Journal of Endocrinological Investigation (2021) 44:2387–2394
https://doi.org/10.1007/s40618-021-01545-0

ORIGINAL ARTICLE

Calcium/calmodulin-dependent kinases can regulate the TSH expression in the rat pituitary

G. G. Altobelli[1] · S. Van Noorden[2] · D. Cimini[3] · M. Illario[4] · D. Sorriento[1] · V. Cimini[1]

Received: 3 December 2020 / Accepted: 26 February 2021 / Published online: 20 March 2021

Abstract

Purpose The endocrine secretion of TSH is a finely orchestrated process controlled by the thyrotropin-releasing hormone (TRH). Its homeostasis and signaling rely on many calcium-binding proteins belonging to the "EF-hand" protein family. The Ca^{2+}/calmodulin (CaM) complex is associated with Ca^{2+}/CaM-dependent kinases (Ca^{2+}/CaMK). We have investigated Ca^{2+}/CaMK expression and regulation in the rat pituitary.

Methods The expression of CaMKII and CaMKIV in rat anterior pituitary cells was shown by immunohistochemistry. Cultured anterior pituitary cells were stimulated by TRH in the presence and absence of KN93, the pharmacological inhibitor of CaMKII and CaMKIV. Western blotting was then used to measure the expression of these kinases and of the cAMP response element-binding protein (CREB). TSH production was measured by RIA after time-dependent stimulation with TRH. Cells were infected with a lentiviral construct coding for CaMKIV followed by measurement of CREB phosphorylation and TSH.

Results Our study shows that two CaM kinases, CaMKII and CaMKII, are expressed in rat pituitary cells and their phosphorylation in response to TRH occurs at different time points, with CaMKIV being activated earlier than CaMKII. TRH induces CREB phosphorylation through the activity of both CaMKII and CaMKIV. The activation of CREB increases TSH gene expression. CaMKIV induces CREB phosphorylation while its dominant negative and KN93 exert the opposite effects.

Conclusion Our data indicate that the expression of Ca^{2+}/CaMK in rat anterior pituitary are correlated to the role of CREB in the genetic regulation of TSH, and that TRH stimulation activates CaMKIV, which in turn phosphorylates CREB. This phosphorylation is linked to the production of thyrotropin.

Keywords CaM kinases · Calmodulin · TSH · Immunochemistry · Rat pituitary · Ca signaling

Introduction

The Ca^{2+}-calmodulin-dependent kinases (CAMK) and transcriptional regulation

Ca^{2+} is an important mediator of cellular signals, and variations in its concentration play a fundamental role in a wide range of biological functions including transcription, cell cycle, apoptosis, protein synthesis, exocytosis, proliferation and differentiation [1]. A calcium-binding protein, calretinin, has been studied in rat pituitary thyrotrophs [2] but the most important intracellular receptor of Ca^{2+} is calmodulin (CaM), a highly conserved ubiquitous protein, sensitive to changes in Ca^{2+} levels. It is composed of two Ca^{2+}-binding globular domains joined to a central connector, is flexible and can wrap around the target proteins in a characteristic way, with the two globular domains grabbing it from both sides [1–4]. When Ca^{2+} is linked to

✉ G. G. Altobelli
ggaltobe@unina.it

✉ V. Cimini
cimini@unina.it

1 Department of Advanced Biomedical Sciences, Medical School, "Federico II" University of Naples, Naples, Italy

2 Department of Histopathology, Imperial College London, Hammersmith Hospital, London, UK

3 Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania Luigi Vanvitelli, Caserta, Italy

4 Department of Public Health, Medical School, "Federico II" University of Naples, Naples, Italy

all four binding sites, calmodulin undergoes a conformational change that activates it and allows it to interact with its target proteins. Most of the effects of the Ca^{2+}/CaM complex are mediated by CaMKs, to which it binds. The kinases include monofactorial CaMKs, such as the myosin light chain kinase (MLCK), phosphorylase kinase and CaMKIII, and multifunctional CaMKs such as CaMKI, CaMKII and CaMKIV. CaMKI and CaMKII are both ubiquitous, while CaMKIV is tissue-specific, and is expressed in the brain, T cells, thymus, cells of the myeloid lineage, testicles, ovaries, adrenal gland and pituitary.

From the structural point of view, the CaMKs have an N-terminal catalytic domain, and a central regulatory region that contains the CaM-binding domain. In the absence of Ca^{2+}/CaM, the kinase is inhibited sterically by an internal autoinhibitory region, which prevents it from binding to both the substrate and ATP. Binding to the Ca^{2+}/CaM complex removes the intrasteric inhibition and initiates enzyme activation [3]. The next step in activation is specific to each CaMK.

Once activated by binding to the Ca^{2+}/CaM complex, CaMKII self-trans-phosphorylates in threonine 286, located in a hydrophobic pocket of the catalytic domain. The return to the inactive state is promoted by protein phosphatases 1 and 2 (PP1 and PP2) which dephosphorylate the residue of threonine 286 [1]. The regulatory properties of the other multifunctional CaMKs show interesting similarities and differences with respect to CaMKII. CaMKI and CaMKIV require phosphorylation by an upstream kinase for their activation, i.e. a CaM kinase–kinase (CaMKK). CaMKI is activated by binding to Ca^{2+}/CaM but, unlike CaMKII and CaMKIV, it has no residual autonomous activity. It is phosphorylated at the threonine 177 autophosphorylation site, which is located inside the activation "loop". The mechanism that leads to the activation of CaMKIV is more complex and takes place in several phases. Once activated, CaMKIV shows activity independent of Ca^{2+}/CaM. Inactivation of the kinase is mediated by the phosphatase PP2A, to which it is found to be constitutively associated [3, 5]. CaMKIV unlike CaMKII does not multimerize and exists in solution as a monomer.

CaMKs are involved in many physiological functions, including cell cycle, smooth muscle contraction, secretion and a number of important neuronal functions. They phosphorylate a wide range of substrates and are involved in transcription control. Their location can be variable [1, 6].

One of the most important nuclear targets of CaMKs is CREB, a transcription factor responsible for the expression of cAMP-containing response element (CRE) genes in their promoter. Initially CREB was identified as a cAMP-dependent factor, which was activated by protein kinase A (PKA). Subsequent studies have shown that Ca^{2+} also generates signals capable of activating CREB, producing events that involve CaMKs [7].

CaMKII and CaMKIV are found both in the nucleus and in the cytoplasm, while CaMKI is mainly in the cytoplasm [6, 8–11] and our unpublished results show that it is not present in the rat pituitary, which expresses both CaMKII and CaMKIV. Nuclear CaMKIV regulates gene expression through the phosphorylation of several transcription factors, including CREB and CREB binding protein (CBP) [12–15]. Furthermore, CaMKIV phosphorylates CREB in Ser 133 and induces the formation of a transcriptional complex at the CRE site of the promoter, thus mediating the formation of a transcriptional complex following increases in concentration of intracellular Ca^{2+} [14]. In response to increased calcium levels, CaMKII also phosphorylates CREB on Ser 133 but, in addition, phosphorylates it at Ser 142. This phosphorylation has an inhibitory and dominant effect, thus suggesting that CamKII is not an activator of CREB in all cellular models. Moreover, phosphorylation of CaMKII nuclear isoforms by CaMKIV has been observed to obstruct the admission of CaMKII to the nucleus; thus, CaMKIV could act as a negative regulator of CaMKII nuclear activity, acting on translocation from the nucleus [14, 16].

Many data indirectly suggest a role of CaMKs in the regulation of gene transcription in pituitary gonadotrophs and somatotrophs. However, a direct demonstration of CaMK involvement in TSH gene regulation has not been shown yet. Since the gene expression of TSH is regulated by CREB, we hypothesized that CaMKIV plays a role in the regulation of TSH gene expression through CREB activation. Therefore, our purpose was to confirm the sub-cellular localization of CaMKs in the rat anterior pituitary and their effect after TSH stimulation with TRH. Finally, we wished to determine the effect of CaMK-specific activation and inhibition on CREB and TSH gene expression in response to TRH. The chosen cellular model was normal rat pituitary cells in primary culture.

Materials and methods

Adult male Wistar rats (about 250 g) were used. Animals were cared for according to the "Guide for the Care and Use of Laboratory Animals" by the US National Institutes of Health (NIH Publication No. 85.23, revised 1996) and to institutional rules for the care and handling of experimental animals of University of Naples Federico II, Italy. Several methodological approaches were used for this study: immunohistochemistry either with fluorescence on frozen sections or with immunoperoxidase on paraffin sections was carried out to localize CaMKs in the rat pituitary gland. Immunofluorescence often seemed the better tool for visualizing the CamKs, which could be present in low quantities.

In vitro experiments used dispersed anterior pituitary cells in culture. The presence of Pit 1 authenticated the origin and viability of these cells which were then used to verify the effect of TRH on CaMK activation as well as the effect of CaMK on CREB and TSH production after TRH stimulation. Measurements were made by Western blotting or RIA.

Immununohistochemistry

Pituitary glands of male Wistar rats were formalin-fixed and paraffin-embedded. Four µm-thick sections were used. An immunohistochemical method, as previously described [17], was performed based on the use of a primary polyclonal rabbit antibody directed against CaMKIV (gift from Dr Jiro Kasahara, 1/500), and a polyclonal rabbit anti-CaMKII (Santa Cruz Biotechnology, cat# sc-9055, RRID:AB_2108768, 1/1000). The secondary antibody, goat anti-rabbit immunoglobulin, was labeled with peroxidase which catalyzes a reaction that forms a brown product in the presence of its substrate and a chromogen. The enzyme was developed in a solution containing the peroxidase substrate, 0.03% H_2O_2, and the chromogen, 0.05%. 3,3′diaminobenzidine tetrahydrochloride dihydrate (DAB).

An immunofluorescence method as previously reported [18] was also carried out on cryostat sections and on cultured cells. Briefly, after postfixation washes, the pituitary glands were placed in a cryoprotective solution of phosphate buffered saline (PBS)/30% sucrose for 24/36 h and subsequently included in O.C.T. and frozen with dry ice. Sections (5 µm-thick) were cut in a cryostat. Rabbit anti-CaMKIV (J. Kasahara) and rabbit anti-CaMKII (RRID:AB_2108768, 1/100), were used for immunofluorescence on cryostat sections. The secondary antibody was conjugated to fluorescein isothiocyanate (FITC), which gave a green fluorescence (495 nm wavelength). The nuclei were counterstained with DAPI to produce blue fluorescence.

Controls

A rabbit anti-ACTH antibody (Dako, cat# A571, 1/4000) was used as a positive method control on sections of rat pituitary glands. PBS was used instead of the primary antibody as a negative control.

Cell cultures

Three animals were used for each of the five experiments and each experiment was repeated at least three times. The anterior lobe of the pituitary gland was separated from the neuro-intermediate lobe under sterile conditions, in a balanced Earle saline solution without calcium and magnesium (EBSS), and finely cut with a sterile scalpel. The tissue was suspended in Hanks' balanced saline solution without calcium and magnesium (HBSS) with 0.25% trypsin and kept at 37 °C with constant mechanical grinding for 10′–15′. After washing, the dispersed cells were cultured at a concentration of $5–10 \times 10^5$ cells per mL^{-1} for up to 6 days, in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEMH) containing fetal bovine serum (FBS). On the day before the experiment the cells were counted, and their vitality tested with Trypan Blue. The cell suspension was placed in DMEMH medium with 0.5% bovine serum albumin (BSA). Cells in culture before the different experiments were suspended at a concentration of $5 \times 10^5 \times mL^{-1}$ of medium. TRH (Sigma-Aldrich, cat# P2161) was always used at a concentration of 1 µM in double distilled H_2O. KN93 (Sigma-Aldrich, cat# K1385), the pharmacological inhibitor of CaMKI, CaMKII and CaMKIV, [19] was used at a concentration of 1 µM in dimethylsulfoxide.

Analysis by western blot

5×10^5 cultured cells were incubated with 1 µM TRH, in the presence and absence of KN93, and then lysed at different timepoints. Cells were lysed in RIPA/SDS buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCL, 1% Nonidet P-40, 0, 25% deoxycholate, 9,4 mg/50 ml sodium orthovanadate, 20% SDS]. Protein concentration was determined using BCA assay kit (Pierce). 50 µg of whole extracts were electrophoresed by SDS/PAGE and transferred to nitrocellulose; targeted proteins were visualized using specific primary antibodies: rabbit anti-CaMKIV (gift from Dr Jiro Kasahara), rabbit anti-CaMKII from Santa Cruz Biotechnology (cat# sc-9055), mouse anti-p-CREB-1 (10E9) from Santa Cruz Biotechnology (cat# sc: 81486) and rabbit anti-Pit1 antibody from Babco (cat# PRB-230C-200, RRID:AB_291590). Primary antibodies were incubated overnight at 4 °C followed by 1 h of incubation with specific HRP-conjugated secondary antibody (Santa Cruz Biotechnology) and standard chemiluminescence (Pierce).

Verification of the cell dissociation procedure

The cell dissociation procedure in whole anterior lobes and in the lysate of cultured pituitary cells obtained by gland dissociation was checked by Western blotting for the expression of Pit1, a pituitary-specific transcription factor that acts on the differentiation of all anterior pituitary secreting cells. Rabbit anti-Pit 1 was used as primary antibody.

CaMK-dependent stimulation of CREB by TRH

To assess CaMK-dependent stimulation of CREB by TRH, 5×10^5 cells were incubated with TRH for 30 min, in the presence and absence of KN93. Cells were subsequently

lysed, and phosphorylated CREB (p-CREB) was visualized after blotting and incubation with the monoclonal antibody p-CREB-1 (10E9), 1/200. The functional effect of TRH stimulation on the cell cultures was verified with time-dependent stimulation to quantify the release of TSH in the culture medium by means of a commercially available R.I.A. (Amersham Biosciences). The cells were removed for analysis at 5, 15, and 30 min intervals.

Effects of stimulation with TRH on the activation of CaMKII and CaMKIV

To evaluate the effects of stimulation with TRH on the activation of CAMKII and CAMKIV, 5×10^5 cultured cells were incubated with 1 µM TRH for 5, 15 and 30 min. Cells were subsequently lysed and CaMKII/CaMKIV activation was determined by western blot and incubation with the same specific antibodies as used for immunohistochemistry.

Effect of CaMKIV on CREB and TSH production by TRH stimulation

Infection with lentivirus

The cells were infected with lentiviral constructs (gift from Dr. Anthony R. Means) [20, 21] coding for CaMKIV-WT (wild type), or CaMKIV-K72M, its negative dominant, or CaMKIV-K71M, as superinfectant. Approximately 5×10^5 cells were subjected to a superinfection with at least 100 (MOI 100) viral particles per cell. The lentiviral vector codes for the enzyme beta-galactosidase (b-gal) which, when developed, gives a blue stain to cells that contain it. An empty virus was used as a control. CREB phosphorylation after TRH stimulation was determined by western blot and phospho-specific antibody. TSH was measured by RIA.

Radioimmunoassay

TSH levels in the culture medium from control and treated cells were measured using a commercially available R.I.A. (Amersham Biosciences). The manufacturer's instructions were followed.

Statistical analysis

The different experimental groups were compared according to paired t test or one-way ANOVA to assess statistical significance. Bonferroni's multiple comparison test was then performed where applicable. A significance level of $p < 0.05$ was assumed for all statistical evaluations. Statistics were computed with GraphPad Prism software (San Diego, CA, USA).

Results

Expression of CaMKII and CaMKIV in rat pituitary

The immunohistochemical investigation, aimed at ascertaining the presence and intracellular localization of the two Ca^{2+} calmodulin-dependent kinases in sections of rat pituitary gland, revealed a fairly widespread distribution of CaMK-containing cells. Scattered immunoreactive CaMKII cells detected by both immunoperoxidase and immunofluorescence showed both nuclear and cytoplasmic localization (Fig. 1a), while CaMKIV seemed to be cytoplasmic only (Fig. 1b).

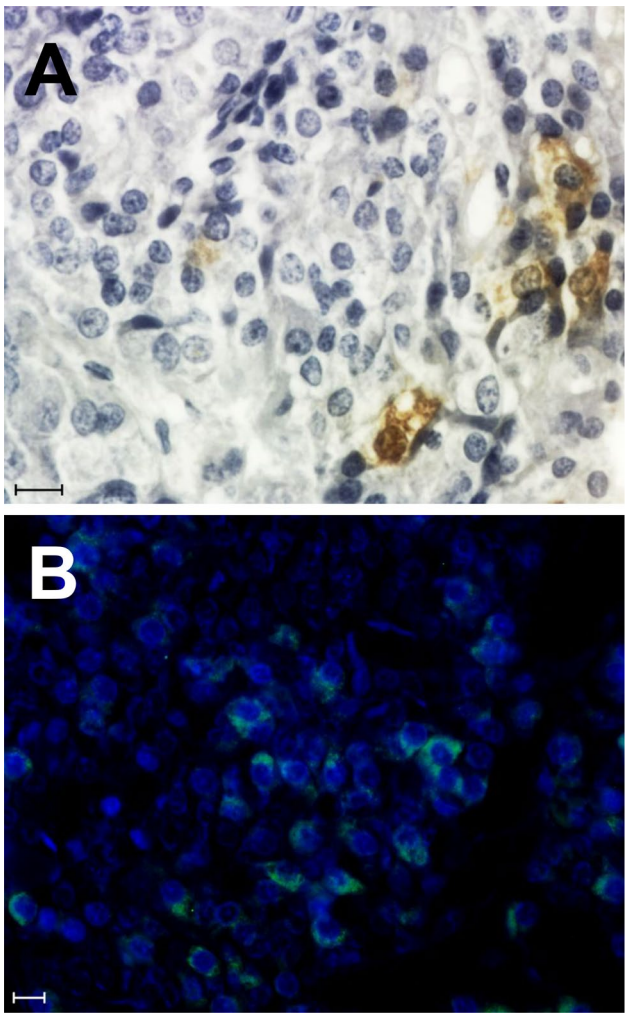

Fig. 1 **a** CaMKII-immunoreactive cells. The brown product of peroxidase reaction shows rat endocrine anterior pituitary cells. Darker nuclear staining indicates that CamKII is present in the nucleus as well as the cytoplasm. Bar, 10 µm. **b** Immunofluorescent CaMKIV-containing rat anterior pituitary cells. Labelling with FITC-conjugated secondary antibody occurs in cytoplasm only. DAPI counterstain. Bar, 10 µm

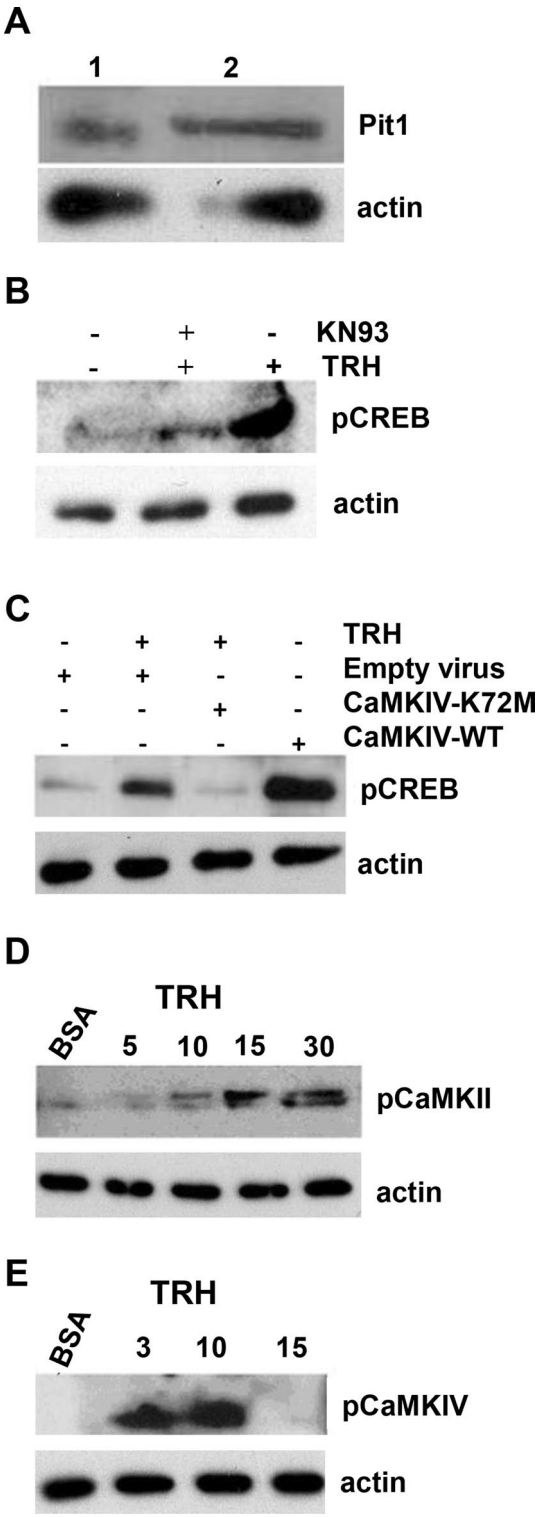

Fig. 2 **a** Western blot analysis of Pit1 expression in rat pituitary cell culture. The expression of whole pituitary Pit1 (1) is comparable to that of isolated pituitary cell lysate (2). **b** Western blot analysis of p-CREB CaMK-dependent stimulation in rat pituitary cells in vitro after TRH administration. After 30 min of TRH stimulation, p-CREB almost dephosphorylates in presence of the CaMK inhibitor KN93, while it significantly increases in the presence of TRH only. **c** Western blot analysis of CaMKIV activity modulation in rat pituitary cell culture. Cells were infected with lentiviral vectors for 30 min. p-CREB immunoreactive blots were positive only in cells infected with empty virus (TRH) and in cells infected but not exposed to TRH (CaMKIV-WT). On the contrary, superinfection of cells with CaMKIV-K72M abolished completely CREB phosphorylation in response to TRH. **d** Western blot analysis of p-CaMKII following stimulation of rat pituitary cells in vitro by TRH. The cells were removed at 5, 10, 15 and 30 min. Synthesis of p-CaMKII was active at 10 min and still evaluable at 30 min. **e** Western blot analysis of p-CaMKIV following TRH stimulation of rat pituitary cells in vitro. Production of p-CaMKIV started at 3 min to reach its peak at 10 and disappeared by 15 min.

Expression of Pit1 in primary pituitary cells

To verify the pituitary cell dissociation procedure, the expression of the Pit1 protein, specific for pituitary cells, was assessed by the western blot method. (Fig. 2a). It is significantly expressed in a comparable measure in the anterior pituitary *in toto* and in the lysate of pituitary cells.

CaMK-dependent stimulation of CREB by TRH

To evaluate the involvement of CaMKs in the regulation of CREB in response to TRH, cells were incubated with TRH in the presence and absence of the CaMK inhibitor KN93.

Stimulation with TRH for 30 min induced significant phosphorylation of CREB, while the presence of KN93 almost completely abrogated it (Fig. 2b).

Modulation of CaMKIV in rat pituitary cells

To specifically assess the effect of CaMKIV activation on CREB in rat pituitary cells, lentiviral vectors coding for CaMKIV-WT (wild type form) or CaMKIV-K72M (dominant negative form) were used. Infection with empty control virus did not modify the basal phosphorylation of CREB. Stimulation with TRH resulted in strong phosphorylation of CREB at 30 min in cells infected with an empty virus (TRH), while stimulation with TRH of cells previously infected and superinfected with CaMKIV-K71M-coding virus completely abrogated the phosphorylation of CREB (TRH-CamKIV). Finally, CaMKIV-WT induced a massive expression of phosphorylated CREB in the absence of TRH (Fig. 2c). These results suggest that CaMKIV regulates the activation of CREB in primary cultures of pituitary cells.

Effects of stimulation with TRH on the activation of CaMKII and CaMKIV

Stimulation with TRH induces CaMKII activation later than CaMKIV. CaMKII is activated significantly after 10 min; maximal stimulation is still visible at 30 min (Fig. 2d).

Stimulation with TRH-activated CaMKIV starting at 3 min and reaching a maximum at 10 min. (Fig. 2e).

TSH production

We evaluated the release of TSH in the culture medium in response to TRH that induced an increase of TSH release in a time-dependent manner (Fig. 3a). While infection with empty control virus (b-gal) did not modify the amount of TSH in the culture medium, stimulation with TRH caused a significant ($p < 0.05$) increase (Fig. 3b). Infection with dominant virus negative for CaMKIV-K72M completely inhibited TRH-induced TSH stimulation, whereas infection with CaMKIV-WT virus significantly increased the amount of TSH in the medium ($p < 0.05$). These results confirm that the stimulation of TSH production in thyrotrophs is positively controlled by CaMKIV.

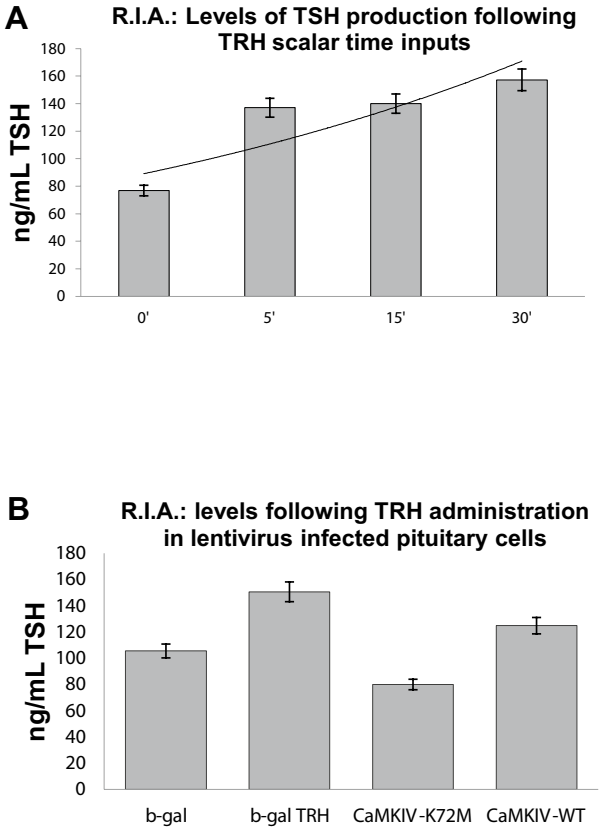

Fig. 3 **a** R.I.A. analysis of TSH production following stimulation of rat pituitary cells in vitro by TRH. Measurements were taken at 5, 15 and 30 min and peaked at 30 min. *P* value < 0.05 vs control. **b** CaMKIV direct effect on TSH production after stimulation of lentivirus-infected thyrotrophs. TRH stimulation of cells infected with empty control viruses (b-gal TRH) increases TSH production with reference to empty control virus (b-gal)-infected cells. CaMKIV-K72M virus-infected cells were unable to produce TRH-stimulated TSH, while cells infected with CaMKIV-WT positively affect TSH production. *P* value < 0.05 vs control.

Discussion

In this study, we evaluated the expression of CaMKs in the rat pituitary and their potential role in the regulation of TSH expression in response to TRH.

We have provided the first morphological evidence for the cellular localization of CaMKs in rat pituitary gland, using specific antibodies. At least some of the immunopositive cells appear to be thyrotrophs, corresponding with the response we obtained after TRH input, but further study is necessary to assign the CaMKs to particular pituitary cell types. The sparse literature available on the differential expression of CaMKII and CaMKIV suggests that each isoform may be involved in different functions [12, 22].

The expression of Pit1 in both whole anterior lobes and primary pituitary cell cultures was demonstrated by western blot. This confirmed that the cells were genuine pituitary cells and that the dissociation and culture procedures had not altered their normal protein expression. Further confirmation for the characterization of anterior pituitary primary cultured cells was that they expressed TSH in several experimental conditions.

TRH is the elective stimulating factor for pituitary thyrotrophs and plays a fundamental role in regulating the expression of genes for TSH subunits. The hypothesis of this study is that CaMKs can regulate the expression of TSH induced by TRH. To define whether TRH had an effect on CaMKII and CaMKIV activity, pituitary cells in primary culture were stimulated with TRH. The results demonstrate that stimulation with TRH induces both CaMKII and CaMKIV activation, although with different kinetics. CaMKIV is phosphorylated at 3 min and is dephosphorylated within 15 min. This rapid dephosphorylation and inactivation is due to protein phosphatase 2 (PP2A) [5, 23]. CaMKII is activated later, starting at 10 min and still detectable at 30 min. These results suggest that CaMKs could play an important role in the signal generated by TRH. Among the intracellular mechanisms regulated by CaMKs is that of transcriptional regulation. Both CaMKII and CaMKIV phosphorylate CREB, with CaMKIV being activating and CaMKII inhibitory [14].

The expression of genes coding for TSH subunits is regulated by CREB. In particular, TRH stimulates the promoter of the TSH beta subunit inducing CREB phosphorylation and CBP recruitment to the transcriptional complex [24]. We, therefore, examined whether stimulation with TRH induces CREB's CaMK-dependent phosphorylation. Stimulation with TRH induced a strong activation of CREB, which was abrogated in presence of the pharmacological inhibitor of CaMK, KN93. Infection with a lentiviral vector encoding a dominant negative of CaMKIV [25, 26] significantly

inhibited both the phosphorylation of CREB and the TRH-induced increase in TSH in the culture medium. These results show for the first time that TRH induces transcriptional activation of CREB through CaMKIV.

A role for CaMKs in the regulation of gene expression by hypothalamic releasing factors has been indirectly suggested: CaMKII is involved in GnRH stimulation of the gonadotropin subunit [27–29], and CaMKIV regulates TRH induction of TSH and PRL gene activity [30], but there has been no direct evidence in pituitary cells of the involvement of a specific CaMK in CREB phosphorylation. A later publication [31] indicated that GH3 cells were not suitable as in vitro models for studying prolactin modulation in rat pituitary. We, therefore, used rat pituitary cells in primary culture in our experiments. Although these may be more difficult to handle, the results should be more reliable. Our results, in fact, directly demonstrate for the first time that:—CaMKII and CaMKIV are expressed in the rat adenohypophysis;—TRH induces phosphorylation of both CaMKII and CaMKIV with different kinetics;—TRH-induced CREB phosphorylation is CaMKIV-dependent;—the specific inhibition of CaMKIV abolishes both the phosphorylation of CREB and the TRH-induced increase of TSH in the culture medium. The presence of TSH in the culture medium shows indirectly that CaMK and TSH beta may coexist. It is not necessary to demonstrate that the target of TRH induction is the thyrotroph. However, a systematic study of the distribution of CaMKs in anterior pituitary cells could help to clarify further the roles of these kinases.

We, therefore, suggest that stimulation with TRH activates CaMKIV, inducing CREB phosphorylation and increasing TSH expression. The reason for the almost simultaneous activation of CaMKII, which negatively regulates CREB, could be explained by its different activation kinetics with respect to CaMKIV: in fact, following stimulation with TRH, CaMKIV would be activated in a short time to phosphorylate CREB; subsequently CaMKII would turn off the activation signal by an additional inhibitory-type CREB phosphorylation event. At present, the data available in the literature do not uniquely define the kinases responsible for CREB activation following stimulation with TRH: in lactotrophs, TRH would induce CREB activation in a PKA-independent manner [32].

Thus, the present results allow us to identify a CaMKIV-dependent signaling pathway responsible for the regulation of TRH-induced gene expression in pituitary cells, and at the same time, raise the question of whether the sub-cellular localization of CaMKII and CaMKIV is a determining element in the regulation of the transcriptional complex through CREB phosphorylation. Our results showing the cytoplasmic localization of CamKIV in pituitary tissue could support a function different that of other tissues, such as brain neurons [33] for example, where the kinase has been reported to be localized in the nucleus. However, a possible explanation could be that CamKIV is present in the cytoplasm of pituitary cells as unphosphorylated kinase, and therefore the catalytic activity required for calcium/calmodulin-dependent protein kinase IV to enter the nucleus [26] has not yet begun. The second possible explanation for the lack of nuclear immunoreactivity in pituitary tissue could be our use of antibody to unphosphorylated CaMKIV. The cellular compartmentalization of kinases in fact controls their activation and their access to substrates: in differentiated smooth muscle cells the targeting of CaMKII is essential for the signal mediated by Erk [34]. In conclusion, these results overall indicate that rat thyrotrophs could be a new model for defining the sub-cellular localization of CaMKII and CaMKIV following stimulation with TRH and for correlating it with their functional role in CREB activation. In addition, they might suggest a role for CaMKs in regulating CREB transcription in thyrotrophs.

Acknowledgements We thank N. Furente, for help in setting up experiments during his apprenticeship for B.Sc., AR Means (Duke University Medical Center) for providing constructs and Jiro Kasahara for the kind gift of CaMKIV antibody. Partial financial support was received from Immunocytochemistry Advanced Course, University of Naples Federico II.

Authors' contributions GGA and VC conceived the study and designed and performed the experiments. DC and GGA interpreted data. GGA and VC wrote the first draft of the manuscript. DS and MI assisted with important, definitive experiments. SVN revised the manuscript critically for important intellectual content. All authors revised and read the manuscript and approved the submitted final version.

Funding The research was supported by funds of Immunocytochemistry Advanced Course, Federico II University of Naples.

Declarations

Conflict of interest Not applicable.

Ethical approval Animals were cared for according to the "Guide for the Care and Use of Laboratory Animals" by the US National Institutes of Health (NIH Publication No. 85.23, revised 1996) and to institutional rules for the care and handling of experimental animals of University of Naples Federico II, Italy.

Consent to participate Not applicable.

Availability of data and materials Not applicable.

Code availability Not applicable.

References

1. Hook SS, Means AR (2001) Ca^{2+}/CaM-dependent kinases: from activation to function. Annu Rev Pharmacol Toxicol 441:471–505
2. Cimini V, Isaacs KR, Jacobowitz DM (1997) Calretinin in the rat pituitary: colocalization with thyroid-stimulating hormone. Neuroendocrinology 65:179–188

3. Means AR (2000) Regulatory cascades involving calmodulin-dependent protein kinases. Mol Endocrinol 14:4–13
4. Anderson KA, Kane CD (1998) Ca^{++}/calmodulin-dependent protein kinase IV and calcium signalling. Biometals 11:331–343
5. Anderson KA, Noeldner PK, Reece K, Wadzinski BE, Means AR (2004) Regulation and function of the calcium/calmodulin-dependent protein kinase IV/protein serine/threonine phosphatase 2A signaling complex. J Biol Chem 279:31708–31716
6. Stedman DR, Uboha NV, Stedman TT, Nairn AC, Picciotto MR (2004) Cytoplasmic localization of calcium/calmodulin-dependent protein kinase I-alpha depends on a nuclear export signal in its regulatory domain. FEBS Lett 566:275–280
7. Dash PK, Karl KA, Colicos MA, Prywes R, Kandel ER (1991) cAMP response element-binding protein is activated by Ca^{2+}/calmodulin- as well as cAMP-dependent protein kinase. Proc Natl Acad Sci USA 88:5061–5065
8. Jensen KF, Ohmstede CA, Fisher RS, Sahyoun N (1991) Nuclear and axonal localization of Ca^{++}/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. Proc Natl Acad Sci USA 88:2850–2853
9. Nakamura Y, Okuno S, Kitani T, Otake K, Sato F, Fujisawa H (2001) Immunohistochemical localization of Ca(2+)/calmodulin-dependent protein kinase kinase beta in the rat central nervous system. Neurosci Res 39:175–188
10. Picciotto MR, Zoli M, Bertuzzi G, Nairn AC (1995) Immunochemical localization of calcium/calmodulin-dependent protein kinase I. Synapse 20:75–84
11. Sakagami H, Umemiya M, Saito S, Kondo H (2000) Distinct immunohistochemical localization of two isoforms of Ca^{++}/calmodulin-dependent protein kinase kinases in adult rat brain. Eur J Neurosci 12:89–99
12. Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GS (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol Cell Biol 14:6107–6116
13. Sheng M, Thompson MA, Greenberg ME (1991) CREB: a Ca^{++} regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science 252:1427–1430
14. Sun P, Enslen H, Myung PS, Maurer RA (1994) Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev 8:2527–2539
15. Sun Z, Means RL, LeMagueresse B, Means AR (1995) Organization and analysis of the complete rat calmodulin-dependent protein kinase IV gene. J Biol Chem 270:29507–29514
16. Sun P, Lou L, Maurer RA (1996) Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca^{2+}/calmodulin-dependent protein kinases type I, II, and IV. J Biol Chem 271:3066–3073
17. Altobelli GG, Van Noorden S, Cimini V (2019) Copper/zinc-superoxide dismutase in human epidermis: an immunochemical study. Front Med 6:258
18. Scuderi S, Altobelli GG, Cimini V, Coppola G, Vaccarino FM (2021) Cell-to-cell adhesion and neurogenesis in human cortical development: a study comparing 2D monolayers with 3D organoid cultures. Stem Cell Rep 6:1–17
19. Illario M, Amideo V, Casamassima A, Andreucci M, di Matola T, Miele C, Rossi G, Fenzi G, Vitale M (2003) Integrin-dependent cell growth and survival are mediated by different signals in thyroid cells. J Clin Endocrinol Metab 88:260–269
20. Illario M, Cavallo AL, Bayer KU, Di Matola T, Fenzi G, Rossi G, Vitale M (2003) Calcium/calmodulin-dependent protein kinase II binds to Raf-1 and modulates integrin-stimulated ERK activation. J Biol Chem 278:45101–45108
21. Illario M, Cavallo AL, Monaco S, Di Vito E, Mueller F, Marzano LA, Troncone G, Fenzi G, Rossi G, Vitale M (2005) Fibronectin-induced proliferation in thyroid cells is mediated by {alpha} v{beta}3 integrin through Ras/Raf-1/MEK/ERK and calcium/CaMKII signals. J Clin Endocrinol Metab 90:2865–2873
22. Fowkes RC, O'Shea L, Sidhu KK, Patel MV, Geddes JF, Burrin JM (2002) Calcium/calmodulin-dependent protein kinases (CAMK'S) in pituitary cell-lines and human pituitary adenomas. 193rd Meeting of the Society for Endocrinology and Society for Endocrinology joint Endocrinology and Diabetes Day. https://www.endocrine-abstracts.org/ea/0004/ea0004p69
23. Westphal RS, Anderson KA, Means AR, Wadzinski BE (1998) A signaling complex of Ca^{2+}-calmodulin-dependent protein kinase IV and protein phosphatase 2A. Science 280:1258–1261
24. Hashimoto K, Zanger K, Hollenberg AN, Cohen LE, Radovick S, Wondisford FE (2000) cAMP response element-binding protein-binding protein mediates thyrotropin-releasing hormone signaling on thyrotropin subunit genes. J Biol Chem 275:33365–33372
25. Kitsos CM, Sankar U, Illario M, Colomer-Font JM, Duncan AW, Ribar TJ, Reya T, Means AR (2005) Calmodulin-dependent protein kinase IV regulates hematopoietic stem cell maintenance. J Biol Chem 280:33101–33108
26. Lemrow SM, Anderson KA, Joseph JD, Ribar TJ, Noeldner PK, Means AR (2004) Catalytic activity is required for calcium/calmodulin-dependent protein kinase IV to enter the nucleus. J Biol Chem 279:11664–11671
27. Haisenleder DJ, Burger LL, Aylor KW, Dalkin AC, Marshall JC (2003) Gonadotropin-releasing hormone stimulation of gonadotropin subunit transcription: evidence for the involvement of calcium/calmodulin-dependent kinase II (Ca/CAMK II) activation in rat pituitaries. Endocrinology 144:2768–2774
28. Haisenleder DJ, Ferris HA, Shupnik MA (2003) The calcium component of gonadotropin-releasing hormone-stimulated luteinizing hormone subunit gene transcription is mediated by calcium/calmodulin-dependent protein kinase type II. Endocrinology 144:2409–2416
29. Haisenleder DJ, Burger LL, Aylor KW, Walsh DAC, HE, Shupnik MA, Marshall JC, (2005) Testosterone stimulates follicle-stimulating hormone beta transcription via activation of extracellular signal-regulated kinase: evidence in rat pituitary cells. Biol Reprod 72:523–529
30. Murao K, Imachi H, Cao WM, Yu X, Tokumitsu H, Inuzuka H, Wong NC, Shupnik MA, Kobayashi R, Ishida T (2004) Role of calcium-calmodulin-dependent protein kinase cascade in thyrotropin (TSH)-releasing hormone induction of TSH and prolactin gene expression. Endocrinology 145:4846–4852
31. van den Brand AD, Rubinstein E, van den Berg M, van Duursen MBM (2019) GH3 and RC-4BC cell lines are not suitable as in vitro models to study prolactin modulation and AHR responsiveness in rat pituitary. Mol Cell Endocrinol 496:110520
32. Coleman DT, Chen X, Sassaroli M, Bancroft C (1996) Pituitary adenylate cyclase-activating polypeptide regulates prolactin promoter activity via a protein kinase A-mediated pathway that is independent of the transcriptional pathway employed by thyrotropin-releasing hormone. Endocrinology 137:1276–1285
33. Nakamura Y, Okuno S, Sato F, Fujisawa H (1995) An immunohistochemical study of Ca^{2+}/calmodulin-dependent protein kinase IV in the rat central nervous system: light and electron microscopic observations. 168:181–194
34. Marganski WA, Gangopadhyay SS, Je HD, Gallant C, Morgan KG (2005) Targeting of a novel Ca^{+2}/calmodulin-dependent protein kinase II is essential for extracellular signal-regulated kinase-mediated signaling in differentiated smooth muscle cells. Circ Res 97:541–549