Adenosine 3′,5′-Monophosphate

A MODULATOR OF EMBRYONIC CHICK CARTILAGE GROWTH

WARNER M. BURCH and HAROLD E. LEBOVITZ, *Departments of Medicine and Physiology, Duke University Medical Center, Durham, North Carolina 27710*

ABSTRACT We tested the hypothesis that cyclic AMP plays a significant role in modulating the growth of embryonic chick cartilage by determining whether cyclic AMP levels change in growing embryonic cartilage and whether cyclic AMP could stimulate embryonic cartilage growth in a long term in vitro organ culture. Cyclic AMP levels were low (0.1 pmol/mg wet wt) in 8-d chick embryo pelvic cartilage, and increased progressively through the 11th d of embryonic development at which time they reached a maximum (1.8 pmol/mg wet weight) and thereafter remained constant. We developed an in vitro organ culture system to determine whether cyclic AMP, a factor known to stimulate radiolabeled precursor incorporation into macromolecules in short-term studies does, in fact, stimulate growth of cartilage. Individual pelvic cartilages were isolated from 9-d chick embryos, placed in serum-free medium (BGJ_b-FJ modification) and incubated for 3 to 5 d during which time they increased in size (39 and 60% in length, respectively), wet weight (90 and 141%, respectively), and content of total soluble protein (30 and 48%, respectively). N^6-monobutyryl cyclic AMP (BtcAMP) added to the medium caused a dose-dependent (0.05 to 1.0 mM) stimulation of growth. After 3 d of incubation, 1.0 mM BtcAMP increased wet weight (125%), [^{14}C]leucine incorporation into protein (75%), and [^{3}H]thymidine incorporation into DNA (48%) compared with control cartilages incubated in medium alone. 1-methyl-3-isobutyl xanthine, a phosphodiesterase inhibitor, also increased cartilage growth above control while sodium butyrate, AMP, and ATP had no effect. Histological examination of cartilage grown in medium was similar to that of cartilage developing *in ovo*, whereas, cartilage grown in medium containing BtcAMP showed marked hypercellularity with many immature chondrocytes. Our observations are compatible with the hypothesis that cyclic AMP can significantly modulate the growth of embryonic cartilage.

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INTRODUCTION

While many hormones and growth factors are known to influence cartilage metabolism, relatively little is known about the basic mechanisms by which cartilage growth is regulated (1–3). Studies with developing embryonic cartilage suggest that cyclic AMP may be an important factor in regulating its growth. In vitro incubation of chick embryo pelvic cartilage with butyrylated cyclic AMP derivatives or agents that elevate intracellular cyclic AMP increases amino acid transport (4), and stimulates proteoglycan, total protein and total RNA synthesis (5). The increase in RNA synthesis is coordinate (6) and the stimulation of poly(A) RNA synthesis occurs by direct nuclear effects of a specific cyclic AMP-cytosolic cyclic AMP binding protein complex (7). Studies with developing limb buds in vitro show that cyclic AMP levels rise as mesenchymal cells are differentiating into cartilage (8). Nanomelic chick embryo cartilage cells respond to somatomedin or pressure stimulation in vitro with rises in intracellular cyclic AMP (9).

If cyclic AMP is a significant positive mediator of embryonic cartilage growth, it should be possible to demonstrate that in vivo growth is associated with increases in intracellular cyclic AMP and that cyclic AMP is able to stimulate growth of embryonic cartilage in long term organ culture in a defined serum-free medium. The present study examines both of these predictions.

METHODS

Measurement of cyclic AMP content of developing cartilage. Pelvic cartilage was removed on consecutive days from 8, 9, 10, 11, 12, 13, and 14-d chick embryos. The cartilages were rapidly cleaned, immediately frozen, and stored in liquid nitrogen until cyclic AMP measurements were made. 80 mg of cartilage were homogenized in 1.0 ml of 2.7% perchloric acid at 0°C. After centrifugation, 600 μl of supernate was removed and neutralized with 100 μl of 2.2 N potassium hydroxide. Following centrifugation, the protein-free supernate was collected and assayed in triplicate for cyclic AMP content by a previously described competitive protein binding

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assay method (10). Cyclic AMP is expressed as picomoles per milligram wet weight.

Prolonged organ culture of pelvic cartilage. Pelvic cartilages from 9-d chick embryos were removed, cleaned aseptically and placed in sterile BGJ_b medium (Fitton-Jackson modification). In vitro organ culture was performed by incubating cartilage in 2 ml medium in 30 mm plastic wells (Linbro Scientific, Inc., Hamden, Conn.) at 37°C in an atmosphere of 10% CO_2:90% room air. No antibiotics were added to the medium. Visible growth of the cartilages was evident after 1 d of culture. On days 1, 2, 3, 4, and 5 of the in vitro incubation (corresponding to total incubation of 10 through 14 d), six cartilages were removed, and the wet weight and length recorded. The medium was changed on day 3. The in ovo wet weight and length of six pelvic cartilages from the same batch of embryos and corresponding to the same days of total incubation (days 10 through 14) were also determined.

The effect of addition of N^6-monobutyryl cyclic AMP (BtcAMP)[1] to the medium was studied as follows: 9-d pelvic cartilages were cleaned and placed individually in 2 ml of medium containing 0, 0.05, 0.125, 0.25, 0.5, 1.0, and 2.0 mM BtcAMP. Three cartilages were used at each concentration of BtcAMP. The cartilages were incubated for 3 d then pulsed for 3 h with 2.0 μCi [^{3}H]thymidine and 0.2 μCi [^{14}C]leucine. The cartilages were measured, weighed, homogenized in 5% trichloroacetic acid, and centrifuged at 10,000 *g* for 15 min. The pellet was solubilized in Protosol and the acid-insoluble counts representing [^{3}H]thymidine incorporation into DNA and [^{14}C]leucine incorporation into protein were determined by liquid scintillation spectrophotometry.

In other experiments, the effect of 1-methyl-3-isobutyl xanthine (MIX) 0.25 mM, BtcAMP 0.5 and 1.0 mM, AMP 1.0 mM, ATP 1.0 mM and sodium butyrate 1.0 mM on growth was determined during 5 d of culture. Medium with fresh additives was changed on the 3rd d. Histology was examined in cartilage incubated in vitro for 3 d in medium alone or in medium containing 0.5 mM BtcAMP. The cartilages were fixed in buffered formaldehyde 3.7%, and tissue sections were prepared from paraffin blocks and stained with Alcian blue, and hematoxylin and eosin.

Total cartilage protein and DNA were determined on cartilages incubated in vitro for 3 and 5 d. Protein determination was done by homogenization of cartilages in 1.0 ml of 0.9% NaCl and 0.2% Triton X-100, centrifugation and measurement of the supernate protein by the method of Lowry et al. (11) using bovine serum albumin as the standard. DNA was measured by the diphenylamine reaction as described by Burton (12).

Materials. BGJ_b (Fitton-Jackson modification) medium was obtained from Gibco Laboratories, Grand Island Biological Co. (Grand Island, N. Y.). N^6-monobutyryl cyclic AMP, MIX, AMP, ATP and butyric acid were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tissue culture plates (76-045-05) were obtained from Linbro Scientific, Inc. Methyl-[^{3}H]-thymidine (50 Ci/mmol), [^{14}C]leucine (55 mCi/mmol), and Protosol were purchased from New England Nuclear (Boston, Mass.).

RESULTS

Cyclic AMP content of embryonic chick pelvic cartilage during in ovo growth. Total cyclic AMP content of embryonic chick pelvic cartilage during *in ovo* development is shown in Fig. 1. The earliest measurement that could be made was in 8-d embryos. Cyclic AMP content that was very low (0.1 pmol/mg wet wt) in pelvic cartilage in 8-d embryos rose progressively and reached a plateau of 1.8 pmol/mg of wet wt in 11-d embryos.

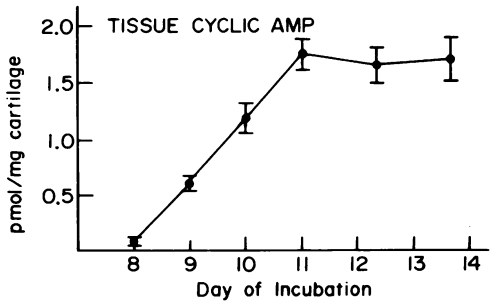

FIGURE 1 Cyclic AMP content of pelvic cartilages removed from 8, 9, 10, 11, 12, 13, and 14-d chick embryos. Cartilages were removed and cyclic AMP measured as described in Methods. Each point is the mean±SEM of three determinations.

In vitro and in ovo growth of embryonic chick pelvic cartilage. The pelvic leaflet cartilage of 9-d chick embryos *in ovo* weighs 4.1±0.1 mg and has a length of 7.0±0.5 mm. This is a convenient stage to initiate in vitro studies because any earlier stage of development makes accurate cleaning of surrounding mesenchymal tissue and preservation of the cartilage size and form of leaflet very difficult.

The organ culture system supports growth of the pelvic cartilage incubated in the medium alone. Over 3 d of in vitro incubation 9-d pelvic leaflets grow as measured by wet weight (7.8±0.5 mg) and length (9.75 ±0.4 mm). These represent increases of 90% in weight and 39% in length. After 5 d in vitro culture there was an increase of 141% in weight and 60% in length (Figs. 2 and 3). This increase in the in vitro weight and length was significantly less than that of the pelvic cartilage corresponding to the same day of incubation in ovo (Figs. 2 and 3). The amount of total protein increases from 81±6 μg/cartilage to 106±4 μg/cartilage after 3 d and 120±5 μg/cartilage after 5 d of in vitro incubation (Table I). Dry weight increases by 90% at 3 d and 118% at 5 d and DNA content increases by 55% at 3 d (Table I).

The gross shape and form of the cartilage grown in vitro for 3 and 5 d are similar to that of the corresponding *in ovo* cartilage. However, they are significantly smaller in size. Microscopic examination of cartilage grown in medium in vitro for 3 d appears similar to *in ovo* embryonic pelvic cartilage of the same age (12 d) (Fig. 4A). The cells are organized in regular patterns within a normal appearing matrix. The nuclei are oval to round with no unusual shapes.

Effects of cyclic AMP on in vitro cartilage growth.

[1] *Abbreviations used in this paper*: BtcAMP, N^6-monobutyryl cyclic AMP; MIX, 1-methyl-3-isobutyl xanthine.

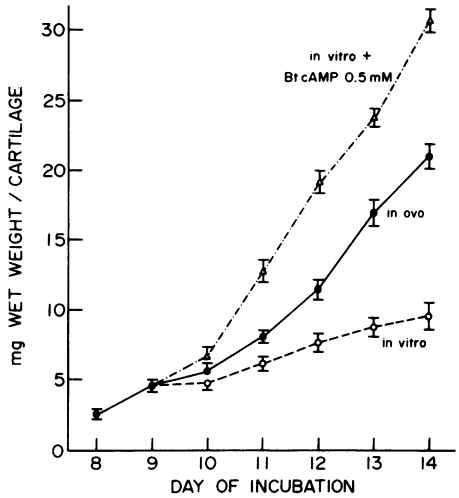

FIGURE 2 Comparison of embryonic chick pelvic cartilage weight with day of incubation *in ovo*, in vitro, and with incubation with BtcAMP 0.5 mM. The day of incubation corresponds to *in ovo* incubation period. At day 9, cartilages were removed and in vitro incubations initiated. The medium was changed on day 12 (3rd d in vitro). Each point represents mean (±SEM) of six cartilages. By day 11 (2nd d of in vitro incubation), there are significant differences in the wet weight (milligrams) among all three groups.

Addition of BtcAMP to the medium markedly increases the rate of growth of cartilage incubated in vitro. The increase is readily apparent by 24 h of incubation. After 3 d of incubation in medium containing 0.5 mM BtcAMP, there is a 340% increase in wet weight over its initial weight as compared with a 90% increase in wet weight of cartilage incubated in medium alone. Incubation of pelvic cartilage with BtcAMP results in a dose-dependent increase in wet weight, [^{14}C]leucine incorporation into protein, and [^{3}H]thymidine incorporation into DNA (Fig. 5). After 3 d of in vitro incubation at a maximal stimulating dose of BtcAMP (1.0 mM), the wet weight increased 125%; [^{14}C]leucine incorporation increased 75%; [^{3}H]thymidine incorporation increased 48% above cartilage incubated in medium alone. Total DNA content and dry weight increased 126 and 90%, respectively (Table I). The increase in length of cartilage grown in medium containing BtcAMP paralleled that of comparable cartilage grown *in ovo* (Fig. 3). After 4 and 5 d of in vitro incubation the length of the cartilage was slightly less than *in ovo*. However, this is not really an accurate assessment of growth since the ends of the cartilage incubated in medium containing BtcAMP curl and have a tendency to fold over toward the center area of the cartilage. This is probably due to loss of supporting matrix that is present *in ovo* but not in vitro. The BtcAMP-treated cartilages are soft and flexible and difficult to pick up with forceps. The weight, however, of these cartilages is significantly greater than that of comparable cartilage grown *in ovo* (Fig. 2). After 5 d of incubation, total soluble protein is 87% greater in cartilage incubated in BtcAMP than in medium alone (Table I). Thus, we have demonstrated that BtcAMP stimulates growth in vitro in a dose dependent matter as measured by changes in wet weight, length, total protein, [^{14}C]leucine incorporation into protein, and [^{3}H]thymidine incorporation into DNA. Corresponding increases in dry weight and DNA content also occur.

Microscopic examination of cartilage incubated in BtcAMP 0.5 mM demonstrate findings that are quite different from cartilage incubated in medium alone (Fig. 4B). Marked hypercellularity exists with nuclei scattered in a disorganized pattern. The predominant nuclear form is spindle-shaped (characteristic of immature chondrocytes). The matrix stains well with alcian blue but appears decreased in relation to the large number of nuclei.

A comparison of the effects of incubating pelvic cartilages for 3 and 5 d in medium alone, medium containing BtcAMP, and medium containing MIX, a phosphodiesterase inhibitor that increases intracellular cyclic AMP (0.25 mM increases cyclic AMP to 2.3±0.2 pmol/mg cartilage as compared to control of 1.4±0.1 pmol/mg cartilage after 3 d of in vitro incubation) are shown in Fig. 6. Incubation in medium containing MIX increases wet weight above controls at 3 and 5 d (50 and 85%, respectively). MIX 0.25 mM also increases DNA content 49±3% above that of cartilage incubated in medium alone. Again BtcAMP increases wet weight

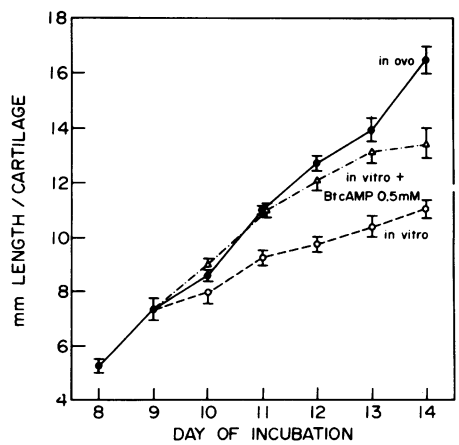

FIGURE 3 Comparison of embryonic chick pelvic cartilage length with day of incubation *in ovo*, in vitro, and with incubation with BtcAMP 0.5 mM. These groups of cartilage are the same as in Fig. 2. Again, by day 11 (2nd d of in vitro incubation), there is significant difference in length (millimeter) among cartilages incubated in vitro as compared to those *in ovo* and with BtcAMP in vitro ($P < 0.02$). By day 14 (day 5 in vitro), there were significant differences among all groups ($P < 0.05$).

TABLE I

Effect of Incubation on Wet and Dry Weight, DNA Content, and Total Soluble Protein

	Day of in vitro incubation				
		3		5	
	0	Medium	Medium + BtcAMP	Medium	Medium + BtcAMP
Wet weight, *mg/cartilage*	3.7±0.4 (8)	6.6±0.2 (8)	15.5±0.4 (8)*	8.5±0.4 (6)	35.9±1.3 (6)*
Dry weight, *mg/cartilage*	0.298±0.017 (8)	0.565±0.019 (8)	1.073±0.042 (8)*	0.65±0.05 (6)	1.96±0.05 (8)*
DNA, *μg/cartilage*	8.0±1.0 (3)	12.4±0.8 (3)	28±1.2 (3)*		
Total soluble protein, *μg/cartilage*	81±6 (3)	106±4 (3)	127±3 (3)‡	120±5 (3)	225±4 (3)*

Wet and dry weight, DNA, and total soluble protein in embryonic pelvic cartilage incubated in medium alone and in medium containing BtcAMP 1.0 mM for 3 and 5 d. Values are expressed as mean±SEM. The number of cartilages per group is in parenthesis. Dry weight was determined after drying to constant weight (2 h at 60°C). DNA and total soluble protein were determined as described in Methods.

* $P < 0.001$ vs. medium alone; ‡ $P < 0.02$.

at 3 and 5 d, respectively. Addition of AMP 1.0 mM, ATP 1.0 mM, or sodium butyrate 1.0 mM to the medium did not increase cartilage wet weight or length above that occurring in medium alone (data not shown). The microscopic exam of cartilage incubated in MIX (0.25 mM) was similar to that of BtcAMP-treated cartilage, but with significantly less hypercellularity. Thus, elevation of intracellular cyclic AMP also increases growth of the pelvic cartilage at 3 and 5 d of in vitro incubation.

DISCUSSION

The data presented are compatible with but do not conclusively prove the hypothesis that cyclic AMP is one of the positive modulators of embryonic cartilage growth. Intracellular cyclic AMP is very low in pelvic cartilage from 8-d chick embryos, increases remarkably from day 9 through 11, then plateaus. The absolute levels achieved are between 1.5 and 2.0 pmol/mg wet wt of cartilage. Since the embryonic pelvic cartilage is ~90% extracellular water (4) this would be equivalent to an intracellular cyclic AMP concentration of 15–20 μM. The remarkable growth response of the pelvic cartilage to BtcAMP in vitro in organ culture in a defined serum-free medium indicates that cyclic AMP can stimulate the growth of this tissue. The minimal in vitro stimulatory concentration of BtcAMP was 50 μM and the dose response range was to 1 mM. Considering barriers to penetration and potential metabolic degradation, these concentrations are not very disparate from the physiological levels measured in rapidly growing cartilage. Further support that physiological elevation of cyclic AMP may indeed stimulate cartilage growth are the in vitro results obtained with 0.25 mM MIX which raises cyclic AMP levels to slightly over 2 pmol/mg wet wt of cartilage.

The marked alteration in morphology of pelvic cartilage incubated in vitro in medium containing BtcAMP or MIX as compared with cartilage developing *in ovo* raise concern as to whether the in vitro organ culture effects of cyclic AMP on growth can be extrapolated to in ovo effects of increased tissue cyclic AMP levels. Thus one alternative interpretation of the data that could be considered is that the in vitro cyclic AMP effects represent transformation of chondrocytes, which leads to morphological and biochemical evidence of growth, but is not representative of any *in ovo* effect on normal cartilage growth. If that were so, then the *in ovo* increases in cyclic AMP levels might be related to cartilage maturation or the onset of ossification, rather than to the stimulation of growth. Our data do not allow us to exclude such alternative possibilities, but the difference in morphology between cyclic AMP stimulated cartilage in organ culture and that in cartilage developing *in ovo* could just as likely be explained by the absence of hormones known to inhibit cartilage growth (glucocorticoids) or stimulate cartilage maturation (thyroid hormones) from the in vitro system. The in vitro serum-free organ culture system is a powerful tool for investigating the mechanisms of specific hormones or factors on cartilage growth and metabolism. The extrapolation to *in ovo* effects will always present difficulties and controversies as do extrapolation of the results of all in vitro studies.

The results of the long term organ culture studies confirm that the increases in radiolabeled precursor incorporation into macromolecules noted with our previous 6- to 18-h incubations of embryonic chick pelvic cartilage with butyrylated cyclic AMP derivatives and

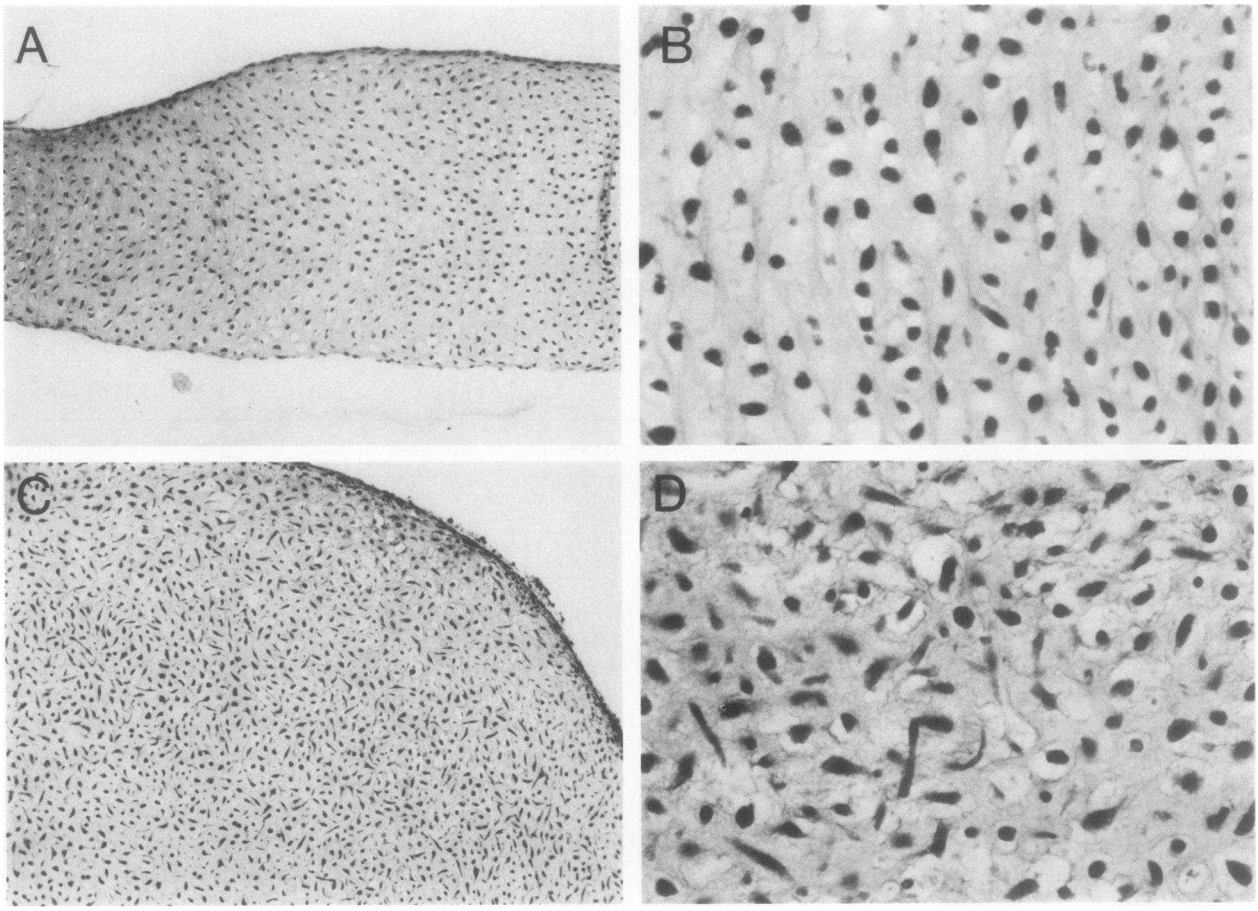

FIGURE 4 Photomicrograph of chick embryonic pelvic cartilage incubated for 3 d in medium alone (A, B) and medium containing BtcAMP 0.5 mM (C, D). (A) The cellular pattern is normal and is comparable to that of *in ovo* cartilage (×100). (B) Higher magnification of A (×400). (C) There is marked hypercellularity and disorganization of nuclear pattern as compared to A (×100). (D) Higher magnification of C (×400) demonstrating spindle-shaped nuclei.

phosphodiesterase inhibitors were accurate predictors of the growth promoting activities of cyclic AMP in cartilage (5, 6). The histologic observations, the total DNA content and the thymidine incorporation data after 3 d of culture in medium containing BtcAMP are conclusive evidence of this growth promoting activity.

Although our data show that cyclic AMP probably modulates embryonic cartilage growth, they neither identify the factors responsible for the increases in intracellular cyclic AMP that occur during development nor define the specific mechanisms by which cyclic AMP stimulates growth. Several years ago we demonstrated that some growth hormone dependent factor in normal serum increases cyclic AMP levels in embryonic chick pelvic cartilage incubated in vitro for 2 to 4 h (4). Partially purified somatomedin C has been reported to inhibit cartilage membrane adenylate cyclase activity (13). However, Bourret et al. (9) demonstrated that somatomedin increased cyclic AMP in nanomelic cartilage cells but not normal ones. Parathyroid hormone (unpublished observations) and prostaglandin E and F increase embryonic pelvic cartilage cyclic AMP during in vitro incubations (14). Whether these factors, various IGF or some unknown hormone is responsible for the *in ovo* rise in pelvic cartilage cyclic AMP must be answered by future studies. The mechanism by which cyclic AMP causes growth is equally elusive. Since cyclic AMP can directly activate gene transcription in embryonic chick pelvic cartilage the growth might result from direct effects on nuclear DNA synthesis and transcription. Another possibility is that cyclic AMP may stimulate the cartilage to make its own growth factor.

The in vitro organ culture system described in this study allows one to study factors that affect growth over a long time interval with definite increases in parameters that are easily quantitated. The pelvic leaflet grows in serum-free medium over 5 d. The growth

of the cartilage in the absence of serum is unusual. In most tissue culture systems there is an obligatory requirement for growth factors present in serum. The fact that this cartilage grows in the absence of any growth factor in the medium may be related to several possibilities. The tissue is embryonic and thus is at a stage that is genetically programmed for growth. Cell to cell interactions are maintained in this organ culture and this may be important in growth. The cartilage itself may make a growth factor that can stimulate growth. In this regard, Klagsburn et al. (15) have isolated a polypeptide growth factor from bovine calf scapular cartilage that stimulates DNA synthesis and cell division of resting bovine chondrocytes and confluent BALB/c 3T3 cells.

The effect of cyclic AMP as a mediator of growth is dependent on the tissue and cell type studied. Most cell and tissue culture systems have demonstrated that cyclic AMP or its derivatives inhibit growth. This is dose dependent as determined by DNA replication and cell death. Ryan and Heidrick (16) reviewed over 20 different cell culture types all of which demonstrated that increasing intracellular cyclic AMP produced inhibition of growth. A notable exception is rapidly responding thymus lymphocytes in which cyclic AMP through a calcium dependent process increases DNA synthesis and cell growth (17).

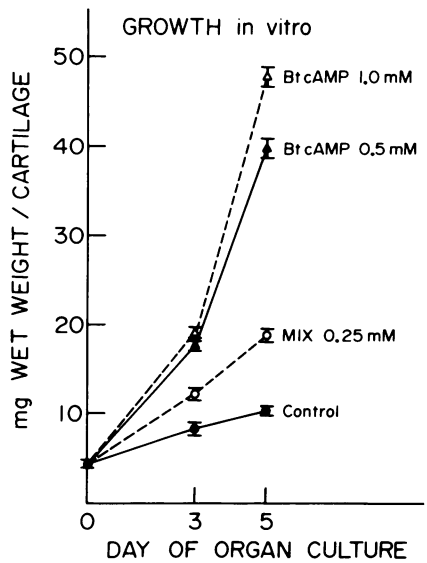

FIGURE 6 Comparison of wet weight of pelvic cartilages incubated in medium alone (control), medium containing MIX 0.25 mM, or BtcAMP for 3 and 5 d. Each point is mean (±SEM) of four cartilages. Medium with fresh additives was changed on 3rd d.

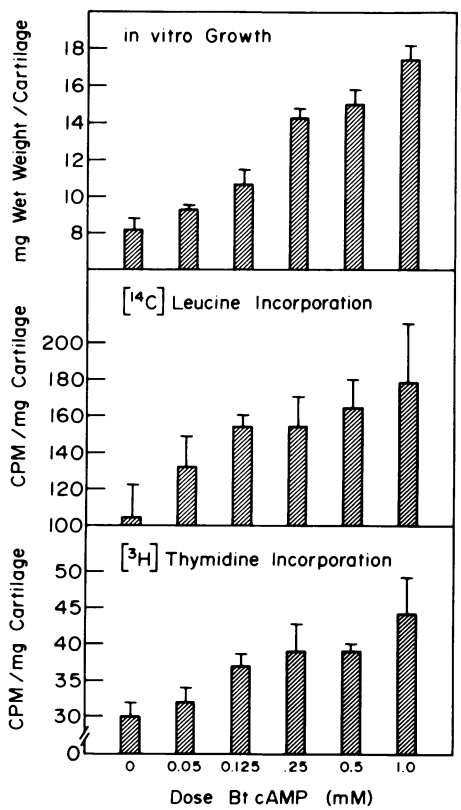

FIGURE 5 Dose response in wet weight, [^{14}C]leucine incorporation, and methyl-[^{3}H]thymidine incorporation of BtcAMP-treated cartilage after 3 d incubation. Cartilages (n = 3) were pulsed for 3 h with 2.0 μCi [^{3}H]thymidine and 0.2 μCi [^{14}C]-leucine for each dose of BtcAMP. The cartilages were weighed (top panel) and acid insoluble incorporation expressed as counts per minute per milligram of wet weight cartilage determined.

Our data demonstrating cyclic AMP as a positive effector of growth are consistent with cyclic AMP-mediated effects in vivo. Two examples in which cyclic AMP stimulates organ growth are isoproterenol-induced cardiac (18) and mouse salivary gland hypertrophy (19). In whole organ systems, most of cyclic AMP anabolic and potential growth effects have been inferred through the action of cyclic AMP in stimulating RNA synthesis. Cyclic AMP increases RNA synthesis in regenerating rat liver (20), ovariectomized rat uterus (21), bovine and porcine thyroid (22, 23), beef adrenal medulla (24), and chick embryonic pelvic cartilage (5, 6). To our knowledge this report of stimulation of growth by cyclic AMP in the embryonic chick pelvic cartilage is unique in that whole organ growth is demonstrated in a serum-free in vitro system.

The microscopic findings of cartilage incubated in medium containing 0.5 mM BtcAMP show that the cells are rapidly dividing. The morphological changes and the increased total DNA content and radiolabeled thymidine incorporation into DNA suggest that cellular hyperplasia is a major component of cyclic AMP stimulated growth in this tissue. Preliminary studies with cartilage incubated with thyroid hormone (25), insulin

and multiplication stimulating activity (unpublished observations) show little or no effects on cellular hyperplasia indicating that the changes seen with cyclic AMP may be unique.

In conclusion, this in vitro organ culture system, which is serum-free, has great potential for defining the effects of various growth factors on cartilage and delineating the mechanisms of their specific effects on cartilage growth and maturation. Our observations that cyclic AMP markedly stimulates cell proliferation and growth of embryonic cartilate as an intact organ suggest that it may be one of the important mediators for growth in this tissue.

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