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Mutations in cyclic AMP-dependent protein kinase and corticotropin (ACTH)-sensitive adenylate cyclase affect adrenal steroidogenesis

(hormone action/Y1 tumor cells/growth control/cell culture)

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ABSTRACT **Two groups of mutant clones were isolated from Y1 adrenocortical tumor cells. One group, Y1(Kin), exhibited altered cytosolic cyclic AMP-dependent protein kinase activity; the second group, Y1(Cyc), exhibited diminished corticotropin-responsive adenylate cyclase activity. Steroidogenic responses to corticotropin and cyclic nucleotides closely paralleled cyclic AMP-dependent protein kinase activity in the Y1(Kin) mutants. In Y1(Cyc) mutants, corticotropin had little effect on steroidogenesis, whereas cyclic nucleotides were fully active. These data imply that adenylate cyclase and cyclic AMP-dependent protein kinase are obligatory components of the corticotropin-stimulated steroidogenic pathway.**

Corticotropin (ACTH) produces various responses in adrenocortical cells, the most notable being an increase in corticosteroid biosynthesis (1). Other responses include stimulation of adenylate cyclase activity with accumulation of cyclic AMP (2), depletion of cholesterol (3) and ascorbic acid content (4), stimulation of cholesterol esterase activity (5), and enhancement of glucose utilization (6). Adrenocortical cells *in vitro* also respond to the hormone with altered morphology (7) and with growth arrest due to inhibition of DNA synthesis (8). Cyclic AMP added to adrenocortical cells elicits many of the same responses as does ACTH, including increased steroidogenesis, suggesting that ACTH action on the adrenal cortex may be mediated by cyclic AMP (9). Inasmuch as ACTH and cyclic AMP increase the activity of the cyclic AMP-dependent protein kinase (10, 11), this enzyme seems to be important in mediating at least some of the actions of ACTH. Certain observations, however, are difficult to reconcile with obligatory roles for cyclic AMP and cyclic AMP-dependent protein kinase in the ACTH-responsive steroidogenic pathway. Low doses of ACTH stimulate steroidogenesis in rat adrenal cells without causing detectable changes in cyclic AMP accumulation (12, 13) or protein kinase activity (11, 14); increasing doses of ACTH beyond that required for maximal steroidogenesis cause further increases in cyclic AMP accumulation (12, 13). An ACTH analog, *o*-nitrophenylsulfenyl-ACTH, stimulates steroidogenesis in rat adrenal cells (13, 15) and Y1 mouse adrenal tumor cells (16) to levels approaching those achieved with ACTH, while causing very little accumulation of cyclic AMP. In contrast, increasing doses of cholera toxin stimulate steroidogenesis and cyclic AMP accumulation in parallel (17, 18). In order to explain these findings, it has been postulated that only a small fraction of the cyclic AMP accumulated in response to ACTH is necessary to evoke the end effects of the hormone and that this active fraction may be localized in a discrete intracellular compartment (12, 19, 20). As an alternative hypothesis, it has been proposed that cyclic AMP and cyclic AMP-dependent protein kinase do not mediate ACTH action, at least at low hormone levels (13, 18, 21). Instead, it has been suggested that ACTH interacts with two distinct receptors on the adrenal cell membrane. Occupancy of one receptor leads to steroidogenesis while occupancy of the other leads to cyclic AMP accumulation (13, 18). Cyclic GMP has been implicated as an intracellular messenger for steroidogenesis at low levels of ACTH (21).

The Y1 mouse adrenocortical tumor cell line (22) provides a useful *in vitro* model system for studies on the mechanism of action of ACTH. The Y1 cell line, in common with normal adrenocortical cells, responds to ACTH or cyclic AMP with increased steroidogenesis, changes in cell shape, and growth arrest (22–25). In a preliminary study, we isolated a mutant of the Y1 cell line 8BrcAMP^r-1, selected for its resistance to the growth inhibitory effects of the cyclic AMP analog, 8-bromoadenosine 3′,5′-cyclic monophosphate (8BrcAMP) (26). The mutant subclone, isolated after mutagenesis with ethyl methanesulfonate, displayed reduced steroidogenic and morphological responses to ACTH and cyclic AMP (26) and was found to have defective cyclic AMP-dependent protein kinase activity (27).

In this report, we describe the selection of two families of 8BrcAMP-resistant mutants after treatment of Y1 cells with the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. In one family of mutants, the cytosolic cyclic AMP-dependent protein kinase activities had reduced apparent affinities for cyclic AMP ranging over two orders of magnitude relative to the Y1 parent. These mutants are designated Y1(Kin). In the second family, designated Y1(Cyc), adenylate cyclase displayed diminished sensitivity to ACTH. In both classes of variants, alterations in steroidogenic responses to ACTH closely accompanied the changes in cyclic AMP-dependent protein kinase or in adenylate cyclase activity.

METHODS AND MATERIALS

Mutagenesis and Selection. The parental, ACTH-responsive cell line used in these studies is a stable subclone of the original Y1 population (22, 28). Details of the routine aspects of cell culture have been described elsewhere (28). Y1 cells (approximately 2×10^6 in 100-mm plastic tissue culture dishes) were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (1.25 μg/ml) in growth medium (nutrient mixture F-10 plus sera and antibiotics) (28) for 2.5 hr. This treatment reduced the plating efficiency of Y1 cells to 30% of control levels and was considered to be effective for induction of mutations (29, 30). Cells were transferred to regular growth medium for 6–9 days to permit expression of the mutation (31, 32) and then were grown for 2–6

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Abbreviations: ACTH, corticotropin (adrenocorticotrophic hormone); 8BrcAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate.

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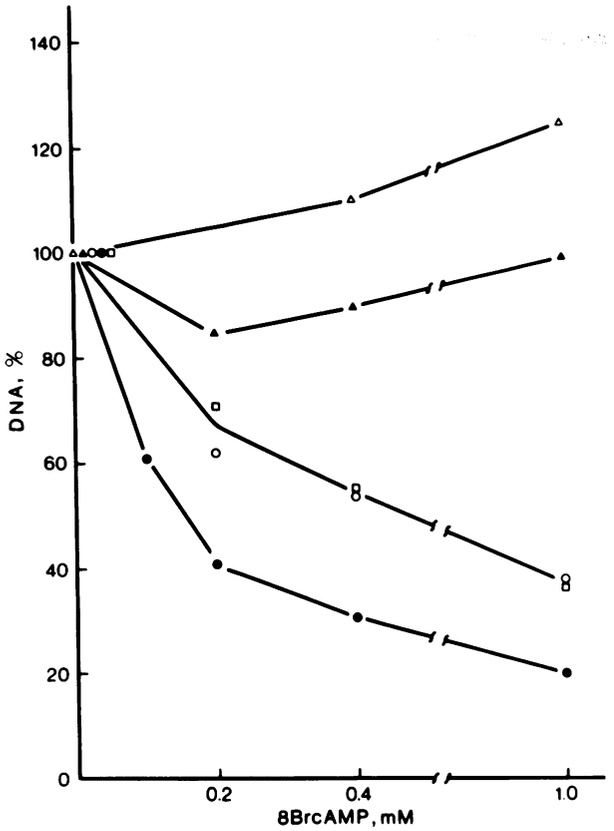

FIG. 1. Inhibition of growth by 8BrcAMP in Y1 (●), Kin 7 (▲), Kin 8 (△), Cyc 101 (○), and Cyc 102 (□). For each cell line, the DNA content of control cultures grown in the absence of 8BrcAMP was set at 100%. The DNA content of cultures grown in the presence of 8BrcAMP was expressed as a percentage of the control.

weeks in the presence of 0.8 mM 8BrcAMP, a concentration which reduced the relative plating efficiency of wild-type cells to $<10^{-5}$ (26). Surviving colonies were isolated and maintained thereafter in regular growth medium without 8BrcAMP.

Growth Inhibition by 8BrcAMP. Cells were grown in 16-mm plastic tissue culture wells in the presence or absence of 8BrcAMP for 100 hr. The DNA content of the monolayers was measured as described by Kissane and Robins (33) after digestion of the cells with 1 M NaOH for 1 hr at 37°C and subsequent neutralization of the digest.

Protein Kinase, Adenylate Cyclase, and Steroidogenic Activities. Cytosolic cyclic AMP-dependent protein kinase (27, 34), adenylate cyclase (35), and steroidogenic activities (27, 36) of cell lines were determined as described.

RESULTS

Growth and Morphological Characteristics of Mutant Clones. As shown in Fig. 1, surviving colonies were of two distinct phenotypes. One type was resistant to the effects of 8BrcAMP on cell shape and remained as well-stretched monolayers in the presence of the cyclic nucleotide. In addition, this phenotype was almost totally resistant to the growth inhibitory effects of 8BrcAMP. Treatment of representative clones from this class (e.g., Kin 7 and Kin 8) with 1 mM 8BrcAMP did not reduce DNA accumulation relative to untreated cultures. In contrast, 1 mM 8BrcAMP reduced DNA accumulation in parental Y1 cells by 80%. The second type of colony that survived the selection retracted from the cell monolayer and assumed a rounded morphology in the presence of 1 mM 8BrcAMP, but was resistant to the effects of ACTH on cell shape. Clones of this second group (e.g., Cyc 101 and Cyc 102) were only partially resistant to growth inhibition by 8BrcAMP. In these cultures, 1 mM 8BrcAMP reduced DNA accumulation by 60% relative to untreated controls. The level of resistance of these clones to growth inhibition by 1 mM 8BrcAMP was about twice that of the Y1 parent and was apparently sufficient to enable them to survive the selection procedure.

Activities of Cyclic AMP-Dependent Protein Kinase and Adenylate Cyclase. Protein kinase activity in the cytosol fraction from Y1 cells was increased up to 10-fold over unstimulated levels by cyclic AMP to a maximal activity of 770 ± 130 (SEM) units/mg of protein (1 unit of kinase activity is defined as the transfer of 1.0 pmol of ^{32}P per min from [^{32}P]-ATP to histone). Half-maximal activity was achieved with 10^{-7} M cyclic AMP, and maximal activity was achieved with 10^{-6} M cyclic AMP (Fig. 2*a*).

The mutant subclones highly resistant to the effects of 8BrcAMP on growth and cell shape had defective cyclic AMP-dependent protein kinase activities. These mutants have been designated Y1(Kin). In Y1(Kin) mutants, from 6- to 600-fold higher concentrations of cyclic AMP were required to activate the protein kinases to the half-maximal level of the enzyme from parent Y1 cells (Fig. 2*a*); Kin 2 was the least affected mutant, Kin 8 was the most severely affected, and Kin 7 displayed an intermediate defect. Y1(Kin) mutants possessed

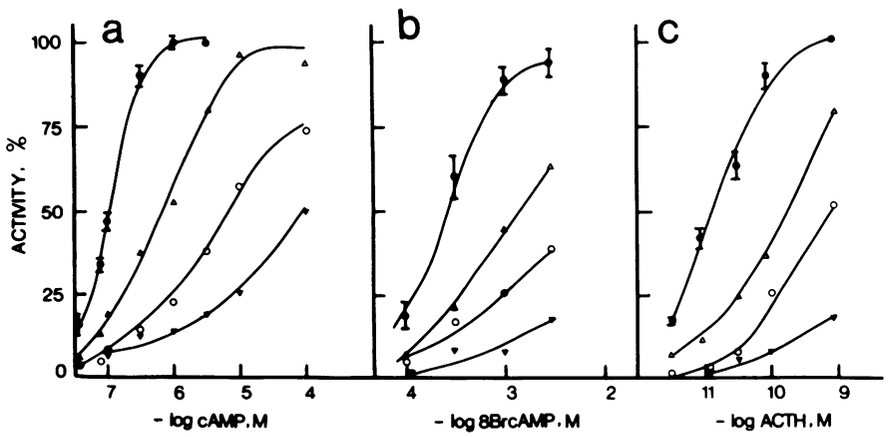

FIG. 2. Protein kinase and steroidogenic activities of Y1(Kin) mutants. Cyclic AMP (cAMP)-dependent protein kinase activity (*a*) and steroidogenesis (*b* and *c*) were measured in Y1 (●) and representative Kin mutants [Kin 2 (△), Kin 7 (○), Kin 8 (▼)]. Activities were expressed as a percentage of the maximal activity achieved with Y1 cells.

Table 1. Adenylate cyclase activity of Y1, Y1(Kin), and Y1(Cyc) mutants

Clone	Adenylate cyclase activity, pmol cAMP/(mg protein·10 min) Basal	ACTH-(1–24) (20 μM)	Fluoride (15 mM)	ACTH* fluoride
Y1	52	3461	2761	1.3
Kin 2	100	4600	3800	1.2
Kin 7	90	2600	1700	1.5
Kin 8	25	1100	1400	0.8
Cyc 103	55	388	1253	0.31
Cyc 102	78	904	3456	0.26
Cyc 101	50	149	3190	0.05

* The ACTH to fluoride activity ratio is described in *Results*.

adenylate cyclase activities that were highly responsive to ACTH as measured in cell homogenates (Table 1). An ACTH to fluoride activity ratio was used as an index of hormone effectiveness in relation to the potential adenylate cyclase activity. For fully responsive Y1 cells, the ACTH to fluoride activity ratio ranged from 1.1 to 1.7 over six experiments. Our Y1(Kin) mutants are comparable to cyclic nucleotide-resistant mutants reported in several other cell systems. A group of S49 lymphoma cell mutants (30, 34), a variant line of rat mammary carcinoma (37–39), a Chinese hamster ovary cell mutant (40), and three mutants of a mouse neuroblastoma cell line (41), all of which were resistant to the growth-inhibitory effects of cyclic AMP analogs, had altered cyclic AMP-dependent protein kinase activities. The isolation of mutants with similar phenotypes from several dissimilar populations of cells implicates the cyclic AMP-dependent protein kinase as a general mediator of growth inhibition by cyclic nucleotides. In contrast to the results described here with Y1 adrenal cells (Fig. 2*a*), treatment of S49 lymphoma cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine resulted in a high frequency of protein kinase mutants with greatly reduced affinities for cyclic AMP (comparable to Kin 8) and no protein kinase mutants (comparable to our Kin 2 clone) with slightly reduced (<12-fold) affinities for cyclic AMP (30). The reason for this difference between cell lines is not readily explained.

The mutant subclones partially resistant to 8BrcAMP had cyclic AMP-dependent protein kinase activities as great as or greater than that of the parent Y1 clone at all the concentrations of cyclic AMP tested (Fig. 3*a*). These subclones characteristically showed little increase in adenylate cyclase activity in response to ACTH, but they responded well to NaF, a general activator of adenylate cyclase (Table 1). The ACTH to fluoride activity ratio of these clones ranged from 0.05 to 0.31. These mutants have been designated Y1(Cyc). The "Cyc" designation is used in the general sense to denote a phenotype defective in regulation of the adenylate cyclase system, and may include mutants with defects in ACTH receptors, catalytic subunits, and molecules that link receptors to the enzyme or otherwise modify adenylate cyclase activity.

Steroidogenesis in Y1(Kin) and Y1(Cyc) Mutants. Maximal steroid production in the parent Y1 clone was 270 ± 30 ng of steroid per mg of protein per hr (n = 17) with ACTH (10^{-9} M) or 8BrcAMP (3×10^{-3} M). Half-maximal activity was obtained with 3×10^{-4} M 8BrcAMP or 10^{-11} M ACTH (Fig. 2 *b* and *c*). In all the mutants of the Y1(Kin) class, steroidogenic responses to 8BrcAMP and to ACTH were reduced in a manner that closely paralleled the loss of apparent affinity of the protein kinase for cyclic AMP (e.g., Fig. 2). The effects of cyclic AMP on steroidogenesis in Y1 and both classes of mutants were qualitatively similar to the effects of the more potent 8BrcAMP. The roles of cyclic AMP and cyclic AMP-dependent protein kinases in the steroidogenic actions of ACTH have been questioned most seriously at concentrations of hormone that stimulate steroidogenesis less than half maximally (12, 16, 21). Accordingly, the agonist concentrations required to stimulate protein kinase activity or steroidogenesis to 25% of the maximum were compared in the Y1 parent and in six Y1(Kin) mutants. As shown in Table 2, the Y1 parent and mutant clones exhibited the same ranked order of potency for activation of protein kinase by cyclic AMP and activation of steroidogenesis by ACTH or 8BrcAMP. These data strongly suggest that the cytosolic cyclic AMP-dependent protein kinase is an obligatory component of ACTH and cyclic AMP action on adrenal steroidogenesis.

In the Y1(Cyc) mutants, steroidogenic responses to 8BrcAMP exceeded those obtained with the Y1 parent (Fig. 3*b*), whereas steroidogenic responses to ACTH were depressed (Fig. 3*c*). The order of potency of ACTH on adenylate cyclase activity in Y1 and in the Y1(Cyc) mutants (Table 1) correlated well with the steroidogenic responses of these cell lines to ACTH (Fig. 3*c*).

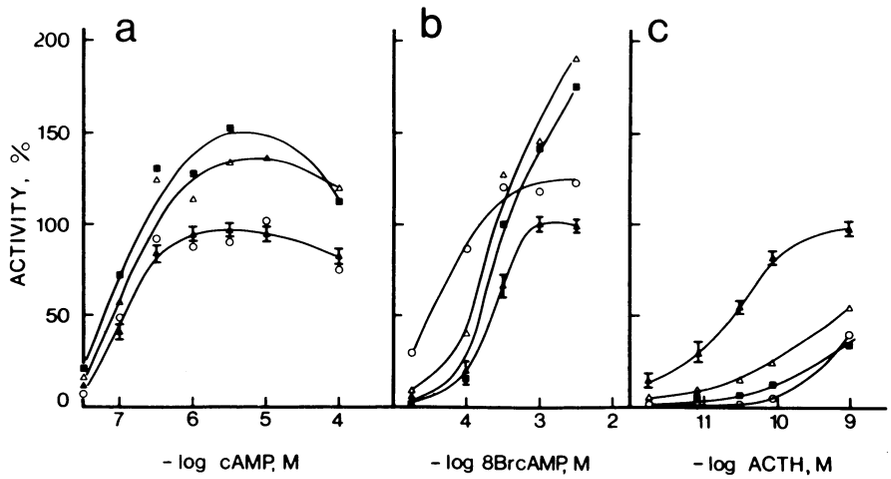

FIG. 3. Protein kinase and steroidogenic activities of Y1(Cyc) mutants. Cyclic AMP (cAMP)-dependent protein kinase activity (*a*) and steroidogenesis (*b* and *c*) were measured in Y1 (▲) and representative Cyc mutants [Cyc 101 (○), Cyc 102 (■), Cyc 103 (△)]. Activities were expressed as described in the legend to Fig. 2.

Table 2. Parallelism between protein kinase activity and steroidogenesis in Y1(Kin) mutants

Clone	cAMP concentration for 25% maximal kinase activity, M	ACTH concentration for 25% maximal steroidogenesis, M	8BrcAMP concentration for 25% maximal steroidogenesis, M
Y1	5.6×10^{-8}	6.0×10^{-12}	1.0×10^{-4}
1*	1.7×10^{-7}	2.2×10^{-11}	3.5×10^{-4}
2	1.7×10^{-7}	2.7×10^{-11}	4.0×10^{-4}
3	4.7×10^{-7}	7.5×10^{-11}	4.5×10^{-4}
4	8.5×10^{-7}	9.4×10^{-11}	4.5×10^{-4}
7	8.5×10^{-7}	1.5×10^{-10}	8.9×10^{-4}
8	5.6×10^{-6}	8.9×10^{-9}	1.0×10^{-2}

* Clone 1, formerly designated 8BrcAMP^r-1, was isolated after mutagenesis with ethyl methanesulfonate. Its isolation and characterization were described (26).

DISCUSSION AND CONCLUSIONS

The study presented here employed a genetic approach to investigate part of the mechanism of action of ACTH in Y1 adrenocortical tumor cells. Our results suggest that enhancement of protein phosphorylation by cyclic AMP-dependent protein kinase is a critical step in the action of ACTH over the entire effective concentration range of hormone from 2×10^{-12} to 10^{-9} M (Fig. 2). Cyclic AMP-dependent protein kinase was shown to phosphorylate ribosomes (42, 43) and enhance cholesterol ester hydrolase activity (44) in adrenocortical preparations and was implicated in the increase of phosphorylase activity (2) and in the inactivation of phosphorylase phosphatase (45); however, the relationship of these reactions to the rate limiting step in steroidogenesis, the conversion of cholesterol to pregnenolone by side-chain cleavage enzyme (46, 47), is uncertain. In bovine corpus luteum, an endocrine tissue which also carries out steroidogenesis, partially purified cytosolic cyclic AMP-dependent protein kinase was found to stimulate conversion of cholesterol to pregnenolone by a reconstituted side-chain cleavage enzyme system (48). The protein kinase concomitantly caused phosphorylation of cytochrome P-450, a component of the side chain cleavage enzyme system. In the adrenal, however, such an effect of the cyclic AMP-dependent protein kinase on the rate-limiting step of steroidogenesis has not yet been demonstrated and the putative substrate of the kinase remains undefined.

The basis of selection of Y1(Kin) mutants conceptually is similar to the selection procedure for cyclic AMP-dependent protein kinase mutants in S49 lymphoma cells (34). In contrast to S49 lymphoma cells, however, Y1 cells do not lyse in the presence of cyclic adenylates. Instead, they exhibit growth arrest and changes in cell shape (23, 25). Inasmuch as Y1(Kin) mutants are resistant to both of these effects of 8BrcAMP, it seems likely that cyclic AMP-dependent protein kinase is a component common to both responses. Our previous demonstration of concomitant defects in cyclic AMP-dependent protein kinase activity and steroidogenesis in a single Y1 mutant (27) did not establish that this phenotype arose from a single mutational event, nor exclude the possibility that a single altered gene product had independently influenced expression of kinase activity and steroidogenesis. The close parallelism between cyclic AMP-dependent protein kinase activity and ACTH- or cyclic AMP-stimulated steroidogenesis in a series of Y1(Kin) mutants greatly minimizes the possibility that the Kin phenotype is the consequence of multiple mutations, and provides compelling evidence for the obligatory role of cyclic AMP-dependent protein kinase in steroidogenesis.

We have isolated Y1(Cyc) mutants by a selection procedure based on resistance to growth inhibition by cyclic nucleotides. The phenotypes of the Y1(Cyc) mutants somewhat resemble those of two spontaneous variants of the Y1 cell line, clones Y6 and OS3 (35, 49). The adenylate cyclase systems of Y1(Cyc) mutants, though responsive to fluoride, display little response to ACTH (Table 1). This lack of sensitivity to ACTH is reflected proportionately in reduced steroidogenic responses to ACTH (Fig. 3*c*). The Y1(Cyc) mutants exhibit normal cyclic AMP-dependent protein kinase activities (Fig. 3*a*) and display normal steroidogenic responses to cyclic adenylates (Fig. 3*b*). The mutant clone, 101, also responds to cholera toxin with increased adenylate cyclase activity and has cyclic AMP phosphodiesterase activity similar to that of Y1 (data not shown). The lesion in the Y1(Cyc) mutants, therefore, appears to be at the level of the plasma membrane, affecting the ACTH receptor or the coupling of the occupied receptor to adenylate cyclase. The mechanism that links the lesion in the ACTH-sensitive adenylate cyclase system to the 8BrcAMP resistance of the Y1(Cyc) mutants is not readily apparent. A defect in the plasma membrane affecting permeability to 8BrcAMP seems to be an inadequate explanation, because steroidogenesis in the Y1(Cyc) mutants is fully responsive to exogenous cyclic nucleotides (Fig. 3*b*). The emergence of the Y1(Cyc) mutants from the selection procedure described here cannot be easily explained, due to the complexity of the peptide hormone receptor–adenylate cyclase system. Further examination of this phenotype, in order to understand the basis of its emergence, also may be expected to clarify our understanding of the regulation of the adenylate cyclase system.

The selective depression of ACTH-sensitive steroidogenic activity in Y1(Cyc) mutants and the correlations between cyclic AMP-dependent protein kinase activity and regulation of steroidogenesis in Y1(Kin) mutants strongly implicate cyclic AMP as an obligatory component of ACTH action on adrenal steroidogenesis. The studies with the Y1(Kin) mutants provide experimental evidence in favor of an obligatory role of cyclic AMP-dependent protein kinase in hormonal control of adrenal steroidogenesis.

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