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Increases in Cytosolic Ca^{++} Down Regulate Thyrotropin Receptor Gene Expression by a Mechanism Different from the cAMP Signal

Motoyasu Saji, Shoichiro Ikuyama, Takashi Akamizu, and Leonard D. Kohn

Section on Cell Regulation, Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Kidney and Digestive Diseases, National Institutes of Health, Bethesda, MD 20892

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Thyrotropin (TSH) receptor mRNA levels in rat FRTL-5 thyroid cells are decreased by treatment with the calcium ionophores, A23187 or ionomycin, as well as with TSH, cholera toxin, forskolin, and 8-bromo-cAMP. Down regulation is, in each case, associated with a decrease in [^{125}I]TSH binding and a decreased ability of TSH to increase cAMP levels. The ionophore does not alter cAMP levels and ethylene glycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid (EGTA) in the medium prevents down regulation of TSH receptor mRNA levels by the ionophore, but not by TSH; the EGTA action is reversed by the simultaneous addition of Ca^{++}. Whereas down regulation by TSH and its cAMP signal requires the presence of insulin and/or serum in the medium; down regulation by a calcium ionophore is still evident in their absence. Down regulation of TSH receptor mRNA levels and receptor desensitization by TSH/cAMP or an ionophore is lost in cells transfected with a full length TSH receptor cDNA devoid of regulatory elements, but able to reconstitute TSH receptor signal generation.

Rat FRTL-5 cells are a line of thyroid cells in continuous culture whose growth and function depend on thyrotropin (TSH) and its ability to activate Ca^{++} as well as cAMP-linked transduction signals (1-3). These actions are followed, within 8 hours, by a decrease in TSH receptor mRNA levels and desensitization of the TSH receptor (4). The decrease in TSH receptor mRNA levels is presumed to be mediated solely by the TSH-induced cAMP increase, since cholera toxin (CT), forskolin (FSK) and 8-bromo-cAMP (8BrcAMP) duplicate the TSH action (4). Nevertheless, other receptors exist in the FRTL-5 cell which increase cytosolic Ca^{++}, for example α_1-adrenergic (3, 5-7) and P_2 purinergic receptors (5, 8, 9); and ligands interacting with these receptors can inhibit the ability of TSH to elevate cAMP levels (6, 8, 9). Some of this inhibition, but not all, has been linked to modulation of the activity of a pertussis toxin-sensitive, "inhibitory" G-protein by the

Abbreviations: TSH, thyroid stimulating hormone; TSHR, TSH receptor; CT, cholera toxin; 8BrcAMP, 8-bromo-cAMP; FSK, forskolin; EGTA, ethylene glycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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Ca^{++} signal (5, 8, 9). In the present report we show that the Ca^{++} signal can, independent of the cAMP signal, also decrease TSH receptor mRNA levels and desensitize the TSH receptor. We also show that Ca^{++} and cAMP are mechanistically different in their ability to decrease TSH receptor mRNA levels; nevertheless, both require more than the structural portion of the TSH receptor to be operative.

MATERIALS AND METHODS

Materials - Bovine TSH was a highly purified preparation obtained from the NIH distribution program (NIDDK-bTSH-I-1, 30U/mg). [^{125}I]cAMP radioimmunoassay kits and [α-^{32}P]dCTP (3000 Ci/mmol) were from Du Pont/New England Nuclear (Boston, MA).

FRTL-5 Cell Culture - The FRTL-5 rat thyroid cells (ATCC CRL# 8305, Interthyr Research Foundation) were a fresh subclone (F_1) with properties previously detailed (1, 2). Cells were grown in Coon's modified F12 medium supplemented with 5% calf serum and a mixture of 6 hormones (6H): TSH ($1X10^{-10}$M), insulin (10 μg/ml), hydrocortisone (1 nM), human transferrin (5 μg/ml), somatostatin (10 ng/ml) and glycyl-L-histidyl-L-lysine acetate (10 ng/ml) (1, 2). They were passaged every 7-10 days and provided fresh media every 2 or 3 days. As appropriate to individual experiments, cells were shifted to 5H medium (no TSH) or to 3H medium (no TSH, no insulin and no hydrocortisone) with either 5% or 0.2% serum, as noted, for 6 days before use.

Stable Transfection of FRT cells with Full Length TSH Receptor cDNA - FRT rat thyroid cells are derived from early, parental FRTL-5 cell cultures (10) but have no functional TSH receptor (10) and no TSH receptor mRNA (4). They are maintained in Coon's modified F12 medium containing 5% serum (10). The full length TSH receptor (T8AFB) cDNA was subcloned into an SV40 promoter-driven pSG5 expression vector (Stratagene, La Jolla, CA) (4) and cotransfected (20 μg) with 2 μg pMC1neo (Stratagene) into $8x10^6$ FRT thyroid cells by electroporation (4). Cells were cultured in Coons' modified F12 medium containing 5% calf serum for 2 days, then maintained in the same medium containing 400 μg/ml G418 (GIBCO, Grand Island, NY). Three weeks after transfection, G418-resistant colonies were isolated and subcloned in the same medium containing 200 μg/ml G418. Subclones were screened for their ability to bind [^{125}I]TSH. The subclone used in this report, T33, exhibited specific TSH binding and TSH-increased cAMP levels (see below).

RNA Isolation and Northern Analysis - Total cellular RNA was isolated and Northern blot analyses performed as described (4). The rat TSH receptor probe was the purified T8AFB insert and represents residues -54 to 2780 of the nucleotide sequence reported for the rat FRTL-5 TSH receptor (4). Rat ß-Actin was kindly provided by Dr. B. Paterson (NCI).

Assays Measuring TSH Binding and cAMP Levels - TSH was radioiodinated and binding measured on cells maintained in 24 well plates with only minor modifications of our previous method (4). Incubations contained $1\text{-}2x10^5$ cpm [^{125}I]TSH (120 μCi/μg), continued for 16-18 h, were at 4° to avoid internalization and degradation, and were terminated by the addition of a diphenylamine solution, which allowed measurements of DNA (1, 2) as well as [^{125}I]TSH bound. Specific binding was calculated by subtracting values obtained in the presence of 10^{-7}M unlabeled TSH (4). Assays measuring intracellular cAMP levels were performed as previously described (2, 4) and lasted 30 min. Absolute ethanol was used to extract the cAMP and diphenylamine solution to measure DNA (2, 4).

All assays were performed in triplicate, on at least three separate batches of cells. Mean values from all three experiments are reported; SE was less than 5%.

RESULTS AND DISCUSSION

When TSH is added to FRTL-5 cells, TSH receptor mRNA levels fall measurably by about 4 hours and maximally between 8 to 24 hours after TSH challenge (Fig 1A, +TSH). As reported (4, 11), and as shown below, the TSH effect can be mimicked by 8-bromo-cAMP, cholera toxin (CT), or forskolin, suggesting the TSH action to decrease receptor mRNA levels is cAMP mediated. Since, however, TSH can also increase Ca^{++} levels, albeit at higher concentrations (3), the possibility existed that this signal could independently cause, or contribute to, the receptor mRNA decline.

To test this possibility, cells were treated with A23187 or ionomycin, two ionophores which increase cytosolic Ca^{++} levels by allowing Ca^{++} to enter the cell from the medium (3, 13, 14). Treatment with either resulted in a decrease in TSH receptor mRNA levels (Fig. 1A). The action of A23187 was measurable at a lower concentration (2 μM) than previously used to induce iodide efflux and release of arachidonic acid metabolites from the cell (10 μM) (3, 13, 14). As with cytosolic Ca^{++} levels (7), the action of ionomycin was concentration dependent between 0.033 and 1 μM and half-maximal at 0.2 μM.

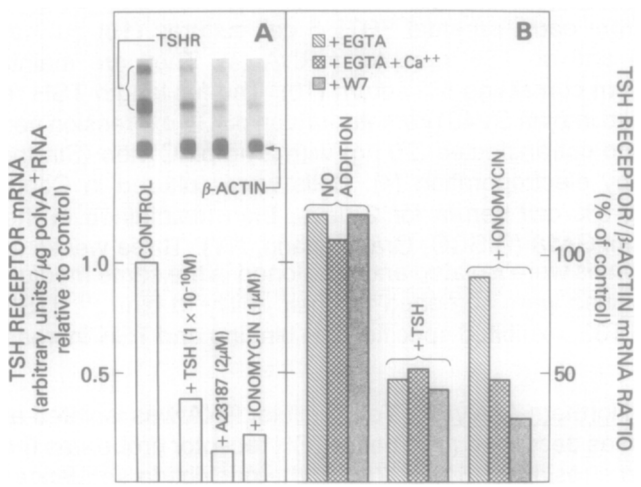

Figure 1. (A) Effect of Ca^{++} ionophores on TSH receptor mRNA levels in FRTL-5 cells by comparison to TSH and (B) the effect of EGTA (5 mM), EGTA (5 mM) plus Ca^{++} (5 mM), or a calmodulin inhibitor, W-7 (50 μM), on this action. Cells were maintained in medium containing insulin and 5% serum but no TSH (5H) for 6 days. TSH (10^{-10}M), A23187 (2 μM), or ionomycin (1 μM), plus or minus EGTA, Ca^{++}, or W7, was then included in the medium as noted and the incubation continued for 24 hours. PolyA$^+$ RNA was prepared and equal amount (5 μg/lane) subjected to Northern analysis using, sequentially, the rat TSH receptor and rat ß-actin cDNA inserts. Quantitation, after autoradiography (gel insert in A), used a LKB Laser densitometer. Densitometry values from cells with no TSH (5H) for 6 days were arbitrarily set to one for both the TSH receptor and ß-actin. Data are referenced to these control data, ratios calculated, and results expressed as the per cent change from the control ratio. The sum of the densitometry values for the 5.6 and 3.3 mRNAs are used for TSH receptor mRNA calculations, as their individual ratios did not vary from their sum (4).

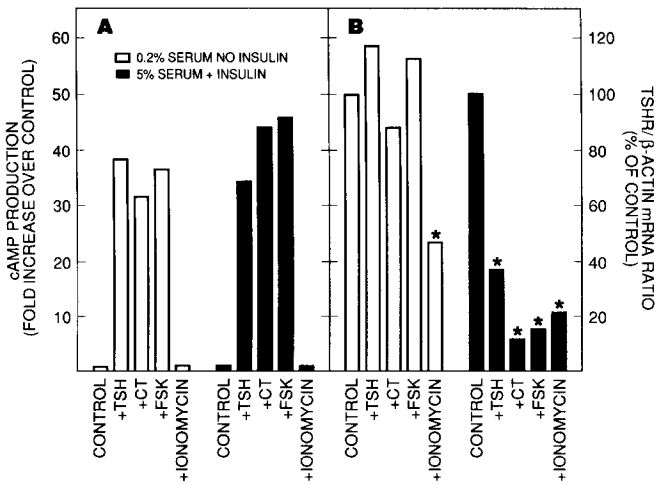

Figure 2. Effect of $1x10^{-10}$M TSH, 10 ng/ml cholera toxin (CT), 10 μM forskolin (FSK) or 1 μM ionomycin on cAMP (A) and TSH receptor mRNA (B) levels in FRTL-5 cells exposed to 3H medium containing no insulin and only 0.2% calf serum (open bars) or to medium containing insulin (100 ng/ml) plus 5% serum (black bars). In (A) cells were maintained 6 days in 24 well plates and in medium with the noted content of insulin and/or calf serum before the ability of TSH, CT, FSK, or ionomycin to increase cAMP production was measured by comparison to no addition (Control). Values presented are relative to the control and are the mean of triplicate experiments; SE was less than 5 %. In (B) cells were also maintained 6 days in medium with the noted content of insulin and/or calf serum; the incubation was then continued in the same medium with or without TSH, CT, FSK, or ionomycin for 24 hours before RNA was prepared and Northern analysis, autoradiography, and quantitation performed exactly as in Figure 1. The (*) signifies a statistically significant ($P<0.01$) decrease.

Introduction of EGTA into the medium reversed the action of the ionomycin but did not affect the ability of TSH to decrease TSH receptor mRNA levels nor did it effect levels in control cells (Fig 1B). The addition of exogenous Ca^{++} with the EGTA reversed the EGTA action and the ionomycin was again able to decrease TSH receptor mRNA levels (Fig. 1B). W7, an inhibitor of calmodulin action (14), had no effect on either the ionophore or the TSH action (Fig. 1B). These data allow several conclusions. First, increases in cytosolic Ca^{++} can decrease TSH receptor mRNA levels. Second, this action does not appear to involve calmodulin nor, as previously reported (4), the c-Kinase, since a phorbol ester will not decrease TSH receptor mRNA levels. Third, the action of TSH to decrease TSH receptor mRNA levels is via the cAMP signal under these conditions, since there is no apparent Ca^{++}-mediated component reversible by EGTA. EGTA can, within minutes, prevent signal transduction activity by hormone-induced increases in cytosolic Ca^{++} derived from intracellular compartments (3) as well as from the extracellular milieu.

Ionomycin had no ability to increase cAMP levels (Fig. 2A). This was true whether cells were in medium with insulin and 5% serum or medium without insulin containing only 0.2% serum. The presence of insulin and 5% serum was, however, required for TSH or

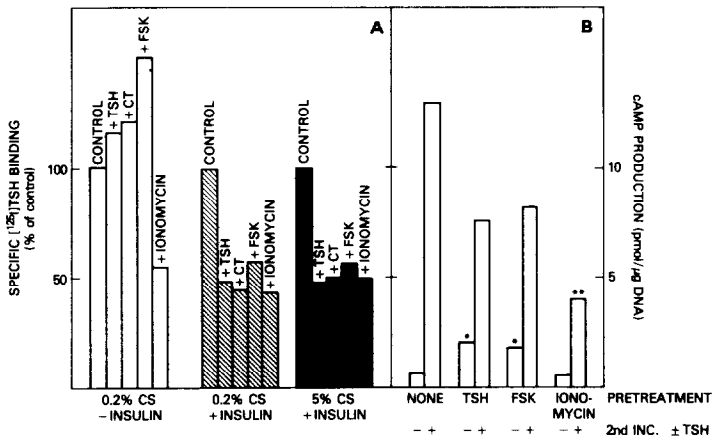

Figure 3. (A) Effects of TSH, cholera toxin (CT), forskolin (FSK) or ionomycin on specific [^{125}I]TSH binding to FRTL-5 cells exposed to 3H medium containing no insulin or TSH and only 0.2% calf serum (open bars), containing insulin (100 ng/ml) but no TSH and only 0.2% serum (cross-hatched bars), or containing insulin (100 ng/ml) and TSH plus 5% serum (black bars); and (B) effect of pretreating cells with TSH, FSK, or ionomycin on cAMP-generation in a subsequent incubation with or without TSH. In (A) cells were maintained 6 days in the medium with the noted content of insulin and/or calf serum and then in the same medium (Control) or in medium containing, in addition, $1x10^{-10}$M TSH, 10 ng/ml CT, 10 μM FSK or 1 μM ionomycin for an additional 24 hours before specific [^{125}I]TSH binding was measured (Materials and Methods). In (B) FRTL-5 cells in 5H medium (insulin, 5% serum, no TSH) for 6 days were exposed to $1x10^{-10}$M TSH, 10 μM FSK or 1 μM ionomycin for a 24 hour period. Cells were then washed and cAMP production measured in the presence or absence of $1x10^{-10}$M TSH. The (*) notes a significant increase, $P<0.05$, in "basal" cAMP generation in cells preincubated with TSH, FSK, or ionomycin by comparison to preincubation with no ligand or ionomycin; the (**) notes a significant decrease, $P<0.01$, in cAMP generation induced by TSH in cells preincubated with ionomycin by comparison to those preincubated with TSH or forskolin. Values (A and B) are the mean of triplicate experiments; SE was <5 % in each case.

cAMP to decrease TSH receptor mRNA levels but not for the action of ionomycin to decrease the level of receptor transcripts. Thus TSH, cholera toxin, or forskolin decreased receptor mRNA levels only when cells were in medium containing insulin and 5% serum (Fig. 2B, black bars). Ionomycin, in contrast, decreased receptor mRNA levels in medium containing no insulin and 0.2% serum (Fig. 2B, open bars), as well as in medium containing insulin plus 5% serum (Fig. 2B, black bars). The sum of these results support the following additional conclusions. The ionomycin action does not involve the cAMP signal, just as TSH does not use the Ca^{++} signal; and the two signals down regulate mRNA levels by different mechanisms, given the dependence of one signal but not the other on insulin and/or serum.

The ability of ionomycin to decrease TSH receptor mRNA levels is paralleled by its ability to decrease [^{125}I]TSH binding (Fig. 3A). As is the case for TSH receptor mRNA levels (Fig. 2B), ionomycin decreases [^{125}I]TSH binding both in cells exposed to insulin and/or 5% serum and in cells exposed to medium with no insulin and only 0.2% serum.

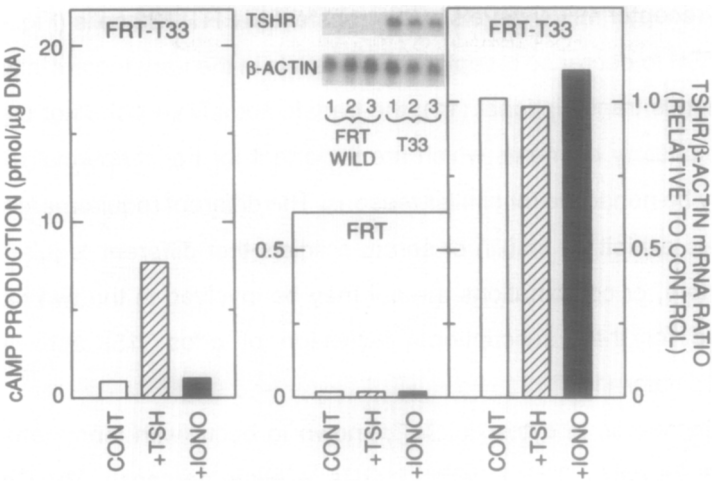

Figure 4. Ability of $1x10^{-10}$M TSH or 1 µM ionomycin to increase cAMP levels (left panel) or decrease TSH receptor mRNA levels (right panel) in FRT-T33 cells transfected with full length TSH receptor cDNA (Materials and Methods). In the left panel cells were maintained 6 days in 24 well plates and in 5H medium with insulin and 5% calf serum before being washed and the ability of TSH or ionomycin to increase cAMP levels measured as in Figure 2. In the right panel cells were maintained 6 days in 5H medium containing insulin and 5% calf serum before the incubation was continued in the same medium with or without TSH or ionomycin for 24 hours. RNA was prepared and Northern analysis, autoradiography, and quantitation performed exactly as in Figure 1. The gels and middle panel show that FRT cells had no TSH receptor mRNA prior to transfection and that TSH or ionomycin do not induce the appearance of TSH receptor transcripts.

TSH, cholera toxin, and forskolin are effective only in the presence of insulin and/or serum (Fig. 3A). The ionomycin-induced decrease in TSH receptor mRNA levels and TSH binding to cell surface receptors is, in turn, associated with a decrease in the ability of TSH to increase cAMP levels. Thus, if cells are pretreated with ionomycin, their ability to respond to TSH in a subsequent incubation is statistically decreased ($P<0.001$) by comparison to control cells (Fig. 3B) and the decrease is even greater than that effected by TSH or forskolin treatment ($P<0.01$). Ionomycin pretreatment does not increase cAMP levels as do TSH and forskolin (Fig. 3B), consistent with data in Figure 2A. In sum, ionomycin induces a decrease in functionally expressed TSH receptors consistent with its effect on TSH receptor mRNA levels.

FRT cells are a line of thyroid cells which have no functional TSH receptor and no measurable TSH receptor mRNA (Fig. 4, gel and middle panel; Ref 4). When SV40-promoter driven full length TSH receptor cDNA is transfected into the FRT cell, the transfected cells, for example FRT-T33, develop a TSH-increased cAMP response (Fig. 4, left panel) not evident in control FRT cells (4, 10), as well as specific TSH binding (data not shown). Although this coincides with the acquisition of measureable amounts of TSH receptor RNA transcripts (Fig. 4, gel and right panel), ionomycin and TSH do not

decrease TSH receptor mRNA levels in the transfected FRT-T33 cells (Fig. 4 right panel). The failure of TSH to decrease receptor mRNA levels in the transfectant is consistent with the fact its action is transcriptional (11); the transfected cDNA does not contain flanking regions with regulatory elements which are important for transcriptional regulation. The Ca^{++} signal may be nonactive for similar reasons. The different requirement that the cAMP and Ca^{++} signals exhibit for insulin or serum predicts that different regulatory elements, transacting factors, or combinations thereof may be involved in the two cases; this has been described for the transcriptional activation of c-fos (15) and urokinase-type plasminogen activator (16).

In sum increases in cytosolic Ca^{++}, known to occur with ionophore treatment of FRTL-5 cells (3, 7), cause TSH receptor mRNA levels to decrease. The Ca^{++} and cAMP signals act independently to decrease receptor mRNA levels and, in both cases, there is an associated loss of functional cell surface receptors. Physiologic concentrations of TSH, i.e. $1x10^{-10}$M, appear to down regulate only via the cAMP signal. Agents which increase cytosolic Ca^{++}, i.e. TSH at higher concentrations (3) or α_1-adrenergic (3, 5-7) and purinergic agonists (5, 8, 9), may also down regulate the receptor at an mRNA level but by a different mechanism; this action would be in addition to perturbing an inhibitory G protein (3, 5, 8, 9).

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