Differential regulation of inhibin/activin α- and β_A-subunit and follistatin mRNAs by cyclic AMP and phorbol ester in cultured human granulosa-luteal cells

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Abstract

Granulosa cell-derived inhibin A (a dimer of α- and β_A-subunits), activin A (a homodimer of β_A-subunits) and the activin-binding protein follistatin are important regulators of human ovarian steroidogenesis. We here studied how 8-bromo-cAMP (8br-cAMP), a protein kinase A activator, and 12-O-tetradecanoylphorbol 13-acetate (TPA), a protein kinase C activator, affect the steady-state levels of α- and β_A-subunit and follistatin mRNAs in cultured human granulosa-luteal cells. 8br-cAMP induced α- and β_A-subunit and follistatin steady-state mRNA levels in a time- and concentration-dependent manner. The levels of α-subunit mRNAs were stimulated by 8br-cAMP in a sustained manner with a maximal induction seen at the time points 24 and 48 h. By contrast, β_A- subunit and follistatin mRNA levels were rapidly and transiently induced by 8br-cAMP with maximal effects observed at 3 h and 8 h, respectively. TPA did not affect basal α-subunit mRNA levels but it rapidly induced β_A-subunit mRNAs at 3 h and the stimulation was still evident at 48 h. TPA induced follistatin mRNA levels with kinetics similar to 8br-cAMP but to a lesser extent. Moreover, 8br-cAMP and TPA stimulated β_A-subunit and follistatin mRNA levels synergistically at 3 h. By contrast, TPA had a potent inhibitory effect on 8br-cAMP- and hCG-induced α-subunit levels. Neither 8br-cAMP nor TPA regulated inhibin/activin β_B-subunit mRNA levels. Taken together, the activation of protein kinase-A and -C by 8br-cAMP and TPA, respectively, lead to clearly differential responses in the steady-state levels of inhibin/activin α- and β_A-subunit and follistatin mRNAs. These results suggest that the inhibin A vs. activin A ratio as well as follistatin levels are regulated by multiple second-messenger pathways in the human ovary.

Keywords: Activin; Inhibin; Follistatin; cAMP; TPA; (Human granulosa cell)

1. Introduction

Inhibins and activins are glycoproteins that were originally purified from bovine [1] and porcine [2–4] ovarian follicular fluids based on their ability to inhibit and stimulate, respectively, FSH secretion from cultured rat anterior pituitary cells. Inhibins are het-erodimers of an α- subunit and one of the two distinct but highly homologous β-subunits (β_A or β_B) forming proteins named inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$). Activins are homo- or heterodimers made up of two β-subunits and are called activin A, activin AB or activin B according to their subunit combinations [5]. Also, an activin β_C-subunit cDNA [6] has been recently cloned from a human liver cDNA library but no information on its biochemical properties or biological activities has been published. Activins and inhibins are members of the transforming growth factor β (TGF-β) superfamily which includes an increasing number of

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structurally related polypeptide growth factors with multiple biological activities in developing embryos as well as in several adult organ systems [5,7].

Follistatin (FS) is a single chain glycoprotein, which was first isolated from bovine [8] and porcine ovarian follicular fluids [9], like inhibin and activin, as an inhibin-like FSH secretion suppressing factor. FS is structurally unrelated to activins and inhibins having three different isoforms derived either by alternative mRNA splicing [10] or proteolytical modification of the core protein [11]. After initial purification FS was found to bind both activin [12] and inhibin through their common β-subunit [13] and neutralize the biological effects of activin [14]. All FS isoforms seem to bind activin with high affinity [11,15] and to prevent the access of the ligand to its cellular receptors [16].

Activin/inhibin subunit and FS mRNAs and proteins are produced in human ovary, where α-, β_A- and β_B-subunits and FS have been localized to the granulosa cells [17,18]. Human follicular fluid has been reported to contain all three isoforms of activin [19] together with inhibin A [20] and FS [19,21]. Activin A has been shown to modulate steroidogenesis in cultured human granulosa-luteal (GL) cells by decreasing basal and gonadotropin-stimulated progesterone secretion and aromatase activity suggesting that activin may have a local antisteroidogenic effect within the human ovary [22,23]. This biological role of activin is also supported by the finding that it inhibits LH-stimulated androgen production in cultured human thecal cells [24]. By contrast, inhibin A has been shown to stimulate human thecal cell androgen production [25]. The effects of activin A on human GL cell steroidogenesis can be antagonized by FS [26].

In cultured human GL cells, inhibin bioactivity is markedly increased by LH [27]. Gonadotropins have also been shown to induce activin A [23] and FS [28,29] protein levels in these cells. We have previously reported the induction of inhibin α- and β_A- subunit and FS mRNA levels by FSH and hCG in cultured human GL cells [28-30]. However, activin A was shown to stimulate human GL cell β_B-subunit mRNA levels which are not induced by hCG [31]. Furthermore, although activin A did not affect β_A-subunit [31] or FS mRNA levels [29], it potently inhibited hCG-induced α-subunit mRNA levels [31]. In order to better understand the role of different second-messenger pathways in the regulation of inhibin/activin subunit and FS expression we now determined how 8bromo-cAMP (8br-cAMP) and TPA affect their steady-state mRNA levels. The results suggest that protein kinase A- and C-dependent pathways may differentially regulate inhibin A vs. activin A ratio as well as FS levels in human ovary.

2. Materials and methods

2.1. Human GL cell cultures

Human preovulatory granulosa cells were obtained from women undergoing hormone treatment for in vitro fertilization (IVF). The cells were enzymatically dispersed and separated from red blood cells by centrifugation through Ficoll-Paque (Pharmacia, Uppsala, Sweden) as previously described [17]. They were plated at a density of 2-5 $\times$ 10^5 cells/well on 35-mm 6-well dishes (Costar, Cambridge, MA, USA) and cultured in 1:1 Dulbecco's Modified Eagle's Medium (DMEM)/ Ham's F-12 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Gibco), 2 mM L-glutamine and antibiotics at 37°C in 95% air/5% CO_2 humidified environment. Cell culture media were changed every other day and treatments were performed at day 6 of culture for different time periods as indicated in text and figure legends.

2.2. Treatments of human GL cells

All experimental incubations were performed in DMEM/Ham's F-12 supplemented with 2.5% FCS. For time course experiments, cells were stimulated with 1.0 mM of 8br-cAMP (Sigma, St. Louis, MO, USA) or with 10 ng/ml of TPA (Sigma) for 3, 8, 24 and 48 h. For concentration-dependence studies the cells were treated with 0.03-3.0 mM of 8br-cAMP or with 0.1-100 ng/ml of TPA for 3, 8 or 48 h. For co-treatments by 8br-cAMP (1.0 mM) or hCG (100 ng/ml; CR-127 preparation, National Hormone and Pituitary Program of the NIDDK, NIH) with TPA (10 ng/ml) the cells were incubated for 3, 8, 24 or 48 h. Cellular RNA was thereafter extracted for dot blot hybridization analyses with gene specific probes. Each experiment was performed at least three times with duplicate or triplicate cultures.

2.3. RNA extraction and preparation of Northern and dot blots

Cytoplasmic RNA was extracted with the modified NP-40 lysis procedure [32] and quantitated by absorbance at 260 nm. For Northern blots 8 or 10 μg of cytoplasmic RNA were size-fractionated in 1.5% agarose gels and transferred to Hybond-N (Amersham, Aylesbury, Buckinghamshire, U.K.) nylon filters. For dot blots, 1-2 μg of cytoplasmic RNA was denatured in 7.5% formaldehyde and 6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at 50°C for 30 min, and spotted onto nylon membranes using a 96-well Minifold device (Schleicher and Schuell, Keene, NH, USA). The RNA blots were baked for 1-2 h at

80°C and UV cross-linked for 6 min with a Reprostar II UV illuminator (Camag, Muttenz, Switzerland).

2.4. Hybridization probes

Human inhibin/activin α-, β_A- and β_B-subunit and FS cDNA clones were derived from appropriate RNA sources by reverse transcription-polymerase chain reaction (RT-PCR) method [33] using oligonucleotide pairs designed according to published human cDNA sequences [10,34]. The primers and cDNA clones obtained have been described in detail previously [17,28,35–37]. A genomic DNA fragment for activin β_C-subunit was derived by PCR performed on human K562 erythroleukemia cell DNA. The 417 bp fragment covers a sequence beginning at the possible cleavage site of the mature region and ending to the 3′ untranslated region nearby the stop codon (nucleotides 825–1242 in ref. [6]). The fragment was subcloned to an EcoRI-HindIII site in pBluescript (KS-) plasmid (Stratagene, La Jolla, CA, USA). A rat glyceraldehyde-6-phosphate dehydrogenase (GAPDH) [38] cDNA probe was used as a loading control.

2.5. Probe labeling, hybridizations and quantification of the data

Double-stranded cDNA inserts were labeled with [32P]-α-dCTP (3000 Ci/mmol; Amersham) using a Prime-a-gene kit (Promega, Madison, Wis, USA). The probes were purified with Nick columns (Pharmacia, Uppsala, Sweden) and used at 1–3 × 10⁶ dpm/ml in hybridization solution containing 50% formamide, 6 × SSC, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 100 μg/ml herring sperm DNA, 100 μg/ml yeast RNA and 0.5% SDS. Dot and Northern blots were hybridized for 16 h at 42°C and washed three times for 20 min with 1 × SSC and 0.1% SDS at 55°C. Filters were exposed to X-ray film with Trimax 16T intensifying screens (3M, Ferrania, Italy) at − 70°C after which the autoradiographic signals were quantified with transmission densitometer (model 331, X-rite Co., Grand Rapids, MI, USA). Alternatively, hybridized filters were analyzed in Fujifilm IP-Reader Bio-Imaging Analyzer BAS 1500 (Fuji Photo co. Ltd., Tokio, Japan) with the MacBas software supplied by the manufacturer.

2.6. Analysis of RNA data

For multiple comparisons, the data were first analyzed by one-way analysis of variance and the statistical significances were determined by the Scheffé's multiple comparison test using the Exstatix program (Select Micro Systems Inc., Yorktown Heights, NY, USA) on a Macintosh personal computer.

3. Results

Our initial hybridization analyses indicated that 8br-cAMP and TPA caused a rapid induction of the specific transcript levels of inhibin/activin β_A-subunit and FS in cultured human GL cells. Inhibin α-subunit mRNA levels were also induced by 8br-cAMP but TPA did not affect its expression. The basal expression levels of inhibin/activin β_B-subunit mRNAs are low in human GL cells [31] and neither 8br-cAMP nor TPA induced their expression. The expression of the recently cloned activin β_C-subunit was also studied but no mRNA signal was detected in any of the human GL cell RNA hybridization analyses performed. As a positive control, however, our probe recognized a transcript of approximately 3.5 kb on a Northern blot of mouse liver RNA (data not shown). Thus, it appears that activin β_C-subunit is not expressed in human GL cells. Fig. 1A shows inhibin α-subunit mRNA levels induced by 0.3 mM 8br-cAMP for 48 h. A single 1.6 kb band is induced similar to our previous report with gonadotropin-stimulated GL cells [30]. Clear inductions of the approximately 6.0-, 4.0-, 2.8- and 1.7-kb transcripts for inhibin/activin β_A-subunit were seen at 3 h by 1.0 mM
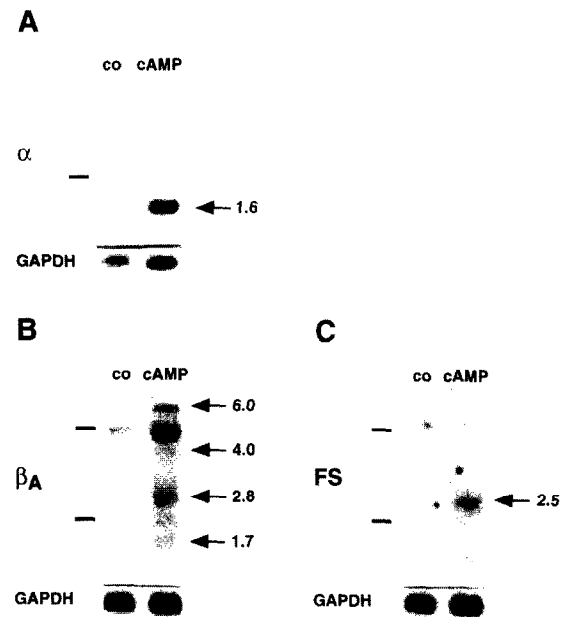

Fig. 1. Northern analyses of inhibin α- and β_A-subunit and FS steady-state mRNA levels induced by 8br-cAMP in human GL cells. The cells were first cultured for 6 days in 10% FCS-containing medium and then treated with 0.3 mM 8br-cAMP for 48 h (A) or with 1.0 mM 8br-cAMP for 3 h (B and C) in 2.5% FCS-containing medium, whereafter cytoplasmic RNA was extracted for Northern blot analyses. Eight (A) or 10 μg (B and C) of isolated RNA was size-fractionated by 1.5% agarose gel electrophoresis, transferred to nylon filters and hybridized to 32P-labeled inhibin α- (A) or β_A-subunit (B), FS (C) and GAPDH cDNA probes. The arrowheads indicate the location of specific transcripts. Migration of the 28S and 18S ribosomal RNAs is shown by dashes.

8br-cAMP as compared to the respective control (Fig. 1B). Also FS mRNA levels were induced by 1.0 mM 8br-cAMP at 3 h as seen in Fig. 1C. Although only a 2.5 kb transcript is evident in the Fig. 1C, an additional 1.5 kb band is observed clearly at later time points (data not shown). To characterize the concentration- and time-dependent effects of 8br-cAMP and TPA on inhibin/activin α- and β_A-subunit and FS mRNA levels, subsequent experiments were performed with duplicate or triplicate cultures and the results were determined by dot blot hybridization analyses.

3.1. Concentration-dependent effect of 8br-cAMP and TPA on inhibin α- and β_A-subunit and FS mRNA levels

8br-cAMP induced inhibin α-subunit mRNA levels maximally with concentrations 0.1 and 0.3 mM at 48 h (Fig. 2A) whereas higher concentrations were less effective. For inhibin/activin β_A-subunit mRNA the maximal induction was observed with 3.0 mM 8br-cAMP at 3 h (Fig. 2B). FS mRNA levels were maximally stimulated by 8br-cAMP at 8 h with concentrations 1.0 and 3.0 mM (Fig. 2C). Thus, α-subunit mRNA levels are induced at a clearly lower concentration range of 8br-cAMP than those of β_A-subunit and FS. The concentration-dependent effect of TPA on inhibin/activin β_A-subunit and FS mRNA levels were studied at 8 h (Fig. 3A and B). The maximal inductions for both β_A-subunit and FS mRNAs occurred with 10 and 100 ng/ml of TPA.

3.2. Time-dependent effect of 8br-cAMP and TPA on inhibin/activin α- and β_A-subunit and FS mRNA levels

Fig. 4A, B and C show two independent experiments in which GL cell cultures have been stimulated with either 8br-cAMP and TPA alone or together for different time periods. The maximal relative inductions of inhibin α-subunit mRNA levels by 8br-cAMP occurred at time points 24 and 48 h (Fig. 4A1 and A2). As the basal inhibin α-subunit mRNA levels steadily decreased during the 48 h culture (which is typical for human GL cells [17,30]), the highest α-subunit mRNA levels were occasionally measured at earlier time points (Fig. 4A2), but also in those experiments the maximal relative induction occurred at 24 or 48 h. 8br-cAMP had a biphasic effect on inhibin/activin β_A-subunit mRNA levels (Fig. 4B1 and B2). It first induced β_A-subunit mRNA levels at 3 h whereafter the transcript levels decreased back to the levels observed in the untreated cells. An additional increase, however, was seen at 48 h. 8br-cAMP induced FS mRNA levels rapidly and transiently. Maximal induction occurred at 8 h after which only moderate or no induction was observed (Fig. 4C1 and C2). TPA did not markedly affect basal inhibin
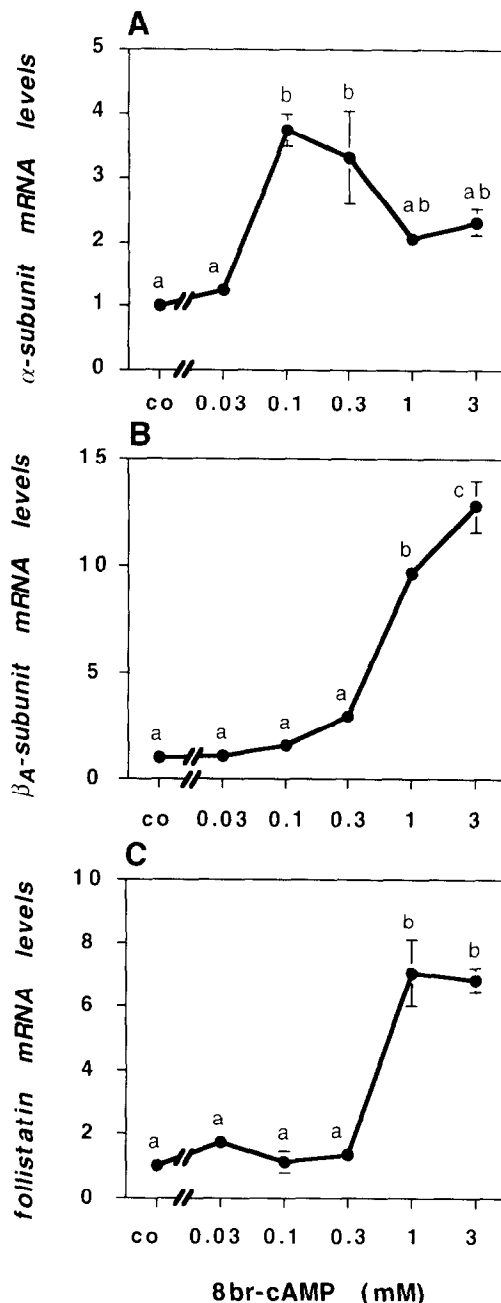

Fig. 2. Concentration-dependent effect of 8br-cAMP on inhibin α- and β_A-subunit and FS mRNA levels in human GL cells. Cells were cultured for 6 days and then treated with increasing concentrations of 8br-cAMP (0.03–3 mM). The effect of 8br-cAMP on α-subunit mRNA levels was detected at 48 h (A), on β_A- subunit mRNA levels at 3 h (B) and on FS mRNA levels at 8 h (C). Cytoplasmic RNA was analyzed by dot blot hybridization with specific ^{32}P-labeled double-stranded α- or β_A-subunit or FS and GAPDH cDNA probes and quantified as indicated in Materials and methods. Results are represented in arbitrary densitometric units ($\pm$ S.E.M.) adjusted to a value of 1.0 for the mean of the respective control. Different letters indicate significant differences between treatments (Scheffé Multiple Comparisons test after one-way ANOVA. $P < 0.05$).

α-subunit mRNA levels but it suppressed the effect of the 8br-cAMP (Fig. 4A1 and A2). TPA caused a sustained induction in β_A-subunit mRNA levels first ob-

served at 3 h and the stimulatory effect was seen up to 48 h (Fig. 4B1 and B2). 8br-cAMP and TPA had a synergistic effect on β_A-subunit mRNA levels most clearly seen at 3 and 48 h. The stimulatory effect of TPA on FS mRNA levels was not seen in all experiments. In six experiments out of ten, TPA was found to increase FS mRNA levels significantly whereas in the remaining experiments no clear stimulatory effect were observed. However, in all these experiments TPA induced inhibin β_A-subunit mRNA levels, indicating that all cultures were responsive to TPA stimulation. Fig. 4C1 and C2 show two individual experiments assessing the time-dependent effects of TPA on FS mRNA levels. The time-dependence of the inductive effect resembled that of 8br-cAMP as the maximal FS mRNA levels were seen at 8 h whereafter they decreased so that only a weak induction was observed at 24 h. 8br-cAMP and TPA had an additive effect on FS mRNA levels when

given together at time point 3 h (Fig. 4C1 and C2).

3.3. Effect of TPA on hCG-stimulated inhibin α- and β_A-subunit and FS mRNA levels

As gonadotropins regulate the studied transcripts in a manner similar to that observed for 8br-cAMP in this study [28,30], we determined how TPA affects hCG-stimulated α- and β_A-subunit and FS mRNA levels. The effect of TPA on hCG-induced α-subunit mRNA levels is shown in Fig. 5A. Although alone it had no effect on basal α-subunit mRNA levels it completely prevented the hCG-stimulated α-subunit mRNA accumulation at 48 h. On the other hand, on hCG-stimulated β_A-subunit or FS mRNA levels TPA had no inhibitory effect (Fig. 5B and C).

4. Discussion

The present study demonstrates that inhibin/activin α- and β_A- subunit mRNA levels are regulated differentially by activation of protein kinase A and C dependent signaling pathways in cultured human GL cells. Both α- and β_A-subunit mRNAs are induced by the cAMP analog 8br-cAMP. Low concentrations of 8br-cAMP (0.1–0.3 mM) were found to induce α-subunit mRNA levels significantly as compared to the controls, whereas high concentrations (1.0–3.0 mM) caused only a weak induction. By contrast, β_A-subunit mRNA levels were induced only with high concentrations of 8br-cAMP. This difference between the effective concentrations of 8br-cAMP resembles our previously reported observation that both hCG and FSH induce α-subunit mRNA levels at lower concentrations than those needed for the induction of the β_A-subunit mRNA levels [30]. Thus it seems that the α-subunit is more readily induced through the cAMP-dependent pathway than the β_A-subunit and this may explain why in vivo α-subunit mRNAs are more highly expressed in the human preovulatory follicles and corpus luteum [17,18,39]. Other common features in gonadotropin and 8br-cAMP regulated inhibin/activin subunit mRNA levels are the kinetics of the inductions. Both 8br-cAMP and gonadotropins stimulated α-subunit transcript levels at time points 24 and 48 h whereas β_A-subunit mRNA levels were increased rapidly at 2–3 h ([30] and this study) after which they decreased back to basal level at 8 h. The additional increase at time point 48 h in β_A-subunit mRNA levels stimulated by 8br-cAMP is not, however, observed in gonadotropin-stimulated cell cultures. The protein kinase C activator TPA did not affect basal α-subunit mRNA levels but it rapidly stimulated the β_A-subunit mRNA levels at 3 h and the induction was sustained up to 48 h. TPA and 8br-cAMP had synergistic effect on β_A-subunit mRNA
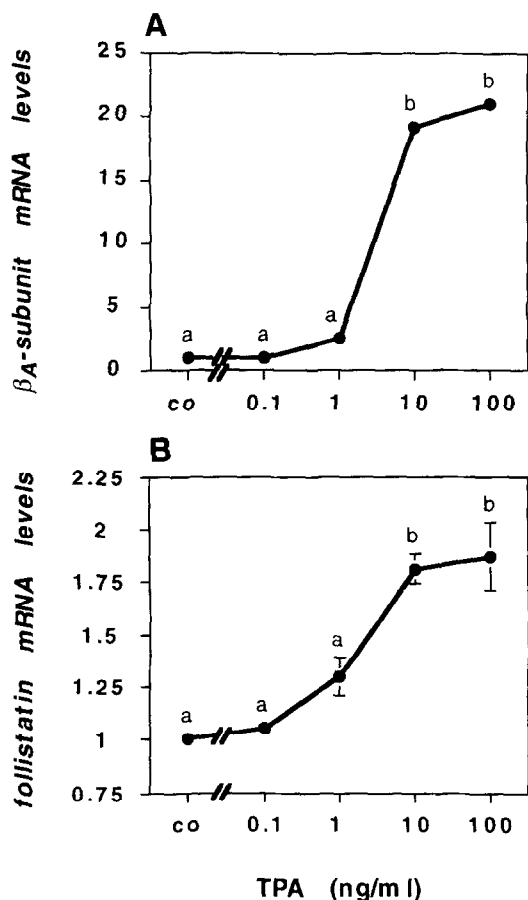

Fig. 3. Concentration-dependent effect of TPA on inhibin/activin β_A-subunit (A) and FS (B) mRNA levels in human GL cells. Cells were cultured for 6 days and then treated with increasing concentrations of TPA (0.1–100 ng/ml) for 8 h. The samples were processed as described in Fig. 1. Results are represented in arbitrary densitometric units (± S.E.M.) adjusted to a value of 1.0 for the mean of the respective control. Different letters indicate significant differences between treatments (Scheffé Multiple Comparisons test after one-way ANOVA, $P < 0.05$).
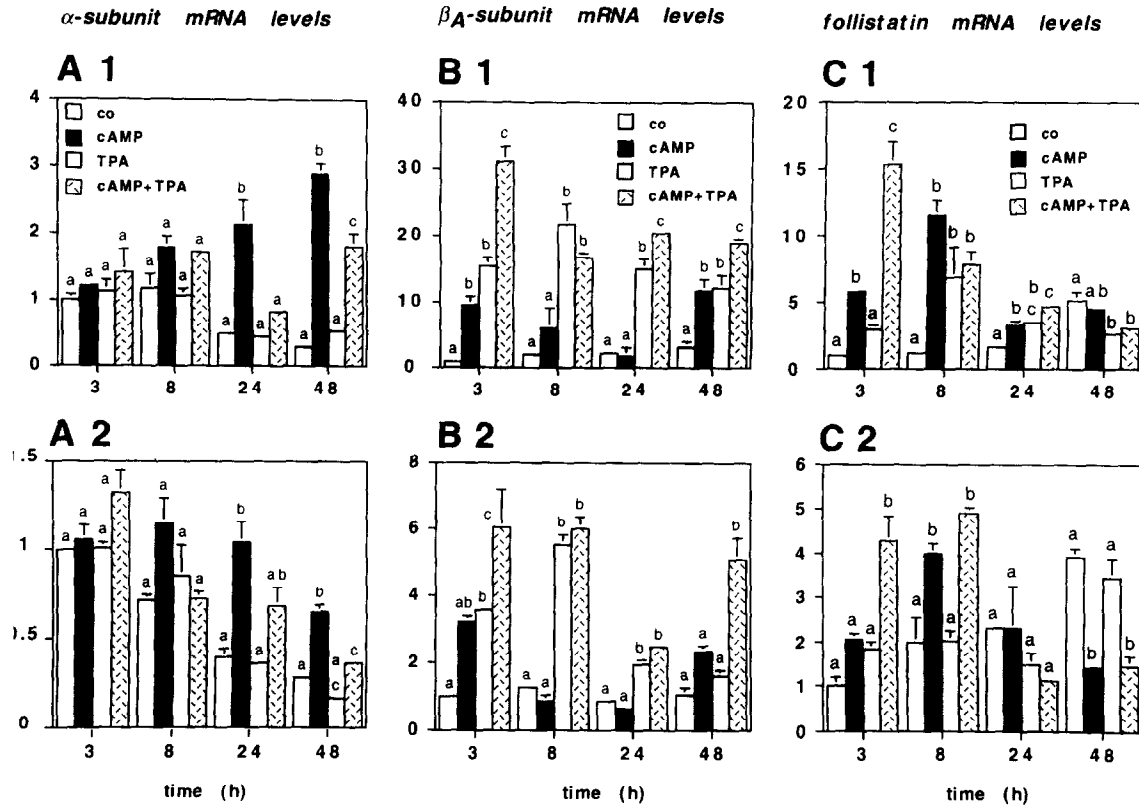
Fig. 4. Kinetics of inhibin/activin α- (A1 and A2) and β_A-subunit (B1 and B2) and FS (C1 and C2) steady-state mRNA levels stimulated by 8br-cAMP and TPA in cultured human GL cells. Cells were cultured for 6 days and then treated with 1 mM 8br-cAMP and 10 ng/ml TPA either alone or together for indicated time periods. Two parallel experiments are shown. Samples were processed as described in Fig. 1. Results are represented in arbitrary densitometric units ($\pm$ S.E.M.) adjusted to a value 1.0 for the mean of the 3 h control. Different letters indicate significancat differences between treatments within each time point (Scheffé Multiple Comparisons test after one-way ANOVA, $P < 0.05$).

levels but, interestingly, TPA was found to suppress the 8br-cAMP-stimulated α-subunit mRNAs. Furthermore, TPA also totally suppressed hCG-stimulated α-subunit mRNA levels.

Although no detailed description of the inhibin α-subunit gene promotor have been reported, analyses of rat [40], mouse [41] and bovine [42] gene promoter regions have revealed that putative cAMP and phorbol ester regulatory elements are located in the 5′-flanking region of the transcription startpoint. Moreover, rat inhibin α-subunit gene promoter activity is regulated by cAMP in cultured rat granulosa cells [43]. cAMP has also been shown to induce inhibin α-subunit mRNA levels in human fetal and adult adrenal cells [35] and in human fetal testicular cells [44]. Parallel to our results on the suppression of hCG- or 8br-cAMP-induced α-subunit mRNA stimulation by TPA in human GL cells, TPA has been shown to inhibit rat granulosa cell FSH-induced inhibin protein expression [45] without affecting its basal level. In cultured human adrenal cells TPA inhibited ACTH- and cAMP-induced α-subunit mRNA levels [35] in a manner similar to that observed in this study for hCG- and cAMP-stimulated α-subunit transcripts in human GL cells. Thus, the inhibin α-sub-

unit mRNAs appear to be induced in ovaries, testes and adrenals of several species in a remarkable similar manner through the cAMP-dependent signaling pathway. Moreover, activation of protein kinase C by TPA appears to counteract the cAMP-dependent induction of α-subunit mRNAs in all species studied so far. However, although the α-subunit gene is similarly regulated in several species through protein kinase A- and C-dependent signaling pathways, recent studies have shown that activin regulates α-subunit mRNAs in a clearly different manner in rodents and human granulosa cells. While activin stimulates inhibin α- and β_A-subunit mRNAs in rat granulosa cells [46], it does not affect their regulation in human GL cells alone [31] but, rather, inhibits hCG-stimulated α-subunit levels in a manner resembling the effect of TPA in the present study.

The human β_A-inhibin/activin subunit gene structure and promoter region have been characterized [47] and the effects of the cAMP [48] and TPA [49] on β_A-subunit expression have been studied, e.g. in human fibrosarcoma HT 1080 cell line. In those studies cAMP was found to stimulate β_A-subunit mRNA levels from 3 h onwards so that maximal inductions were seen at 24

and 48 h [48]. These observations differ from our results with human GL cells where 8br-cAMP induced β_A-subunit expression rapidly at 3 h and then the transcript levels decreased back to the basal level within a few hours. In the HT 1080 cell line TPA was found to increase β_A-subunit mRNA levels rapidly similar to our experiments but the induction was transient and only a weak induction was observed at time points 24 and 48

Fig. 5. Time-dependent stimulation of inhibin/activin α- (A) and β_A-subunit (B) and FS (C) steady-state mRNA levels stimulated by hCG and TPA in cultured human GL cells. Cells were cultured for 6 days and then treated with 100 ng/ml hCG and 10 ng/ml TPA either alone or together for indicated time periods. Samples were processed as described in Fig. 1. Results are represented in arbitrary densitometric units ($\pm$ S.E.M.) adjusted to a value 1.0 for the mean of the 3 h control. Different letters indicate significant differences between treatments (Scheffé Multiple Comparisons test after one-way ANOVA, $P < 0.05$).

h [49] whereas in our studies the induction was sustained up to 48 h. In human adrenal cells ACTH, cAMP and TPA stimulate β_A-subunit transcripts up to 48 h [35]. Thus, regulation of human β_A-subunit mRNAs by protein kinase A and C signaling pathways seems to differ in distinct cell types suggesting tissue-specific regulation of the β_A-subunit gene.

Both 8br-cAMP and TPA induced FS mRNA levels in cultured human GL cells. The effect of the 8br-cAMP was rapid and transient like those of the gonadotropins FSH and hCG described in our previous reports [28,29]. The kinetics of the effect of TPA were similar to those seen for cAMP but the effect was clearly weaker and sometimes absent. A study characterizing rat FS gene promoter has revealed that both cAMP and TPA have regulatory elements in the 5′-flanking region of the rat FS transcription startpoint and that these elements act coordinately [50]. In rat granulosa cell cultures forskolin, an adenylate cyclase activator, and TPA have been shown to induce FS protein production [51]. Also, Tano et al. [52] reported recently that in cultured rat granulosa cells TPA and cAMP induced FS mRNA levels with kinetics similar to that we observed in this study for human GL cells. In porcine granulosa cell cultures TPA induced FS mRNA levels rapidly at 2 and 6 h [53] whereas forskolin causes a sustained effect on FS mRNA levels with a maximal stimulation occurring at 72 h [54]. These findings demonstrate that there are species specific differences in FS regulation. We conclude that in human GL cells FS mRNAs are more strongly stimulated by the cAMP-dependent signaling pathways than by activation of protein kinase C.

Although human inhibin/activin β_B-subunit gene is known to have cAMP regulatory elements in its promoter sequence [55] and β_B-subunit transcripts are induced by cAMP in human testicular cell cultures [44] it is not regulated by 8br-cAMP in human GL cells. No induction of β_B-subunit transcripts was seen by TPA treatment either. In a previous study we have shown that human GL cell activin/inhibin β_B-subunit mRNA levels are induced by activin A [31] which is known to transmit its signal through heterodimeric serine/threonine kinase receptors [56,57]. Obviously, the signal transduction pathway activated by activin receptors leading to β_B-subunit mRNA induction in human GL cells does not activate protein kinase A- and C-dependent pathways.

The inhibin α-subunit mRNA is highly expressed in human granulosa cells of dominant follicles and corpus luteum as detected by in situ hybridizations [17,18]. As compared to the α-subunit the expression of the β_A-subunit is moderate whereas β_B-subunit mRNA expression levels are low [18] suggesting that the most preponderant of bioactive inhibin and activin dimeric proteins formed in human dominant follicle and corpus
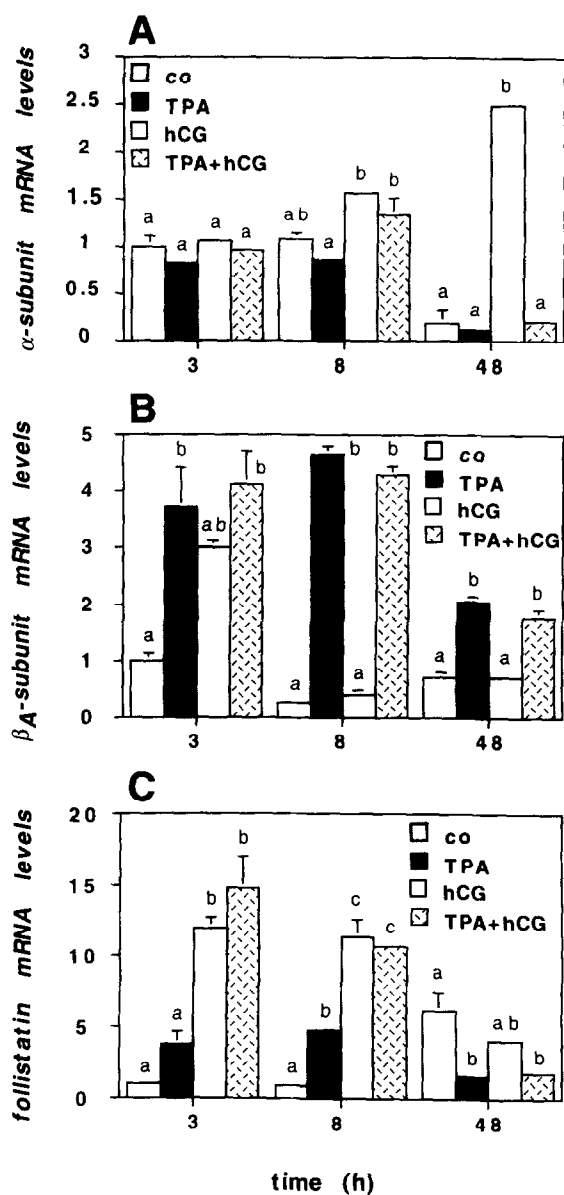
luteum is likely to be inhibin A. In our studies with cultured human GL cells we have observed that basal inhibin α-subunit mRNA levels are much higher than basal inhibin/activin β_A mRNA levels [17] and that the basal expression of β_B-subunit mRNA is hardly detectable [31]. This expression pattern resembles the situation observed in vivo. Gonadotropins [30] and 8br-cAMP cause an induction in β_A-subunit mRNA levels in human GL cell cultures but it is unlikely that this would cause clear changes in formation of bioactive inhibin/activin dimers as inhibin α-subunit mRNAs are induced at the same time. Thus, signals mediated through protein kinase-A dependent pathway are likely to induce human GL cell inhibin A production. Interestingly however, staining of cultured human GL cells with an activin A dimer-specific antibody has indicated that gonadotropin treatment also increases activin A production in these cells to some extent [23]. The fact that activin-binding protein FS expression is regulated in a very similar manner to that seen for the β_A-subunit might be understood as a mechanism ensuring the neutralization of antisteroidogenic activity of activin at the time of corpus luteum formation. The present study together our previous observations on the regulation of inhibin/activin subunits and FS mRNAs in human GL cells [28,29,31] have layed a basis for a better understanding of the complex control of these local regulators of human ovarian function. Further efforts are needed to establish how the regulation of the specific transcripts is reflected to the protein levels and how the respective changes in local inhibin and activin bioactivities affect ovarian physiology.

Acknowledgements

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