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SUPERINDUCTION OF ORNITHINE DECARBOXYLASE BY ACTINOMYCIN D AND CORDYCEPIN

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Cordycepin and Actinomycin D when added 2.0 - 3.5 hrs. following the addition of serum or serum and dibutyryl cAMP to quiescent cultures of Chinese hamster ovary cells, resulted in "superinduction" of ornithine decarboxylase at 4 hrs. If these agents were added from 0 to 2 hrs after stimulation, ornithine decarboxylase induction was diminished. In contrast, similar treatments with Puromycin or Cycloheximide did not enhance ornithine decarboxylase above serum or dibutyryl cAMP stimulated levels, but always attenuated its induction at all time intervals tested. Dibutyryl cAMP induced ornithine decarboxylase above the serum stimulated levels only when added during the initial 2 hrs. after stimulation with serum. These data and other observations presented suggest that transcriptional processes leading to the induction of ornithine decarboxylase occur primarily during the initial 2 hrs. after refeeding with serum and that the major action of dibutyryl cAMP in the induction of this enzyme appears to be at pretranslational sites.

Ornithine decarboxylase (ODC) (L-ornithine carboxylase E.C. 4.1 1.14) is the rate limiting enzyme for the biosynthesis of polyamines (1). It is found in almost all tissues and has the shortest half-life of any known mammalian enzyme (2,3). This enzyme is rapidly induced during the growth and proliferation of tissues presumably to produce polyamines which play a role in RNA and protein synthesis (4,5). The transcriptional and/or translational control of ODC may be mediated by a cAMP-dependent mechanism in a variety of tissues since the cAMP stimulated activity of this enzyme is sensitive to inhibitors of both RNA and protein synthesis and formation of antibody precipitable ODC molecules is enhanced by intracellular cAMP elevation (6-8).

Clark (9) reported on the superinduction of ODC by Actinomycin D but not by Cordycepin in serum stimulated 3T3 cells. Beck et al. (10) have reported that Puromycin administration to rats stimulated hepatic ODC activity and potentiated the activity due to dibutyryl (dB) cAMP administration. In the

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present study, the effects of RNA and protein synthesis inhibitors on the induction of ODC by serum or serum and dB cAMP were investigated using Chinese hamster ovary (CHO) cells. These inhibitors and dB cAMP were added at various times following serum refeeding, in an attempt to understand their temporal effects on processes leading to the induction of ODC.

MATERIALS AND METHODS

CHO cells were maintained in monolayer cultures as previously described (11-13) and routinely checked for mycoplasma contamination by the method of Peden (14). Confluent cultures were placed in serum free McCoys 5a media for 16-18 hrs. The cells were stimulated with fresh McCoys 5a media (15) containing 20% fetal bovine serum (Gibco, Inc.) in the absence and presence of dB cAMP. At various times after this stimulation cells were scraped with a rubber policeman from plates, (150x20mm) collected by centrifugation, and homogenized by sonication in 0.70 ml of 0.05 M phosphate buffer pH 7.2 containing 1mM dithiothreitol and 0.1mM EDTA. The homogenate was centrifuged at 50,000 xg for 10 min. and 0.17 ml of the resulting supernatant solution was used for assay. Ornithine decarboxylase activity was assessed by measuring the liberation of $^{14}CO_2$ from [1-^{14}C] DL-ornithine (New England Nuclear 40-50 m Ci/m mol). The reaction contained 0.05 M phosphate buffer pH 7.2, 25μM pyridoxyl phosphate, 1mM dithiothreitol, 1mM[1-^{14}C] L-ornithine (containing 1.25 cpm/pmol L-ornithine) 0.1mM EDTA in a final volume of 0.20 ml. The reaction was carried out in 15 ml conical glass centrifuge tubes, which were sealed with a rubber stopper supporting a polyethylene well containing a strip of Whatman 3MM filter paper, impregnated with 50μL of Protosol (New England Nuclear, 0.5 M). The reaction was allowed to proceed at 37°C for 60 min. and was terminated by injection of 2 ml of 1 M citric acid through the wells. The tubes were incubated at 37°C for an additional 30 min. and then the filter paper was counted in 5 ml of an Omnifluor-toluene mixture using a Packard liquid scintillation counter. In some experiments, cells were pulsed for 30 min. with [^{3}H] thymidine or [^{3}H] leucine or [^{3}H] uridine around the time intervals shown in the tables. The incorporation of these radiolabeled precursors into trichloroacetic acid insoluble material was determined by Millipore filtration.

RESULTS

Increases in ODC activity were apparent 2 hrs. following stimulation with serum or serum and dB cAMP (Table 1). Maximal ODC levels were found 4 hrs. after stimulation and enzyme activity declined rapidly to basal levels 5-6 hrs. after refeeding. The simultaneous addition of dB cAMP and serum enhanced ODC activity 2 fold above the serum stimulated levels. (Table 1) From 5-7 hrs. following refeeding with serum, a period of DNA synthesis occurred as suggested by the increase in thymidine uptake (Table 1). The increase activity of ODC produced by serum or serum containing dB cAMP is markedly diminished by placing either Cycloheximide, or Puromycin or Actinomycin D or Cordycepin in the stimulation media at 0 hrs. (Table 2). However, if these agents are placed in the media at

TABLE 1
CHANGES IN ORNITHINE DECARBOXYLASE ACTIVITY AND THYMIDINE UPTAKE
FOLLOWING SERUM AND dB cAMP STIMULATION

Time after stimulation (hrs.)	Serum		Serum and dB cAMP
	ODC Activity (nmol Co_2/60 min /mg protein)	[^{3}H methyl] thymidine uptake (cpm/10^6 cells)	ODC Activity (nmol Co_2/60 min /mg protein)
0	0.231 ± 0.031	1,216	0.289 ± 0.011
1	0.242 ± 0.014	1,304	0.311 ± 0.021
2	0.821 ± 0.052	1,373	1.013 ± 0.061
3	1.013 ± 0.073	1,510	1.928 ± 0.087
4	1.261 ± 0.113	1,492	2.413 ± 0.058
5	0.538 ± 0.032	4,312	0.512 ± 0.061
6	0.401 ± 0.017	7,039	0.358 ± 0.022
7	0.231 ± 0.015	10,321	0.316 ± 0.016
8	0.301 ± 0.022	11,191	0.333 ± 0.029
9	0.341 ± 0.016	10,873	0.288 ± 0.011

Confluent monolayers of CHO cells were placed in serum free McCoy's 5a media (15) for 16-18 hrs. The cultures were then fed with fresh media containing 20% fetal bovine serum in the absence and presence of 1mM dB cAMP. Enzyme activity was assessed at hourly intervals. (See Methods section for details.) Cultures were also pulsed with 1μci/ml of [^{3}H-methyl] thymidine for 30 min around the time intervals shown in the table and the incorporation of radiolabel percursor into trichloroacetic acid insoluble material was determined by millipore filtration. Each number for ODC activity represents the mean ± S.E.M. of 3 determinations for each of 2 separate plates. The [^{3}H-methyl] thymidine incorporation is the mean of 3 determinations from 1 plate.

various times after the initial stimulation, the effects seen differed. For example, by 1.5-2 hrs. after serum or serum and dB cAMP stimulation, Actinomycin D or Cordycepin no longer depressed ODC activity assayed at 4 hrs. In fact, after this initial 2 hrs. the addition of these two agents enhanced ODC activity above the serum or dB cAMP stimulated levels (Table 2). In contrast, ODC activity was depressed when Puromycin or Cycloheximide were added at all time points after stimulation (Table 2). Optimal superinduction of ODC by Actinomycin D or Cordycepin was found when these drugs were added 3 hrs. after stimulation and the enzyme assayed at 4 hrs (Table 2).

The superinduction of ODC by either Actinomycin D or Cordycepin was concentration dependent, whether the cells were stimulated by serum or serum containing dB cAMP (Table 3). Enhancement of [^{3}H] leucine uptake by Actinomycin D and Cordycepin was also concentration dependent, while [^{3}H] uridine uptake was markedly depressed by addition of these drugs at 3 hrs. following serum stimulation (Table 3).

TABLE 2

EFFECT OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS ON THE SERUM AND dBcAMP STIMULATION OF ORNITHINE DECARBOXYLASE ACTIVITY

Inhibitor	Time Interval (hrs)	Ornithine Decarboxylase Activity (nmol Co_2 liberated/60 min/mg protein)	
		Serum	Serum and dBcAMP
Cycloheximide (25 µg/ml)	0-4.0	0.111	0.171
	1.0-4.0	0.124	0.287
	2.0-4.0	0.512	0.831
	3.0-4.0	0.983	1.247
	2.5-4.0	1.011	1.493
Puromycin (50 µg/ml)	0-4.0	0.066	0.060
	1.0-4.0	0.091	0.113
	2.0-4.0	0.267	0.419
	3.0-4.0	0.711	1.238
Actinomycin D (4 µg/ml)	0-4.0	0.238	0.317
	1.0-4.0	0.999	2.332
	1.5-4.0	1.781	2.965
	2.0-4.0	1.952	3.356
	2.5-4.0	1.952	3.356
	2.5-4.0	2.014	4.123
	3.0-4.0	2.142	5.319
	3.5-4.0	1.914	3.278
Cordycepin (25 µg/ml)	0-4.0	0.161	0.179
	1.0-4.0	0.872	1.389
	1.5-4.0	1.431	2.287
	2.0-4.0	2.212	3.621
	2.5-4.0	2.487	4.381
	3.0-4.0	3.101	5.591
	3.5-4.0	1.421	3.413
Control (1)	No inhibitors (0-4 hrs)	1.411	2.631
Control (2)	No inhibitors (0-4 hrs)	1.321	2.512

Confluent cultures were stimulated with serum in the absence and presence of dBcAMP (1mM, see Methods). The cultures were incubated with the drugs shown in the table for the time intervals after serum stimulation indicated in the table. ODC activity was assessed 4 hrs. after serum stimulation as described in the legend to Table 1. Each number shown in the table represents the mean of 3 determinations from 2 separate plates. S.E.M.,for the numbers shown in the table (when expressed as a percentage of the mean) ranged from 3.21% - 11.68%.

Cycloheximide inhibited [^{3}H] leucine uptake about 90% and [^{3}H] uridine uptake by about 40%.

The enhancement of ODC activity by dB cAMP depended on the time at which this agent was added (Table 4). For example, if dB cAMP was placed in the media within the initial 2 hrs. after serum stimulation, ODC activity was elevated above the levels produced by serum alone (Table 4). However, if this agent was added after the initial 2 hrs. no enhancement of ODC activity was found at 4 hrs. (Table 4). The stimulation of ODC activity by dB cAMP occurred only when added during the initia

TABLE 3

EFFECTS OF CORDYCEPIN AND ACTINOMYCIN D ON ORNITHINE DECARBOXYLASE ACTIVITY, [^{3}H] LEUCINE AND [^{3}H] URIDINE UPTAKE

Drug	[^{3}H] Leucine Uptake cpm/10^6 cells	[^{3}H] Uridine Uptake cpm/10^6 cells	Ornithine Decarboxylase Activity at 4 hrs. nmol CO_2/60 min /mg protein	
			Serum	Serum and dBcAMP
No drug	452 ± 32	2934 ± 121	1.321 ± 0.071	2.432 ± 0.168
Actinomycin D (0.5 µg/ml)	514 ± 41	---	1.532 ± 0.083	3.096 ± 0.092
1.0 g/ml	623 ± 22	---	1.678 ± 0.042	3.824 ± 0.171
2.0 g/ml	678 ± 30	212 ± 16	1.937 ± 0.041	4.321 ± 0.162
4.0 g/ml	709 ± 43	203 ± 12	2.148 ± 0.053	5.413 ± 0.189
8.0 g/ml	804 ± 16	---	2.271 ± 0.062	6.164 ± 0.203
Cordycepin 1 µg/ml	672 ± 27	---	2.212 ± 0.092	3.892 ± 0.177
5 µg/ml	778 ± 43	862 ± 47	2.810 ± 0.103	4.913 ± 0.236
25 µg/ml	829 ± 38	412 ± 22	3.103 ± 0.079	5.532 ± 0.168
Cycloheximide (25 µg/ml)	46 ± 5	1821 ± 111	0.712 ± 0.102	1.103 ± 0.049

Confluent serum deprived cultures were stimulated with fresh serum. For ODC measurements the cultures were stimulated with serum in the absence and presence of dB cAMP. At 3 hrs. after this stimulation the drugs shown in the table were added and ODC activity determined 1 hr. later (see Methods section for assay procedures). In some cases the cells were pulsed for 3.5-4.0 hrs. with 0.2µCi/ml of [^{3}H] Leucine or [^{3}H] uridine. The incorporation of these isotopes into trichloroacetic acid insoluble material was determined by Millipore filtration. Each number represents the mean of 3 determinations from 3 separate plates. The dashed lines indicate that these conditions were not tested.

2 hrs., even if enzyme activity was determined at 5 or 6 or 7 hrs. following the initial stimulation with serum.

DISCUSSION

A number of interesting concepts and considerations about the mechanisms of cAMP-mediated induction of ODC in synchronous cell proliferation are presented. The concept of a critical time interval (early G_1) where cellular events are primed for the cAMP-mediated induction is suggested by the data shown in Table 4. Only during this critical time interval did dB cAMP enhance the induction of ODC above the serum stimulated levels. The concept that critical events are occurring during this time interval is further suggested by the effects of RNA and protein synthesis inhibitors on ODC activity. The opposite effects of Actinomycin D and Cordycepin addition prior to and following this

TABLE 4

Enhancement of Ornithine Decarboxylase Activity by dB cAMP Addition at Various Time Intervals Following Serum Stimulation

Time Interval (hrs.) with dB cAMP (1mM)	Ornithine Decarboxylase Activity nmol CO_2 Liberated/60min/mg Protein
Serum (0-4.0)	1.39
0 ↔ 4.0	3.00
0.5 ↔ 4.0	2.88
1.0 ↔ 4.0	3.11
1.5 ↔ 4.0	2.89
2.0 ↔ 4.0	2.99
2.5 ↔ 4.0	1.91
3.0 ↔ 4.0	1.21
3.5 ↔ 4.0	1.31
Serum (0-5.0)	0.61
0 ↔ 5.0	1.18
1.0 ↔ 5.0	1.22
1.5 ↔ 5.0	1.30
2.0 ↔ 5.0	1.07
3.0 ↔ 5.0	0.53
4.0 ↔ 5.0	0.36
Serum (0-6.0)	0.34
0 ↔ 6.0	0.47
1.0 ↔ 6.0	0.56
1.5 ↔ 6.0	0.61
2.0 ↔ 6.0	0.53
2.5 ↔ 6.0	0.27
3.0 ↔ 6.0	0.23
4.0 ↔ 6.0	0.14
5.0 ↔ 6.0	0.28
Serum (0-7.0)	0.28
0 ↔ 7.0	0.32
3.0 ↔ 7.0	0.24
4.0 ↔ 7.0	0.26

Confluent cultures of CHO cells were serum deprived for 16-18 hrs. These cultures were stimulated with fresh McCoys 5a media containing 20% fetal bovine serum. Some of the cultures received 1mM dB cAMP concomittant with serum addition while other cultures were allowed to incubate for various times prior to the addition of dB cAMP. Enzyme activity was assessed at 4, 5, 6 and 7 hrs. after serum stimulation. Each point represents the mean of 3 assays from 1 culture dish of cells.

critical time interval (see Table 2) suggest that ODC activity is regulated at transcriptional levels during early G_1 while after this time interval the events

regulating ODC activity are more complex and the effects of Actinomycin D and Cordycepin may involve similar processes attributed to "superinduction" such as depression of an inhibitor (10), increased half-life of the enzyme (9) or perhaps stimulation of protein synthesis. The latter point is interesting since, either Puromycin or Cycloheximide depressed ODC activity at all time intervals tested and Actinomycin D or Cordycepin enhanced [^{3}H] leucine uptake when added at 3 hrs. following serum stimulation (Table 3). Other investigators have observed in vitro stimulation of protein synthesis by Actinomycin D or Cordycepin in cell free protein synthesis systems (17).

Collectively, the above observations suggest that cAMP acts at the transcriptional level during the initial 2 hrs. following serum stimulation to induce ODC. An examination of what related events might determine why cAMP can only act during this critical time interval to enhance ODC induction, may relate to the cell cycle specific expression of type I cAMP-dependent protein kinase during this same time interval (11-13,16). The type I cAMP-dependent protein kinase is the predominant form of kinase present during early G_1 and there is only a small amount of type II cAMP-dependent protein kinase activity expressed during this time interval. After this time period, from mid to late G_1, the type II cAMP-dependent protein kinase is the predominant form. Since the major action of cAMP in cells is thought to be the activation of cAMP-dependent protein kinase, it is suggested that during this time interval cAMP activates type I cAMP-dependent protein kinase which then may phosphorylate nuclear proteins (18,19), which in turn enhances the transcription of ODC.

While these events seem to be very important and closely linked to the cAMP-mediated induction of ODC, other yet unknown processes may contribute significantly to the induction of ODC as well. However, continued studies of the involvement of cAMP in the induction of ODC should focus on the possible role of type I cAMP-dependent protein kinase regulating the transcription of ODC.

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