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Role of Calcium Influx in mediating the TRH-induced c-*fos* Gene Expression

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TRH (Thyrotropin-Releasing Hormone) is known to regulate the transcription of the TSH (Thyroid-Stimulating Hormone) gene in pituitary cells, but little is understood about the mechanism(s) involved. The present study was attempted to elucidate the role of Ca^{2+} movement through the voltage-gated channels in the regulation of TSH gene transcription. The c-*fos* is one of immediate early genes and used as model system for the investigation of signaling pathways involved in various stimuli. The changes of c-*fos* mRNA levels were determined after treatment of various agents using Northern and slot hybridization analysis. The c-*fos* mRNA was rapidly and transiently induced by TRH (about 3-fold) in GH_3 cells and this induction was repressed by calcium chelating agent (EGTA), calcium channel blocker (verapamil) and protein kinase C inhibitor (aminoacridine). The abilities of forskolin (adenylate cyclase activator), PMA (protein kinase C activator), and A23187 (calcium ionophore) to affect c-*fos* gene transcription, either alone or in combination with TRH were tested in the same cells. All of them significantly increased the level of c-*fos* mRNA. However, no additive relationship was observed in all combined treatments except forskolin. These results suggest that TRH action on the c-*fos* gene activation is mediated by calcium influx as well as through protein kinase C.

KEY WORDS: TRH, TSH, c-*fos* gene, GH_3 cell, calcium influx, protein kinase C

During the past several years, the action mechanism of Thyrotropin Releasing Hormone (TRH) has been intensively studied in GH cells, the cloned rat mammotropic tumor cell lines, which contain the receptors for TRH. In GH_3 cells, TRH stimulates the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) by phospholipase C to yield inositol triphosphate (IP_3) and 1,2-diacylglycerol (DG) at the initial step in the sequence of intracellular events leading to many processes (Martin and Tashjian, 1977; Gourdji *et al.*, 1982). Recently, the role of movement of Ca^{2+} through voltage-gated channels in the regulation of prolactin gene transcription by TRH was examined (Hinkle *et al.*, 1988; Day and Maurer, 1990; Enyeart *et al.*, 1990). Calcium specifically stimulates prolactin synthesis in GH_3 cells (White *et al.*, 1981). In the same cells, the response to TRH was not altered by addition of the Ca^{2+} channel agonist, suggesting similar mechanism may mediate these responses (Day and Maurer, 1990). However, it is unclear whether voltage gated calcium channel is related to activation of Thyroid Stimulating Hormone (TSH) gene expression. The TRH action mechanism on TSH gene expression is difficult to study directly, because there are no cell lines which secrete endogenous TSH.

On the other hand, c-*fos* is one of those immediate early genes rapidly induced in neuronal

cells as well as in endocrine cells by neurotransmitter and membrane depolarization (Greenberg and Ziff, 1984; Morgan and Curran, 1986; Saffen *et al.*, 1988). Therefore, the c-*fos* gene has been studied extensively to investigate the intracellular signaling pathways involved in the expression of several genes by diverse set of stimuli. In PC 12 cells, signaling pathways that lead to the increase in intracellular Ca^{2+} and cAMP levels appear to activate the c-*fos* gene transcription through phosphorylation of a common transcription factor, known as the cAMP response element-binding protein (CREB) (Sheng and Greenberg, 1990; Sheng *et al.*, 1990). Activation of immediate early genes (IEGs) by phorbol esters is mediated by a second phosphoprotein known as a serum response factor (Sheng *et al.*, 1988). Phorbol esters activate protein kinase C, thereby mimicking the endogenous activator, DG (Nishizuka, 1984).

In the present study, the role of Ca^{2+} movement in the mediation of TRH action was investigated. Northern and slot blot hybridization analysis were performed to analyze the interactions among TRH, the phorbol ester 12-myristate 13-acetate (PMA), A23187 (Ca^{2+} ionophore) and forskolin (adenylate cyclase activator) on c-*fos* gene expression. To determine the mechanism of TRH action, various inhibitors of calcium channel and protein kinase C were used in combination with TRH treatment: calcium channel blocker (verapamil), calcium chelating agent (EGTA) and protein kinase C inhibitor (aminoacridine).

Materials and Methods

Cell culture and treatment of agents

GH_3 cells, cloned by Tashjian *et al.* (1968), were maintained in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Prior to stimulation with TRH or other compounds the cells were kept in F10 medium containing 15% horse serum and 1% fetal bovine serum for one day. Experiments were generally performed about a week after plating 1: 10 dilution. All agents were prepared from highly concentrated stock solution and were subjected to successive dilution in conditioned medium.

RNA isolation

Total cellular RNA was prepared from GH_3 cells by the method of Maniatis *et al.* (1989) with minor modifications. The ninety millimeter-dish culture of GH_3 cells were washed twice with 7 ml of ice-cold phosphate-buffered saline (PBS) lacking calcium and magnesium ions. Two milliliter of 10 mM EDTA (pH 8.0) and 0.5% SDS were added to the cells and lysate was scraped and transferred to a clean tube. The plate was then rinsed with 2 ml of 0.1 M sodium acetate (pH 5.2) and 10 mM EDTA (pH 8.0), and the solution was transferred to the tube containing the cell lysate. The content of the tube was mixed with 4 ml of phenol (equilibrated with water) and centrifuged at 5,000 rpm for 10 mins at 4°C in Sorvall SS34 rotor. The upper (aqueous) phase was extracted with phenol twice and transferred to a fresh tube. RNA was ethanol-precipitated at −20°C for 2 hrs after the addition of 440 μl of ice-cold 1 M Tris-Cl (pH 8. 0), 180 μl of 5 M NaCl and 10 ml of ice-cold ethanol. The RNA was collected by centrifugation at 4°C and redissolved in 60 μl of TE (pH 8.0) for quantitation.

Radiolabeling

Labeling of DNA was carried out by the random primer extension method, as described by Feinberg and Volgelstein (1984).

Northern Blot Analysis

Isolated RNAs were fractionated by electrophoresis through 1.2% agarose gel containing 3% formaldehyde (Lehrach *et al.*, 1977). Each lane contained 20 μg of total cellular RNA. The RNA was then transferred to Nitrocellulose filter (Thomas, 1980) by capillary action. The filter was baked for 2 hrs at 80°C *in vacuo* and prehybridized in hybridization buffer [50% (v/v) deionized formamide, 5X Denhardt's solution (1 mg/ml of ficoll, 1 mg/ml of polyvinylpyrrolidone and 1 mg/ml of bovine serum albumin), 6X SSC, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA] for 12-14 hrs at 42°C. ^{32}P-labeled probe (5×10^6 cpm) was added to fresh hybridization buffer and hybridization

proceeded for 16-24 hrs at 42°C. The filter was then washed for 10 mins at room temperature in 2X SSC, followed by two washes of 10 mins each at room temperature in 0.5X SSC. The filter was then wrapped in clean plastic wrap and exposed to X-ray film (Fuji NIF-RX) with intensifying screen for 2-4 days at −70°C.

Slot Blot Analysis

Twenty microgram of total cellular RNA was denatured by incubating for 15 mins at 60°C in a solution of TE containing 6X SSC and 7.5% (v/v) formaldehyde. The nitrocellulose filter was immersed in DEPC-treated water and then 20X SSC before being mounted in the slot blot apparatus (S & S Minifold II). The denatured samples were applied into slots of the apparatus with slight vacuum applied, and each well was then washed with 100 μl of 20X SSC. The nitrocellulose filter was dried at room temperature before baking for 2 hrs at 80°C *in vacuo*. Conditions for hybridization and filter washing were the same as Northern Blotting.

Result

The specificity of c-*fos* gene expression in response to TRH treatment was examined. Total RNA was extracted from GH_3 cells treated with 10nM TRH in varying amount of time and analyzed by Northern and slot blot techniques using specific probe, labeled rat c-*fos* cDNA. As shown in Fig. 1 (A), the level of c-*fos* mRNA increased at 30 mins after the addition of 10 nM TRH and returned to the basal level seen in unstimulated GH_3 cells within 90 mins. In contrast, the levels of mRNA hybridizing to the γ-actin probe as internal control were constant for several hours after TRH induction. Densitometric analysis of slot blots also revealed about 3-fold increase in the levels of c-*fos* mRNA upon treatments with 10nM TRH for 30 mins {Fig. 1 (B), (C)}.

In an attempt to elucidate which one of the intracellular signal transduction pathways is/are involved in c-*fos* gene activation by TRH stimulus, a variety of compounds, known to affect

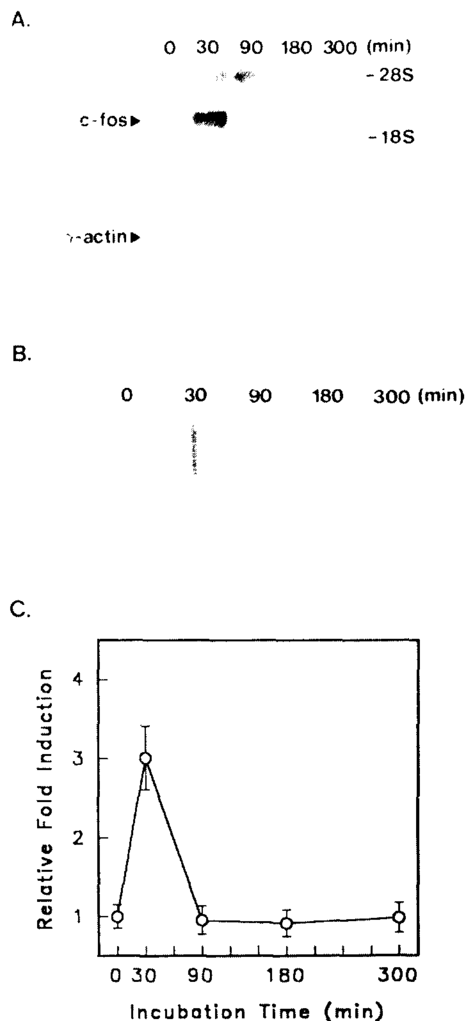

Fig. 1. Time course of c-*fos* gene expression in GH_3 cells stimulated with TRH. Northern and slot blot analysis of total RNA were performed in time course after treatments of 10 nM TRH. (A). Northern blot analysis: the size of the transcript for c-*fos* (2.2kb) is indicated by arrow and the minor RNA species visible above c-*fos* mRNA (2.2kb) in all blots represent immature c-*fos* RNA (3.7kb). γ-actin was used as a control and two bands were appeared (upper band may represent immature γ-actin RNA). The positions of 28S and 18S markers are indicated. (B). Slot blot analysis (C). Relative fold induction: data are means for densitometric scans from three-independent slot-blots. Induction values were calculated by scanning densities of bands relative to that of unstimulated samples. The bars represent the mean ± SE.

intracellular Ca^{2+} concentration, protein kinase C activity or cAMP level, were treated to GH_3 cells either alone or in combination (Fig. 2). TRH action on cellular process has been proposed to occur via protein kinase C- (Drust and Martin, 1984), inositol phosphate- or Ca^{2+}-mediated pathways (Tan and Tashjian, 1981), with little or no contribution from cAMP (Gershengorn *et al.*, 1980). All four agents which are TRH, PMA (PKC activator), A23187 (Ca^{2+} ionophore) and cAMP (PKA activator) stimulated the synthesis of c-*fos* mRNA. However, additive relationships were not observed among these four agents in combination except forskolin; combined treatment with forskolin and TRH increased the level of c-*fos* mRNA approximately to the sum of that observed with either agent alone. No additive effects were detectable between PMA and TRH or A23187 and TRH. These observations suggested that the activation of protein kinase C and Ca^{2+} influx may be involved in TRH signal transduction leading to c-*fos* gene expression but not cAMP-related pathways. It was important to investigate a possible role for Ca^{2+} influx on c-*fos* gene expression. In view of this, GH_3 cells were stimulated with calcium ionophore, A23187, in different concentrations. Figure 3 shows the dependence of the rapid induction kinetics of c-*fos* gene upon A23187 concentration. The c-*fos* gene expression was increased with as little as 0.5 μM A23187 and the levels of induction increased with dose-dependent manner until 1.5 μM and reached plateau.

To determine whether TRH action on the transcriptional induction of c-*fos* gene might occur via pathways similar to those used in PMA or A23187 action, specific inhibitors were used. Figure 4 showed that protein kinase C inhibitor, aminoacridine suppressed the level of c-*fos* mRNA induced by TRH. Densitometric analysis of slot blots revealed that 3-fold induction by TRH was inhibited to the basal level in the presence of aminoacridine, demonstrating that activation of protein kinase C is required for TRH signaling pathway on c-*fos* gene expression.

On the other hand, very similar result was obtained when verapamil and EGTA were used as inhibitor of Ca^{2+} influx. Verapamil is a calcium channel blocker and EGTA is a calcium chelating agent. Slot blot analysis revealed that TRH-induced c-*fos* gene expression (about 3.5-fold) was completely abolished by verapamil or EGTA (Fig. 5). This result strongly suggests that Ca^{2+} influx also mediates TRH signal on c-*fos* gene activation.

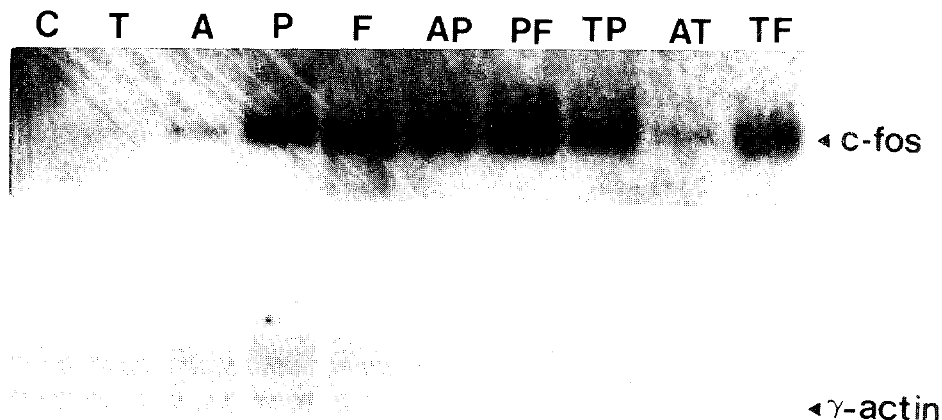

Fig. 2. Dose response of c-*fos* gene activation to A23187. The expression of c-*fos* in total cellular RNA from GH_3 cells treated for 30 mins with different doses of A23187 (0-2.0 μM) was determined by Northern and slot blot analysis. (A). Northern blot analysis (B). Slot blot analysis (C). Relative fold induction: densitometric scanning was performed, as in FIg. 1 (C)

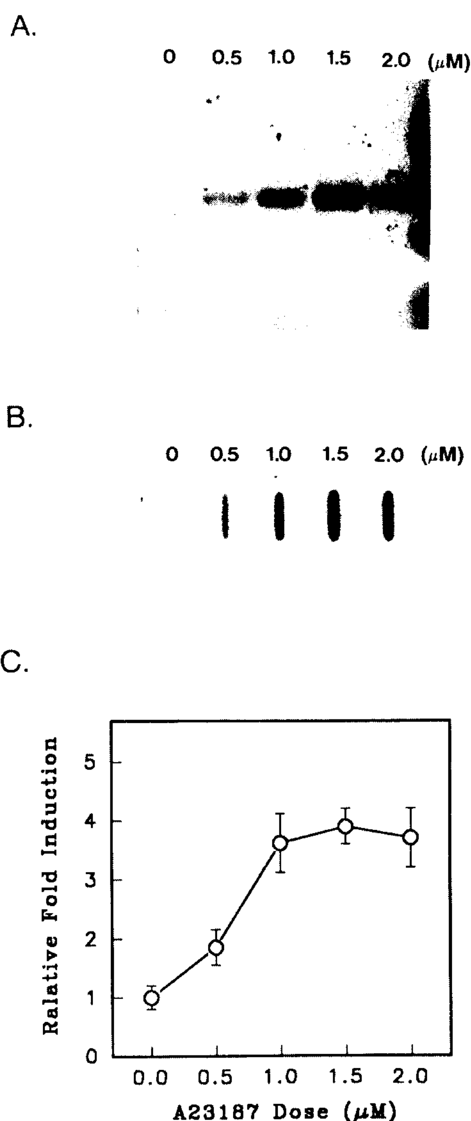

Fig. 3. Induction of c-*fos* gene expression in GH_3 cells by diverse set of agents. Quiescent GH_3 cells (C) were treated for 30 mins with the indicated agents, either alone or in combination: T; 10 nM TRH, A; 5 μM A23187, P; 100 nM PMA, F; 2 μM forskolin, AP; A23187 plus PMA, FP; forskolin plus PMA, TP; TRH plus PMA, AT; A23187 plus TRH, FT; forskolin plus TRH. c-*fos* mRNA were quantitated by Northern blot analysis of 20 μg samples of total RNA. γ-actin was used as internal control.

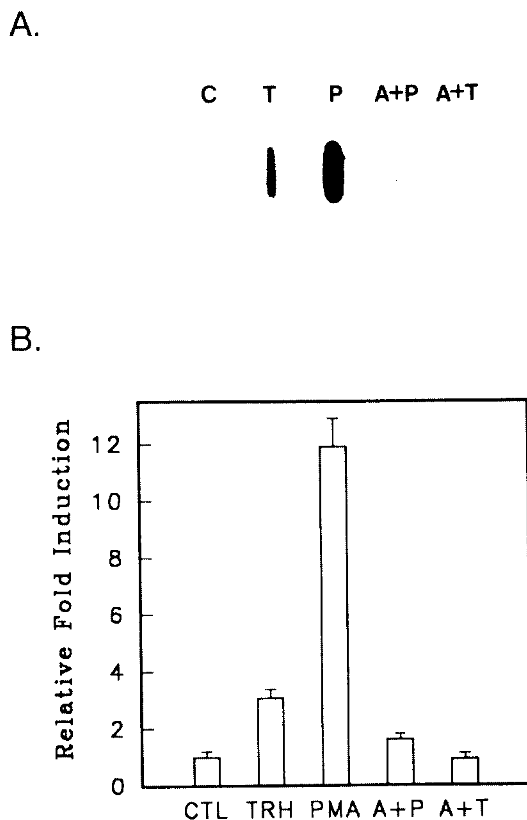

Fig. 4. Effect of aminoacridine on TRH- or PMA-induced c-*fos* gene expression. Quiescent GH_3 cells (C) were treated with TRH (10 nM) or PMA (100 nM) for 30 mins either in the absence or presence of aminoacridine (100 μM). (A). Total RNA were isolated and slot blot analysis was carried out. (B). Quantitation of the three-independent slot blot autoradiograms were carried out by comparing band intensities.

Discussion

The pathways linking interaction of TRH with its specific receptor to the subsequent synthesis and secretion of TSH by GH_3 cells or primary cultured cells have been extensively investigated and shown to involve activation of phosphoinositide metabolism (Hinkle, 1988), protein kinase C and increase of intracellular calcium levels (Gershengorn, 1982). While the rapid elevation of $[Ca^{2+}]_i$ by IP_3 (first phase

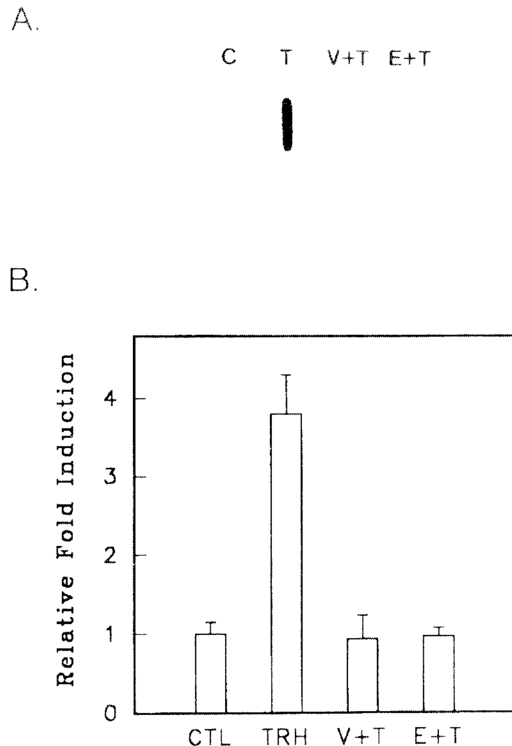

Fig. 5. Specific inhibition of TRH-induced *c-fos* gene expression by calcium channel blocker. (A). Quiescent GH_3 cells (C) were treated as follows for 30 mins: 10 nM TRH (T) or preincubated with 75 μM verapamil (V+T) and 1 mM EGTA (E+T). Total RNA were isolated and slot-blot analysis was performed. (B). *c-fos* mRNA levels were quantitated by densitometric scanning of the autoradiograms. Data are means for densitometric scans from three independent slot blots.

elevation) (Drummond, 1985) couples stimulus to burst secretion, second phase elevation extended by a delayed but prolonged TRH-induced enhancement of extracellular Ca^{2+} influx are still unclear in its molecular events. Unfortunately, since there are no cell lines which are established from pituitary thyrotrophs and produce endogenous TSH, TRH action mechanism on TSH gene expression has been studied indirectly or using primary cell culture. In our laboratory, TRH response elements (TRHREs) were characterized in the promoter region of the human TSH α gene and gene-proximal proteins that transmitted TRH signal to these elements were partly characterized. However, these studies were performed using GH_3 cells which were heterologous systems without producing endogenous TSH. Thus, more detailed studies on biochemical pathway of TRH action was required using homologous system.

On the other hand, in neural crest-derived PC12 cell, Ca^{2+} channel activation by depolarization strongly induced the expression of immediate early genes (Sheng *et al.*, 1990). But, in endocrine cells, this type of analysis has been less well studied. Electrically excitable endocrine cells, including anterior pituitary cells and established cell lines, possess all of the signaling pathways known to activate the expression of IEGs in other cells. Interestingly, *c-fos* is one of those IEGs and rapidly induced in endocrine cells as well as in neuronal cells by membrane depolarization and neurotransmitters. In GH_3 cells, the *c-fos* gene expression was stimulated at early stages (within 30 mins) and responded not only to TRH but also to various agents which activates protein kinase C or elevated intracellular calcium or cAMP levels, as in other cell lines (Kruijer *et al.*, 1984; Morgan and Curran, 1986; Morgan *et al.*, 1987). The more detailed pathway of TRH signaling on *c-fos* gene activation was studied using combined treatments of diverse set of inducing agents and specific inhibitors. The transient induction of *c-fos* mRNA level by TRH was suppressed by verapamil or EGTA as well as aminoacridine. The *c-fos* gene also responded with increased level of transcription to PMA, A23187 or forskolin without additive fashion except forskolin. These results suggest that both activation of protein kinase C and Ca^{2+} influx are necessary for transcriptional activation of *c-fos* gene by TRH.

In addition, TRH, which activates protein kinase C, also increase the frequency of Ca^{2+}-dependent action potentials in GH_4C_1 cells (Duchemin *et al.*, 1992). This Ca^{2+} influx may be closely related with the activation of protein kinase C, which is currently unclear. However, electrophysiologists have described a small number of instance in which TPA (phorbol ester) modulates ion channel activity. In *Aplysia* abdominal ganglion bag cell neurons, TPA enhanced the magnitude of inward

current and evokes calcium action potentials and these actions of TPA were mimicked by addition of exogenous protein kinase C (DeRiemer *et al.*, 1985), suggesting that modulation of ion channels by TPA occurred via activation of protein kinase C and phosphorylation of the channel or a regulatory protein. Recently, it was reported that interaction between the Ca^{2+} and phorbol ester-activated pathways of IEG expression might occur at several levels in excitable cells (Doerner *et al.*, 1990). Consistent with these observations, Gammon *et al.* (1989) demonstrated that 2 μM PMA increased the frequency of Ca^{2+}-dependent action potentials in GH_3 cells by inhibiting K^+ channels, indirectly. Consequently, it seems likely that Ca^{2+} influx may be closely related to the activation of protein kinase C. Alteration in $[Ca^{2+}]_i$ is a crucial step in the initiation of a variety of cellular process including possible transcription of target genes (White, *et al.,* 1981). Indeed, other studies have found that changes in the level of intracellular Ca^{2+} may mediate hormonal regulation of prolactin gene expression (Laverriere *et al.*, 1988).

Taken together, above results obtained from studies of regulation of c-*fos* gene expression, may be applied to the activation of TSH α gene by TRH. Indeed, similar results were obtained in the studies of transient expression of chimeric reporter gene construct, TSHα-Luc in GH_3 cells in our laboratory.

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갑상선자극 분비 호르몬에 의해 유도되는 *c-fos* 유전자 발현에서 Ca^{2+}의 역할에 관한 연구

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뇌하수체 전엽에서 분비되는 갑상선 자극 호르몬(TSH)의 작용기작이 voltage-gated channel을 통한 Ca^{2+}의 이동과 어떠한 관계를 가지고 있는지를 밝히고자 *c-fos* 유전자의 전사유발을 그 model system으로 사용하였다. GH_3 세포에서 *c-fos* mRNA는 갑상선 자극 분비 호르몬(TRH)에 의해 짧은 시간 내에(30분) 그 양이 처리전에 비해 약 3배가 증가하였으며, 이러한 증가는 A23187(calcium ionophore), PMA(protein kinase C activator), forskolin(adenylate cyclase activator)의 처리에서도 발견 되었다. 그러나 이들 약물을 갑상선자극 분비 호르몬(TRH)과 함께 처리하였을 때는 forskolin을 제외하고는 모두 단독처리 때와 같은 정도의 증가를 보였으며, 또한 갑상선자극 분비 호르몬(TRH)에 의한 *c-fos* mRNA의 증가는 protein kinase C의 활성 저해물질인 aminoacridine은 물론 Ca^{2+} inflix의 저해물질들인 verapamil과 EGTA에 의해서도 억제 되었다. 이러한 결과들은 *c-fos* 유전자 발현에 있어서, 갑상선자극 분비 호르몬(TRH)의 작용에는 기존에 알려져 왔던 protein kinase C 뿐만 아니라 Ca^{2+} influx 또한 관여하고 있음을 시사한다.