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CALCIUM, ASPARAGINE AND cAMP ARE REQUIRED FOR ORNITHINE DECARBOXYLASE ACTIVATION IN INTACT CHINESE HAMSTER OVARY CELLS

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Asparagine specifically activated ornithine decarboxylase activity 5-7 fold by 7-8 h in confluent cultures maintained with a salts/glucose medium. When dibutyryl cAMP was added with asparagine, a 40-50 fold stimulation of ornithine decarboxylase activity was produced. Ornithine decarboxylase activation in the salts/glucose medium was not sensitive to actinomycin D. Omission of Ca^{++} and Mg^{++} from the medium abolished the ability of asparagine and/or dibutyryl cAMP to stimulate enzyme activity. Calcium was essential for the asparagine and dibutyryl cAMP mediated stimulation of ornithine decarboxylase activity.

Introduction

Ornithine decarboxylase (L-ornithine decarboxylase E.C.4.1.1.17, ODC), the rate limiting enzyme for biosynthesis of polyamines has been extensively studied (1). This enzyme is of interest because: 1) it is rapidly induced during cell growth, proliferation, and transformation (2-4); 2) under certain conditions it displays one of the shortest half-lives of any known mammalian enzyme (2); 3) it is involved with biosynthesis of polyamines which function in RNA, DNA, and protein synthesis (5,6); and 4) its induction (de novo synthesis) appears to be directly under cAMP control in many (7-9) but not all systems studied (10).

Chen, Canellakis, and other workers have shown that the amino acids glutamine and asparagine stimulate ODC activity (11-13). In earlier studies

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Abbreviations used: ODC, ornithine decarboxylase; dB cAMP, dibutyryl cyclic AMP; CHO, Chinese Hamster ovary; IBMIX, 1-methyl, 3-isobutylxanthine; ASN, asparagine; PGE_1, Prostaglandin E_1.

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glutamine was used in complex medium (11,12); however, more recently, asparagine was found to enhance ODC activity in cultures maintained with a salts/glucose medium better than glutamine (13). Due to the complexity of tissue culture growth medium, the exogenous addition of a specific amino acid which stimulated ODC activity did not establish a cause and effect relationship as well as when the same experiments were performed with medium composed of only salts and glucose (11-13).

In this report we have studied the regulation of ODC activity by asparagine and cAMP in Chinese Hamster ovary (CHO) cells maintained with a salts/glucose medium. Optimal ODC stimulation in this system required the presence of asparagine, calcium, and dB cAMP.

MATERIALS AND METHODS

CHO cells (doubling time 14-16 h) were maintained in McCoy's 5a medium (14) as previously described (15-17). Confluent cultures of CHO cells grown in 100 x 20 mm Petri dishes were placed in serum free medium for 16-18 h and then transferred to the salts/glucose medium (Earle salts, Gibco, Inc.) which contained: $CaCl_2$:200mg; KCl:400mg; $MgSO_4$:97.7mg; NaCl:6,800mg; $NaH_2PO_4.H_2O$: 140mg; $NaCHO_3$:2,200mg; and glucose:1,000mg in 1 liter of water. Cells were incubated in this medium with or without other additions. In some cases the salts/glucose medium was prepared without calcium and/or magnesium to test the divalent ion requirement for ODC stimulation.

Ornithine decarboxylase activity was determined by measuring the liberation of $^{14}CO_2$ from [1-^{14}C] L-ornithine (15). Cells were isolated from the monolayer by scraping with a rubber policeman and collected by centrifugation at 2500 xg. Cell pellets were initially frozen in liquid nitrogen and then stored at -20°C overnight without loss of ODC activity. The cells were homogenized by freeze-thawing in 0.70 ml of 0.05mM phosphate buffer pH 7.2 containing 1mM dithiothreitol and 0.1mM EDTA. The homogenate was centrifuged at 50,000 xg for 10 minutes and 0.17 ml of the resulting supernatant was used for the enzyme assays as previously described (15). The decarboxylation of ornithine by ODC does not require O_2 while other pathways which could decarboxylate ornithine require O_2. Since there was no depression of enzyme activity by incubation in a nitrogen atmosphere it was concluded that the decarboxylation of ornithine was produced directly by ODC and not by other oxidative indirect pathways. CHO cells were routinely checked for mycoplasm contamination by the method of Peden (18). Protein determinations were made by the method of Lowry *et al.*, (19) using bovine serum albumin as the standard.

RESULTS

The activity of ODC was increased when asparagine was added to the salts/glucose medium (Fig. 1). However, more ODC activity was produced following incubation with both asparagine and dB cAMP (Fig. 1). The cAMP mediated stimulation of ODC however, required the presence of asparagine since dB cAMP addition alone produced little stimulation of ODC activity (Table 1). Other

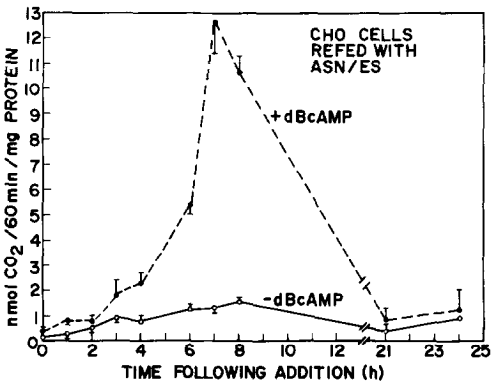

Fig. 1 Changes in ornithine decarboxylase activity following addition of asparagine and/or dB cAMP to cultures maintained with a salts/glucose (Earle salts) medium. Confluent cultures were placed in serum free medium for 16-18 h. The cultures were washed with Earle salts (ES) and then incubated in this medium supplemented with 10mM asparagine in the absence or presence of dB cAMP (1mM). At hourly intervals the cells were isolated as described in the methods section and ODC activity determined. Each point in the figure is the mean $\pm$ S.E.M. of 6 determinations from 2 separate experiments.

agents which increased cAMP levels such as IBMIX or PGE_1 also stimulated ODC activity but only in the presence of asparagine (Table 1). Note the synergistic enhancement of ODC activity following incubation with dB cAMP, IBMIX, and asparagine (Table 1). The enhancement of ODC activity by dB cAMP was concentration dependent from 0.05 to 1.0mM (Table 1). Maximal ODC activity was produced by 1mM dB cAMP in the presence of saturating asparagine (10mM) (Table 1). The addition of spermidine inhibited the dB cAMP and asparagine mediated stimulation of ODC activity (Table 1). The ODC stimulated by asparagine and/or dB cAMP in cells maintained with salts/glucose medium was not attenuated by continuous exposure to actinomycin D (4ug/ml) but was inhibited by cycloheximide (Table 2). Addition of fetal bovine serum to the salts/glucose medium also stimulated ODC activity alone and synergistically with asparagine, dB cAMP, and IBMIX. The stimulation of ODC activity produced in conjunction with serum was sensitive to both actinomycin D and cycloheximide (Table 2). The addition of asparagine from 1mM to 40mM could not substitute for the cAMP mediated enhancement of ODC activity (20).

Table 1

Effect of Various Agents
on Ornithine Decarboxylase Activity
in Cells Maintained with a Salts/glucose Medium

Other Agents Added	ASN(10mM)	Ornithine Decarboxylase Activity nmol/h/mg protein
-	-	0.18 ± 0.01
-	+	1.12 ± 0.10
dB cAMP(1.0mM)	-	0.37 ± 0.02
dB cAMP(0.05mM)	+	1.66 ± 0.04
dB cAMP(0.10mM)	+	1.79 ± 0.08
dB cAMP(0.50mM)	+	2.71 ± 0.10
dB cAMP(1.0mM)	+	4.34 ± 0.11
dB cAMP(2.0mM)	+	4.24 ± 0.27
Sodium butyrate(1mM)	+	1.30 ± 0.19
IBMIX(0.1mM)	+	1.89 ± 0.04
dB cAMP(0.05mM) and IBMIX(0.1mM)	+	7.66 ± 0.51
PGE_1(10uM)	+	4.21 ± 0.13
IBMIX(0.1mM)	-	0.28 ± 0.08
PGE_1(10uM)	-	0.31 ± 0.12
dB cAMP(0.5mM) and spermidine(50uM)	+	0.18 ± 0.01
PGE_1(10uM) and IBMIX(0.1mM)	+	6.94 ± 0.61

Confluent cultures of CHO cells were placed in serum free medium for 16-18 h. The cultures were washed two times with Earle salts and then incubated in this salts/glucose medium with the additions indicated in the table for 7.5 h ± 30 min After this incubation the cells were isolated by scraping them from the monolayer with a rubber policeman and ODC activity determined as described in the methods section. Each number shown in the table is the mean ± S.E.M. of 6 determinations from two separate plates.

Asparagine and dB cAMP could not stimulate ODC activity in a Mg^{++} and Ca^{++} free salts/glucose medium. Addition of Mg^{++} alone was not sufficient to permit asparagine and dB cAMP to stimulate ODC activity (Table 3). However, addition of only Ca^{++} allowed for the asparagine and dB cAMP mediated stimulation of ODC activity (Table 3).

Table 4 confirms that calcium but not magnesium was required for enhancement of ODC activity. When various levels of calcium were added to the Mg^{++}, Ca^{++} free salts/glucose medium, the activation of ODC by asparagine and dB cAMP

Table 2

Serum, dB cAMP, and ASN Stimulation of
ODC Activity and its Sensitivity to
Actinomycin D and Cycloheximide
in Cells Maintained with a Salts/glucose Medium

Additions	ODC Activity (nmol CO_2/h/mg protein)					
	4 h			8 h		
	No Inhibitor	Actinomycin D (4ug/ml)	Cycloheximide (25ug/ml)	No Inhibitor	Actinomycin D (4ug/ml)	Cycloheximide (25ug/ml)
ES	0.20	0.04	0.13	0.17	0.03	0.09
ES + ASN	0.42	0.40	0.29	0.97	1.04	0.78
ES + ASN + dB cAMP + IBMIX	1.21	1.15	0.71	3.23	2.68	1.73
ES + 10% fbs	0.37	0.25	0.27	0.71	0.09	0.24
ES + ASN + 10% fbs	0.86	0.23	0.21	3.22	0.27	0.16
ES + ASN + 10% fbs + dB cAMP + IBMIX	5.04	0.22	0.13	12.69	0.29	0.25

ES = Earle salts

fbs = fetal bovine serum

Confluent serum deprived cultures of CHO cells were washed two times with a salts/glucose medium and then placed in this Earle salts medium. Cultures were treated with the various agents shown in the table for 4 h or 8 h and then the cells were isolated and ODC activity determined as described in the methods section. The following concentrations of the agents shown in the table were added: ASN = 10mM; dB cAMP = 0.5mM; IBMIX = 0.1mM. Each number shown in the table is the mean of 3 determinations from one plate.

Table 3

Effect of Calcium and Magnesium
on the Enhancement of ODC Activity by ASN and cAMP

Conditions	Ornithine Decarboxylase Activity (nmol CO_2/h/mg protein)			
	$MgCl_2$(0.87mM) $CaCl_2$(1.82mM) (controls)	$CaCl_2$ (1.82mM)	$MgCl_2$ (0.87mM)	No Other Additions
Earle salts without Ca^{++} and Mg^{++}	0.28	0.26	0.25	0.28
Earle salts without Ca^{++} and Mg^{++} + ASN (10mM)	0.79	0.69	0.74	0.56
Earle salts without Ca^{++} and Mg^{++} + ASN (10mM), dB cAMP (0.5mM) and IBMIX (0.1mM)	5.48	4.54	1.21	0.98

Confluent cultures of CHO cells were placed in serum free medium for 16-18 h. The cultures were washed 2 times with Earle salts without Ca^{++} or Mg^{++}. Control cultures were incubated in complete Earle salts medium and some of the cultures were treated with ASN or ASN with dB cAMP (0.5mM) and IBMIX (0.1mM). Other cultures were placed in Ca^{++}, Mg^{++} free Earle salts medium and supplemented with the agents shown in the table. ODC activity was determined 7.5 h $\pm$ 30 min after these additions. Each number is the mean of 6 determinations from 2 separate experiments.

was dependent on the levels of Ca^{++} (Table 4). The ODC activity resulting from incubation with Ca^{++}, asparagine and dB cAMP was comparable to the levels produced by incubation with both Ca^{++} and Mg^{++}.

Additional studies were conducted to understand the mechanism and specificity of the asparagine and dB cAMP mediated stimulation of ODC. Incubation of cells with agents such as ornithine, aspartic acid, and NH_4Cl_2 could not substitute for asparagine in activating ODC with or without dB cAMP addition. Additionally, a number of attempts were made to activate either stimulated or unstimulated crude preparations of ODC isolated from CHO cells. However, *in vitro* incubation of ODC with ATP, Ca^{++}, Mg^{++}, cAMP, asparagine, protein kinase, and others, alone or in combination, did not enhance or depress enzyme activity.

DISCUSSION

The activation of ODC by asparagine and cAMP in cells maintained with the salts/glucose medium differed from its induction reported in other systems (2,

Table 4

Dose-Dependent Effects of Calcium on ODC Activity in Cells Maintained with a Magnesium and Calcium Free Salts/glucose Medium

$CaCl_2$ Added (mM)	Asparagine (10mM)	dB cAMP (0.5mM) IBMIX (0.1mM)	ODC Activity (nmol CO_2/h/mg protein)
0.0	-	-	0.30
0.0	+	-	0.46
0.0	+	+	0.28
0.1	+	+	0.62
0.5	+	+	1.97
1.0	+	+	2.89
2.0	+	+	5.26
5.0	+	+	4.68
10.0	+	+	0.48
2.0	+	-	0.82

Confluent cultures were placed in serum free McCoy's medium for 16-18 h. The cultures were washed 2 times with Ca^{++}, Mg^{++} free Earle salts and placed in this medium with the additions indicated in the table. After 7.5 h ± 30 min the cells were isolated and ODC activity was determined as described in the methods section. Each number is the mean of 3 determinations from one experiment.

3,18,21,22). As a reference point for discussion, the induction of ODC activity by serum addition to quiescent cultures (15) will be compared with its stimulation by asparagine (see results section) (13). Serum stimulation in complete medium enhanced ODC activity maximally by 3-4 h (15) while incubation of cells maintained with salts/glucose medium supplemented with asparagine and dB cAMP resulted in maximal ODC activity by 7-8 h. The ODC stimulation was extremely sensitive to inhibitors of RNA and protein synthesis added with serum (15, Table 2) while the asparagine or dB cAMP stimulation of ODC in the salts/glucose medium was not sensitive to similar treatment with actinomycin D (Table 2). However, some sensitivity of ODC activity to cycloheximide was found in the salts/glucose medium supplemented with asparagine and dB cAMP. These differences suggest that the mechanism of enzyme stimulation differed in these two systems. The enhancement of ODC by serum probably involved de novo synthesis

of the enzyme (15) while with the salts/glucose medium, asparagine and dB cAMP probably increased the half-life of the enzyme. In fact, Chen and Canellakis (13) have found that removal of asparagine from the salts/glucose medium caused the rapid disappearance of ODC activity having the usual half-life of 12.5 minutes while the half-life of ODC in the presence of asparagine was 200-400 minutes (13). Our findings also agree very well with their observations.

The mechanism by which asparagine, dB cAMP, and calcium enhance the ODC half-life remains unclear. These agents do not affect significantly total cellular RNA, DNA, or protein synthesis or the rates of protein degradation in this system (13, data not shown), suggesting some selectivity and specificity. We have tried to stimulate ODC activity using a number of agents related to asparagine in this system, such as aspartic acid, ornithine, and NH_4Cl_2, but were unable to mimic the specificity of asparagine in regulating and permitting the cAMP mediated stimulation of ODC activity. We also could not stimulate ODC activity by adding a variety of agents including asparagine directly to the isolated enzyme. It would appear that the increase in ODC half-life requires the integrity of the intact cell and probably involves one or more indirect mechanism(s). Further work is required to understand the mechanism by which asparagine, calcium, and cAMP exert their effects on ODC activity in cells maintained with a salts/glucose medium.

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