Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 109-116

ORNITHINE DECARBOXYLASE AND POLYAMINE LEVELS ARE REDUCED IN CHO CELLS DEFICIENT IN cAMP-DEPENDENT PROTEIN KINASE

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Received June 8, 1982

A Chinese hamster ovary cell mutant with greatly reduced catalytic activity of cAMP-dependent protein kinase was compared with wild type cells having normal kinase activity for differences in biosynthesis, uptake and conjugation of polyamines. The inducibility of ornithine decarboxylase in response to cAMP, serum, human chorionic gonadotropin, asparagine and phorbol esters was greatly reduced in the mutant cells. Putrescine, spermidine and spermine levels rose 2-6 fold in wild type cells but in kinase mutant cells the basal and stimulated levels were generally lower. The cellular uptake and conjugation of radiolabelled putrescine and spermidine were reduced 5-10 fold in the kinase mutant cells. These results provide further evidence of the positive regulatory control exerted by cAMP-dependent protein kinase on polyamine biosynthesis.

Previous studies have suggested that the induction of ornithine decarboxylase (ODC) activity is regulated by a cAMP-dependent protein kinase mechanism (1,2). This hypothesis is based primarily upon the observations that elevations of cellular cAMP levels result in the activation of cAMP-dependent protein kinase followed by induction of ornithine decarboxylase activity and increased formation of cellular polyamines (1-3,5,6). While these studies provide a link between cAMP-dependent protein kinase and ODC induction, the actual association and mechanisms of regulation are currently unknown. Cyclic AMP-dependent protein kinase may activate the transcription of mRNA coding for ODC enzyme protein, however due to the complexity of ODC regulation this process may be only a small part of the entire regulatory scheme (1,3,6). Recently a polyamine dependent protein kinase capable of phosphorylating a 70,000 dalton nonhistone chromosomal protein has been described (7-9). This polyamine kinase activity is stimulated 80 fold by spermidine and spermine (7-9). In the phosphorylated state this protein binds to

Abbreviations used: ODC, ornithine decarboxylase; HCG, Human chorionic gonadotropin; SGM, salts/glucose medium; HPLC, high performance liquid chromatography. TPA, 12-O-tetradeconoyl phorbol-13-acetate; dBcAMP, dibutyryl cAMP.

0006-291X/82/130109-08$01.00/0

rDNA sites in the nucleolus and stimulates rRNA synthesis 5 fold. Polyamines are also conjugated to cellular proteins by transglutaminase (10,11) and nuclear putrescine conjugation correlates with the activation of cell growth (10), however the cellular role of this reaction is unknown.

In the present study, we have reconsidered the importance of cAMP-dependent protein kinase in regulating the polyamine biosynthetic pathway in mutant cells selected for a deficiency in the catalytic activity of cAMP-dependent protein kinase. Our results demonstrate that a reduction of cAMP-dependent protein kinase activity is associated with lowered induction of ODC, with reduced cellular levels of polyamines and finally with reduced uptake of polyamines from the culture medium.

MATERIALS AND METHODS

Parental CHO cells were maintained in either McCoy's 5a medium or αMEM medium supplemented with 10% v/v fetal bovine serum (Gibco, Inc.) at 37°C in a 5% CO_2/95% air atmosphere. CHO mutants (10260) were obtained from Dr. M. Gottesman (NCI, Bethesda, MD) and grown in αMEM medium fortified with 10% fetal bovine serum. Growth of wild type cells in McCoy's medium did not influence the results reported in the present manuscript. For all of the experiments reported here confluent cells were incubated in serum free medium for 16 to 18 hrs prior to their use. This allowed for optimal activation of ODC.

Ornithine decarboxylase activity was determined as previously described (12). High performance liquid chromatography (HPLC) procedures were used for measuring cellular polyamine levels. Detailed description of the method will be presented elsewhere (Lin et al, submitted to Anal. Biochem. 1982). Briefly, 1,10-diaminodecane (2.5 μg) was added to 1 ml of cell suspension and acidified with $HClO_4$ (0.4M final concentration). This mixture was homogenized, centrifuged for 15 min at 2400 xg and the supernatant neutralized with KOH. A 0.2 ml portion of this supernatant was mixed with 50 μl Na_2CO_3 saturated water and 100 μl of dansyl chloride solution (25 mg/ml acetone) in a Teflon lined screw cap vial. The vials were heated at 70°C for 30 min and then the mixture was evaporated to dryness by a N_2 stream. To the residue was added 0.5 ml of H_2O and 2.5 ml toluene. After vigorous mixing and centrifuging, the organic layer was removed, evaporated to dryness, reconsituted in 400 μl acetone and subjected to HPLC analysis. HPLC separation was made using a reverse phase C_{18} μ-Bondapack column. Mobile phases A and B were composed of acetonitrile, water, glacial acetic acid and tributylamine in the ratio of 40:60:0.02:005 and 95:5:0.02:0.005, respectively. Elution was carried out at a flow rate of 1 ml/min with a gradient starting at 80% A and 20% B and increasing in B strength to 85% in 25 min. Measurements of polyamines in the cells was made with fluorescence (Schoeffels GM 970 monochromator), by comparing with a polyamine standard curve the ratios of polyamine to 1,10-diaminodecane peak heights.

Radiolabelled polyamines were added to the medium to determine their uptake. Polyamine conjugation was measured by determining the trichloroacetic acid insoluble radioactivity associated with cells (10,11). Protein concentrations were determined using the Biorad Coomassie blue reaction and bovine serum albumen was used as the standard. Cell number was determined by a Coulter counter particle size analyzer.

TABLE 1

INDUCTION OF ORNITHINE DECARBOXYLASE IN WILD TYPE AND cAMP-DEPENDENT PROTEIN KINASE DEFICIENT CHO CELLS

ODC Activity (nmol CO_2/h/mg protein)

Time (h)	SGM[a]		SGM, ASN dBcAMP		SGM, ASN dBcAMP, HCG		Complete medium ASN, dBcAMP, HCG	
	WT[b]	PK(-)[c]	WT	PK(-)	WT	PK(-)	WT	PK(-)
2	0.02	0.03	0.13	0.20	0.22	0.09	3.11	0.02
4	0.05	0.02	0.34	0.29	0.60	-	34.50	0.48
6	0.02	0.02	1.68	0.56	4.61	0.91	-	0.49
8	0.03	0.03	4.75	0.60	16.20	0.26	62.11	0.86

[a] SGM, salts/glucose medium.

[b] WT = wild type CHO cells.

[c] PK(-) = CHO cells deficient in cAMP-dependent protein kinase activity (10260) (see ref. 15).

Wild type or kinase mutant (10260) CHO cells were grown to confluency, and then incubated in serum free media for 16-18 h . Cultures were then placed in the various solutions indicated in the Table. ODC activity was determined as described in the Methods section. SGM = 50 mM Hepes Buffer pH 7.2, 5 mM glucose, 100 mM NaCl, 2 mM $CaCl_2$ and 5 mM KCl. ASN = 10 mM, dBcAMP=0.5 mM, HCG = 20 µg/ml complete medium: αMEM containing 10% fetal bovine serum. Each value shown in the table is the mean of 3 determinations.

RESULTS

Previous studies have suggested two distinct mechanisms of ODC activation in intact cells (13). The activation of ODC obtained in conjunction with the addition of serum was markedly sensitive to inhibitors of RNA and protein synthesis while that obtained in the absence of serum was less sensitive to these inhibitors (13). Significant enhancement of ODC activity in cells maintained with a salts/glucose medium required the addition of any one of several amino acids (i.e. ASN, GLN, GLY, SER) and agents that elevate cellular cAMP levels (14). Table 1 shows the activation of ODC in wild type and in CHO cells deficient in cAMP-dependent protein kinase activity (15). ODC activity in wild type cells was enhanced minimally by ASN but more activation resulted following the addition of agents that elevate cellular cAMP levels while the addition of serum with ASN and agents that increase cAMP levels resulted in striking activation of ODC (Table 1).

TABLE 2

CELLULAR POLYAMINE LEVELS FOLLOWING ACTIVATION OF ODC IN WILD TYPE AND PROTEIN KINASE MUTANT CHO CELLS

Incubation time (h) (complete medium, ASN dBcAMP and HCG)	Putrescine nmol/mg protein		Spermidine nmol/mg protein		Spermine nmol/mg protein	
	WT	PK(-)	WT	PK(-)	WT	PK(-)
2	1.99	1.42	5.62	2.41	7.99	1.49
4	8.51	1.74	7.38	1.86	8.46	4.21
6	12.85	2.48	11.94	3.39	11.47	0.83
8	7.39	2.91	12.98	3.02	-	1.65

Wild type and protein kinase mutant cells were grown to confluency and incubated for 16-18 h in serum free medium. Complete medium supplemented with ASN (10mM), dBcAMP (0.5 mM), IBMX (0.1 mM), and HCG (20 µg/ml) was added for the indicated time periods. Polyamine levels were determined as described in the Methods section. Each value shown in the Table is the mean of 3 determinations.

In contrast to the wild type cells, ODC activity was only marginally enhanced by these conditions in the mutant cells (Table 1).

Phorbol esters enhanced wild type ODC activity 5-10 fold above those levels produced by conditions that activate this enzyme in the salts/glucose medium, (see Table 1) while in mutant cells only a 3.8 fold enhancement was obtained with the most ideal conditions. For example ODC activity (nmol CO_2/h /mg protein) obtained in the SGM with ASN, dBcAMP and HCG containing 0, 10, 100 µM TPA, respectively, was as follows, wild type: 10.55, 13.00, 67.72, and 105.86; mutants: 0.93, 1.24, 2.21, and 3.83.

The deficiency in protein kinase activity in 10260 CHO mutants appears to reside in the catalytic subunit rather than the regulatory subunit of the holoenzyme (15,16). Our own studies of protein kinase activity in these mutant suggest that they have approximately 10-15% of the cAMP-dependent protein kinase activity of wild type cells based upon activation of enzymatic activity by cAMP and effects of the Walsh protein kinase inhibitor (17). These findings are in agreement with the characterization of these mutants reported by Gottesman and colleagues (15,16).

TABLE 3

COMPARISON OF THE UPTAKE AND CONJUGATION OF PUTRESCINE AND SPERMIDINE IN WILD TYPE AND PROTEIN KINASE MUTANT CELLS

Cell Type	[^{3}H] Polyamine added (total incubation time)	Uptake and Conjugation (cpm/10^4 cells)					
		SGM		SGM & ASN		SGM, ASN, dBcAMP and HCG	
		Total	Conjugated	Total	Conjugated	Total	Conjugated
Wild Type	Putrescine (4h)	7,562	1,653	11,704	567	19,166	693
	Putrescine (6h)	10,190	672	24,420	889	16,589	1,280
	Spermidine (4h)	40,238	3,779	22,874	2,247	25,127	1,867
	Spermidine (6h)	25,512	1,606	58,336	3,668	44,582	719
Protein kinase mutant cells	Putrescine (4h)	1,066	285	3,433	170	3,028	113
	Putrescine (6h)	632	129	889	146	5,211	141
	Spermidine (4h)	9,742	691	10,887	613	13,176	561
	Spermidine (6h)	10,363	591	10,888	471	16,871	603

Confluent cultures of wild type or protein kinase mutant cells were placed in serum free medium for 16-18 h . Cultures were transferred to the salts/glucose medium conditions indicated in the Table (see Legend to Table 1 for abbreviations and concentrations). Upon transfer to the SGM, 1 μCi/ml of [^{3}H] polyamine was added to the cells for the time intervals shown in the Table. Following incubation, the monolayer was washed 3 times; cells were detached by trypsin treatment and the radioactivity associated with the cells determined. The conjugated polyamine was determined by measuring the radioactivity associated with the trichloroacetic acid insoluble materials following Millipore filtration. Each value shown in the table presents the mean of 2 plates. Similar results were obtained in 3 additional experiments.

Since the deficiency in cAMP-dependent protein kinase was associated with a similar lack of inducibility of ODC, we studied polyamine levels in both the wild type and mutant cells (Table 2). The levels of all three polyamines in wild type cells was increased 2-6 fold by serum, ASN, dBcAMP, and HCG. However, there was a much less striking elevation of polyamine in mutant cells in agreement with the lower inducibility of ODC associated with a protein kinase deficiency.

To further characterize the lower levels of polyamines in mutant cells, we studied polyamine uptake and conjugation. Putrescine and spermidine uptake was generally 5-10 fold higher in normal cells (Table 3). Similarly, polyamine conjugation was higher in normal cells compared with the mutants. The experiments shown in Table 3 were performed in a salts/glucose medium, however, similar results were observed by culturing cells in complete growth media.

DISCUSSION

Mutation resulting in reduced catalytic activity of cAMP-dependent protein kinase was associated with a similar reduction in the biosynthesis and accumulation of polyamines in intact cells. These findings are not surprising since ODC induction is thought to be mediated by a cAMP-dependent mechanism (1,2). However, the findings in this paper strengthen the validity of the link between cAMP-dependent kinase and polyamine biosynthesis. The lower level of polyamines in mutant cells is probably due to a deficiency in ODC inducibility and to a diminished uptake of polyamines from the medium. The role of polyamine transport and conjugation to proteins is currently not well understood. It has been proposed that polyamine conjugation may be related to cell growth in a manner similar to the relationship of ODC induction and cell growth (10). Addition of polyamines to cells inhibits the induction of ODC; the polyamines may induce a protein that inhibits enzymatic activity or they may attenuate ODC by activating a polyamine dependent protein kinase that specifically phosphorylates tyrosine residues on the ODC moiety (19). Recent evidence suggests that the ODC enzyme is complexed with the polyamine dependent protein kinase constituting the 70,000 dalton non-histone protein whose phosphorylation results in the activation of rRNA synthesis (7-9, 19).

Our laboratory has been particularly interested in the mechanism of activation of ODC by selected amino acids in cells maintained with a salts/glucose medium (14). Certain amino acids such as ASN permit the cAMP mediated activation of ODC and this activation does not appear to involve de novo enzyme synthesis but is dependent upon Ca^{2+} in the medium (13,14). At the present time, the mechanism for this activation is not understood however ODC activity may be regulated both

by post-translational modification and by transcriptional control (1,19). The polyamine dependent kinase (19) inactivates ODC activity directly while the role of cAMP-dependent protein kinase remains obscure. Direct covalent modification of the enzyme by cAMP-dependent protein kinase remains a possible regulatory control point, however this has not been demonstrated.

The extremely low inducibility of ODC by a variety of stimuli such as phorbol esters, serum, hormones, amino acids, and cAMP indicates a general deficiency in the ability of the kinase mutant cells to produce an active form of the enzyme either by induction or by other proposed mechanisms of ODC activation (13). These results strongly suggest that ODC induction even by phorbol esters is dependent upon cAMP-phosphorylation.

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