DOES CYCLIC-3',5'-AMP INDUCE GLUTAMINE SYNTHETASE IN EMBRYONIC NEURAL RETINA?

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SUMMARY: The inducibility of retinal glutamine synthetase (GS) by dibutyryl cyclic-3',5'-AMP (DB-cAMP) was re-examined in view of conflicting reports. Various lots of DB-cAMP were compared for a) ability to induce GS in cultures of embryonic chick neural retina, and b) their composition as visualized by paper chromatography. Chromatographically purified DB-cAMP did not induce retinal GS, nor did cAMP, DB-cGMP, epinephrine, or norepinephrine; none of these enhanced the induction of GS by hydrocortisone. Some of the agents occasionally caused small increases in GS activity; however, these were invariably below the GS levels induced consistently by hydrocortisone. A single lot of DB-cAMP was found which significantly raised GS activity in the retina; it contained a contaminant which when isolated was found to be responsible for this effect.

A rapid and multifold increase in the level of glutamine synthetase (GS) in the embryonic neural retina of the chick is associated with the onset of functional differentiation and maturation in this tissue (1). This enzymic increase can be induced prematurely and specifically in the retina in vitro by hydrocortisone (HC) or related 11ß-hydroxycorticosteroids added to primary cultures of embryonic retina tissue (1-7). The hormonal induction of retinal GS has been extensively studied as a model system of differentiation: it is a rapid, tissue-specific, gene-controlled increase in the rate of synthesis of the enzyme which does not involve ongoing cell replication or increase in overall protein synthesis (1-9). Concerning the effects of cyclic-3',5'-AMP (cAMP) and its N^6, $O^{2'}$- dibutyryl derivative (DB-cAMP) in this system, we found some time ago (2) that their additions to retina cultures did not raise GS activities to induced levels and did not enhance the induction of GS by hydrocortisone. However, more recently it was reported that cAMP and especially DB-cAMP induced GS in cultured embryonic chick retina (10). In view

of the significance of this general problem and the importance of the retinal GS system in studies on differentiation and gene regulation, we have re-examined this matter in detail. Our results showed conclusively that neither cAMP, DB-cAMP, nor N^2, $O^{2'}$ - dibutyryl cyclic-3',5'-GMP (DB-cGMP) increased GS levels in the retina to an extent characteristic of induction by corticosteroids. Furthermore, epinephrine and norepinephrine which reportedly increase cAMP activity in nervous tissue (13) did not significantly raise GS levels in cultured embryonic retina.

MATERIALS AND METHODS

Culture Methods: Neural retina tissue isolated aseptically from 12-day chick embryos was cultured in Erlenmeyer flasks on a gyratory shaker, as described before (3). Embryonated eggs were obtained locally or from Cobb farms in Littleton, Mass. While in our original experiments on this problem (2) our standard culture medium (3) was used, in most of the present experiments the culture medium was that used by Chader (10) in which reportedly there was induction of GS by DB-cAMP; it consisted of Eagle's basal medium with Hank's salt solution, glutamine, 5% dialyzed fetal bovine serum (Gibco), and 50 units/ml each of penicillin and streptomycin. Epinephrine, norepinephrine, theophylline, and DB-cGMP were from Sigma Chemical Co. Various lots of DB-cAMP were obtained from Sigma, Calbiochem, and Schwarz/Mann; three were kindly supplied by Dr. G. J. Chader (see Table 1). GS in retina cultures was induced by hydrocortisone (9.2×10^{-7} M; Pfizer Co.) added to the appropriate culture flasks at the beginning of cultivation.

Enzyme Assay: Glutamine synthetase specific activities were determined colorimetrically by our standard procedure (3,11) and according to a modification (12) used by Chader (10). Since both assay procedures yielded comparable ratios of experimental to control specific activities, the experiments reported here are only those assayed by the latter method.

Chromatographic Methods: Each lot of DB-cAMP used in this study was subjected to chromatography on Whatman 3MM chromatographic paper. The separ-

Table 1

The Effects of Various Lots of DB-cAMP on GS Specific Activity in Retina Cultures

Compound Number	Lot Number	Supplier	GS Specific Activity		Ratio	
			Average	Range	Average	Range
-	No Additions (Control)	-	2.2	1.5-3.6	1.0	0.7-1.6
1	71C-7171	Sigma	3.2	2.2-5.5	1.5	1.0-2.5
2	71C-7172	Sigma	3.0	2.6-3.3	1.4	1.2-1.5
3	41C-7373	Sigma	3.5	2.9-3.8	1.6	1.3-1.7
4	91C-7200	Sigma	4.2	2.7-5.1	1.9	1.2-2.3
5	91C-7062	Sigma	2.4	2.2-2.5	1.1	1.0-1.1
6	91C-7201	Sigma	2.3	1.8-2.7	1.1	0.8-1.2
7	91C-7360	Sigma	3.8	2.0-5.2	1.7	0.9-2.4
8	W-3528	Schwarz/Mann	4.0	3.2-4.8	1.8	1.5-2.2
9	60C-7374	Sigma	4.7	3.5-5.2	2.1	1.6-2.4
10	010255	Calbiochem	3.3	2.5-5.4	1.5	1.1-2.5
11	41C-7372	Sigma	16.4	11.4-24.5	7.5	5.2-11.1
-	Hydrocortisone	Pfizer	16.6	13.0-21.4	7.6	5.9-9.7

10^{-7} Concentration of DB-cAMP was 8×10^{-4} M. Concentration of HC was 9.2×10^{-7} M. Compounds 1-8 were from suppliers as indicated; compounds 9-11 were generously provided by Dr. G. J. Chader, Howe Laboratory, Harvard Medical School. Retinas were cultured for 20-24 hours, harvested, and assayed for GS activity (10,12). Units of specific enzyme activity are μmoles $\times 10^2$ gamma-glutamyl hydroxamate formed per mg protein per minute at 37°C. Values given are means from 3-8 experiments (3-4 retinas per experiment).

$$\text{Ratio} = \frac{\text{Experimental GS Specific Activity}}{\text{Average Control GS Specific Activity}} .$$

ated materials were localized under ultraviolet light. DB-cAMP spots were eluted with triple-distilled water; isolated impurity spots were eluted with 50% ethanol. The eluates were clarified by centrifugation, Millipore-filtered, and dried in vacuo at 30°C. The residual crystalline material was dissolved in culture medium and appropriate amounts were added to cultures to assay for GS induction.

Table 2

The Effects of Various Additives on GS Specific Activity in Retina Cultures

Additions	GS Specific Activity	Ratio
No Additions (Control)	1.48	1.00
cAMP	1.55	1.04
DB-cGMP	1.60	1.08
Theophylline	1.62	1.09
Epinephrine	2.19	1.48
Epinephrine + Theophylline	2.30	1.55
Norepinephrine	1.79	1.21
Norepinephrine + Theophylline	1.81	1.22
HC (Hydrocortisone)	18.63	12.59
HC + cAMP	18.59	12.56
HC + DB-cGMP	18.76	12.68

The following concentrations were used: cAMP and theophylline, 10^{-3} M; DB-cGMP, 8×10^{-4} M; epinephrine and norepinephrine, 10^{-4} M; HC, 9.2×10^{-7} M. Other conditions were as in Table 1.

RESULTS AND DISCUSSION

Table 1 summarizes the results of testing eleven different lots of DB-cAMP for ability to induce GS in the retina. Although our experimental conditions reproduced exactly those reported to give maximum GS induction with DB-cAMP (10), no GS inductions were observed with the lots tested, with the exception of the compound designated in Table 1 as number 11. In these particular experiments (Table 1) 8×10^{-4} M DB-cAMP was used since this concentration was reported to be inductive (10); however, extensive tests with other concentrations also gave negative results. Similarly, no GS inductions were obtained by cAMP, DB-cGMP, epinephrine, and norepinephrine (Table 2), nor did they enhance the typical induction of GS by hydrocortisone. Some of these chemicals occasionally caused minor increases of GS specific

activity; however these increases were always considerably below the magnitude characteristic for the induction of GS by hydrocortisone (see Tables 1,2).

Our results with compound number 11 (Table 1) were exceptional in that this particular sample of DB-cAMP caused a significant increase in GS specific activity of a magnitude similar to that reported by Chader (10). However, our negative results with all the other lots of DB-cAMP raised the possibility that the effect of number 11 might have been due to an unsuspected contaminant.

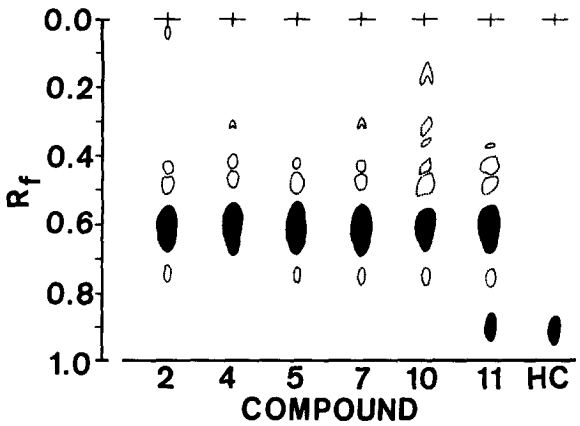

Figure 1. Paper Chromatography of several lots of DB-cAMP. Samples were dissolved in triple-distilled water and Millipore-filtered. Hydrocortisone (HC) samples were dissolved in absolute ethanol and filtered. The following quantities were chromatographed: 1000 µg DB-cAMP, 50 µg HC. The papers were equilibrated for 1.0-1.5 hours with 64% pyridine and then developed for 13 hours at 24-26°C in a descending fashion with a mixture of 45:25:40 parts n-butanol, pyridine, and distilled water. Light spots indicate trace impurities. DB-cAMP spots (R_f=0.61), the unique impurity in compound number 11 (R_f=0.90), and HC (R_f=0.91) are dark. Compound numbers are as in Table 1.

This possibility was tested first, by comparing various lots of DB-cAMP by paper chromatography and second, by assaying chromatographic eluates of materials isolated from number 11 for their ability to induce GS.

Figure 1 illustrates the chromatographic variants of several lots of DB-cAMP. All the non-inducing lots of commercial DB-cAMP tested exhibited trace amounts of impurities if large samples (500-1000 µg) were chromatographed. However, compound number 11 contained an additional distinct u.v.-absorbing spot (R_f=0.90) that was absent in the non-inducing lots of DB-cAMP.

Table 3

The Effects of Chromatographically Isolated DB-cAMP and Contaminant on GS Specific Activity in Retina Cultures

Additions	Concentration (μg/ml)	GS Specific Activity
None (Control)	-	2.23
DB-cAMP*(Not Purified)	250	14.10
Chromatographically Isolated DB-cAMP*	250	4.60
Chromatographically Isolated Contaminant	1	12.65

*The DB-cAMP used was compound number 11 (Table 1) - Sigma Lot Number 41C-7372. Other conditions were as in Table 1.

The solvent system employed effectively separated this contaminant from DB-cAMP (R_f=0.61). As shown in Figure 1, in this solvent system the R_f value of this particular contaminant in compound number 11 was very close to that of hydrocortisone.

Spots were eluted from the chromatograms of compound number 11 and tested for induction of GS. The results (Table 3) showed that the unique contaminant present in this batch of DB-cAMP caused a major increase in GS specific activity while the DB-cAMP spot eluted from the same chromatogram did not.

Thus, we have established that the only sample of DB-cAMP thus far found to elicit a significant increase in GS activity in retina cultures contained a contaminant which, when isolated and added to retina cultures at low concentrations caused, like hydrocortisone, a very significant increase in GS specific activity. We therefore conclude that this unidentified contaminant accounts for the "induction" of retinal GS previously attributed to DB-cAMP (10) While this by no means excludes the possibility that endogenous cAMP may play some role in the regulation of GS activity or turnover, our evidence does not indicate that this metabolite is causally involved in the induction of retinal GS. In this connection, it is also of interest that cAMP does not seem to

play a direct role in the steroid induction of tyrosine aminotransferase in cultures of hepatoma cells (14).

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