EXPERIMENTAL STUDY

TSH/cAMP up-regulate sarco/endoplasmic reticulum Ca^{2+} -ATPases expression and activity in PC Cl3 thyroid cells

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Abstract

Objective: We recently reported that the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) 2b is the SERCA form preferentially expressed in rat thyroid. Moreover, SERCA2b expression dramatically decreases in virally transformed, highly tumorigenic, PC Cl3 thyroid cells. These results suggest that, in the thyroid, SERCA2b, in addition to its housekeeping role, is linked to differentiation and is a regulated gene. We therefore sought to study the effect of TSH, the main regulator of thyroid function, on SERCA2b expression and activity.

Methods: PC Cl3 cells were hormone starved in low-serum medium and stimulated for long (48 h) or short (1, 2 and 4 h) times. SERCA2b expression and activity were evaluated by Northern and Western blots, Ca^{2+} -ATPase activity and Ca^{2+} store content.

Results: In PC Cl3 cells, SERCA2b mRNA and protein were induced twofold by a 48-h long treatment with TSH. Long-term elevation (48 h) of intracellular cAMP levels, by forskolin or 8-Br-cAMP, had similar effects on SERCA2b mRNA and protein. We also measured Ca^{2+} -ATPase activity and Ca^{2+} store content. Both long (48 h) and short (0.5–1 h) treatments with TSH, forskolin or 8-Br-cAMP induced a marked increase of SERCA2b activity. This effect was completely abolished by H89, a specific inhibitor of cAMP-dependent protein kinase A (PKA). TSH and 8-Br-cAMP increased Ca^{2+} store content after both long (48 h) and short (1–2 h) treatments.

Conclusions: These data suggested that TSH/cAMP acts as an important regulator of both SERCA2b expression and activity in the thyroid system, through PKA activation.

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Introduction

Modulation of Ca^{2+} homeostasis is a mechanism common to signal transduction pathways that regulate a wide range of cell phenomena. Cytosolic Ca^{2+} is known to control numerous cell functions, including contraction, gene expression and cell growth (1). Maintenance of Ca^{2+} levels within the lumen of the endoplasmic reticulum (ER) is another critical factor for cell growth (2) as well as for the synthesis, processing and folding of ER translated proteins (3). Sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCAs) play a fundamental role in regulating cytosolic Ca^{2+} signals, as well as in accumulating and maintaining Ca^{2+} within the ER and the sarcoplasmic reticulum against a steep concentration gradient (4). Three SERCA genes (SERCA1, SERCA2 and SERCA3) are known which, by alternative splicing, can give rise to several protein isoforms (5).

Regulation of SERCA expression and/or activity appear to be rather complex, being developmentally

regulated but also occurring in a tissue-specific manner (4). Moreover, several factors have been described to regulate the expression of SERCAs. In aortic smooth muscle cells, platelet-derived growth factor (PDGF) up-regulates SERCA2a preferentially (6). SERCA2a is differentially modulated by thyroid hormone and norepinephrine in ventricular myocytes in culture (7). Corticosteroids decrease the expression of SERCA1a and SERCA2a in the diaphragm (8). Growth hormone increases expression of SERCA2a in rat cardiomyocytes (9). Nitric oxide (NO) increases SERCA-dependent uptake of intracellular Ca^{2+} (Ca_i^{2+}) both in primary cultures of aortic smooth muscle cells and in platelets (10, 11). On the other hand, SERCA1 activity of skeletal muscle is inhibited by NO (12). A large body of evidence therefore indicates that the expression of SERCAs is a regulated phenomenon. However, there is a scarcity of data concerning the regulation of SERCA2b. Several SERCA-modulated cellular functions in the thyroid have been studied by

our group (13–19). Very recently, we reported that SERCA2b is the main SERCA form expressed in thyroid and that SERCA2b expression is down-regulated by neoplastic transformation of the thyroid cell line PC Cl3 (20). These data suggest that, in the thyroid, SERCA2b expression is a regulated phenomenon.

Thyrotropin (TSH) is the main hormone controlling thyroid function. It up-regulates expression of critical genes involved in thyroid hormone formation, such as the sodium iodide symporter (NIS), thyroid peroxidase (TPO) and thyroglobulin (Tg) (21, 22). The best known signaling pathway of TSH, identified long before its receptor, involves an increase in cyclic AMP (cAMP) production (23). Since then, however, other TSH-signaling pathways have been described (24–26).

The aim of this study was therefore to verify if TSH or cAMP modulate the expression and activity of SERCA2b in fully differentiated thyroid cells (PC Cl3) (27, 28). Following a 48 h treatment, we found that TSH and cAMP agonists and analogs were able to approximately double SERCA2b expression in PC Cl3 cells at the mRNA and protein levels. Ca^{2+} -ATPase activity and Ca^{2+} store content showed a similar up-regulation. Moreover, following short treatments (0.5–1–2 h) with TSH or cAMP agonists and analogs, Ca^{2+} -ATPase activity and Ca^{2+} store content were significantly increased. The increase of Ca^{2+} -ATPase activity, following both 1 h and 48 h treatments with TSH or 8-Bromide-cAMP (8-Br-cAMP), was no longer detectable when cells were stimulated in the presence of H89, a specific inhibitor of protein kinase A (PKA) activity (29). Thus, TSH, through cAMP signaling and PKA activation, is an important regulator of both SERCA2b expression and activity in PC Cl3 cells.

Materials and methods

Cell lines

PC Cl3 is a rat differentiated thyroid cell line, grown in Coon's modified Ham's F-12 medium (Sigma, St Louis, MO, USA) supplemented with 5% calf serum (Sigma) and a mixture of hormones and growth factors (insulin, 1 µg/ml; TSH, 1 mU/ml; glycyl-histidyl-L-lysine, 10 ng/ml; human transferrin, 5 µg/ml; cortisone, 10 nM; somatostatin, 10 ng/ml) (six hormone mixture; Sigma) (27).

RT-PCR assays

RT-PCR experiments were performed in accordance with standard methods (30). cDNA (0.5 µg) was amplified in 50 µl PCR buffer (Promega, Madison, WI, USA) containing 15 pmol SERCA2b or SERCA3 specific forward and reverse primers (see below). After a first step of denaturation for 2 min at 95 °C, 25 or 35 cycles of cDNA amplification were performed as follows:

30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C with a final extension of 5 min at 72 °C. The last 20 cycles of amplification were carried out adding 15 pmol rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward and reverse specific primers (20) to the PCR mixture. The following SERCA primers were used: forward 5'-TCT ACC AGC TGA GTC ATT TC-3' (bp 2075–2094) and reverse 5'-TAA AGT TAG TGT CTG TGC TG-3' (bp 2589–2570) for rat SERCA2b (31); forward 5'-CCT GCT CTC GGC TGC CGA CG-3' (bp 15–34) and reverse 5'-GTG GAC TTG ATC TCG ATG AG-3' (bp 483–464) for rat SERCA3 (32). The products of the PCRs were analyzed by electrophoresis on 1% agarose gels and u.v. visualized by ethidium bromide staining. Measurements of SERCA2b and SERCA3 mRNA levels in rat tissues and cell lines were performed at two cDNA concentrations at least and normalized to the GAPDH mRNA content.

RNA extraction and Northern blot experiments

Total RNA was extracted from cells by the Chomczynski & Sacchi method (33). Total RNA (20 µg) was analyzed by electrophoresis on a 1.2% formaldehyde agarose gel and blotted onto nitrocellulose membranes (BioRad, Hercules, CA, USA). Filters were washed twice in 10 ml 2 × SSPE (0.3 M NaCl, 0.02 M NaH_2PO_4 and 0.002 M EDTA), incubated for 5 h at 42 °C in 10 ml pre-hybridization buffer (50% formamide, 5 × SSPE, 2 × Denhardt's, 0.2% SDS and 100 µg/ml sonicated denatured salmon sperm DNA) and then for 16 h in 10 ml of the same buffer with 1×10^7 c.p.m./ml of a [^{32}P]-dATP-labeled specific probe encompassing bp 2074–2590 of rat SERCA2b cDNA (31). After the hybridization step, filters were washed twice at room temperature in 10 ml 2 × SSPE and 0.2% SDS and once at 42 °C in the same washing buffer. Detection was by autoradiography. Bands were quantitated by densitometry with a desk scanner (Pharmacia Discovery system, Uppsala, Sweden) and RFLPrint software (PDI, Huntington Station, NY, USA). Only gel exposures within the linear range of the film and the scanner were measured.

Western blot assay

Western blot was performed on microsomal fractions as previously reported (20). Isolation of microsomal fractions was carried out as previously described (34). After evaluation of protein content, 100 µg microsomal proteins were boiled in loading buffer containing 2% SDS and 5% β-mercaptoethanol and analyzed by SDS-PAGE. Proteins were then electroblotted onto a polyvinylidene difluoride (PVDF) sheet (Millipore, Bedford, MA, USA) which was blocked for 15 h at 4 °C with Tris-buffered saline–Tween 20 (TBST) buffer (10 mM Tris–HCl, pH 8.0, 0.1% Tween 20 and 150 mM NaCl) containing 10% non-fat dry milk. It was then incubated in TBST buffer for 2 h at room

temperature with a 1:100 dilution of the goat polyclonal Ab-SERCA2 (N-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After TBST washing, the blot was incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-goat antibody (BioRad) diluted 1:3000 in TBST buffer; detection was by enhanced chemiluminescence (ECL) (Amersham, Little Chalfont, Bucks, UK).

Assay of Ca^{2+} -dependent ATPase activity of SERCAs

We previously developed a method to determine the Ca^{2+} -dependent ATPase activity which is sensitive enough to be used not only on purified microsomal fractions but also on total cellular lysates (20). In this study we therefore used total cellular lysates to analyze the regulated expression of SERCA2b in the thyroid. To prepare total cellular lysates, cells were washed twice with phosphate-buffered saline, then swollen by incubation with 5 mM K-(3-[N-morpholino] propanesulfonic acid) (KMOPS), pH 7.0, for 15 min on ice. After the addition of 0.1 mM phenylmethylsulfonyl fluoride and 100 units/ml trasylol, cells were homogenized with 50 strokes in a glass Dounce homogenizer. ATPase activities were determined at 37 °C (35) by measuring Pi production by a modification of the method of Fiske and Subbarow (36). This method is based on the reaction between phosphate and molybdate to give the yellow molybdate phosphoric acid, which contains a molybdate (Mo) (VI), which is then reduced to Mo (V) present in a blue-colored heteropolyacid compound. This blue compound is directly measured by reading the absorbance at 700 nm. Typically, 100 µg total cell lysates were incubated in 25 mM KMOPS, 100 mM KCl, 5 mM MgCl_2 , 4 mM ATP, 0.11 mM EGTA, 107 µM CaCl_2 , pH 7.0 and 5 µM [Ca]-free, for 10 min at 37 °C. The free Ca^{2+} concentration was calculated using the CHELATOR program ((Th Schoenmakers, Nijmegen, The Netherlands) implemented in Turbo Pascal 5.5 (Botland Software Corp., Scotts Valley, CA, USA) for an IBM PC) (20). The phosphate produced was determined directly by reading the absorbance at 700 nm of a standard curve. Activity either in the presence of EGTA alone or in the presence of 25 nM thapsigargin (Calbiochem, Darmstadt, Germany) (37) (which gave equivalent results) was subtracted. Inclusion in the buffer of sodium azide or *p*-trifluoromethoxyphenylhydrazide, potent inhibitors of mitochondrial Ca^{2+} uptake, did not modify the results. Reactions were linear with respect to time and protein concentrations.

[Ca^{2+}]_i measurements

[Ca^{2+}]_i was measured using a microfluorimetric technique, as previously reported (38). Briefly, the cells grown on glass coverslips were loaded with 5 µM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzo-furan-5-oxy]-2-(2¹-amino-5¹-methylphenoxy)-ethane-N,N,N¹,N¹-tetraacetic acid pentaacetoxymethyl ester

(fura-2 AM) in Krebs–Ringer saline solution (5.5 mM KCl, 160 mM NaCl, 1.2 mM MgCl_2 , 1.5 mM CaCl_2 , 10 mM glucose and 10 mM Hepes-NaOH, pH 7.4) for 1 h at room temperature. At the end of the fura-2AM loading period, the coverslip was introduced into a microscope chamber (Medical System, Co., Greenvale, NY, USA) on an inverted Nikon Diaphot fluorescence microscope. The cells were kept in Krebs–Ringer saline solution throughout the experiment. All the drugs tested were introduced into the microscope chamber by fast injection. A 100 W Xenon lamp (Osram, München, Germany) with a computer-operated filter wheel bearing two different interference filters (340 and 380 nm) illuminated the microscopic field with u.v. light, alternating the wavelengths at an interval of 500 ms. The interval between each pair of illuminations was 1 s, and the interval between filter movements was 1 s. Emitted light was passed through a 400 nm dichroic mirror, filtered at 510 nm, and collected by a CCD camera (Photonic Science, Robertsbridge, East Sussex, UK) connected to a light amplifier (Applied Imaging Ltd, Gateshead, Tyne and Wear, UK). Images were digitized and analyzed with a Magiscan image processor (Applied Imaging Ltd). Using a calibration curve, the AUTOLAB software (RBR Altair, Florence, Italy) calculated the [Ca^{2+}]_i corresponding to each pair of images from the ratio between the intensity of the light emitted when cells were illuminated at both 340 and 380 nm. At the end of each experimental session, the calibration was performed according to the procedure described by Grynkiewicz *et al.* (38). In particular, cells were lysed with ionomycin (2–10 µM), in the presence of 1.5 mM extracellular Ca^{2+} . The addition of ionomycin produced a rapid increase in fluorescence intensity that allowed calculation of the maximal ratio (R_{max}) value. To determine the R_{min} value, cells were subsequently exposed to a Ca^{2+} -free solution containing 1–20 mM EGTA. Given that the K_d for Ca^{2+} of fura-2 is 224 nM at 37 °C, the R_{min} and R_{max} values were introduced into the Grynkiewicz *et al.* (38) formula to convert the values of fluorescence ratio between 340 and 380 nm into [Ca^{2+}]_i. The values were subtracted for background fluorescence obtained from images taken from a region of the coverslip devoid of cells. No interference was detected between any of the compounds utilized in the present study and the excitation or the emission spectra of fura-2.

Statistical analysis

Data are expressed as means ± S.E.M. The statistical significance of differences between various treatments was determined using a two-tailed Student's *t*-test of the STAT VIEW program (Abacus Concepts Inc., Berkeley, CA, USA). A level of $P < 0.05$ was considered statistically significant.

Results

TSH, forskolin and 8-Br-cAMP up-regulate SERCA2b mRNA expression in PC Cl3 cells

We recently showed, by RT-PCR and Northern blot, that SERCA2b is the SERCA form preferentially expressed in rat thyroid (20). Figure 1A shows that SERCA2b is the isoform preferentially expressed in the fully differentiated thyroid cell line PC Cl3. Semi-quantitative RT-PCR on total RNA from PC Cl3 cells grown in standard conditions (5% calf serum and the six hormone mixture) shows that, after 25 cycles of PCR amplification, a band of the expected size (514 bp), corresponding to SERCA2b, was detected in PC Cl3 cells (Fig. 1A, panel one). The intensity of the 514 bp band amplified from PC Cl3 RNA was much higher than that from spleen RNA samples (Fig. 1A, panel one). On the other hand, under the same experimental conditions, no expression of SERCA3 mRNA was detected

in PC Cl3 cells, while a SERCA3 cDNA fragment of the expected size (468 bp) was amplified in spleen (Fig. 1A, panel two). By increasing the number of PCR cycles (35 cycles versus 25), a faint SERCA3 band appeared in PC Cl3 cells, with a size corresponding to the band amplified from spleen RNA (Fig. 1A, panel three). RNA levels of GAPDH, a housekeeping gene used as an internal control to normalize SERCA2b and 3 mRNA content, were identical in all samples (Fig. 1A, panel four). Since TSH, through cAMP, acts as the main regulator of thyroid function, we wished to determine whether TSH/cAMP was able to modulate SERCA2b mRNA expression. With this aim, since the SERCA2b mRNA is easily detected in PC Cl3 cells by Northern blot (20), we decided to use this assay. PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated or stimulated for 48 h with 10 mU/ml TSH, 1 μ M forskolin or 1 mM 8-Br-cAMP. Total RNA

Figure 1 The effect of TSH, forskolin and 8-Br-cAMP on SERCA2b mRNA levels in PC Cl3 cells. (A) RT-PCR assays with specific SERCA2b (25 cycles), SERCA3 (25 and 35 cycles) or GAPDH (20 cycles) primers were performed on total RNA from PC Cl3 cells grown in standard conditions (5% calf serum and the six hormones mixture) and spleen extracts. Amplification products were analyzed by 1% agarose gel electrophoresis and u.v. visualized by ethidium bromide staining. (B) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated (control) or exposed to 10 mU/ml TSH, 1 μ M forskolin or 1 mM 8-Br cAMP for 48 h. Purified total RNA from control and treated PC Cl3 cells was subjected to Northern analysis and resulting blots were hybridized with a specific SERCA2b 32 P-labeled cDNA probe (upper panel). After the hybridization step, filters were washed twice at room temperature in 10 ml $2 \times$ SSPE and 0.2% SDS and once at 42°C in the same washing buffer. Detection was by autoradiography. SERCA2b transcript sizes and ribosomal 28S and 18S RNA are indicated (upper panel). Ethidium bromide staining of 28S and 18S ribosomal RNA is shown (lower panel). (C) Bands corresponding to 7.7 kb, 6.2 kb and 4.6 kb transcripts were quantitated by densitometry with a desk scanner and RFLPrint software and normalized to 28S and 18S ribosomal RNA content. Only gel exposure within the linear range of the film and the scanner were measured. Longer exposures were usually used for photography. Results are means $\pm$ S.E.M. of at least three separate experiments. Forsk., forskolin; 8-Br, 8-Br-cAMP.

was extracted and Northern blot experiments were performed. Figure 1B shows that SERCA2b mRNA was markedly increased by TSH stimulation as well as by forskolin and 8-Br-cAMP. All three spliced isoforms of SERCA2b mRNA (7.7 kb, 6.2 kb and 4.6 kb) (32) were detectable, as indicated by transcript sizes (Fig. 1B, upper panel). The mRNA isoforms arise from alternative splicing of the 3'-untranslated region of SERCA2b mRNA (32). These results were confirmed by using SERCA2b-labeled probes encompassing different regions of SERCA2b full-length cDNA (data not shown), to exclude any possible cross-hybridization with SERCA3 isoforms, given the high grade of homology between SERCA2b and SERCA3 cDNA sequences (32). 28S and 18S ribosomal RNA were used to normalize SERCA2b mRNA (39) (Fig. 1B lower panel and C). SERCA3 mRNA, barely detectable in thyroid and PC Cl3 cells by RT-PCR even at a high number of cycles (20 and Fig. 1A), did not vary after TSH, forskolin or 8-Br-cAMP treatments (data not shown).

TSH, forskolin and 8-Br-cAMP up-regulate SERCA2b protein expression in PC Cl3 cells

PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated or stimulated for 48 h with 10 mU/ml TSH, 1 μ M forskolin or 1 mM 8-Br-cAMP and Western blot experiments were performed. As shown in Fig. 2, expression of SERCA2b protein was increased following TSH, forskolin or 8-Br-cAMP treatments. The SERCA2b protein induction by TSH, forskolin, 8-Br-cAMP reflected that observed at the mRNA level. The SERCA2b protein content was normalized to microsomal protein content (Fig. 2B).

TSH, forskolin and 8-Br-cAMP increase Ca^{2+} -ATPase activity of the SERCA2b pump in PC Cl3 cells. PKA activation mediates the TSH/cAMP stimulatory effect on SERCA2b expression and activity

The method used here to measure the Ca^{2+} -ATPase activity of SERCA2b was sensitive and reproducible. Ca^{2+} -ATPase activity increased linearly with increasing amounts of total cell lysates from 5 to 400 μ g. PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated or exposed for 48 h to 0.1, 1 or 10 mU/ml TSH, 0.1, 1 or 10 μ M forskolin or 0.1, 0.5, 1 or 5 mM 8-Br-cAMP and Ca^{2+} -ATPase activity was measured. As shown in Fig. 3A, SERCA2b activity measured in PC Cl3 cells treated with all the stimuli markedly increased with respect to untreated cells in a concentration-dependent manner. Thus, the up-regulation of SERCA2b Ca^{2+} -ATPase activity by TSH/cAMP mimicked the increase in SERCA2b mRNA and protein levels by the same agents. To address whether TSH/cAMP was able to induce SERCA2b activity independently from increased protein expression, PC Cl3 cells were starved as described above and stimulated for 0.5, 1 or 4 h with 10 mU/ml TSH, 1 μ M forskolin or 1 mM 8-Br-cAMP. As shown in Fig. 3B, SERCA2b activity was significantly up-regulated by TSH or forskolin as early as 30 min and by 8-Br-cAMP as early as 1 h. This difference may reflect the time required for 8-Br-cAMP to diffuse inside the cells. Western blot experiments demonstrated that, after 30, 60 and 120 min of TSH/cAMP stimulation, the SERCA2b protein levels were constant (not shown). Therefore, TSH/cAMP stimulation resulted in up-regulation of both expression and activity of SERCA2b in PC Cl3 cells. The effects of TSH/cAMP on activity and

Figure 2 The effect of TSH, forskolin and 8-Br-cAMP on SERCA2b protein levels in PC Cl3 cells. (A) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated (control) or exposed to 10 mU/ml TSH, 1 μ M forskolin or 1 mM 8-Br-cAMP for 48 h. Total protein (100 μ g) from microsomal fractions was electrophoresed on 10% SDS polyacrylamide gels, blotted on PVDF membrane and incubated with the Ab-SERCA2 (N-19). SERCA2b reactivity was revealed by incubation with horseradish peroxidase-conjugated antibodies and ECL. (B) Relative levels of SERCA protein in control and treated cells. Results are means $\pm$ S.E.M. of at least three separate experiments.

Figure 3 Ca^{2+} -ATPase activity of the SERCA2b pump in PC Cl3 cells following TSH, forskolin and 8-Br-cAMP treatments. (A) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated (control) or exposed to 0.1, 1 or 10 mU/ml TSH, 0.1, 1 or 10 μM forskolin or 0.1, 0.5, 1 or 5 mM 8-Br-cAMP for 48 h. Ca^{2+} -ATPase activity of the SERCA2b pump in total cell lysates was determined at 37°C (35) by measuring Pi production by a modification of the method of Fiske and Subbarow (36). Results are means $\pm$ S.E.M. of at least three separate experiments. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$ versus the control group. (B) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, PC Cl3 cells were left untreated or exposed for 0.5, 1 or 4 h to 10 mU/ml TSH, or 1 μM forskolin (Forsk.) or 1 mM 8-Br cAMP. Results are means $\pm$ S.E.M. of at least three separate experiments. * $P < 0.05$ and *** $P < 0.0005$ versus the control group.

expression were temporally separated, since they were not additive at 48 h. It is very likely that initially there is an effect on activity and then on expression.

To address whether the TSH/cAMP-dependent increase of SERCA2b expression and activity was mediated by PKA activation, PC Cl3 cells were starved as described above and stimulated for 1 h (Fig. 4A) or 48 h (Fig. 4B) with 10 mU/ml TSH or 1 mM 8-Br-cAMP in the absence or presence of 30 μM (Fig. 4A) or 10 μM (Fig. 4B) of the PKA inhibitor H89 (Calbiochem, Darmstadt, Germany), added to cells 30 min before TSH or cAMP. These concentrations of H89 have been proved to be effective in inhibiting forskolin- and cAMP-induced neurite outgrowth in PC12 pheochromocytoma cells (29). As shown in Fig. 4, the TSH/cAMP-dependent increase of SERCA2b expression

and activity was completely blocked by the previous addition of H89. Thus, the up-regulation of SERCA2b Ca^{2+} -ATPase expression and activity by TSH/cAMP appears to be modulated by PKA activation.

TSH, forskolin and 8-Br-cAMP increase Ca_i^{2+} store content in PC Cl3 cells

To determine the functional consequence of the TSH/cAMP stimulation of SERCA2b expression and activity we decided to verify if TSH/cAMP was able to increase the (Ca_i^{2+}) store content of PC Cl3 cells. The cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated or stimulated for 48 h with 10 mU/ml TSH, 1 μM forskolin

Figure 4 Ca^{2+} -ATPase activity of the SERCA2b pump in PC Cl3 cells following TSH and 8-Br-cAMP treatments in the presence of H89. (A) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated (control) or exposed to 30 μM H89 for 30 min. Then, 10 mU/ml TSH or 1 mM 8-Br-cAMP (8Br) were added for 1 h in the presence of H89. Results are means $\pm$ S.E.M. of at least three separate experiments. ** $P < 0.005$ and *** $P < 0.0005$ versus the control group. (B) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. After starvation, cells were left untreated (control) or exposed to 10 μM H89 for 30 min. Then, 10 mU/ml TSH or 1 mM 8-Br-cAMP (8Br) were added for 48 h in the presence of H89. Results are means $\pm$ S.E.M. of at least three separate experiments. ** $P < 0.005$ versus the control group.

or 1 mM 8-Br-cAMP. To evaluate (Ca_i^{2+}) store content, cells were finally exposed to the SERCA ATPase inhibitor thapsigargin (1 μM) in the absence of extracellular Ca^{2+} (Ca_e^{2+}) and in the presence of the Ca^{2+} chelator EGTA (2 mM). This experimental condition achieved the depletion of (Ca_i^{2+}) stores (40) as shown in Fig. 5A. In TSH-, 8-Br-cAMP- and forskolin-treated cells, the Ca^{2+} release from thapsigargin-sensitive stores was much higher than that observed in untreated cells (Fig. 5). To address whether TSH/cAMP can also regulate SERCA2b activity, besides its expression, cells were stimulated in the same experimental conditions but for short times (1 or 2 h). As shown in Fig. 6, PC Cl3 cells stimulated for a short time with TSH, 8-Br-cAMP or forskolin showed a higher Ca^{2+} release from thapsigargin-sensitive stores than that observed in untreated cells, as a result of increased SERCA2b activity. Therefore, the TSH/cAMP up-regulation of both expression and activity of SERCA2b resulted in increased (Ca_i^{2+}) stores in PC Cl3 cells.

Discussion

The results of the present study have demonstrated that SERCA2b, the predominant SERCA isoform in thyroid

and in fully differentiated PC Cl3 cells, is up-regulated by TSH, forskolin and cAMP. To our knowledge, this is the first report showing a hormonal regulation of the SERCA2b isoform.

The lack of data in the literature on the hormonal regulation of SERCA2b reflects a more general deficiency of information about the regulation of SERCA2b expression. This fact may reflect the generally accepted concept that SERCA2b serves cellular housekeeping duties. At variance with SERCA2b expression, SERCA3 expression has been linked to differentiation. Therefore, the expression of SERCA3 has been shown to vary in response to various pathophysiologic states. In platelets of spontaneously hypertensive rats, SERCA3 expression has been found to decrease while SERCA2b expression was unchanged (41). The expression of SERCA3 is lost in human colon carcinomas and dramatically reduced in colonic and gastric carcinoma cell lines, while the expression of SERCA2b is not altered (42). The housekeeping role of SERCA2b has been also suggested by data obtained in cells that co-express SERCA2b and SERCAs different from SERCA3. For example, aortic smooth muscle cells (SMC) co-express SERCA2a and SERCA2b. During PDGF-induced SMC proliferation, SERCA2a expression was up-regulated while SERCA2b expression was unchanged (6). However, the concept of an exclusive housekeeping role of SERCA2b has recently been questioned as it has become clear that SERCA2b could be the only SERCA isoform expressed by highly differentiated cells (31). Recent results from our laboratory support this new standpoint: the thyrocyte is similar to enamel cells in that it is a highly differentiated cell expressing almost exclusively SERCA2b (20). Moreover, in the thyrocyte, SERCA2b expression appears to be down-regulated by neoplastic transformation (20). The view that SERCA2b expression and activity can be modulated by physiopathologic conditions is further supported by the findings of the present study, in that the expression and activity of thyroid SERCA2b is regulated by TSH, the most important hormone in the physiopathology of the thyroid gland. TSH regulates both differentiated functions and growth of thyroid cells. TSH up-regulates the expression of Tg, TPO and NIS, essential proteins involved in thyroid hormone synthesis (21, 22). Since all these proteins are cargo (secretory or membrane) proteins, their folding occurs in the ER. Therefore, TSH increases the ER load of newly synthesized proteins. It is well known that protein folding in the ER requires a high Ca^{2+} concentration (3). Moreover, we have directly demonstrated a Ca^{2+} requirement for the folding of Tg (17–19). On this basis, it could be expected that an increased ER protein load would correspond to an increased capability to take up Ca^{2+} in the ER lumen, i.e. an increased expression and activity of the SERCA ATPases. This is, in fact, the case. However, TSH also stimulates the growth of thyroid cells. Maintenance of Ca^{2+} levels

Figure 5 Calcium release from intracellular stores of PC Cl3 cells following a 48-h treatment with TSH, forskolin or 8-Br-cAMP. (A) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. Representative single-cell traces for the effect exerted by thapsigargin (TG; 1 μ M) in the presence of EGTA (2 mM) and in the absence of Ca_e²⁺ on PC Cl3 cells left untreated (control) or exposed for 48 h to 10 mU/ml TSH, 1 mM 8-Br-cAMP or 1 μ M forskolin. The traces are representative of 40 to 60 cells for each experimental group studied in at least three different experimental sessions. (B) Quantification of the effects of thapsigargin (1 μ M) on Ca²⁺ release from intracellular stores in control (Ctrl) condition, and after 48-h exposure to TSH, 8-Br-cAMP or forskolin. The results are reported as [Ca²⁺]_i increase (% peak/basal). *** P < 0.0005 versus the control group.

within the ER lumen is a critical factor for cell growth (2) so, again, it could be expected that a growth factor would up-regulate SERCA expression. This has been already reported in the SMC system. During PDGF-induced SMC proliferation, SERCA2a expression was up-regulated while SERCA2b expression was unchanged (6). In thyroid cells that express mostly SERCA2b, growth factor (TSH) stimulation up-regulates SERCA2b expression. In this respect, the recent

interesting finding of a reduced expression of SERCA proteins in the heart of CREM $-/-$ mice (43) suggests a cAMP-dependent transcriptional control of the SERCA2 gene.

The signaling pathways through which TSH stimulates growth and differentiation of thyroid cells begin with a seven transmembrane-spanning domain receptor that is coupled to the heterotrimeric G proteins. Activation of Gs leads to the stimulation of

Figure 6 Calcium release from intracellular stores of PC Cl3 cells following 1- or 2-h treatments with TSH, forskolin or 8-Br-cAMP. (A) PC Cl3 cells were starved for 4 days in low serum (0.2%)-containing medium, in the absence of hormones/growth factors. Representative single-cell traces for the effect exerted by thapsigargin (TG; 1 μM) in the presence of EGTA (2 mM) and in the absence of Ca_e^{2+} on PC Cl3 cells left untreated or exposed for 1 or 2 h to 10 mU/ml TSH, 1 mM 8-Br-cAMP or 1 μM forskolin. The traces are representative of 30 to 50 cells for each experimental group studied in at least three different experimental sessions. (B) Quantification of the effects of thapsigargin (1 μM) in the presence of EGTA (2 mM) and in the absence of Ca_e^{2+} on Ca_i^{2+} store release in control (Ctrl) conditions and after 1- and 2-h exposure to TSH, 8-Br-cAMP and forskolin. The results are reported as $[\text{Ca}^{2+}]_i$ increase (% peak/basal). *** $P < 0.0005$ versus the control group.

adenylyl-cyclase, the production of the second messenger cAMP and activation of the cAMP-dependent protein kinase (PKA). Many of the effects of TSH on proliferation and differentiation can be mimicked by forskolin and cAMP analogs, indicating that these effects are mediated through cAMP (44). However, it is known that p21 Ras is required in TSH growth

signal pathways (24). Our data strongly suggest that the up-regulation of SERCA2b by TSH is exerted through cAMP, and that p21 Ras is not implicated in this pathway, since in cells that present an activation of the ras pathway (E1A + Py and E1A + raf) (45) the expression of SERCA2b is dramatically decreased, rather than up-regulated (20). Moreover,

the up-regulation of SERCA2b Ca^{2+} -ATPase expression and activity by TSH/cAMP appears to be modulated by PKA, since it was completely blocked by the previous addition of H89, a specific inhibitor of PKA. Therefore, TSH up-regulation of SERCA2b in the thyroid is exerted by a pathway that closely resembles that used to control thyroid differentiation. Thus, it appears that SERCA2b is linked to the thyroid differentiated state by several standpoints. First, the function of SERCA2b, to maintain a high Ca^{2+} concentration in the lumen of the ER, is essential for the folding of Tg and possibly other cargo proteins involved in hormonogenesis (TPO and NIS). Secondly, the expression of SERCA2b is up-regulated by TSH, which is the main stimulator of the expression of the other thyroid proteins involved in hormonogenesis, by a pathway (cAMP–PKA) that is similar to that used to up-regulate Tg, TPO and NIS. Thirdly, the expression of SERCA2b is down-regulated by neoplastic transformation of thyroid cells (20), again underscoring the similarities between expression of SERCA2b and that of the proteins characteristic of the thyroid differentiated state (27).

More efforts are required to precisely dissect the pathways that mediate the TSH/cAMP control of SERCA2b expression and activity in the thyroid described in this study. However, the demonstration of such a control could help to better understand several aspects of thyroid physiopathology.

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