed to activate adenylate cyclase directly, could also stimulate TIS1 expression in isoprenaline-pretreated cells. As both isoprenaline and forskolin presumably act through a cyclic AMP-dependent pathway, the result indicates that the primary mechanism responsible for the loss of responsiveness to isoprenaline occurred before the adenylate cyclase step, and that receptor desensitization may contribute significantly to the transient nature of the TIS1-induction response to isoprenaline.

Respective homologous desensitization of the TIS1 induction response by forskolin and PMA

As stimulation of TIS1 expression by agents such as forskolin and PMA which bypass cell-surface receptors also occurred in a transient manner [4,5], receptor desensitization cannot be the only mechanism for terminating the TIS1-induction response. To find out if the transient kinetics of TIS1 expression under such circumstances are also due to a specific loss of responsiveness to the particular inducing agent, we pretreated cells with either PMA or forskolin for 4 h and then challenged them again with either of the two agents individually. Figure 3 shows that pretreatment with either agent inhibited only the restimulation by the respective agent. Induction of TIS1 by agents that activate an alternative signal-transduction pathway was largely unaffected and might even be enhanced. For example, the PMA-induced TIS1 mRNA level in forskolin-pretreated cells was 2.6-fold higher than that achieved in cells without forskolin pretreatment. This apparent reciprocal potentiation of TIS1 induction with agents activating different signalling pathways thus provides a marked contrast with the attenuation of responsiveness to the homologous agents and suggests that the attenuation of the TIS1-induction response occurs in a signal-transduction-pathway-specific manner.

Rapid desensitization of the induction response and slow recovery

We proceeded next to analyse the time course of the desensitization process in more detail. First, we shortened the length of forskolin pretreatment to 5 min. The cells were rinsed to remove forskolin from the medium, and challenged 4 h later by readdition of the agent. As shown in Figure 4(a), 5 min of forskolin pretreatment was apparently sufficient to block subsequent induction of TIS1 by this agonist. In contrast, stimulation by bFGF was only partially affected, whereas PMA stimulation was again enhanced. The PMA-induced TIS1 mRNA level after pretreatment with forskolin was 3.6-fold higher than that observed in cells that had not been pretreated. To determine whether TIS1 induction remains desensitized during chronic exposure to forskolin, C2C12 cells were treated with this agent for various periods of time from 4 to 30 h, and the inducibility of TIS1 was assessed by rechallenging the cells with additional forskolin for 1 h. As indicated in Figure 4(b), maintaining the cells in forskolin for up to 30 h still substantially reduced the

cellular RNA was harvested and analysed by Northern-blot hybridization. Lane P, cells were pretreated with DMSO (solvent control) for 4 h, rinsed and re-fed with growth medium; forskolin stimulation (1 h) was performed 45 h after the medium change. Lane C, cells were treated with DMSO for 4 h, rinsed, fed with growth medium and then harvested for RNA analysis without additional treatment 45 h after the medium change.

subsequent inducibility of TIS1 by forskolin. Although some inducibility returned by 19–30 h of forskolin pretreatment, the level of induction was less than that observed in cells that had not been previously exposed to forskolin. We examined next the recovery of TIS1 inducibility after forskolin removal. Cells were pretreated with the latter for 4 h, then rinsed and fed with growth medium without forskolin. The inducibility of TIS1 by forskolin was assessed from 4 to 45 h after the medium change. As shown in Figure 4(c), the recovery of TIS1 inducibility appeared to be a rather slow process. Although some induction of TIS1 was evident by 8 h after removal of forskolin, TIS1 inducibility was not completely restored until about 40 h after the medium change. Interestingly, a substantial return of TIS1 inducibility was observed by 36 h after the start of forskolin pretreatment even if the medium was left unchanged, presumably because of the gradual inactivation of forskolin in the incubation medium (results not shown).

DISCUSSION

The primary-response gene TIS1 encodes a nuclear receptor-like transcription factor that is rapidly induced by growth factors and a variety of other agents [7,17–19]. Results from a number of studies suggest that altered expression of TIS1 in response to external stimuli may have important modulating influences on the expression of tissue-specific genes [10,11]. We report in this study that TIS1 can be induced in the C2C12 skeletal-muscle cell line in response to the β -adrenergic agonist isoprenaline (Figure 1). As chronic stimulation by adrenergic agonists has been shown to cause skeletal-muscle hypertrophy and alter the metabolism of specific muscle fibre types in experimental animals [20], the inducibility of TIS1 by isoprenaline in C2C12 cells suggests that TIS1 may mediate some of the secondary transcriptional responses to β -adrenergic stimulation in muscle tissues.

Consistent with the induction characteristics of a primary-response gene, the induction of TIS1 by isoprenaline was transient (Figure 1a). Moreover, as addition of fresh isoprenaline failed to re-induce TIS1 gene expression (Figure 2), the cells appeared to have become refractory to the stimulatory effect of the adrenergic agonist. This refractoriness to further stimulation has at least two alternative explanations. First, induction of the early-response gene TIS1 can be repressed at a step after the convergence of various signal-transduction pathways such that it can no longer be stimulated by any inducing ligand. Alternatively, the transcription of TIS1 may be repressed in a pathway-specific manner. Our data (Figure 2) showing that agents that presumably stimulate TIS1 expression through non-cyclic AMP-signalling pathways could induce TIS1 in isoprenaline-pretreated cells indicate that the attenuation of the TIS1-induction response is pathway-specific. In addition, as bypassing the cell-surface receptors by directly activating adenylate cyclase with forskolin also induced TIS1 in isoprenaline-pretreated cells (Figure 2), the transient nature of TIS1 induction by isoprenaline may be due in part to the desensitization of the β -adrenergic receptor. A number of distinct mechanisms have been shown previously to cause similar desensitization of adrenergic and other G-protein-coupled receptors (reviewed in ref. [21]).

Additional data suggest that the signal-transduction cascade leading to TIS1 expression could also be uncoupled at some step(s) distal to the cell-surface receptor. We showed in Figure 3, for example, that pretreatment with agents such as forskolin and PMA, which act intracellularly to bypass membrane receptors, nonetheless resulted in the 'desensitization' of TIS1 induction. The desensitization again occurred in a ligand-specific manner, and the desensitized cells could still be induced to express TIS1

in response to agents that activate other signal-transduction pathways. The desensitization process occurred very rapidly and persisted for up to 11 h in the presence of forskolin (Figures 4a and 4b). In contrast, removing the inducing ligand resulted in a slow recovery of the induction response (Figure 4c).

Relatively little attention has been paid to the mechanisms responsible for the attenuation of the transcription of primary-response genes. Gius et al. [22] reported that expression of the primary-response gene *egr-1* can be down-regulated by over-expression of *c-fos*, and suggested that induction of *c-fos* may serve as a feedback mechanism to prevent the continuous activation of *fos* and other primary-response genes. This model of inhibition, however, does not readily explain the pathway-specific nature of the attenuation observed in our experiments. As *c-fos* is induced in common by a large number of different stimulating agents, transcriptional repression by *c-fos* would not be expected to discriminate among inducers triggering different signal-transduction pathways. More recently, Hagiwara et al. [23] reported that the attenuation of cyclic AMP-induced gene induction may be due to the dephosphorylation of cyclic AMP-responsive element (CRE)-binding protein (CREB) by protein phosphatase 1. According to their data, okadaic acid, which is a potent inhibitor of this enzyme, potentiated CRE-mediated gene induction, but had little or no effect on gene expression regulated through the PMA-responsive element. Similarly, Wadzinski et al. [24] also demonstrated that okadaic acid can enhance the cyclic AMP-mediated transcriptional response of the phosphoenolpyruvate carboxykinase promoter but argued on the basis of other evidence that protein phosphatase 2a rather than protein phosphatase 1 may be responsible for CREB dephosphorylation and attenuation of the inductive response. The TIS1 5' flanking region, however, does not contain any consensus CRE [9,19,25]. Although it is possible that a non-consensus CRE might be involved in TIS1 expression, there is no direct evidence to date demonstrating the involvement of CREB in the cyclic AMP-dependent induction of TIS1. It is thus not clear whether the previously reported dephosphorylation of CREB would necessarily be applicable to the attenuation of TIS1 induction after forskolin pretreatment. In this regard, the slow recovery of TIS1 inducibility after removal of forskolin suggests that the desensitization phenomenon may have resulted from processes other than rapidly reversible post-translational modification.

The precise mechanism responsible for the pathway-specific desensitization of TIS1 response after the short (4 h) PMA pretreatment is also not clearly understood. Although it is well established that prolonged treatment with PMA causes the down-regulation of protein kinase C activity [26] and degradation of the enzyme [27], the rapidity of the loss of TIS1 inducibility presumably cannot be accounted for by the down-regulation of the classic protein kinase C isoenzymes. That some novel isoforms of protein kinase C that are down-regulated more rapidly might be responsible for the PMA-mediated induction and desensitization of TIS1 expression cannot at present be completely ruled out.

In a related study, Yu et al. [28] recently demonstrated that overexpression of oncoproteins involved in the tyrosine kinase signalling pathway also resulted in the reduced serum inducibility of several early-response genes including TIS1. Thus stimulation of many different signal-transduction pathways leading to early-response-gene expression can all result in the desensitization of the induction response. Data from the present study indicate that the desensitization occurs in a signal-transduction-pathway-specific manner. These observations emphasize the importance of the attenuation process in fine-tuning the expression of primary-response genes in response to different stimuli. The

pathway-specific nature of the desensitization ensures that the early-response genes will not be overexpressed in response to a single type of stimulus but yet remain poised to respond to other types of signal that might further impinge on the cell, thus allowing the cell to integrate the incoming signals to mount a coherent physiological response.

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