

Attenuation of cAMP-mediated Responses in MA-10 Leydig Tumor Cells by Genetic Manipulation of a cAMP-Phosphodiesterase*

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In order to assess the effect of increased cAMP degradation on the responsiveness on an endocrine cell, we have obtained stable transfectants of MA-10 Leydig tumor cells that overexpress a mammalian cAMP-phosphodiesterase. Two novel cell lines, designated MA-10(P⁺8) and MA-10(P⁺29), that express high levels of the transfected enzyme were characterized. Although the basal levels of cAMP in the mutant cell lines are comparable to those of the wild-type cells, the increase in cAMP accumulation elicited by human chorionic gonadotropin (hCG) is severely blunted. Further studies with MA-10(P⁺29) show that the ability of hCG to stimulate adenyl cyclase activity is normal. The failure of MA-10(P⁺29) cells to accumulate cAMP in response to hCG can be correlated with a similar reduction in hCG-stimulated steroidogenesis. On the other hand, the maximal steroidogenic response of MA-10(P⁺29) cells to dibutyryl cAMP, a cAMP analogue that is fairly resistant to phosphodiesterase degradation, is normal. We also show that the ability of these cells to respond to hCG with increased cAMP accumulation and steroid synthesis can be restored with a specific phosphodiesterase inhibitor. These results demonstrate that overexpression of a cAMP-phosphodiesterase in MA-10 cells limits the levels of cAMP attained under hCG stimulation and suppresses the steroidogenic response of these cells to hCG. Since gonadotropins increase the cAMP-phosphodiesterase activity in their target cells, these findings also provide evidence that this regulation plays a major role in the modulation of cell responsiveness. Last, these new cell lines should be valuable in the study of the actions of cAMP because they express a conditional and reversible cAMP-resistant phenotype.

on molecular events that lead to decreases in the synthesis of cAMP (see Refs. 1 and 2 for recent reviews), one additional mechanism of desensitization that has received less attention involves stimulation of the rate of degradation of cAMP. For example, follitropin and cAMP analogues have been shown to increase cAMP-phosphodiesterase activity in Sertoli cells (3) and granulosa cells (4), while other agents that increase cAMP accumulation (such as forskolin, prostaglandins, or β -adrenergic agonists) have been shown to increase cAMP-phosphodiesterase activity in platelets, pineal, glioma, fat, and lymphoma cells (5–10). Moreover ligands that do not elicit increases in cAMP accumulation, such as insulin and muscarinic cholinergic agonists, have also been shown to increase cAMP hydrolysis in such diverse tissues as fat cells (10), the thyroid (11), and astrocytoma cells (12).

Cyclic nucleotide phosphodiesterases are a complex family of enzymes that localize to different cellular compartments and whose activity can be modulated by a number of hormones, neurotransmitters, and second messenger analogues (reviewed in Refs. 13 and 14). Although this complexity provides the basis for the multiplicity of regulatory phenomena described above, the molecular mechanisms responsible for the regulation of cAMP-phosphodiesterase activity and the consequences of such regulation are just beginning to be explored. With regard to the molecular basis of the regulation of cAMP-phosphodiesterase activity, it seems clear that this can be accomplished by activation of pre-existing levels of the enzyme(s), and/or by increases in the actual levels of enzyme. The former may be accomplished by phosphorylation of the enzyme(s) as it appears to occur in adipocytes and platelets (8–10, 15), while the latter seems to involve changes in the cognate mRNA as reported to occur in Sertoli and glioma cells (16).

One of our laboratories has been involved in studying the hormonal modulation of cAMP-phosphodiesterases and its involvement in the desensitization of cAMP-mediated responses (3, 4, 16, 17). Although the effects of increased cAMP-phosphodiesterase activity on cAMP metabolism have been studied in some detail, the impact that this change may have in cellular responses elicited by cAMP has been difficult to distinguish from changes occurring at the level of cAMP synthesis. One approach to this problem would be to produce mutant cell lines with increased cAMP-phosphodiesterase activity and to study the impact of this mutation on cAMP metabolism and actions. Although mutant cell lines with increased cAMP-phosphodiesterase activity have been isolated (18), or produced by transfection with the yeast cAMP-phosphodiesterase (19, 20), the parental lines (and therefore the mutants) do not express cAMP-mediated responses that are readily amenable to quantitation.

With this aim in mind we have taken a Leydig tumor cell

Desensitization of hormonal responses is a process whereby cellular responses wane in spite of the continuous presence of the hormone. Some of the most complete studies of desensitization have been performed using ligands that use cAMP as their second messenger. Although most of the recent studies on desensitization of cAMP-mediated responses have focused

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line (designated MA-10, see Ref. 21) that exhibits an easily measurable cAMP-mediated response (*i.e.* steroid biosynthesis) and using an expression vector that encodes for the rat cAMP-phosphodiesterase 3 (16, 17), we have produced stable transfectants that overexpress cAMP-phosphodiesterase. In this paper we describe the production of these transfectants and show that they display a reduction in hormone-stimulated cAMP levels and cAMP-mediated responses.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The origin and handling of MA-10 cells have been described (21). They were maintained in Waymouth MB752/1 modified to contain 1.1 g/liter of NaHCO₃, 20 mM Hepes,¹ 50 µg/ml gentamicin, and 15% horse serum, pH 7.4 (growth medium), in a humidified atmosphere containing 2.5% CO₂.

Cells to be transfected were plated in 10-cm dishes at a density of 2×10^6 cells/dish and used 1 day later. DNA transfection was performed during an overnight incubation with CaPO₄ as described by Chen and Okayama (22) or during a 4-h incubation with CaPO₄ as described by Parker and Stark (23). In either case each dish received 18 µg of a rat phosphodiesterase 3 expression vector (pCMV5-ratPDE3, see Ref. 16) and 2 µg of the selectable plasmid pSV₂Neo. When the transfection lasted only 4 h, the cells were also shocked with 15% glycerol (in Hanks' balanced salt solution) for 2 min at room temperature at the end of the incubation with the plasmid/CaPO₄ mixture. Following transfection, the cells were extensively washed with Hanks' balanced salt solution and reincubated in growth medium for 1 day. The cells were trypsinized (21), plated in several 10-cm dishes at a density of $0.5\text{--}1.0 \times 10^6$ cells/dish, and reincubated in growth medium for 1 day. The medium was then replaced with growth medium supplemented with 200 µg/ml G418 alone, or together with 5 µM Ro 20-1724 (added from a 200-fold concentrated stock in dimethyl sulfoxide) as indicated in the text. The cells were maintained in these media for about 2 weeks with twice weekly media changes. Resistant colonies were isolated by gentle scraping and suction with a P1000 Pipetman and transferred to individual wells of 24-well plates. These wells were maintained in 1 ml of the appropriate medium (see above) and were expanded into T25 flasks as they became confluent. As these flasks became confluent, the cells were subcultured into 10-cm dishes and assayed for cAMP-phosphodiesterase activity (see below).

Stock cultures of the stable transfectants have always been maintained in the presence of G418 alone or G418 plus Ro 20-1724 as indicated below.

Assay of cAMP-Phosphodiesterase—Experimental cultures were plated (day 0) in 10-cm dishes at a density of $1\text{--}2 \times 10^6$ cells/dish. MA-10 cells and MA-10(P⁺8) cells were plated in growth medium (see above), while MA-10(P⁺29) cells were plated in growth medium supplemented with 5 µM Ro 20-1724. The media were replaced on day 2 or 3, and the experiments were performed on day 3 or 4, respectively. On the day of the experiment, the dishes were placed on ice and allowed to cool for about 30 min. They were then washed twice with 5-ml portions of a cold buffer containing 0.25 M sucrose, 50 mM Tris-Cl, pH 7.4, scraped into a minimal amount of the same buffer and recovered by centrifugation. The pellets were resuspended in 50 mM Tris-Cl, pH 8.0, containing 20 µM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 mM mercaptoethanol, 10 mM NaF, and 50 mM benzamide. After a 5-min incubation on ice, a cell lysate was prepared by forcing the cell suspension 20 times through a syringe fitted with a 22-gauge needle. The lysate was centrifuged at $800 \times g$ for 10 min and the pellet was resuspended in buffer, homogenized, and centrifuged again as described above. The two supernatants were combined and used to assay for cAMP-phosphodiesterase activity.

Assays were done basically as described by Thompson and co-workers (24). The reactions were performed in a total volume of 300 µl containing 25–100 µg of protein in 50 mM Tris-Cl, pH 8.0, 1 mM mercaptoethanol, 10 mM MgCl₂, a constant amount of [³H]cAMP (about 200,000 cpm) and the indicated concentrations of cAMP. After a 15–60-min incubation at 30 °C, the tubes were transferred to a boiling water bath for 45 s, and cooled in an ice bath for 5 min. After

addition of 0.07 units of 5' nucleotidase, the tubes were incubated at 30 °C for an additional 25 min. The reactions were terminated by addition of 1 ml of methanol, and the [³H]cAMP and generated [³H] adenosine were separated by ion-exchange chromatography as described by Thompson and co-workers (24).

RNA Blot Analysis—RNA was extracted by the method of Chirgwin (25) and purified by centrifugation on cesium chloride (26). Twenty µg of total RNA were then glyoxylated during a 1-h incubation (50 °C) in 10 µl of a solution containing 50% (v/v) dimethyl sulfoxide, 20% (v/v) glyoxal, and 20 mM sodium phosphate, pH 6.8. The glyoxylated RNA samples were resolved on a 1% agarose gel, blotted onto nylon membranes (Biotrans) and baked for 1 h at 80 °C under vacuum. The blots were prehybridized for 4 h at 42 °C and hybridized for 16 h at 42 °C using 10^6 cpm/ml of a ³²P-labeled (27) ratPDE3 cDNA in a solution containing 50% formamide and $5 \times$ SSC (saline sodium citrate). After hybridization the blots were rinsed with $2 \times$ SSC, 0.1% sodium dodecyl sulfate and washed twice for 20 min at 68 °C in $0.1 \times$ SSC, 0.1% sodium dodecyl sulfate. The washed blots were exposed to x-ray film at -70 °C with intensifying screens. After removal of this ratPDE3 probe, the membranes were rehybridized with a ³²P-labeled actin cDNA probe.

Ion-exchange Chromatography—Cells were cultured as described above, cooled on ice, washed three times with 5-ml portions of ice-cold phosphate-buffered saline, and scraped into a minimal amount of a buffer containing 20 mM Tris-Cl, pH 8.0, 10 mM NaF, 1 mM EDTA, 0.2 mM EGTA, 50 mM benzamide, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, and 0.12 TIU/ml aprotinin. Phenylmethylsulfonyl fluoride was then added to a final concentration of 2 mM, and the cells were homogenized using the tight fitting pestle of a Dounce homogenizer (30 strokes). Following centrifugation at $14,000 \times g$ for 30 min, the supernatant was adjusted to 200 mM sodium acetate, pH 6.5, and filtered through a 0.2-µm filter. Aliquots of the supernatants (9–33 mg of protein) were applied to a high performance liquid chromatography DEAE column (7.5 × 75-mm DEAE 5PW from Waters) pre-equilibrated with 200 mM sodium acetate, pH 6.5, 5 mM 2-mercaptoethanol, 10 mM NaF, 1 mM EDTA, 0.2 mM EGTA, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride. After washing with this buffer (1 ml/min) bound proteins were eluted with a linear sodium acetate gradient (200–750 mM) at the same flow rate. Each fraction (1 ml) was adjusted to 33% (v/v) ethylene glycol, and additional leupeptin (1 ng), pepstatin (1.4 ng), phenylmethylsulfonyl fluoride (400 nmol), aprotinin (0.12 TIU), and lima bean trypsin inhibitor (10 µg) were added from concentrated stocks. Triplicate 15-µl aliquots from each fraction were assayed for phosphodiesterase activity (using 1 µM cAMP as substrate) during a 15-min incubation at 34 °C. The activity recovered from the columns was corrected for the amount of protein loaded.

Progesterone and cAMP Levels—Experimental cultures were plated (day 0) in 6×35 -mm wells at a density of $2\text{--}3 \times 10^5$ cells/well. MA-10 cells and MA-10(P⁺8) cells were plated in growth medium (see above), while MA-10(P⁺29) cells were plated in growth medium supplemented with 5 µM Ro 20-1724. The media were replaced on day 2 or 3, and the experiments were performed on day 3 or 4, respectively. On the day of the experiment the cells were washed four times with 2-ml portions of warm assay medium (Waymouth MB752/1 modified to contain 1.12 g/liter of NaHCO₃, 20 mM Hepes, 1 mg/ml bovine serum albumin, pH 7.4) and then incubated in 2 ml of assay medium containing the additions indicated in the text. Progesterone and cAMP were measured by radioimmunoassay as previously described (21, 28, 29).

Other Methods—The procedures used to prepare cell membranes and to measure adenylyl cyclase activity have been described (30). Protein was measured by the method of Bradford (31) using bovine serum albumin as a standard. The different parameters that describe concentration-response curves were calculated with the aid of the computer program ALLFIT (32).

Hormones and Supplies—Purified hCG (CR-125, 11, 900 units/mg) was obtained from the National Hormone and Pituitary Agency of the National Institute of Diabetes, Digestive and Kidney Diseases (National Institutes of Health). Bt₂cAMP was purchased from Sigma. Ro 20-1724 was obtained from Biomol, and radioactive compounds were purchased from Du Pont-New England Nuclear. All tissue culture supplies were from GIBCO, and tissue culture plasticware was from Falcon or Corning. The antiserum to cAMP was generously provided by Dr. David Garbers (Howard Hughes Medical Institute, University of Texas at Dallas) or Dr. Robert Ryan (Mayo Clinic). The pSV₂Neo plasmid was kindly provided by Dr. Neal Musto (Georgetown University).

¹ The abbreviations and trivial names used are: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenetriamino)]tetraacetic acid; hCG, human chorionic gonadotropin; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolizidone.

RESULTS

Isolation and Initial Characterization of Transfectants—In order to obtain stable transfectants with high levels of cAMP-phosphodiesterase activity, MA-10 cells were cotransfected with an expression vector encoding for the rat cAMP-phosphodiesterase 3 (pCMV5-ratPDE3) and pSV₂Neo. One set of transfectants was selected in the presence of G418 only, while another set was selected with G418 and a cAMP-phosphodiesterase inhibitor (Ro 20-1724). While the concentration of G418 used (200 μ g/ml) is lethal to MA-10 cells, the concentration of Ro 20-1724 used (5 μ M) is not lethal and is similar to the EC₅₀ required to enhance the hCG-induced increase in cAMP accumulation in MA-10 cells.² We chose to include a phosphodiesterase inhibitor as a selective agent because it was possible that overexpression of cAMP-phosphodiesterase could be deleterious to the growth of MA-10 cells. Two clones, isolated from the two different transfections and designated MA-10(P⁺8) and MA-10(P⁺29), were chosen for further analysis. MA-10(P⁺8) was selected with medium containing 200 μ g/ml G418, while MA-10(P⁺29) was selected with medium containing 200 μ g/ml G418 and 5 μ M Ro 20-1724. Although stock cultures have always been maintained with G418 or G418 plus Ro 20-1724, we always remove the G418 from experimental cultures 3–4 days prior to the experiment. When using MA-10(P⁺29), however, experimental cultures are maintained in the presence of Ro 20-1724 until the beginning of the experiment. At this time the inhibitor is removed by extensively washing the cells. This protocol was adopted because the presence of Ro 20-1724 until the beginning of the experiment does help maintain the mutant phenotype in MA-10(P⁺29) (see below). It should also be noted that if there were some residual inhibitor left during the experiment, it would lead to an underestimation of the effect of increased cAMP-phosphodiesterase on the phenotype of the mutant cells.

RNA blot analysis of the wild-type and mutant cells (Fig. 1) shows that in contrast to the wild-type cells, MA-10(P⁺8) and MA-10(P⁺29) express the mRNA for the rat cAMP-phosphodiesterase 3 (*i.e.* the mRNA derived from the transfected cDNA, see above). Densitometric scanning of the autoradiograms (after normalization for the amount of total RNA present in each lane as judged by the levels of actin mRNA, see *right panel* of Fig. 1) revealed that the level of mRNA for rat cAMP-phosphodiesterase 3 is 1.5-fold higher in MA-10(P⁺29) than in MA-10(P⁺8). The lack of a signal for the wild-type cells in the left panel of Fig. 2 is explained by the finding that these cells do not express the homologue of the rat cAMP-phosphodiesterase 3, and the levels of mRNA for the endogenous enzyme (*i.e.* equivalent of the rat cAMP-phosphodiesterase 2) are very low.³

Additional information about the relative expression of the endogenous and transfected enzymes was obtained by chromatography on a DEAE-high performance liquid chromatography column. The results presented in Fig. 2 show that while the cAMP-phosphodiesterase activity of the wild-type cells, which is the mouse equivalent of the rat cAMP-phosphodiesterase 2,³ elutes as a single peak centered around fraction 17, the two mutants also display an additional peak centered

² Experiments were done in which intracellular cAMP levels were measured in MA-10 cells incubated with 50 ng/ml hCG alone or together with increasing concentrations (2.5–100 μ M) of Ro 20-1724 for 30 min. Half-maximal and maximal enhancement of the hCG-induced cAMP accumulation were obtained at 5 and 50–100 μ M Ro 20-1724, respectively.

³ S. Denegri, J. V. Swinnen, M. Ascoli, and M. Conti, unpublished observations.

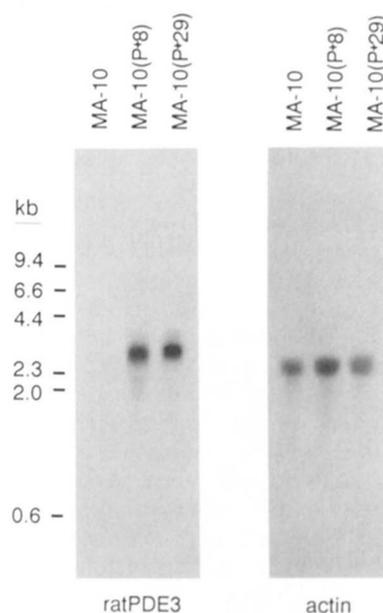


FIG. 1. RNA blot analysis of cAMP-phosphodiesterase mRNA in wild-type and mutant MA-10 cells. A blot containing 20 μ g of total RNA/lane isolated from the indicated cells was hybridized with radiolabeled ratPDE3 cDNA (*left panel*) or actin cDNA (*right panel*). Size markers were *Hind*III-digested lambda phage DNA fragments. The results of a representative experiment are shown.

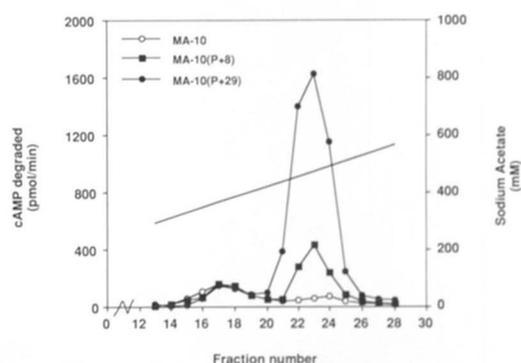


FIG. 2. Ion-exchange chromatography of cAMP-phosphodiesterases in wild-type and mutant MA-10 cells. Cells (plated in 10-cm dishes) were washed and extracts were prepared, fractionated on a DEAE column, as described under "Experimental Procedures." The amount of phosphodiesterase activity present in each fraction was measured as described under "Experimental Procedures" and was corrected for the amount of protein loaded on the column. The *solid line* shows the salt gradient used to elute the column. The results of a representative experiment are shown.

around fraction 23. This is the expected elution position of the transfected enzyme (*i.e.* the rat cAMP-phosphodiesterase 3).⁴ These results also show that the level of transfected enzyme activity is about 4-fold higher in MA-10(P⁺29) than in MA-10(P⁺8). The overall level of cAMP-phosphodiesterase activity (*i.e.* endogenous + transfected) is about 2- and 7-fold higher in MA-10(P⁺8) and MA-10(P⁺29), respectively, than in the wild-type cells.

Effects of hCG and Ro 20-1724 on cAMP Levels and Adenylyl Cyclase Activity—In order to determine the impact of increased cAMP-phosphodiesterase activity on cAMP accumulation, we incubated the wild-type and the two mutant cell lines with a saturating concentration of hCG and measured

⁴ M. Conti, J. Odeh, J. V. Swinnen, and M. E. Svoboda, submitted for publication.

the accumulation of cAMP in the cells and in the medium at different times after addition of the hormone. These results are presented in Fig. 3 and show that although the basal levels of cAMP are comparable in all cell lines, the ability of hCG to increase cAMP accumulation in MA-10(P⁺8) and MA-10(P⁺29) is drastically impaired. The maximal levels of intracellular and extracellular cAMP attained by MA-10(P⁺29) cells incubated with hCG reach only 10–20 and 5%, respectively, of those attained in the wild-type cells. The cAMP responses of MA-10(P⁺8) cells are 1.5–2-fold higher than those of MA-10(P⁺29) cells but are still much lower than those detected in the wild-type cells. These results are consistent with the levels of cAMP-phosphodiesterase activity detected in the wild-type cells and the two mutants (*cf.* Fig. 2). In order to maximize the difference between the wild-type and transfected cells, however, all subsequent studies were done with MA-10(P⁺29) only.

If the changes summarized above are entirely due to the increased levels of cAMP-phosphodiesterase, one would predict that MA-10(P⁺29) should have normal levels of adenylyl cyclase activity. Moreover, the cAMP responsiveness of MA-10(P⁺29) cells should be restored by addition of a cAMP-phosphodiesterase inhibitor.

The first prediction is met by the results presented in Table I which show that the basal, hCG-, and NaF-stimulated adenylyl cyclase activities of MA-10(P⁺29) cells are very similar (or identical) to those of the wild-type cells.

The second prediction was tested in the experiments sum-

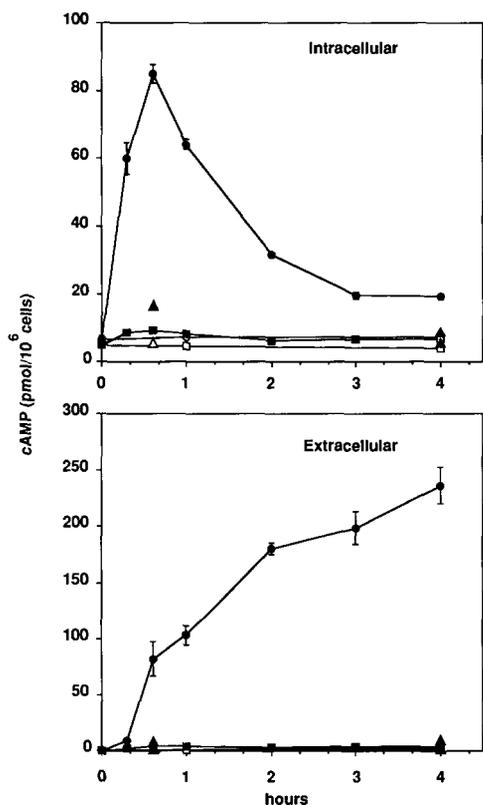


FIG. 3. Time course of hCG-stimulated cAMP accumulation in wild-type and mutant MA-10 cells. Cells (in 6 × 35-mm wells) were washed four times with 2-ml portions of assay medium and then incubated in 2 ml of assay medium containing buffer only (basal) or 50 ng/ml hCG as indicated. Each point represents the mean ± S.E. of four determinations (duplicate determinations in two independent experiments). ○, MA-10, basal; ●, MA-10, hCG; □, MA-10(P⁺29), basal; ■, MA-10(P⁺29), +hCG; △, MA-10(P⁺8), basal; ▲, MA-10(P⁺8), +hCG.

TABLE I

Adenylyl cyclase activity in MA-10 and MA-10(P⁺29) cells

Membranes were prepared and assayed for adenylyl cyclase activity under basal conditions or when stimulated with 1 μg/ml hCG and 100 μM GTP, or with 10 mM NaF as described under "Experimental Procedures." Each value represents the average ± S.E. of 12 determinations (four independent experiments with triplicate determinations in each).

Cell type	cAMP formed		
	Basal	hCG and GTP	NaF
	<i>pmol/min × mg</i>		
MA-10	1.8 ± 0.1	13.3 ± 0.4	144.2 ± 6.5
MA-10(P ⁺ 29)	2.0 ± 0.1	12.4 ± 0.3	128.2 ± 5.9

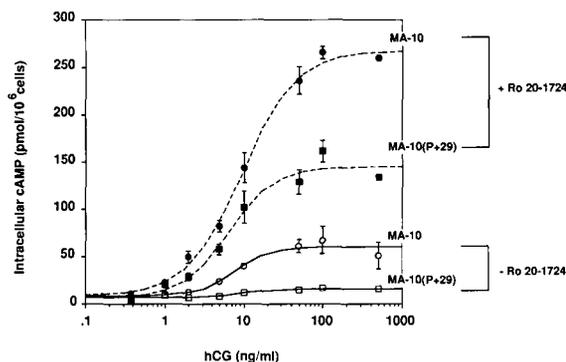


FIG. 4. Concentration-response curves for the hCG-stimulated cAMP accumulation in wild-type and mutant MA-10 cells. Cells (in 6 × 35-mm wells) were washed four times with 2-ml portions of assay medium. Each well received 2 ml of assay medium with or without 50 mM Ro 20-1724 as indicated. After addition of increasing concentrations of hCG, the wells were incubated for 30 min, and intracellular cAMP was measured as described under "Experimental Procedures." Each point represents the mean ± S.E. of four determinations (duplicate determinations in two independent experiments). The curves drawn were calculated as described under "Experimental Procedures."

marized in Fig. 4, in which the levels of intracellular cAMP were measured in cells incubated for 30 min with increasing concentrations of hCG in the presence or absence of a maximally effective concentration of Ro 20-1724. The results presented show that addition of Ro 20-1724 greatly enhances the levels of cAMP attained by MA-10(P⁺29) cells incubated with all concentrations of hCG tested. In the presence of Ro 20-1724, the response of MA-10(P⁺29) cells is higher than that detected in MA-10 cells incubated without the inhibitor, but it is still lower than the response of MA-10 cells incubated with the inhibitor. Thus, Ro 20-1724 increases the maximal response of MA-10 and MA-10(P⁺29) cells from 60 to 268 and from 16 to 145 pmol/10⁶ cells, respectively. The respective EC₅₀ values (reported in ng/ml) for the aforementioned conditions changed from 7.7 to 9.5 in MA-10, and from 10 to 6.4 in MA-10(P⁺29) cells.

Taken together these results show that the attenuation of cAMP responses detected in MA-10(P⁺29) cells is entirely due to the increased expression of cAMP-phosphodiesterase.

Steroidogenic Responses—One advantage of using MA-10 cells as a model to study the impact of increased cAMP-phosphodiesterase on hormonal responsiveness is their ability to respond to hCG and cAMP analogues with increased progesterone synthesis (21, 33, 34). Inasmuch as the steroidogenic effects of hCG on MA-10 cells are largely (or totally) mediated by cAMP (28, 35, 36), we predicted that MA-10(P⁺29) would have a reduced steroidogenic response to hCG.

The results presented in Fig. 5 show concentration-response curves for the steroidogenic responses of MA-10 and MA-

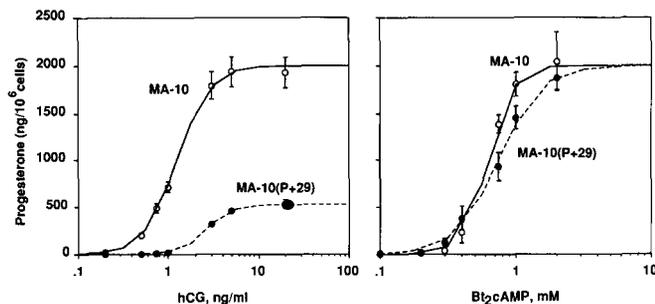


FIG. 5. Steroidogenic responses of wild-type and mutant MA-10 cells. Cells (in 6×35 -mm wells) were washed four times with 2-ml portions of assay medium. Each well was then incubated in 2 ml of assay medium with increasing concentrations of hCG (*left panel*) or Bt_2cAMP (*right panel*) for 6 h. Extracellular progesterone levels were measured as described under "Experimental Procedures." Each point represents the mean \pm S.E. of four determinations (duplicate determinations in two independent experiments). The curves drawn were calculated as described under "Experimental Procedures." Basal levels of progesterone were 1.9 ± 0.3 and 1.5 ± 0.3 ng/ 10^6 cells for MA-10 and MA-10(P⁺29) cells, respectively.

10(P⁺29) cells to hCG and Bt_2cAMP . As can be seen in the *left panel* of Fig. 5, the maximal steroidogenic response to hCG in MA-10(P⁺29) cells is only 27% of the maximal response attained in MA-10 cells (531 and 1998 ng of progesterone/ 10^6 cells, respectively). The EC_{50} for hCG in MA-10(P⁺29) cells is about 2-fold higher than that measured in MA-10 cells (2.6 and 1.2 ng/ml, respectively). In contrast to the results obtained with hCG, the results presented in the *right panel* of Fig. 5 show that the steroidogenic response of MA-10(P⁺29) cells to Bt_2cAMP is only minimally affected. Thus, the maximal amount of progesterone produced in response to Bt_2cAMP is the same in MA-10 and MA-10(P⁺29) cells (1998 ng/ 10^6 cells), and this in turn is the same as the maximal steroidogenic response of MA-10 cells to hCG (compare *left and right panels*). The only difference noted in the steroidogenic response of the wild-type and mutant cells to Bt_2cAMP is the EC_{50} which is somewhat higher in MA-10(P⁺29) than in MA-10 cells (630 and 740 μ M, respectively). The minimal difference between the steroidogenic responses of the wild-type and mutant cells to Bt_2cAMP presumably reflects the relative insensitivity of this cAMP analogue to degradation by cAMP-phosphodiesterase (37).

In the last series of experiments, we tested the effects of Ro 20-1724 on the steroidogenic response of MA-10(P⁺29) cells. These experiments are summarized in Fig. 6 and show that Ro 20-1724 reduces the EC_{50} for the stimulation of steroidogenesis by hCG (from 2.0 to 0.72 ng/ml) in the wild-type cells, but does not change the maximal steroidogenic response (2295 ng of progesterone/ 10^6 cells). On the other hand, while this inhibitor reduces the EC_{50} for the stimulation of steroidogenesis by hCG (from 2.7 to 0.72 ng/ml) in MA-10(P⁺29) cells, it also increases the maximal steroidogenic response from 756 to 2295 ng of progesterone/ 10^6 cells. The end result of these changes is that, in the presence of Ro 20-1724, the concentration-response curves for the hCG-induced steroidogenesis are identical in MA-10 and MA-10(P⁺29) cells.

These results also show that regardless of the sensitivity and magnitude of the cAMP response, the maximal steroidogenic response to hCG is always attained when the levels of cAMP reach a set level. Thus, in MA-10 cells, a maximal steroidogenic response is attained at about 10 ng/ml hCG (*cf.* Fig. 6), a hormone concentration that increases the intracellular levels of cAMP to about 40 pmol/ 10^6 cells (*cf.* Fig. 6). In MA-10 and MA-10(P⁺29) cells incubated with Ro 20-1724,

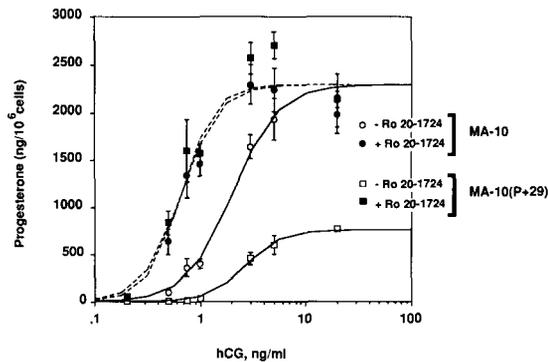


FIG. 6. Effects of Ro 20-1724 on the steroidogenic responses of wild-type and mutant MA-10 cells. Cells (in 6×35 -mm wells) were washed four times with 2-ml portions of assay medium. Each well received 2 ml of assay medium with or without 50 mM Ro 20-1724 as indicated. After addition of increasing concentrations of hCG, the wells were incubated for 6 h, and extracellular progesterone was measured as described under "Experimental Procedures." Each point represents the mean \pm S.E. of four determinations (duplicate determinations in two independent experiments). The curves drawn were calculated as described under "Experimental Procedures."

TABLE II

Effect of supplementing the growth medium with a cAMP-phosphodiesterase inhibitor on cAMP levels in MA-10(P⁺29) cells

MA-10 and MA-10(P⁺29) cells were plated (day 0) in 6×35 -mm wells containing growth medium supplemented with or without 5 μ M Ro 20-1724 as indicated. The medium was replaced on day 2 and the cells were used on day 3. After extensive washing (four times with 2-ml portions of assay medium) each well received 2 ml of assay medium. Intracellular cAMP was measured at the end of a 30-min incubation with buffer or 50 ng/ml hCG as indicated. Each number represents the average \pm S.E. of six determinations (triplicate determinations in two independent experiments).

Cell type	Additions to growth medium	Intracellular cAMP	
		Basal	hCG
<i>pmol/10⁶ cells</i>			
MA-10	None	6.5 \pm 0.1	50.8 \pm 5.2
MA-10(P ⁺ 29)	None	5.8 \pm 0.6	24.4 \pm 1.9
MA-10(P ⁺ 29)	Ro 20-1724	4.2 \pm 0.4	11.2 \pm 0.7

the maximal steroidogenic response is identical to that of MA-10 cells incubated without the inhibitor, but is attained at about 2 ng/ml hCG (*cf.* Fig. 6). As shown in Fig. 6, this concentration of hCG raises the intracellular levels of cAMP to 50 and 30 pmol/ 10^6 cells, respectively, in MA-10 or MA-10(P⁺29) cells incubated with Ro 20-1724.

Taken together, the results presented show that Ro 20-1724 completely restores the steroidogenic response of MA-10(P⁺29) cells.

Effect of Culture Conditions on the cAMP Responses of the Mutant Cells—As summarized above, the ability of MA-10(P⁺29) cells to respond to hCG with increased cAMP accumulation can be (at least partially) restored by including Ro 20-1724 during the incubation. Paradoxically, the inclusion of a low concentration of the same inhibitor in the medium used to maintain and propagate the cells seems to be necessary to maintain a stable mutant phenotype. This phenomenon is illustrated by the data presented in Table II. In these experiments MA-10(P⁺29) cells were plated in growth medium supplemented with or without a low concentration of Ro 20-1724 and cultured for 3 days. The cells were then washed and incubated in assay medium with or without a saturating concentration of hCG for 30 min, and intracellular

cAMP was measured. The results presented in Table II show that maintaining MA-10(P⁺29) cells in the absence of the inhibitor has little effect on the basal levels of cAMP but results in a 2-fold increase in their ability to respond to hCG with increased cAMP accumulation. Other experiments (not presented) showed that the maximal steroidogenic response (to hCG) of MA-10(P⁺29) cells maintained without Ro 20-1724 for 3 days was also higher than the response of the cells continuously maintained in the presence of the inhibitor. In additional experiments we measured cAMP-phosphodiesterase activity after high performance liquid chromatography separation of cell-free extracts prepared from MA-10(P⁺29) cells maintained without Ro 20-1724 for 2-3 days. Our results (not presented) showed that while the levels of cAMP-phosphodiesterase 2 activity remain unchanged, the levels of cAMP-phosphodiesterase 3 activity declined to levels comparable to those detected in MA-10(P⁺8), which is always maintained in the absence of the inhibitor.

Although we have not yet sought an explanation for these findings, it is clear that MA-10(P⁺29) cells must be kept in the presence of a phosphodiesterase inhibitor in order to maintain high levels of activity of the transfected enzyme.

DISCUSSION

The results presented here show that transfection of MA-10 Leydig tumor cells with an expression vector encoding for the rat cAMP-phosphodiesterase 3 readily yields stable cell lines with increased levels of cAMP-phosphodiesterase activity. Two novel cell lines, designated MA-10(P⁺8) and MA-10(P⁺29), were established and characterized. These cell lines have increased levels of cAMP-phosphodiesterase activity, which is due to expression of the transfected cDNA as judged by RNA blot analysis, and ion-exchange chromatography (Figs. 1 and 2). Although the levels of cAMP-phosphodiesterase 3 activity are about 4-fold higher in MA-10(P⁺29) than in MA-10(P⁺8), the levels of the cognate mRNA are only slightly higher in the former than in the latter. Differences in the rate of synthesis and/or degradation or in protein phosphorylation may contribute to this phenomenon. Moreover, it should be noted that the inclusion of a low concentration of Ro 20-1724 in the medium used to maintain and propagate MA-10(P⁺29) cells seems to be (at least partially) responsible for the increased levels of cAMP-phosphodiesterase activity and reduced cAMP responses detected in this cell line once the inhibitor is removed (see "Results" and Table II). Future experiments will further examine the molecular basis of the differential expression of cAMP-phosphodiesterase activity between these two cell lines.

Although the basal levels of cAMP are comparable in the wild-type and mutant cells (Fig. 3, Table II), the elevation of cAMP levels that occurs following hCG stimulation are severely limited, and this limitation varies in parallel with the levels of cAMP-phosphodiesterase activity. Thus, the cAMP responses to hCG stimulation are more blunted in MA-10(P⁺29) than in MA-10(P⁺8) (Fig. 3), and the response of MA-10(P⁺29) can be manipulated with a phosphodiesterase inhibitor in a predictable fashion (Fig. 4). These results indicate that while the basal levels of cAMP do not reflect phosphodiesterase activity, the hormone-stimulated levels of cAMP attained by MA-10 cells are, to a large extent, dictated by the activity of this enzyme. This is true even in the wild-type cells where the inclusion of phosphodiesterase inhibitors such as Ro 20-1724 (Fig. 4) or 3-isobutyl-1-methylxanthine (33, 38) have little or no effect on the basal levels of cAMP, but greatly enhance the hCG-induced cAMP-accumulation. A similar phenomenon was also noted in Chinese hamster

ovary cells transfected with the yeast cAMP-phosphodiesterase. These cells have normal basal levels of cAMP but show a greatly reduced response to prostaglandin E₁ and cholera toxin (19).

One notable advantage of using MA-10 cells for these experiments is that they respond to hCG and cAMP analogues with a robust increase in steroid synthesis (21, 33, 34). Moreover, as discussed elsewhere (28, 35, 36) the steroidogenic response to hCG is largely (or totally) mediated by cAMP. Thus, the reduced ability of MA-10(P⁺29) to respond to hCG with increased cAMP accumulation can be easily correlated with a similar reduction in hCG-stimulated steroidogenesis (Figs. 5 and 6). In contrast to the large reduction in the maximal steroidogenic response to hCG, the maximal steroidogenic response of MA-10(P⁺29) cells to Bt₂cAMP is normal (Fig. 5), presumably because this cAMP analogue is a poor substrate for the cAMP-phosphodiesterase (see "Results"). An additional illustration of the importance of increased cAMP-phosphodiesterase activity in limiting cAMP-mediated responses in MA-10(P⁺29) cells is provided by the finding that addition of Ro 20-1724 during the incubation with hCG restores the cAMP and steroidogenic responses (Figs. 4 and 6).

The finding that the reduced responsiveness of MA-10(P⁺29) cells to hCG can be overcome with cAMP analogues that are relatively insensitive to phosphodiesterase degradation, or by addition of a phosphodiesterase inhibitor, establishes these new cell lines as rather flexible experimental paradigms. Together with another stable transfectant of MA-10 cells (designated MA-10(K3)) that have reduced levels of cAMP-dependent protein kinase activity (36), MA-10(P⁺29) cells provide us with valuable tools to examine the metabolism of cAMP and to assess the role of cAMP as a mediator of steroid synthesis or other actions of hCG (39).

It has been proposed that the refractoriness of Sertoli cells that occurs following treatment with follitropin is due, at least in part, to a 10-fold increase in the activity of a cAMP-phosphodiesterase similar to the rat cAMP-phosphodiesterase 3 (3, 40). Since desensitization of adenylyl cyclase occurred within the same time frame, conclusive proof of the role of phosphodiesterase activation in the refractoriness of the cells could not be obtained. Since the data presented here show that a 5-7-fold increase in cAMP-phosphodiesterase activity causes an 80-95% reduction in cAMP accumulation, it can be inferred that the 10-fold increase in phosphodiesterase activity previously shown to occur in Sertoli cells must have a profound effect on cAMP accumulation. Thus, our data support the notion that activation of a cAMP-phosphodiesterase plays a pivotal role in the follitropin-induced desensitization of Sertoli cell responsiveness.

Last, since in several cell types hormone-mediated increases in cAMP accumulation lead to an increase in the levels of a cAMP-phosphodiesterase similar to the transfected rat phosphodiesterase 3 (3-7, 16), our results provide evidence that this regulation limits cell responsiveness.

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