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THE CYTOSOL AS SITE OF PHOSPHORYLATION OF THE CYCLIC AMP-DEPENDENT PROTEIN KINASE IN ADRENAL STEROIDOGENESIS

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Summary—The mitochondria, the microsomes and the cytosol have been described as possible sites of cAMP-dependent phosphorylation. However, there has been no direct demonstration of a cAMP-dependent kinase associated with the activation of the side-chain cleavage of cholesterol. We have investigated the site of action of the cAMP-dependent kinase using a sensitive cell-free assay. Cytosol derived from cells stimulated with ACTH or cAMP was capable of increasing progesterone synthesis in isolated mitochondria when combined with the microsomal fraction. Cytosol derived from cyclase or kinase of negative mutant cells did not. Cyclic AMP and cAMP-dependent protein kinase stimulated *in vitro* a cytosol derived from unstimulated adrenal cells. This cytosol was capable of stimulating progesterone synthesis in isolated mitochondria. Inhibitor of cAMP-dependent protein kinase abolished the effect of the cAMP. ACTH stimulation of cytosol factors is a rapid process observable with a half maximal stimulation at about 3 pM ACTH. The effect was also abolished by inhibitor of arachidonic acid release. The function of cytosolic phosphorylation is still unclear. The effect of inhibitors of arachidonic acid release, and the necessity for the microsomal compartment in order to stimulate mitochondrial steroidogenesis, suggest that the factor in the cytosol may play a role in arachidonic acid release.

INTRODUCTION

In the current model of adrenocorticotropin (ACTH) action, it is proposed that the hormone binds specifically to receptors on the plasma membrane and at least in part, stimulate, adenylate cyclase activity, with accumulation of adenosin 3′,5′-cyclic monophosphate (cAMP) and activation of a cAMP-dependent protein kinase. Mitochondria isolated from steroidogenic tissues of rats pretreated with the corresponding hormone have an increased capacity to synthetize pregnenolone when compared with controls. This cAMP-dependent acceleration of steroidogenesis by ACTH is cycloheximide sensitive [1, 2]. Consequently, it is generally believed that cAMP enhances the adrenal content of some labile translation product which is important, either directly or indirectly, for intramitochondrial cholesterol movement. However, the question still remains as to how a cAMP-dependent phosphorylation can transmit a specific signal to its mitochondrial site of action. Several phosphoproteins have been proposed as intermediate, but their function and relationship remain unclear [3–7]. Epstein *et al.* [3] have identified a 28,000 Da phosphoprotein in rat mitochondrial adrenal cortex. It is produced with a time-course, stimulant dose–response, and translation-dependence which parallel those of stimulated steroidogenesis. Pedersen and co-authors [4, 5] have isolated a factor, steroidogenic activator factor (SAP), from rat adrenocortical and Leydig cell tumor tissues. They have proposed that one point of regulation of the cAMP-dependent protein kinase could be a novel protease, that cleaves the putative precursor-product of SAP, the glucose regulated protein 78 (GRP78). Deviller *et al.* [6] have described a correlation between dephosphorylation of a cytoplasmic protein with an apparent molecular weight of 20,000 Da and activation of steroidogenesis. Podesta *et al.* [7] have described the phosphorylation of a cytoplasmic protein APS150 in intact isolated cells stimulated by ACTH with stimulant dose–response, which parallels those of stimulated steroidogenesis. In recent reports it

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has also been suggested that cAMP activation of a phospholipase that releases arachidonic acid from a microsomal pool, may be an obligatory step in hormone–induced steroidogenesis [8, 9]. Thus, the mitochondria, the microsomes and the cytosol have been described as possible sites of cAMP-dependent phosphorylation. However, in none of these studies a direct demonstration of a cAMP-dependent kinase associated with the activation of the side-chain cleavage of cholesterol has been shown. It is clear that the identification of the subcellular site of the cAMP-dependent protein kinase would elucidate the understanding of the post-translational control by cAMP of regulation of cholesterol side-chain split. In the present study we have investigated the site of action of the cAMP-dependent protein kinase using a sensitive cell-free assay system [10]. The results demonstrated that a soluble protein is at least one of the putative sensitive sites of action of the cAMP-dependent protein kinase, and that this protein appears to play a role at the level of the microsomal compartment inducing the release of arachidonic acid.

MATERIALS AND METHODS

Animals

Adrenal zona fasciculata were obtained from male rats maintained with dexamethasone in the drinking water [7–12].

Isolation and treatment of the zona fasciculata with ACTH

The zona fasciculata was obtained from the decapsulated fraction of the adrenal gland. The zona fasciculata from one gland was quartered and incubated with or without 10^{-12} M ACTH. at the end of the incubation corticosterone in the medium was assayed by radioimmunoassay as previously described [7–12]. The adrenal zona fasciculata was homogenized gently in a glass tube with Teflon pestle in 1 ml of medium A (270 mM mannitol/1 mM Tris–HCl pH 7.4) and processed as indicated in "Preparation of subcellular fractions".

Zona fasciculata-reticularis type cells preparation

Zona fasciculata-reticularis type cells preparation and incubations followed published procedures [7–12]. Incubations (37°C) with and without 3-isobutyl 1-methylxanthine (MIX) (10^{-4} M) and ACTH (10^{-12} M) were done in duplicate or triplicate in 12 × 75 mm Falcon plastic tubes under O_2/CO_2 (95%: 5%) with shaking (100 cycles/min). Each tube contained 0.5 ml of a cell suspension of about 10^5 cells (150 μg of protein) in Krebs–Ringer bicarbonate solution pH 7.4, 0.01 M glucose, 0.5% bovine serum albumine (RIA grade, Sigma Chem. Co.). The incubation was stopped by cooling the tubes in ice/water and all further processing was done at 0–4°C. In some experiments, the inhibitor of arachidonic acid release, bromophenacyl bromide (BPB) was added before the addition of ACTH and MIX.

Adrenal tumor cells

The Y-1 cells and the two families of mutants were generously provided by Dr Bernard Shimmer, Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada.

The Y-1 cell line has an average doubling time of 30–40 h, has a plating efficiency of 4–10%, and reaches saturation density at approximately 2.7×10^5 cells/cm^2. Y-1 cells behave like normal mouse adrenal cells in several aspects: they increase the rate of steroidogenesis 4–10-fold in response to ACTH; they concentrate cholesterol and ascorbic acid from their growth medium. The cell line, in the presence of maximally effective concentration of ACTH, produces steroids at the rate of 0.72 ± 0.13 μg/mg of protein per h. The major steroid produced is progesterone and 20α-hydroxyprogesterone. Two families of 8-BrcAMP-resistant mutants were described [13, 14] after treatment of Y-1 cells with the mutagen *N*-methyl-*N'*-nitrosoguanidine. In one family of mutants, the cytosol cAMP-dependent protein kinase activities had reduced apparent affinities for cAMP ranging over two orders of magnitude relative to the Y-1 parent. These mutants were designated Y-1-(kin) [14]. In the second family, designed Y-1 (cyc$^-$), adenylyl cyclase displayed diminishing sensitivity to ACTH [13, 14]. In both variants, alterations in the steroidogenic response to ACTH closely accompanied the changes in cAMP-dependent protein kinase or in adenylate cyclase activity. In the present study the cells were grown in 82.5% nutrient mixture F10 [14], 15% horse serum, and 2.5% fetal calf serum.

Preparation of postmitochondrial and mitochondria fractions

After incubation as described above, adrenal zona fasciculata tissue was homogenized gently

in a glass tube with Teflon pestle and centrifuged at 800 *g* for 10 min. The pellets containing cell debris and membranes (premitochondrial fractions from unstimulated or ACTH-stimulated adrenal) were washed once with 0.5 ml of medium A. The mitochondrial fractions were obtained by recentrifugation of the 800 *g* supernatant at 9000 *g* for 10 min, rinsing without disturbing the pellets in medium A and then resuspending them in fresh medium A (200 μl per adrenal). The 9000 *g* supernatant was recentrifuged at 105,000 *g* for 60 min to obtain the 105,000 *g* pellet and cytosol. The subcellular fractions used throughout the work are designated as follows: PMF = 9000 *g* supernatant, cytosol = 105,000 *g* supernatant, microsomes = 105,000 *g* pellet and mitochondria = 9000 *g* pellet [12].

Assay of extramitochondrial steroidogenic factors

In order to measure progesterone as a final product, mitochondrial steroidogenesis was allowed to proceed in the presence of 0.27 mM metopyrone [15] and 1.6 mM 1-(β-guanidinoethyl)-3-(4-pyridyl)indol sulfate (Ba 40.028) [16] as inhibitors of 11-,18-,19-hydroxylation, and 21-hydroxylation, respectively, without addition of an electron donor. For the incubation, siliconized glass scintillation vials were loaded with a postmitochondrial fraction (500 μg of protein) and supplemented with medium A to a total volume of 250 μl. The two inhibitors of hydroxylation were added in 350 μl of medium B (1 vol of 0.15 M Tris–HCl, pH 7.4/2 vol of medium A/1 vol of 0.3 M KCl/1 vol of 0.02 M $MgCl_2$) according to [12]. The concentration of metopyrone base and Ba 40028 were 60 and 600 μg, respectively, per ml of the final incubation mix. The mix was completed by adding 100 μl of mitochondrial fraction (320 μg of protein) from control adrenals or 100 μl of medium A to a final volume of 700 μl. After warming it in a water bath at 37°C for 2.5 min, the mixture was incubated for 7.5 min with shaking (100 cycles per min). The incubation was stopped by cooling the tubes in ice/water and by adding of 2 ml of cold methanol/2.5 mM HCl (1:1, v/v) per tube. Steroids were extracted and assayed as previously described [12]. The net formation of progesterone + pregnenolone was calculated in one of the following ways: (A) Incubations with and without PMF at 37°C were performed with or without mitochondria. Progesterone produced by PMF without mitochondria plus progesterone produced by mitochondria alone was subtracted from the progesterone produced during the complete incubation. (B) Incubations of the complete mixture were performed in parallel at 37 and 0°C. The progesterone value obtained from the latter incubation was subtracted from the former. (C) In order to correct for some residual pregnenolone in the adrenal fractions before and after incubation, pregnenolone and progesterone were determined separately in the complete incubations, done at 37 and 0°C. The sum of the two steroids in the later incubation was then subtracted from the sum in the incubation at 37°C. This procedure gave the net formation of both pregnenolone and progesterone and was obviously the most accurate one. However, the results obtained by all three procedures were equivalent in terms of fold increases.

RESULTS

We have previously showed that PMF (9000 *g* supernatant) derived from cells stimulated with ACTH or cAMP was capable of increasing progesterone synthesis in isolated mitochondria using the adrenal cell-free assay [9]. Mutant clones of Y-1 adrenocortical tumor cells (adenylate cyclase, cyc^-, or cAMP-dependent protein kinase, kin^-, deficient) were used to validate the use of the adrenal cell-free assay to study the site of action of the cAMP-dependent protein kinase.

The PMFs derived from rat adrenal cells, Y-1 cells, Y-1 cyc-, kin^- stimulated with ACTH or 8-Br-cAMP were incubated with mitochondria from nonstimulated rat adrenal or Y-1 adrenal cells. Progesterone synthesis was measured at the end of the incubation. The results are shown in Table 1. PMFs from ACTH-stimulated adrenal or ACTH-stimulated Y-1 cells increased net progesterone synthesis 10- and 20-fold, respectively, compared with PMF from unstimulated adrenal or Y-1 cells. PMFs derived from ACTH-stimulated Y-1 cyc^- or kin^- were not able to increase net progesterone synthesis. On the other hand, PMF derived from 8-Br-cAMP-stimulated Y-1 cyc^- cell increased net progesterone synthesis approximately 20-fold compared with PMF from unstimulated cells. However, PMF derived from 8-Br-cAMP-stimulated Y-1 kin^- was not able to increase progesterone synthesis compared with PMF from Y-1 cyc^-. These results suggest that in

Table 1. Net progesterone production in mitochondria from Y-1 adrenal tumor cells incubated with ACTH-dependent, cAMP-dependent postmitochondrial fraction (PMF) from Y-1 adrenal tumor cells, Y-1, cyc$^-$ and kin$^-$. Values are expressed as mean ± SD, $n = 3$

PMF source	Progesterone (ng/incubation)
Rat adrenal cells	
Control	3.7 ± 0.3
ACTH	41.4 ± 2.9
Y-1 cells	
Control	0.4 ± 0.3
ACTH	8.8 ± 0.6
Y-1 cyc$^-$ cells	
ACTH	0.9 ± 0.7
cAMP	7.9 ± 0.5
Y-1 kin$^-$ cells	
ACTH	0.2 ± 0.2
cAMP	0.4 ± 0.1

Cells were stimulated with ACTH (10^{-12} M) or 8-Br-cAMP (10^{-5} M) or without (control) for 90 min. The cells were homogenized and the subcellular fractions were obtained and incubated as described in "Materials and Methods".

Table 3. Production of steroidogenic factors by the recombination, in a cell-free system assay, of particulate fraction and mitochondria from nonstimulated rat adrenal with cytosol from ACTH-treated adrenal fasciculata cells: effect of inhibitor of a cAMP-dependent protein kinase in the cell-free assay. Values are expressed as mean ± SD, $n = 3$

	Progesterone (ng/incubation)
Control	9.5 ± 0.3
PKI	7.8 ± 0.9
ACTH	32.5 ± 4.5
ACTH + PKI	31.7 ± 0.4

Adrenal zona fasciculata cells were treated with ACTH (10^{-10} M) or without (control). After the incubation, the cells were homogenized and their cytosol fractions were obtained as indicated in "Materials and Methods". The fractions were recombined in the presence or in the absence of PKI (70 μg/ml) with PF (particulate fraction) and mitochondria from control cells; progesterone production was determined by radioimmunoassay.

the PMF fraction there is a cAMP-dependent process activated by ACTH. In order to determine the participation of the cAMP-dependent kinase effect in this step we study the action of the specific protein kinase inhibitor from rabbit muscle (PKI Sigma P-5015). The results are shown in Table 2. PMF derived from ACTH-stimulated adrenal increased net progesterone synthesis 10-fold in the presence or in the absence of PKI. Thus, the phosphorylation process occurs before the isolation of the PMF fraction. To investigate this possibility the post-mitochondrial fraction was further divided into the 105,000 *g* supernatant (cytosol) and the 105,000 *g* pellet (microsomal fraction). Cytosol from ACTH-stimulated adrenal is able to activate the mitochondrial progesterone synthesis when is recombined with the microsomal fraction obtained from unstimulated adrenal (Table 3). In this assay the PKI has also no effect. This result suggested that in the cytosol, obtained from stimulated adrenal the phosphorylation process also already occurred. To confirm this possibility, next experiments were carried out to induce the phosphorylation of cytosol proteins *in vitro*. Cytosol from non-stimulated adrenal was incubated with increasing concentrations of cAMP (10^{-7}–10^{-3} M) for 10 min at 37°C, followed by 9.5 min incubation in the presence of the 105,000 *g* pellet and the mitochondria from nonstimulated adrenals. The results are shown in Fig. 1. In the presence of cAMP, cytosol obtained from nonstimulated adrenal was able to increase progesterone synthesis. There is a dose-dependent effect of cAMP, reaching 4-fold stimulation with 10^{-3} M cAMP. In this assay it is now possible to see an effect of the PKI. Figure 2 shows the inhibition by PKI (70 μg/ml) upon the stimulation with progesterone synthesis obtained with 10^{-6} M cAMP.

Table 2. Net progesterone production in mitochondria from rat adrenal zona fasciculata incubated with ACTH-dependent postmitochondrial fraction (PMF) from adrenal zona fasciculata. *In vitro* effect of inhibitor of cAMP-dependent protein kinase. Values are expressed as mean ± SD, $n = 3$

PMF	Progesterone (ng/incubation)
Control	3.7 ± 1.3
PKI	3.1 ± 0.8
ACTH	34.3 ± 2.4
ACTH + PKI	33.2 ± 3.2

Adrenal zona fasciculata cells were stimulated with ACTH (10^{-12} M) or without (control) for 90 min. The cells were homogenized and the subcellular fractions were obtained and incubated as described in "Materials and Methods" in the presence or in the abscence of PKI (70 μg/ml).

As suggested by the recombination experiments in the foregoing sections, the PKA activity in the cytosol and the phosphorylation of a protein in the cytosol fraction is rate limiting to produce the activation of the 105,000 *g* pellet. In order to check this assumption, the 105,000 *g* pellet derived from nonstimulated adrenal was incubated with cAMP and cAMP in combination with a cAMP-dependent protein kinase. In both cases the 105,000 *g* pellet was not able

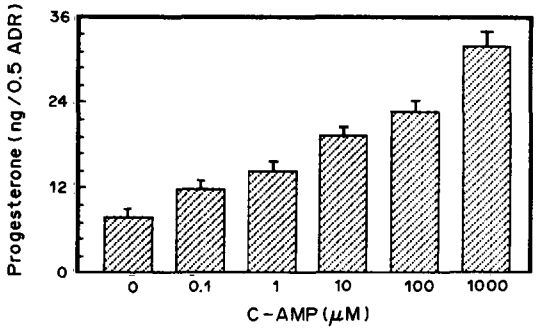

Fig. 1. Cyclic AMP stimulation of progesterone mitochondria neosynthesis. Mitochondria were isolated, as described in "Materials and Methods" from adrenal zona fasciculata. The 105,000 *g* supernatant from adrenal zona fasciculata was incubated with cyclic AMP during 10 min. After the incubation this fraction was recombined with the 105,000 *g* pellet and the mitochondria (corresponding to 0.5 adrenal) according to the assay for extramitochondrial steroidogenic factors for 9.5 min, as described in "Materials and Methods". Progesterone synthesis was measured by radioimmunoassay and expressed as ng/0.5 adrenal, equivalent to ng/incubation.

to stimulate mitochondria isolated from nonstimulated adrenal (Table 4).

Bromophenacyl bromide (BPB), inhibitor of arachidonic acid release [17–21], has been shown to inhibit the ACTH stimulation of corticosterone synthesis and the effect of the PMF fraction on progesterone synthesis on nonstimulated mitochondria [8, 9]. Therefore we studied the effect of BPB on specific intracellular steps regulated by a cAMP-dependent kinase. For this purpose, adrenal cells zona fasciculata were incubated in the presence or

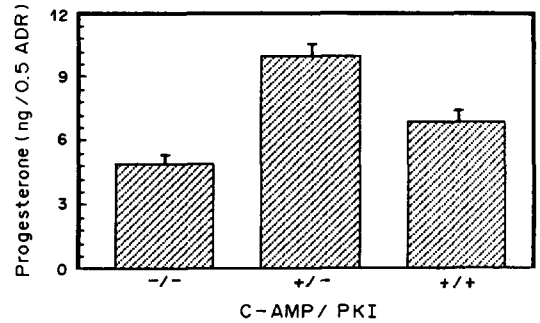

Fig. 2. Effect of PKI on the cyclic AMP stimulation of progesterone mitochondria neosynthesis. Mitochondria were isolated, as described in "Materials and Methods" from adrenal zona fasciculata. incubation with cyclic AMP (1 μM) in the presence or in the absence of 70 μg/ml of PKI was done as described in Fig. 1. After the incubation the 105,000 *g* supernatant was recombined with the 105,000 *g* pellet and the mitochondria (corresponding to 0.5 adrenal) according to the assay for extramitochondrial steroidogenic factors for 9.5 min, as described in "Materials and Methods". Progesterone synthesis was measured by radioimmunoassay, and expressed as ng/0.5 adrenal, equivalent to ng/incubation.

Table 4. Production of steroidogenic factors by the recombination, in a cell-free system assay, of 105,000 *g* pellet and mitochondria from nonstimulated rat adrenal: effect of cAMP and cyclic AMP-dependent protein kinase (from rabbit muscle Sigma P-4890, PKA) in the cell-free assay. Values are expressed as mean ± SD, $n = 3$

	Progesterone (ng/incubation)
Control	9.5 ± 1.8
cAMP	13.3 ± 3.5
cAMP + PKA	16.0 ± 4.2

Adrenal zona fasciculata was homogenized and the 105,000 *g* fraction and the mitochondria were obtained as indicated in "Materials and Methods". The fractions were recombined in the presence or in the absence of cAMP (10^{-3} M) and or PKA (150 μg); progesterone production was determined by radioimmunoassay.

absence of the inhibitor for 15 min at 37°C before the addition of 8-Br-cAMP (10^{-5} M). The incubation was continued for 60 min. The medium was kept for assay of corticosterone and the cells homogenized to obtain PMF. PMF from cells stimulated with 8-Br-cAMP in the presence of inhibitor was unable to activate mitochondrial steroidogenesis (Table 5). These results were confirmed by measurement of total corticosterone production in the incubation medium of the cells (pg corticosterone/105 cells; mean ± SD, $n = 3$: no addition, 83 ± 5; 8-Br-cAMP, 360 ± 30; BPB plus 8-Br-cAMP, 53 ± 2). To control for nonspecific effects of this inhibitor in the cell-free assay we performed two experimental approaches as follows: (a) PMF was obtained from cells incubated with ACTH and the inhibitor was added to the cell-free assay. The inhibitor had no direct effect on mitochondrial progesterone synthesis in response to PMF from ACTH-stimulated cells, (ng progesterone/incubation; mean ± SD, $n = 3$: no addition, 3.7 ± 0.3; ACTH, 91.4 ± 2.9; ACTH plus BPB, 94.6 ± 5.0. (b) In the

Table 5. Net progesterone production in mitochondria from rat adrenal zona fasciculata cells incubated with cAMP-dependent postmitochondrial fraction (PMF): effect of preincubation of the cells with inhibitor of arachidonic acid release. Values are mean ± SD, $n = 3$

Inhibitor	Progesterone (ng/vial) Control PMF	cAMP-dependent PMF
None	4.2 ± 0.4	89.5 ± 3.7
BPB	3.0 ± 0.3	5.6 ± 0.4

Adrenal zona fasciculata was incubated for 20 min at 37°C with or without bromophenacyl bromide (BPB) (10^{-11} M), followed by a 90 min incubation in the presence or in the absence of 10^{-5} M 8-Br-cAMP. The cells were homogenized and the PMF fraction was obtained and incubated with mitochondria fraction as described in "Materials and Methods".

presence of BPB and a PMF fraction obtained from nonstimulated cells, we studied the effect of exogenous arachidonic acid (10^{-5} M). Arachidonic acid was able to stimulate progesterone synthesis by adrenal mitochondria (28.2 ± 2.2 vs 3.7 ± 0.3 ng progesterone/incubation, in the presence vs in the absence of arachidonic acid, respectively). Arachidonic acid in the absence of PMF does not stimulate progesterone synthesis.

DISCUSSION

Studies with mutant clones of Y-1 adrenocortical tumor cells (adenylate cyclase and cAMP-dependent protein kinase deficient) provide strong evidence that cAMP and cAMP-dependent protein kinase are obligatory components of the ACTH-stimulated steroidogenesis [14]. These mutant clones were used to validate the use of the adrenal cell-free assay [9] to study the site of action of the cAMP-dependent protein kinase. Because cAMP-dependent protein kinases do not appear to have a high substrate specificity and catalyze the phosphorylation of numerous substrates, it is important to achieve specificity by intracellular localization. In this context, the characterization of the site of action of the cAMP-dependent protein kinase would substantially enlarge the possibility of characterization of an endogenous specific substrate(s). This could be provided by the phosphorylation of a physiological protein substrate(s) in intact cells in response to physiological ACTH concentration. However, because of the lack of an appropriate cell-free assay, no direct link between the specific protein phosphorylation and the activation of mitochondrial steroidogenesis has been established.

The results in the present paper demonstrate that stimulation of the adrenal zona fasciculata with ACTH or cAMP promote the intracellular formation of steroidogenic factors capable of stimulating mitochondrial progesterone synthesis. The results also demonstrated that at least one site of action of the cAMP-dependent protein kinase is in the cytosol. The cytosol as a physiological site for phosphorylation and intermediate in ACTH-stimulated mitochondrial progesterone synthesis has to fulfill several requirements: (1) cytosol derived from cells stimulated with ACTH or cAMP should be capable of increasing progesterone synthesis when is combined with the appropriate fraction; (2) cytosol derived from cyclase or kinase negative mutant cells should not; (3) cAMP or cAMP and cAMP-dependent protein kinase should phosphorylate *in vitro* a cytosol derived from nonstimulated adrenal cells and transform it into a cytosol capable of stimulating progesterone synthesis in mitochondrial from nonstimulated adrenal; (4) inhibitors of cAMP-dependent kinase should inhibit the *in vitro* effect of cAMP or cAMP-dependent protein kinase. Cytosol derived from cells stimulated with ACTH indeed fulfilled the criteria mentioned above. ACTH stimulation of cytosol factors is a rapid process observable with a half maximal stimulation at about 3 pM ACTH. The alteration of a cytosolic cAMP-dependent protein kinase activity in a mutant Y-1 adrenocortical tumor cell line follows the alteration in the steroidogenic response to ACTH or cAMP and in the activation of the cytosol. These observations imply that the activation of protein kinase during hormone action is a cytoplasmic event. The finding of a soluble protein in the cytosol as intermediate in the ACTH-stimulated mitochondrial steroidogenesis would strongly argue that, both, activation of protein kinase and phosphorylation, are restricted to the cytoplasmic compartment. We have isolated a soluble protein with such a characteristic [22]. These findings are in contrast to the hypothesis proposing the mitochondrion as a target for phosphorylation [3]. However, there is still the possibility of activation of other kinases as a cascade of events initiated in the cytosol with the phosphorylation of a cAMP-dependent substrate.

It is unlikely that the factor in the cytosol represents the free catalytic subunit of the cAMP-dependent kinase or the autophosphorylated kinase as described in other tissues because, the kinase in the absence of cytosol is not able to stimulate mitochondrial progesterone synthesis. The function of a cytosolic phosphorylation is still unclear. The inhibitory effect of BPB on the activation of mitochondrial steroidogenesis and the necessity of the microsomal compartment in order to stimulate mitochondrial steroidogenesis would suggest that the factor in the cytosol may play a role in arachidonic acid release. The fact that the inhibitors of arachidonic acid release can block the effect of the cytosol on microsomal compartment and mitochondrial steroidogenesis suggest that the phosphoprotein may be an activator of the ACTH-dependent phospholipase. This con-

cept is supported by recent publications on the role of phospholipase activation in the control of steroids synthesis [23–28]. Thus, the cytosol as a possible site of cAMP-dependent phosphorylation implies a direct demonstration of a cAMP-dependent kinase associated with the activation of arachidonic acid release and the activation of the side-chain cleavage of cholesterol. There is evidence from several experimental systems that a phosphoprotein, which can be phosphorylated *in vivo* or *in vitro* could be a phospholipase inhibitor, the activity of which can be regulated by phosphorylation [29]. It is clear that the identification of the putative substrate of the cAMP-dependent protein kinase would open a light in the understanding of the posttranslational control by cAMP upon the regulation of the cholesterol side-chain split.

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