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Cyclic AMP Stimulation of Macromolecular Synthesis in Reaggregates of Embryonic Organs (39819)

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Nerve presides over a variety of developmental phenomena. The most outstanding examples are the trophic influence of nerve on the development and maintenance of muscle and the dependence on nerve for normal limb regeneration in the newt (1, 2). The possibility that nerve may exert a more general regulatory role in organogenesis was explored and it was found that nerve influences the regrowth of embryonic parenchymal organs in culture (3). The nature of the chemical and physical factors derived from nerve which are responsible for these effects is not known.

It has been shown that cyclic AMP and related compounds increase neurite outgrowth in explanted chick embryo dorsal root ganglia (4, 5). In order to stimulate axonal elongation and perhaps further enhance the growth-promoting effect of nerve, the influence of N^6,$O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP) on dispersed spinal and sympathetic ganglia, liver, and kidney cells was investigated (6). It is thought that DBcAMP probably exerts its effect by elevating intracellular levels of cyclic AMP. The present study reveals that the stimulatory effect of nerve on the growth and proliferation of kidney and liver reaggregates may be mimicked by low concentrations of DBcAMP. This lends supports to the notion that cyclic AMP plays a critical role in the regulation of embryonic growth and development (6, 7) and may itself be one of the "trophic" factors provided by nerve (8).

Materials and methods. Dorsal root and sympathetic chain ganglia and liver were obtained from 8-day chick embryos. Metanephros was removed from 11-day chicks. The tissues were dissociated with 0.25% trypsin (Gibco). The cells were counted on a hemocytometer and a total of 5×10^6 cells in 5 ml of medium 199 containing 5% horse serum, 5% embryo extract, 100 units/ml of penicillin, and 100 μg/ml of streptomycin was placed in 25-ml Erlenmeyer flasks. The flasks were gassed and incubated at 37° on a rotary shaker set at 80 rpm (9). Medium was changed after 2 days and the tissues were harvested at the end of 4 days.

The sodium salt of DBcAMP, obtained from Sigma Chemical Co., was added to the medium when the cells were first placed in culture and once again when the medium was changed. Duplicate flasks were set up for each concentration of drug in each tissue tested.

The flasks were double labeled with 5 μCi[^{3}H]thymidine and 0.5 μCi of [^{14}C]-leucine (0.36 Ci/mmole and 180 mCi/mmole, respectively) on Day 3 and harvested 18 hr later. The aggregates were collected by centrifugation, washed several times, and dissolved in 1 *N* NaOH at 37° for 1/2 hr. Protein and DNA were precipitated with TCA and the pellet was dissolved in 0.2 *N* NaOH. An aliquot was removed for counting in Bray's solution. Protein (10) and DNA (11) were determined colorimetrically in the remainder of the sample.

Results. The presence of 5 m*M* DBcAMP in the medium, a concentration reported to stimulate neurite outgrowth in explanted dorsal root ganglia (5), inhibits DNA synthesis by 96% and protein synthesis by 41% in reaggregates of dorsal root and sympathetic nerve cells. This concentration of DBcAMP also has an inhibitory effect on kidney and liver reaggregates; both DNA and protein synthesis are depressed 70 to 96% in these tissues. However, with successively lower concentrations of the nucleotide, enhancement in macromolecular synthesis is observed in all three tissues (Fig. 1). Nerve growth factor, used previously to enhance neurite outgrowth (3), has no effect on precursor incorporation into kidney

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and liver reaggregates.

Histologic examination of reaggregates exposed to 0.5–5 m*M* DBcAMP reveals many dead or vacuolated cells, large empty spaces with cell debris scattered throughout, and a great many loosely arranged spindle-shaped cells. Controls and cultures submitted to low concentrations of the cyclic nucleotide show the expected organization into compact aggregates composed of either characteristic renal tubules or typical hepatic parenchyma.

Whereas macromolecular synthesis is severely inhibited in reaggregates exposed to high concentrations of DBcAMP, low concentrations of the cyclic nucleotide enhance incorporation of labeled precursors into DNA and protein. In kidney and liver reaggregates, 1.5- to 3-fold increments in synthesis of both macromolecules are achieved with concentrations of DBcAMP as low as 5×10^{-3} and 5×10^{-4} m*M* (Fig. 1). The concentration of DBcAMP which most effectively enhances macromolecular synthesis in ganglia reaggregates is 5×10^{-2} m*M*. In general, DBcAMP appears to influence the incorporation of thymidine to a greater degree than it does the incorporation of leucine. In all cases, 5 m*M* DBcAMP depresses DNA synthesis over 90%, while protein synthesis is inhibited to a lesser degree.

Concomitant with the enhanced incorporation of radioactive precursors in the presence of low concentrations of DBcAMP, the amount of chemically measured DNA and protein per flask aliquot also increases (Table I). The increment in DNA in cultures treated with cyclic AMP is 51 to 95% above controls, whereas protein per flask aliquot is increased to a lesser extent (27 to 71%) when compared to control cultures grown in the absence of the cyclic nucleotide. Since increased incorporation of labeled precursors is paralleled by an increase in chemically measured DNA and protein, it seems likely that low concentrations of cyclic AMP stimulate growth and proliferation in reaggregates of chick embryonic liver, kidney, and ganglia.

5′-AMP and butyrate at concentrations of 0.05 to 0.0005 m*M* have no effect on macromolecular synthesis. Protein and DNA synthesis in mixed aggregates of nerve and

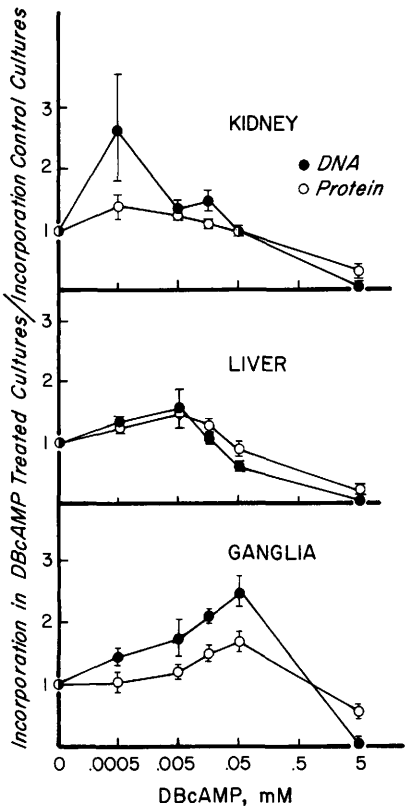

FIG. 1. DNA and protein synthesis in cultured reaggregates of kidney, liver, and ganglia exposed to various concentrations of DBcAMP is compared. The ratio of incorporation of [^{3}H]thymidine and [^{14}C]-leucine in cultures exposed to DBcAMP to incorporation in controls grown without the drug is plotted on the ordinate. The points on the graph represent the averages of several experiments, and the standard errors of the mean are indicated by the bars.

parenchymal cells are not consistently stimulated by low concentrations of DBcAMP, although the inhibitory effect at high concentrations is similar to that observed in the single tissue aggregates.

Discussion. DNA and protein synthesis are severely inhibited in chick embryo kidney, liver, and nerve reaggregates exposed to 0.5–5 m*M* DBcAMP. Although these concentrations are well within the range used by other investigators on cultured mammalian cells (12–14) and chick embryo dorsal root ganglia in culture (5), they appear to be toxic to reaggregates of chick embryo cells. In the experiments reported here, the cyclic nucleotide is added to the

TABLE I. THE EFFECT OF DBcAMP ON TOTAL PROTEIN AND DNA.[a]

DNA and protein content per flask aliquot (μg)	DBcAMP concentration (mM)				
	0.01	0.005	0.0005	0	R^b
Kidney					
DNA			2.42	1.60	1.51
Protein			177.0	137.8	1.28
		230.8		167.0	1.38
Liver					
DNA			2.65	1.69	1.57
		2.90		1.87	1.55
Protein	195.5			153.8	1.27
		293.1		222.6	1.32
Ganglia					
DNA		1.42		0.73	1.95
		1.33		0.85	1.56
Protein	187.8			131.1	1.43
		138.6		80.9	1.71

[a] Each flask was initially innoculated with 5×10^6 cells. The results are expressed per 10^6 cells initially innoculated or one-fifth the flask content.

[b] R is the Ratio of DNA or protein in cultures treated with DBcAMP to that in controls grown without added cyclic nucleotide.

medium when the cells are initially dispensed and again when the medium is changed. The aggregates are maintained in culture with DBcAMP for 4 days, similar to the time that cultured mammalian cells have been exposed to the cyclic nucleotide (13, 15). Macintyre and her colleagues (16) have also observed the cytotoxicity of DBcAMP concentrations above 0.3 mM on human tumor astrocyte lines.

Low concentrations of DBcAMP enhance macromolecular synthesis in chick embryo kidney, liver, and ganglia reaggregates. Similarly, low concentrations of DBcAMP (0.01 to 0.001 mM) have been reported to stimulate [^{14}C]leucine incorporation into newt limb regeneration blastemas *in vitro* (8). The findings presented here lend support to the notion of a dual role for cyclic AMP in the control of cell growth.

A stimulatory as well as inhibitory role for cyclic AMP in control of growth has been reported for other cell types. Physiological concentrations of cyclic AMP (10^{-8} to 10^{-6} M) stimulate DNA synthesis in cultures of rat thymic lymphocytes, but concentrations greater than 10^{-6} M inhibit growth (17). High concentrations of DBcAMP inhibit the growth of cultured melanoma cells, whereas lower concentrations of the nucleotide (10^{-5} M) enhance growth (18).

Originally, DBcAMP was added to mixed aggregate cultures in order to stimulate neurite outgrowth and perhaps further enhance the growth-promoting effect of nerve. However, macromolecular synthesis in mixed aggregates was not accelerated by exposure to low levels of the cyclic nucleotide. It may be that cell–cell interactions between nerve and parenchymal cells alter the intracellular cyclic AMP concentration such that the addition of small amounts of exogenous DBcAMP is not sufficient to cause a further detectable stimulation of synthesis.

There is a striking similarity between the increase in macromolecular synthesis when kidney or liver is grown in the presence of nerve (3) and the present finding of growth enhancement in the same tissues exposed to low concentrations of DBcAMP. Mixed aggregates of kidney and nerve cells synthesize from 1.5 to 3 times as much DNA, RNA, and protein as controls of each tissue alone or kidney and nonneural cells (3). Stimulation of thymidine incorporation exceeds that of leucine in parenchymal reaggregates cultured with nerve (3) similar to reaggregates exposed to low concentrations of DBcAMP. Thus, the stimulatory effect of nerve on the growth and proliferation of kidney and liver reaggregates is mimicked by very low concentrations of DBcAMP, suggesting that one neurotrophic factor may be cyclic AMP itself or another molecule which alters the intracellular levels of cyclic AMP in the target cell.

The similarity in effect between cyclic AMP and a nerve-related influence has been observed previously. Dissociated embryonic rat brain undergoes morphological transformation from epithelial cells devoid of cell processes to multipolar cells resembling mature astrocytes by exposure to either DBcAMP or extracts from adult rat brain (19). Greengard and his associates have accumulated a vast body of evidence indicating that cyclic AMP plays a role in synaptic transmission in sympathetic ganglia (20–22). It has also been suggested that the importance of neurotransmitters in development lies in their ability to regulate the synthesis of cyclic AMP (7).

Many investigators have suggested that cyclic AMP occupies a pivotal position in the regulation of embryonic growth and development (7, 23, 24). Rutter and his colleagues (23) have proposed that growth and differentiation may be inversely regulated by the same effectors. An increase or decrease in cyclic AMP levels may permit changes to occur between proliferation and differentiation in some tissues (24). This report suggests a possible role for cyclic AMP in the growth processes of embryonic parenchymal organs and sensory nerve. Further analyses of mixed aggregates which exhibit enhanced growth may provide useful information as to whether intracellular levels of cyclic AMP change when cells undergo active developmental transitions, such as during the course of organ resynthesis or regeneration.

Summary. The effect of DBcAMP on reaggregates of dispersed spinal and sympathetic ganglia, kidney, and liver cells is examined in order to define the nature of the growth acceleration that occurs when nerve is cultured with parenchymal tissue. Single cell suspensions prepared from chick embryo tissue are permitted to reaggregate on a rotary shaker. Measurement of the incorporation of labeled precursors into DNA and protein is used to monitor the effect of DBcAMP on the cultured reaggregates. The stimulatory effect of nerve on the growth and proliferation of kidney and liver reaggregates is mimicked by low concentrations of DBcAMP, suggesting a possible role for cyclic nucleotides in the growth processes of embryonic parenchymal organs.

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