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Steroidogenesis and early response gene expression in MA-10 Leydig tumor cells following heterologous receptor down-regulation and cellular desensitization

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ABSTRACT

The Leydig tumor cell line, MA-10, expresses the luteinizing hormone receptor, a G protein-coupled receptor that, when activated with luteinizing hormone or chorionic gonadotropin (CG), stimulates cAMP production and subsequent steroidogenesis, notably progesterone. These cells also respond to epidermal growth factor (EGF) and phorbol esters with increased steroid biosynthesis. In order to probe the intracellular pathways along with heterologous receptor down-regulation and cellular desensitization, cells were preincubated with EGF or phorbol esters and then challenged with CG, EGF, dibutryl-cyclic AMP, and a phorbol ester. Relative receptor numbers, steroid biosynthesis, and expression of the early response genes, *JUNB* and c-*FOS*, were measured. It was found that in all cases but one receptor down-regulation and decreased progesterone production were closely coupled under the conditions used; the exception involved preincubation of the cells with EGF followed by addition of CG where the CG-mediated stimulation of steroidogenesis was considerably lower than the level of receptor down-regulation. In a number of instances *JUNB* and c-*FOS* expression paralleled the decreases in receptor number and progesterone production, while in some cases these early response genes were affected little if at all by the changes in receptor number. This finding may indicate that even low levels of activated signaling kinases, e.g. protein kinase A, protein kinase C, or receptor tyrosine kinase, may suffice to yield good expression of *JUNB* and c-*FOS*, or it may suggest alternative pathways for regulating expression of these two early response genes.

1. Introduction

Studies on homologous and heterologous receptor down-regulation and cellular desensitization have provided considerable information on cell regulation by exogenous ligands. The mouse Leydig tumor cell line, MA-10, is responsive to human chorionic gonadotropin (hCG) via the cell surface luteinizing hormone/chorionic gonadotropin G protein-coupled receptor (LHR), resulting in the stimulation of progesterone biosynthesis [1]. In the canonical pathway, the hCG-LHR complex leads to activation of protein kinase A (PKA) and increased intracellular cAMP, resulting in steroidogenesis [2]. Acting via the cell surface epidermal growth factor (EGF) tyrosine kinase receptor (EGFR), EGF also activates steroidogenesis, but at a much lower level than that observed with hCG [3]. Phorbol esters, intracellular activators of protein kinase C (PKC), increase steroidogenesis as well [4,5], but, as with EGF, the stimulation is much less than that achieved with hCG.

Ascoli and colleagues showed a number of years ago that the three cell stimuli, hCG, EGF, and phorbol esters, reduced the number of LHRs, and thus responsiveness to hCG-mediated steroidogenesis, by reducing the level of LHR mRNA [6,7], in particular by decreasing transcription of the LHR gene [8]. Other reports demonstrated that, following homologous LHR down-regulation

Abbreviations: AP-1, activator protein 1; BSA, bovine serum albumin; CREB, cAMP response element binding protein; db-cAMP, dibutryl cAMP; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERG, early response gene; ERK1/2, extracellular signal regulated kinases; EPAC, exchange protein directly activated by cAMP; hCG, human chorionic gonadotropin; IBMX, isobutylmethylxanthine; IP3, 1,4,5-inositol trisphosphate; LHR, luteinizing hormone receptor (also binds hCG); 4α-PE, 4α-phorbol 12,13-didecanoate (inactive phorbol ester); PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate (active phorbol ester); RACK1, receptor for activated C kinase 1; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 15 mM sodium citrate; TPA, 12-O-tetradecanoyl phorbol 13-acetate (active phorbol ester)

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with hCG [9], cAMP-mediated steroidogenesis was decreased due to a depletion of cholesterol [10].

Not surprisingly, there is cross-talk between these three signaling pathways in MA-10 cells. For example, EGF reduces hCG-responsive cAMP accumulation by inhibiting adenylyl cyclase activity [11,12], and hCG-mediated LHR activation has been shown to lead to the phosphorylation of EGFR and extracellular signal regulated kinases (Erk1/2) [13] via two independent pathways, an intracellular one mediated by PKA and an extracellular mediated by Fyn [14]. EGF, in turn, was shown to attenuate hCG-mediated cAMP synthesis by inhibiting adenylyl cyclase activity [3,11].

Various signaling pathways lead to the induction of the FOS or JUN family of proteins, homodimers or heterodimers that form the activator protein 1 (AP-1) transcription factor complex [15–17]. Genes encoding these proto-oncogenes and other transcription factors are referred to as early response genes (ERGs), also known as primary response genes or immediate early genes. Results with MA-10 cells from our lab [18,19] and from others [20,21] demonstrated that hCG induced several ERG mRNAs, including *c-FOS*, *FOSB*, *c-JUN, JUNB*, *JUND*, as well as *c-MYC*. Also, other ligands, e.g. endothelin-1 [22] and tumor necrosis factor-α [23], were found to increase the mRNAs of several ERGs in MA-10 cells. In addition to extracellular ligands, it was further shown that phorbol esters increased the protein levels of certain ERGs [5,24]. Complementing these studies on Leydig tumor cells, others have reported that non-transformed Leydig cells, not surprisingly, also respond both in vitro and in vivo to hCG with increased gene expression of several ERGs [25–27].

These investigations have provided a solid framework for extended studies on heterologous receptor down-regulation and cellular desensitization, with a particular focus on the LHR-protein kinase A (PKA) axis. In the present study cells were pretreated with EGF and with the phorbol ester, 12-O-tetradecanoyl-phorbol-13 acetate (TPA), followed by a challenge of the LHR-PKA axis with hCG and with dibutryl-cAMP (db-cAMP). This paradigm permits an evaluation of the effects of signaling cross-talk, heterologous receptor down-regulation, and cellular desensitization with EGF (and the resulting receptor tyrosine kinase pathway) and with PKC (as mediated by the PKC-1,4,5-inositol trisphosphate (IP_3)/1,2-diacylglycerol/calcium pathway) on subsequent hCG/cAMP-mediated actions. In addition to assessing the cellular responses to hCG and cAMP following EGF and TPA pretreatment, the responses to a re-challenge with EGF and TPA were also evaluated. The goal of this work is to gain a better understanding of how the two differentiated functions of MA-10 cells, steroidogenesis and ERG expression, are coupled.

2. Materials and methods

2.1. Supplies

Sources of the supplies are given in the Supplement.

2.2. Cell culture

MA-10 cells, kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA), were grown in Waymouth's 752/1 medium, without phenol red, supplemented with 15% horse serum, 25 mM Hepes, and 50 μg/mL gentamicin at 37 °C in humidified air containing 5% CO_2 as recommended [1] and described elsewhere [18]. The cells were plated and grown as follows for the various experiments. For the progesterone assays, 1.5×10^5 cells/well in 12-well tissue culture plates, each well containing 2 mL medium; for receptor binding assays, 5×10^5 cells/well in 6-well tissue culture plates, each well containing 3 mL medium; and for RNA preparations 4×10^6 cells/well in T175 flasks, each flask containing 35 mL medium. The serum-containing medium was changed every other day until the cells reached 70% confluency. On the day before the experiment, the serum-supplemented medium was removed and replaced with Waymouth's medium containing 1 mg/mL bovine serum albumin (BSA) with or without one of the factors given below. On the day of the experiment, the cells received just buffer (control), 40 ng/mL hCG, 50 ng/mL EGF, 20 ng/mL TPA, 20 ng/mL 4α-phorbol 12,13-didecanoate (4α-PE), or 2 mM db-cAMP plus 1 mM isobutylmethylxanthine (IBMX). Unless stated otherwise, the experiments were terminated at 0 (control), 15, 30, 45, 60, and 240 min.

2.3. Measurement of secreted progesterone

The medium was removed from control or pre-treated cells (16 h incubation with 40 ng/mL hCG, 2 mM db-cAMP-plus-1 mM IBMX, 50 ng/mL EGF, 20 ng/mL TPA, or 20 ng/mL 4α-PE) on the day of the experiment, followed by washing the cells and then addition of medium alone or one of the above at the same concentration, and then incubated for 4 h at 37 °C. The progesterone concentration in the medium was determined via a standard radioimmunoassay using [1,2,6,7-^{3}H] progesterone and rabbit anti-progesterone. Each experiment was performed independently in triplicate (with excellent reproducibility), and the results are presented as mean ± SEM for a representative experiment. An unpaired Student's *t*-test was used to determine statistical significance.

2.4. Measurement of hCG and EGF binding

Cells were treated as above, and on the day of the experiment the medium was removed and replaced with medium containing either 0.3 ng/mL [^{125}I]hCG or [^{125}I]EGF, and then incubated for 2 h at 37 °C. Nonspecific binding was measured in the presence of a 200-fold excess of unlabeled ligand. After incubation, the medium was removed and the cells washed twice with phosphate-buffered saline, then trypsinized and counted in a γ-counter (LKB Instruments, Inc., Rockville, MD). Each experiment was performed independently two (hCG) or three (EGF) times demonstrating reproducibility. The data, normalized to 100% binding in control cells, are given as mean ± SEM for a typical experiment ($n=3$), and statistical significance was determined using an unpaired Student's *t*-test. With the reasonable assumption that the K_d does not change for either ligand, this paradigm provides a good relative estimate of the number of available receptors for binding ligand when comparing different conditions and time points.

2.5. Northern blots of selected ERGs

Cells were incubated with 40 ng/mL hCG, 2 mM db-cAMP-plus-1 mM IBMX, 20 ng/mL TPA, or 50 ng/mL EGF for 0, 15, 30, 45, 60, and 240 min followed by Northern analysis. In other experiments, cells were incubated for 16 h (overnight) with either hCG, TPA, or EGF at the concentration given above, then washed, and incubated again with one of the agents, again at the same concentration denoted above. To isolate total cellular RNA, the classical guanidine isothiocyanate method [28] was used with modifications as described [18]. At the end of each experiment, the medium was removed and the cells washed with ice-cold phosphate-buffered saline. Then 7 mL of 4 M guanidine isothiocyanate/40% CsCl was added to the flasks with DNA being sheared by passing the solution through an 18-gauge needle 12 ×. A detailed description of the method is provided in the Supplement, along with the techniques used for transfer and hybridization. Quantitation of the blots was via densitometric scanning, and the data are presented

as fold-increase over control (0 min), corrected using β-actin mRNA (not affected by the treatments used) as an internal standard. Since the control values were often very low and difficult to measure with high precision, occasionally requiring over-exposure of the blots to estimate the basal level, the absolute values of the fold-increases are subject to error; however, the inherent error is constant for a given time course. Thus, the fold-increases in mRNA levels should, in general, be considered more qualitative than quantitative. Unless noted otherwise each experiment was performed independently twice, and the results are presented as mean ± range.

3. Results

3.1. Stimulation of steroidogenesis and ERG expression

To establish appropriate baselines, cellular responses of progesterone and selected ERG mRNAs, *JUNB*, *c-FOS*, and *c-JUN*, to hCG, db-cAMP, TPA, and EGF were determined. Progesterone synthesis increased with each of the two extracellular ligands, hCG and EGF, and with each of the two intracellular activators, db-cAMP and TPA (Table 1A). Steroid synthesis was much greater in cells incubated with hCG or db-cAMP than in those treated with TPA or EGF.

A few representative Northern blots are given in Supplement Fig. 1, and the results of densitometric scans showing the relative temporal expressions of *JUNB* and *c-FOS* in response to hCG, db-cAMP, TPA, and EGF are shown in Fig. 1. Expression of these two ERGs was low in the basal state and increased significantly after incubation with each of the above factors. There are, however, interesting temporal differences in the responses of *JUNB* and *c-FOS* to hCG, db-cAMP, TPA, and EGF. For example, in response to hCG the expression of *JUNB* increases at 15 min and reaches a maximum at 30–45 min, and then begins decreasing, returning to the basal level at 240 min. With db-cAMP, *JUNB* expression begins at about 30 min and increases gradually, reaching a maximum at 60 min (or later) and also declines to the basal level by 240 min. TPA leads to a gradual time-dependent increase in *JUNB* expression up to at least 60 min, after which the expression is just above the basal level at 240 min. Incubation with EGF results in increasing fold-expression of *JUNB* up to 30 min, where it remains elevated for another 30 min and by 240 min is slightly above basal levels. The kinetics with *c-FOS*, on the other hand, exhibit maxima at 30 min for hCG and EGF, at 45 min for TPA, and at 60 min or later for db-cAMP. The increase in *c-JUN* mRNA was modest in response to these cellular stimuli, e.g. 2–6-fold, and for this reason the results with *c-JUN* are not shown for subsequent experiments.

3.2. The inactive phorbol ester

As shown in Table 1A, 4α-PE is not steroidogenic; moreover, preincubation of MA-10 cells with the inactive phorbol ester had no appreciable effect on subsequent stimulation of progesterone synthesis by hCG, db-cAMP, or EGF (Table 1B). Radioreceptor assays demonstrated that preincubation of the cells with 4α-PE had no significant effect on the apparent number of hCG and EGF receptors (Table 2). Thus, the effects of TPA are specific for the active compound.

3.3. Effects of pretreatment with TPA

Overnight incubation of MA-10 cells with TPA led to a reduction of over 40% in the binding of [^{125}I]hCG (Table 2). This result is in reasonable agreement with the report by Wang et al. [7] in which hCG binding was determined over a 48 h time course in the presence of PMA; after 16 h the reduction in binding was about 20–30%. Whereas hCG binding was reduced 40–45% by TPA, hCG-mediated steroidogenesis was diminished by more than 80% (Table 1C). In addition to heterologous LHR down-regulation, these results are strongly indicative of cellular desensitization. Of comparative interest with hCG, there was also a significant reduction

Table 1
Progesterone synthesis by MA-10 cells in response to hCG, db-cAMP, 4α-PE, TPA, and EGF (A) and following overnight incubation with 4α-PE (B), TPA (C), and EGF (D) with subsequent stimulation with hCG, db-cAMP, and either TPA or EGF.[a]

A. Overnight incubation in medium only followed by incubation with hCG, db-cAMP, TPA, 4α-PE, and EGF

Treatment	Control	hCG	db-cAMP	4α-PE	TPA	EGF
Prog. (ng/mL)	0.7 ± 0.1	46.7 ± 3.3	43.9 ± 6.3	0.8 ± 0.1[b]	10.6 ± 1.0[b]	4.8 ± 0.5[b]
Percentage	0	100	94	0.2	22	9

B. Overnight incubation with 4α-PE and subsequent incubation with hCG, db-cAMP, and EGF

Treatment	Control	hCG	db-cAMP	EGF
Prog. (ng/mL)	0.7 ± 0.1	52.1 ± 5.9	52.1 ± 5.9	4.6 ± 0.3
Percentage	0	112	119	95

C. Overnight incubation with TPA and subsequent with hCG, db-cAMP, and EGF

Treatment	Control	hCG	db-cAMP	EGF
Prog. (ng/mL)	0.8 ± 0.1	8.5 ± 0.1[b]	10.0 ± 1.3[c]	2.4 ± 0.2[c]
Percentage	0	17	21	39

D. Overnight incubation with EGF and subsequent incubation with hCG, db-cAMP, and TPA

Treatment	Control	hCG	db-cAMP	TPA
Prog. (ng/mL)	1.3 ± 0.1	37.9 ± 10.6	47.8 ± 8.6	5.7 ± 0.3[c]
Percentage	0	80	108	44

[a] Accumulated medium progesterone (Prog.) was measured 4 h after addition of stimulus. In panel A the percentages shown in parentheses refer to the accumulated progesterone concentration in the medium with overnight incubation in medium followed by addition of hCG as the base value, i.e. 100%. In panels B, C, and D the percentages refer to the respective values in panel A. For example, the progesterone concentrations elicited by hCG in panels B, C, and D are compared to that of hCG in panel A; likewise for db-cAMP in panels B, C, and D compared to that of db-cAMP in panel A; also for EGF in panels B and C compared to that of EGF in panel A; and for TPA in panel D with that of TPA in panel A; and The various percentages were determined as follows from the values in ng/mL: [(progesterone concentration produced by stimulus-respective control concentration)/(progesterone concentration of the stimulus in panel A – respective control concentration)] × 100%.

[b] $P < 0.0001$.

[c] $P < 0.01$.

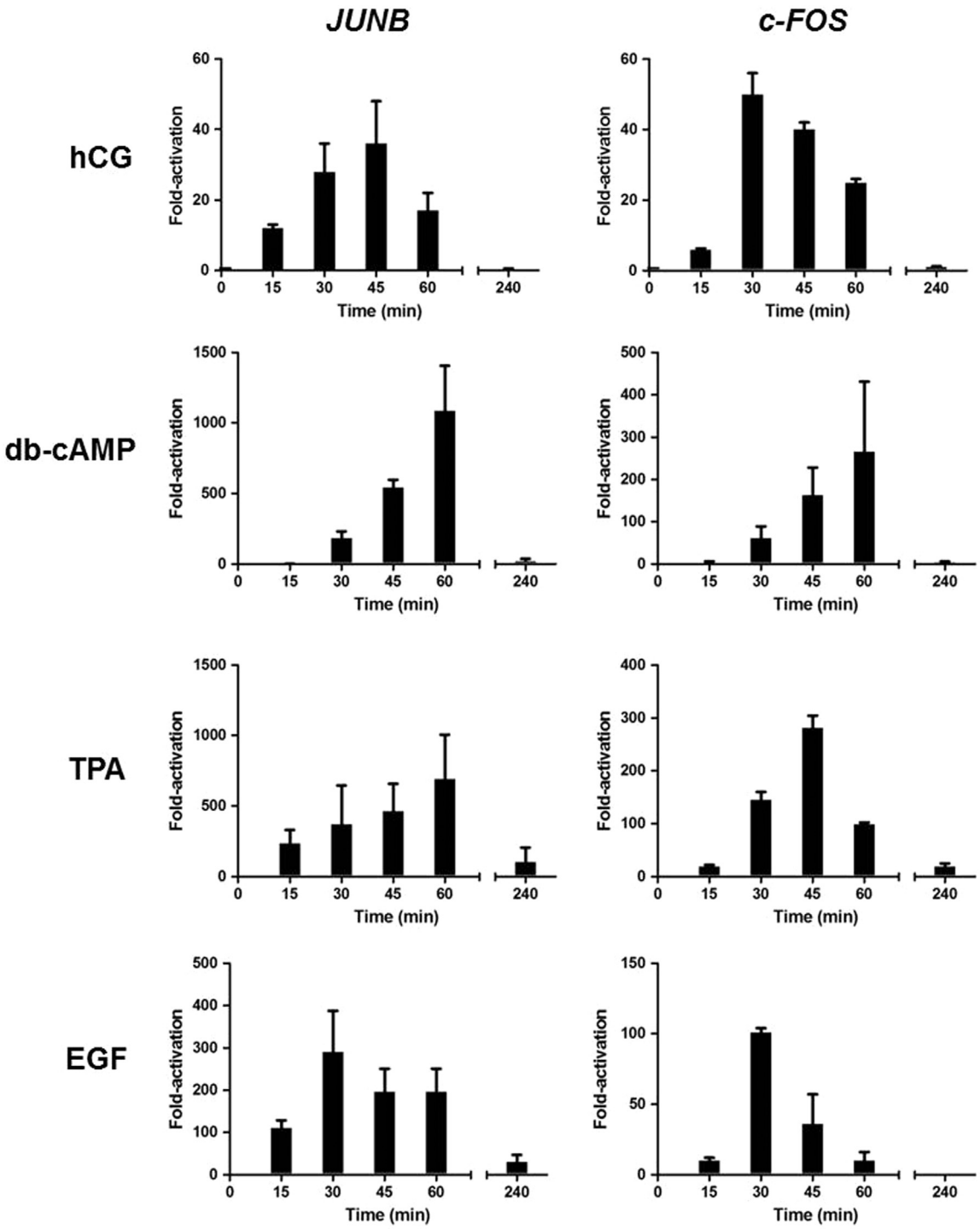

Fig. 1. Temporal expression of *JUNB* and *c-FOS* mRNAs in MA-10 cells. Responses to hCG (40 ng/mL), db-cAMP (2 mM-plus-1 mM IBMX), TPA (20 ng/mL), and EGF (50 ng/mL) are shown, and the data are presented as fold-increase over control (0 min). Each point represents the mean ± range of integrated intensities from two independent experiments.

of progesterone production (also ca. 80%) in the cellular response to db-cAMP in TPA-treated cells (Table 1C).

[^{125}I]EGF binding to cells preincubated with TPA was reduced some 65% (Table 2), and progesterone synthesis was also diminished about 60% as well (Table 1C). These findings indicate that under these conditions receptor number and steroidogenic responsiveness are closely coupled. One caveat, however, is that the relatively low levels of EGF-mediated progesterone synthesis preclude high accuracy.

Desensitization with TPA followed by a challenge with hCG, cAMP, and EGF (Fig. 2) resulted in some interesting similarities and differences compared to the results in Fig. 1. hCG-mediated expression of *JUNB* was similar to that of cells that had not been previously incubated with TPA, except that expression remained elevated for 60 and 240 min. The temporal responses of *JUNB* expression to db-cAMP and EGF were similar to those presented in Fig. 1 except that the EGF response seemed blunted. The fold-activation of *c-FOS* following prior incubation with TPA was similar to that given in Fig. 1 following incubation with hCG and db-cAMP; with EGF the response appears somewhat blunted and the fold-

Table 2
Radioreceptor assays following incubation of MA-10 cells with either. medium only or medium containing 4α-PE, TPA, or EGF.[a]

Overnight incubation	[^{125}I]hCG binding (%)	[^{125}I]EGF binding (%)
Medium	100	100
4α-PE	94.9 ± 0.6	98.4 ± 4.4
TPA	57.3 ± 3.3[b]	34.4 ± 1.3[b]
EGF	69.5 ± 3.5[b]	4.5 ± 0.9[b]

[a] Control cells were incubated overnight in medium only followed by measurements of specific binding with radiolabeled hCG and EGF as described in Materials and Methods. All results are expressed as % specific binding relative to control.
[b] $P < 0.0001$.

activation remains elevated and nearly constant for 45 min. Preliminary results indicated that challenging TPA-pretreated cells with TPA yielded greatly reduced levels of both *JUNB* and *c-FOS* (data not shown).

3.4. Effects of pretreatment with EGF

Measurement of bound [^{125}I]EGF to MA-10 cells that had been incubated overnight with a saturating concentration of unlabeled EGF indicated a 95% reduction in EGFR (Table 2). Thus, under the conditions used, EGFR down-regulation was achieved. [^{125}I]hCG binding to these EGF-pretreated cells suggested a 30% reduction in LHR (Table 2), in reasonable agreement with the reports by Ascoli and colleagues [6,7,29], who also showed that after 24–48 h incubation with EGF, hCG binding was reduced some 80%. Consistent with our binding data, addition of hCG to the EGF-treated cells led to a 20% reduction in progesterone synthesis compared to that of untreated cells (Table 1D). As also presented in Table 1D, addition of db-cAMP produced essentially the same amount of progesterone in the EGF-treated and control cells, while the TPA response was about 55% less following EGF pretreatment. These results on hCG and db-cAMP are, by and large, in qualitative agreement with earlier studies in which it was reported that following 48 h of preincubation with EGF, hCG-mediated progesterone synthesis was reduced about 90% while 8-Bromo-cAMP-mediated progesterone synthesis was some 108% of that in the absence of EGF pretreatment [6].

Pretreatment of the cells with EGF followed by a challenge with either hCG or TPA resulted in, if anything, a small increase in *JUNB* expression and no significant change in *c-FOS* expression with hCG (relative to that produced by hCG without a preincubation), and no change in expression of either *JUNB* or *c-FOS* expression in response to TPA (Fig. 3). As also shown in Fig. 3, incubation of MA-10 cells with fresh EGF, following preincubation with EGF, resulted in

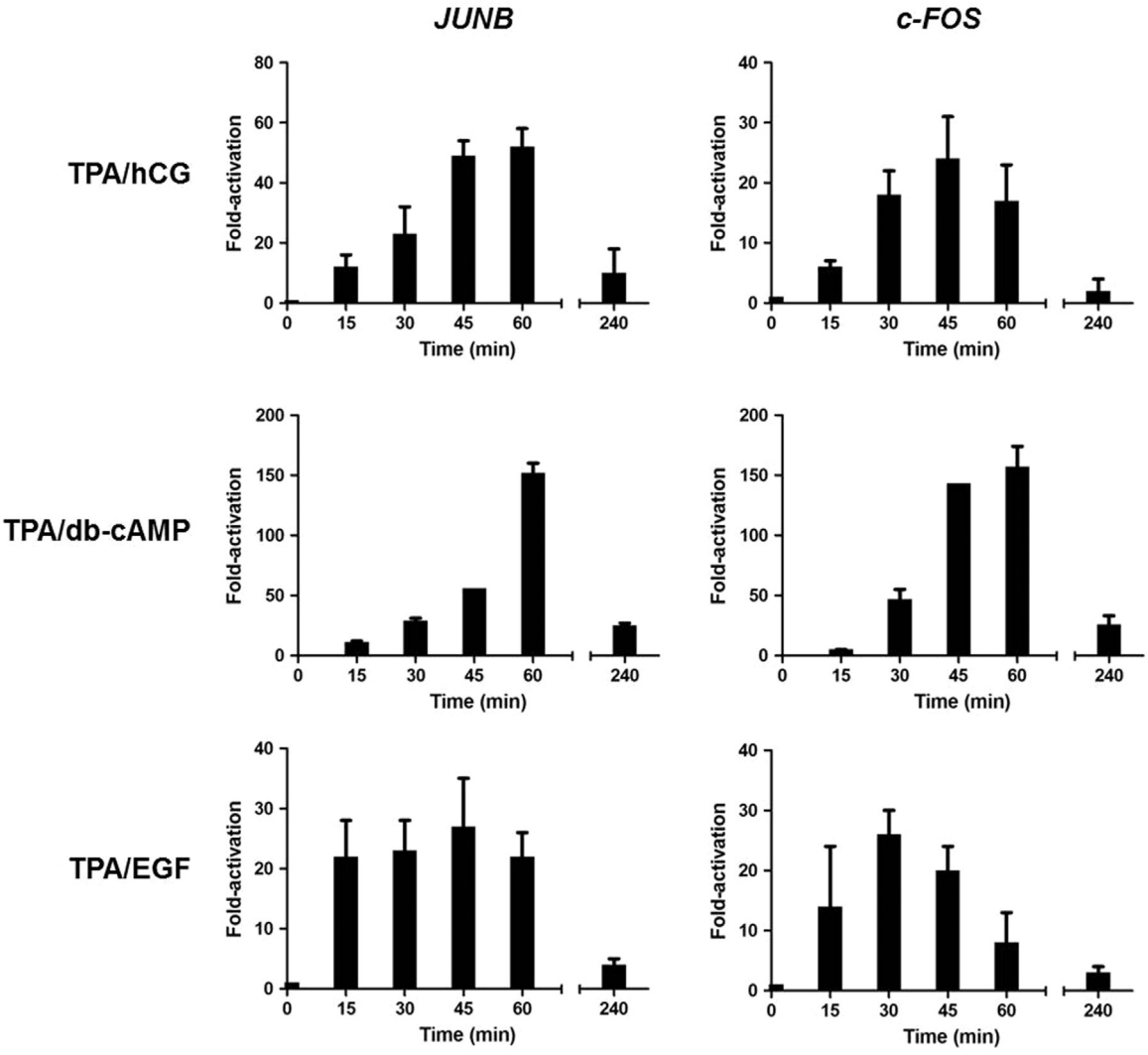

Fig. 2. Temporal expression of *JUNB* and *c-FOS* mRNAs. The MA-10 cells were incubated overnight with TPA (20 ng/mL), then washed and incubated with hCG (40 ng/mL), db-cAMP (2 mM-plus-1 mM IBMX), and EGF (50 ng/mL). See Fig. 1 legend for further details.

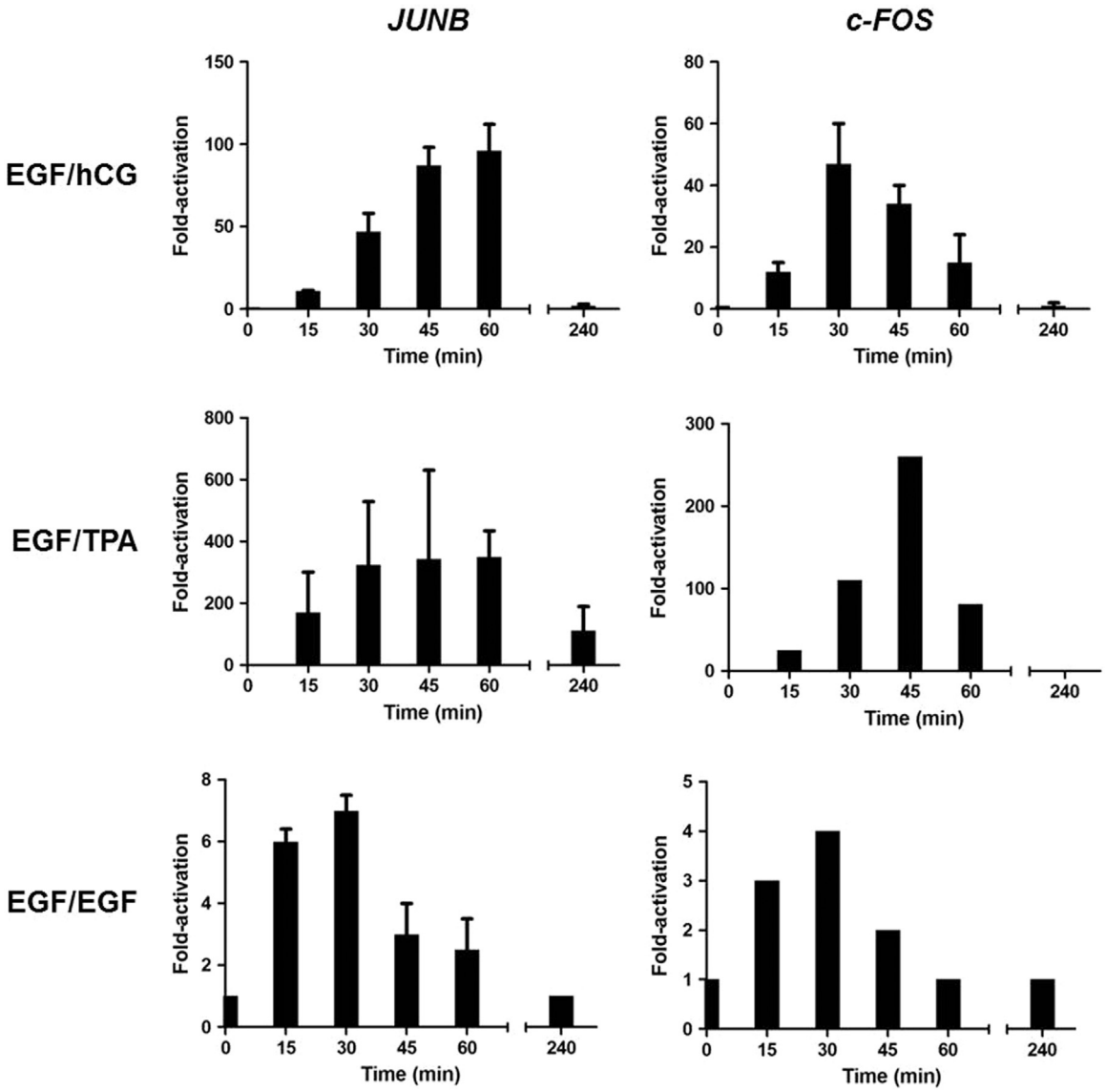

Fig. 3. Temporal expression of *JUNB* and *c-FOS* mRNAs. The MA-10 cells were incubated overnight with EGF (50 ng/mL), then washed and incubated with hCG (40 ng/mL), TPA (20 ng/mL), and EGF (50 ng/mL). See Fig. 1 legend for further details; the only exception is that the data for *c-FOS* in the EGF/TPA panel represents a single measurement at each time point. Note the low ordinate values in the EGF/EGF panels compared to the others.

greatly reduced levels of *JUNB* mRNA; preliminary results suggested that *c-FOS* expression was also significantly diminished.

4. Discussion

The findings reported herein have shown that steroidogenesis and expression of *JUNB* and *c-FOS* in MA-10 cells can be stimulated not only by hCG and db-cAMP, but also by TPA and EGF, albeit at much lower levels. Although some portions of this work have been reported by others, this is the first report in which steroidogenesis and ERG expression have been determined under the same conditions. Ascoli et al. [3] originally showed that EGF was steroidogenic for these cells, although they reported a lower increase in progesterone production compared to hCG than that found herein. Interestingly, Jo et al. [30] reported that phorbol-12-myristate 13-acetate (PMA) failed to activate steroidogenesis unless supplemented with a low concentration of db-cAMP, although increased progesterone production in response to PMA, with no added db-cAMP, was found earlier [4].

Our findings are in agreement with those of Wang et al. [7] who also found that overnight incubation of the cells with TPA and with EGF reduced the number of LHRs and EGFRs. Preincubation of cells with TPA diminished the ability of hCG, db-cAMP, and EGF to stimulate steroid production, while preincubation with EGF also reduced steroid synthesis by hCG and TPA, but not db-cAMP. Interestingly, the degree of EGF-mediated down-regulation of LHR (ca. 30%) roughly corresponds to the reduction in the degree of hCG-mediated steroidogenesis in these cells (ca. 20%), and the degree of TPA-mediated down-regulation of EGFR (about 65%) correlates with the reduction in the ability of EGF to stimulate steroidogenesis (ca. 60%). These results, collected under the defined experimental conditions, suggest a correlation between receptor number and steroidogenesis. An exception is TPA-mediated down-regulation of LHR (ca. 40–45%) and the reduction in the amount of steroidogenesis elicited by hCG in these pretreated cells (about 80–85%). In this case the decrease in steroidogenesis exceeds that expected from receptor number alone, indicating both receptor down-regulation and cellular desensitization.

The ERG expression data agree, at least qualitatively, with other

reports on one or both of these ERGs [5,20,21,23,24], although different conditions and methods of collecting/reporting the data, e.g. showing blots only or using immunoblots of protein, prevent a detailed comparison. Others also reported a minimal effect of cAMP on *c-JUN* mRNA [23] and a 2–4-fold increase in c-JUN protein elicited by PMA [5,24]; in contrast, Suzuki et al. [20] found a time-dependent hCG-mediated increase in *c-JUN*. We have no explanation for these disparate results. Of the receptor down-regulation and cellular desensitization protocols used, the most dramatic change in ERG expression was the large decrease in both *JUNB* and *c-FOS* expression following EGFR down-regulation by EGF with a subsequent challenge with EGF. In a similar vein, preliminary data suggest that desensitization with TPA followed by a TPA challenge led to major reductions in *JUNB* and *c-FOS* expression (data not shown).

The results presented herein show that while receptor down-regulation and desensitization of ERG expression generally correlate quantitatively, in certain circumstances this is not the case. Supplement Table 1 shows a summary of the results on receptor number, steroid production, and ERG expression. In a number of instances following preincubation with either TPA or EGF and a subsequent challenge with hCG, db-cAMP, TPA, or EGF, there is a correlation between receptor number, steroid production, and *JUNB*/c-*FOS* expression. In other cases ERG expression may not change dramatically while receptor number and progesterone production diminish. These results may suggest that low levels of activated signaling intermediates such as protein kinase A, protein kinase C, or tyrosine kinase may suffice to yield good expression of *JUNB* and c-*FOS*; it may also indicate alternative pathways for regulating expression of these two early response genes. Our results do not permit a distinction between the two possibilities. Also, we have no information on the cAMP response element binding protein (CREB) and the CREB binding protein in these paradigms. CREB can be phosphorylated not only by PKA but also by PKC [31], representing another level of cross-talk of the intracellular signaling pathways.

Recent studies on the transcriptome of SKOV-3 human ovarian cancer cells stably expressing LHR showed that luteinizing hormone-mediated activation of LHR resulted in only a modest up-regulation of *JUNB* (1.2-fold) and *c-FOS* (3.0-fold) in 60 min [32], fold-activations less than that observed herein with MA-10 Leydig tumor cells. Moreover, a comparison of the different gonadotropin-mediated fold-activations of ERGs in rat Leydig cells [25,27] and pig Leydig cells [26] suggests species differences, although this must be tempered by the differences reported using a single cell type, the MA-10 Leydig tumor line [5,18,19,20,21]. qRT-PCR would probably resolve at least some of the issues; however, even in a single species cancer cells exhibit a shift in the dimer composition of AP-1 [33] and hence differences in ERG expression can be expected between non-transformed and transformed cells from the same species. Thus, ERG expression in response to LHR activation is, not surprisingly, cell/species specific.

Interactions of the PKA pathway with other signaling systems are well documented [34], including the potential action(s) of cAMP independent of PKA, e.g. via the exchange protein directly activated by cAMP (EPAC) [35]. Also, in addition to the phosphorylation of CREB as mentioned above, PKC signaling can impact on PKA-mediated actions via the receptor for activated C kinase 1 (RACK1), a scaffold protein for PKC and the cAMP-specific phosphodiesterase, PDE_4D_5 [36]. Interestingly, one isoform of PKC activates PDE_4D_5 when it is bound to RACK1, thus reducing cellular cAMP. The roles of EPAC and RACK1 in MA-10 cells have not, to the best of our knowledge, been explored.

5. Conclusions

The MA-10 cells are an attractive tumor cell line in that one of the differentiated functions of the cells, namely steroidogenesis, can be investigated using multiple signaling pathways: PKA, PKC, and tyrosine kinase. The present study has demonstrated that activation of these three pathways by hCG, TPA, and EGF, respectively, leads to increases in both progesterone biosynthesis and *JUNB* and c-*FOS* expression. Heterologous receptor down-regulation and cellular desensitization, followed by challenges with hCG, db-cAMP, TPA, and EGF indicate that, under the experimental conditions used, there is a close parallel between receptor number and steroidogenesis in most cases investigated. An exception is that of preincubation with TPA followed by challenge of the cells with hCG, in which case progesterone production is greater than that expected from the loss of receptors alone. Expression of *JUNB* and c-*FOS* often, but not always, paralleled the decreases in receptor number and steroidogenesis. Thus, low levels of intracellular second messengers and kinases activated may suffice to render responses comparable to those achieved in the absence of preincubation.

Acknowledgments

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Appendix A. Transparency Document

Transparency Document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.01.005.

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