In Vitro Stimulation of Alkaline Phosphatase Activity in Immature Embryonic Chick Pelvic Cartilage by Adenosine 3′,5′-Monophosphate

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

ABSTRACT Cyclic AMP content in embryonic chick pelvic cartilage increases significantly as the embryo ages from 8 to 10 d. This *in ovo* elevation in cyclic AMP content precedes maximal cartilage alkaline phosphatase activity by some 24 h. We studied whether this temporal relationship may be causally related, using an in vitro organ culture. Incubation of pelvic cartilage from 9- and 10-d embryos in medium containing monobutyryl cyclic AMP (BtcAMP) resulted in significant increases in alkaline phosphatase activity (220 and 66%, respectively) as compared to that of cartilages incubated in medium alone. This stimulation was both concentration- and time-dependent with maximal response at 0.5 mM BtcAMP and 4-h incubation, respectively. Similar incubations of cartilage in medium containing 1-methyl-3-isobutyl xanthine (MIX), 0.25 mM, also resulted in increased alkaline phosphatase activity (114%). However, pelvic cartilage from 11-d embryos incubated in medium containing BtcAMP or MIX showed no increase in alkaline phosphatase activity. We postulated that developmental age was the factor responsible for this difference in response and that immature cartilage (that with little or no alkaline phosphatase activity) would respond to BtcAMP whereas mature cartilage (that with significant alkaline phosphatase activity) would not. This was tested by incubating end sections of 11-d cartilage, which have little alkaline phosphatase activity, and center sections, which have significant alkaline phosphatase activity, with both BtcAMP and MIX. Alkaline phosphatase activity in end sections (immature cartilage) was stimulated by BtcAMP and MIX, whereas it was not stimulated in the center sections. Actinomycin D and cycloheximide inhibited BtcAMP and MIX stimulation of alkaline phosphatase activity. Thus, the in vitro data suggest that cyclic AMP is a mediator for the stimulation of alkaline phosphatase activity in embryonic cartilage.

The growth and maturation of embryonic cartilage is a time-dependent and orderly process that is regulated by a variety of hormones and metabolic factors. Several lines of evidence suggest that cyclic AMP may be one of the more important agents controlling these processes. In vitro incubation of embryonic chick pelvic cartilage with butyrylated cyclic AMP derivatives or phosphodiesterase inhibitors (which elevate intracellular cyclic AMP) stimulate RNA and protein synthesis (1–3). Cyclic AMP's stimulation of the synthesis of macromolecules in this tissue appears to result from a direct action of a cyclic AMP-cyclic AMP binding protein complex in the nucleus to induce mRNA synthesis (4). Whether the action of cyclic AMP is only to increase the synthesis of the same species of mRNA already being made or to induce the synthesis of new species of mRNA is not known.

Studies of the *in ovo* development of pelvic cartilage from 8- to 14-d chick embryos show that cyclic AMP content, which is quite low at 8 d, rises dramatically between 9 and 10 d and then plateaus. Pelvic cartilage alkaline phosphatase activity is barely measurable at 9 d but rises rapidly at 10 d and peaks at 11 d (Burch and Lebovitz. Manuscript submitted for publication). This temporal relationship of rising cyclic AMP levels followed by increasing alkaline phosphatase activity suggests that cyclic AMP may be a mediator in the induction of alkaline phosphatase activity in developing chick embryonic cartilage.

If cyclic AMP induces alkaline phosphatase activity in pelvic

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cartilage *in ovo*, it should be possible to demonstrate that in vitro incubation of immature chick embryo pelvic cartilage (that with little or no alkaline phosphatase activity) with butyrylated cyclic AMP derivatives or phosphodiesterase inhibitors increases alkaline phosphatase activity whereas incubation of mature cartilage (that already induced *in ovo* and having high alkaline phosphatase activity) has little or no effect on alkaline phosphatase activity. This report describes the results of such studies.

MATERIALS AND METHODS

In Vitro Incubation Procedures

Pelvic cartilages of chick embryos were removed, cleaned, and incubated in RPMI 1640 medium. Incubations were performed in a gyrorotary shaker bath at 37°C in an atmosphere of 5% CO_2:95% room air.

Initial studies to determine the effect of time of incubation on alkaline phosphatase activity were done by incubating twelve pelvic cartilages from 10-d chick embryos in 15 ml of medium alone or in medium containing 0.5 mM N^6-monobutyryl cyclic AMP (BtcAMP). Three cartilages were removed from each incubation flask at 1, 2, 4, and 7 h and assayed for protein content and alkaline phosphatase activity. A dose-response curve for BtcAMP (0–1.0 mM) was done by incubating four pelvic leaflets from both 9- and 10-d embryos at each dose of BtcAMP for 4 h, followed by measurement of alkaline phosphatase activity and protein.

After determination of the optimal incubation time and the dose of BtcAMP to elicit the maximal response, all subsequent experiments were performed with a 4-h incubation time and 0.5 mM BtcAMP. No alkaline phosphatase activity was detectable in the medium after any incubation, and changes occurring in the incubated cartilages could not be attributed to alterations in enzyme extrusion into the medium.

Elevation of endogenous cyclic AMP was achieved by incubating 10-d pelvic cartilage for 4 h in medium containing the phosphodiesterase inhibitor, 1-methyl-3-isobutyl xanthine (MIX), 0.25 mM. Tissue cyclic AMP was measured in these cartilages by a previously described competitive protein binding assay (5).

Measurement of Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured by a modification of the method of Bessey et al (6) using *p*-nitrophenyl phosphate (*p*NP) as the substrate. Pelvic cartilages from embryos (9-, 10-, 11-d) were homogenized in 0.9% NaCl and 0.2% Triton X-100 at 4°C and centrifuged at 10,000 *g*. The supernate, which contained 95% of the total activity, was assayed as follows: 2 to 20 μg of protein extract containing the enzyme was added to 1 ml of reaction mixture containing 0.5 mM *p*NP, 0.5 mM $MgCl_2$, and 0.5 M Tris-HCl pH 9.0 and incubated at 22°C for 30 min. Hydrolysis of *p*NP was monitored as the change in A_{410} on a Zeiss spectrophotometer. The rate of hydrolysis was linear over the 30-min interval. Enzyme activity is expressed as micromoles of *p*-nitrophenol released per mg protein per h based on a measured molar extinction coefficient of the product of $1.6 \times 10^4\ M^{-1}\ cm^{-1}$. The enzyme activity measured in the chick embryo pelvic cartilage is characteristic of bone alkaline phosphatase, in that activity is inhibited by L-homoarginine (7) and levamisole (8). Protein determinations were done by the method of Lowry et al. (9) using bovine serum albumin as the standard.

Alkaline phosphatase activity was identified histochemically by staining of individual pelvic cartilage leaflets from 11-d embryos using Sigma histochemical kit #85 (Sigma Chemical Co., St. Louis, MO). The product of this reaction, naphthol AS-MX, when coupled to a diazonium salt forms an insoluble visible precipitant that stains the cartilage.

Materials

p-Nitrophenyl phosphate, alkaline phosphatase histochemical stain kit #85, N^6-monobutyryl cyclic AMP, 1-methyl-3-isobutyl xanthine, and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI medium was purchased from Gibco (Grand Island Biological Co., Grand Island, NY). Actinomycin D was purchased from P-L Biochemicals, Inc. (Milwaukee, WI). All other chemicals were reagent grade. Embryonated chick eggs (*Gallus gallus*) were obtained from Pittsboro Hatchery, Pittsboro, NC.

RESULTS

In Vitro Stimulation of Alkaline Phosphatase Activity

Pelvic cartilages from 10-d chick embryos were incubated in vitro with and without added BtcAMP for 7 h as described in Materials and Methods. Fig. 1 depicts the results of this experiment. Cartilage incubated in medium alone had no significant alteration in its alkaline phosphatase activity. In contrast, cartilage incubated in medium containing BtcAMP showed a rise in alkaline phosphatase activity at 2 h and reached maximum activity (85% increase above control) at 4 h. Alkaline phosphatase activity was not increased after incubating cartilages in 1 mM ATP, AMP, or cyclic GMP.

Several dose-response curves of the effect of BtcAMP on alkaline phosphatase activity were done using pelvic cartilage from either 9- or 10-d embryos and a 4-h incubation. Fig. 2 shows such a study with cartilages from 9-d embryos and BtcAMP concentrations of 0 to 1.0 mM. Significant increases in alkaline phosphatase activity occurred with BtcAMP 0.1 mM. Maximal stimulation was observed at BtcAMP 0.5 mM, and higher concentrations were associated with significantly lesser stimulation.

In six separate experiments performed at optimum incubation conditions, BtcAMP stimulated alkaline phosphatase activity in pelvic cartilage from 10-d embryos by 66%. The alkaline phosphatase activity for cartilage incubated in medium alone was 9.7 ± 0.8 micromoles of *p*-nitrophenol released/mg protein per h and for the BtcAMP-treated cartilage 16.1 ± 0.7

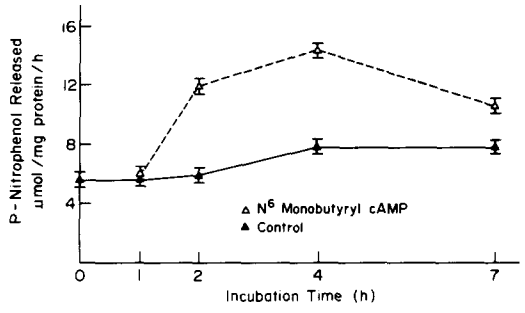

FIGURE 1 Stimulation of alkaline phosphatase activity in vitro in pelvic cartilage from 10-d chick embryos: time-course experiments. The cartilages were incubated in medium alone (▲) (control) or in medium containing 0.5 mM N^6-monobutyryl cyclic AMP (Δ) as described in Materials and Methods. At the indicated times, cartilages were removed and alkaline phosphatase activity was determined. Specific activity is represented on the ordinate. Each point represents the mean of three determinations (± SEM).

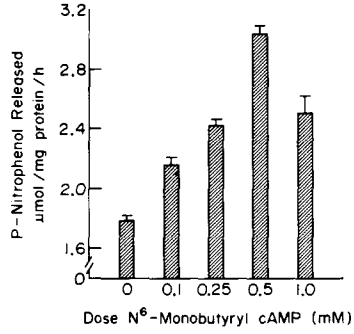

FIGURE 2 Stimulation of alkaline phosphatase activity in vitro in pelvic cartilage from 9-d chick embryos: dose response with N^6-monobutyryl cAMP (BtcAMP). Cartilages were incubated for 4 h in medium containing the indicated concentrations of BtcAMP followed by determination of alkaline phosphatase activity. Each point is the mean of three determinations (± SEM).

micromoles of *p*-nitrophenol released/mg protein per h ($P < 0.001$).

To determine whether increasing pelvic cartilage cyclic AMP content in vitro would increase alkaline phosphatase activity, we carried out a series of experiments in which pelvic cartilages from 10-d embryos were incubated in medium containing the phosphodiesterase inhibitor, 1-methyl-3-isobutyl xanthine (MIX). MIX, 0.25 mM, increased cyclic AMP from 1.58 ± 0.04 pmol/mg wet weight to 2.29 ± 0.15 pmol/mg wet weight ($P < 0.05$), which represents a 45% increase in cartilage cyclic AMP. MIX, 0.25 mM, added to the incubation medium also increased alkaline phosphatase activity in the pelvic cartilage. At 4 h the activity was 114% greater than that of cartilage incubated in medium alone. In these experiments the mean alkaline phosphatase activity was 10.9 ± 1.3 in control cartilage and 20.8 ± 1.7 in MIX-treated cartilage ($P < 0.01$).

Thus, exogenous addition of cyclic AMP in the form of BtcAMP or elevation of endogenous cyclic AMP by the phosphodiesterase inhibitor MIX stimulates alkaline phosphatase activity in vitro in pelvic cartilage from 10-d chick embryos.

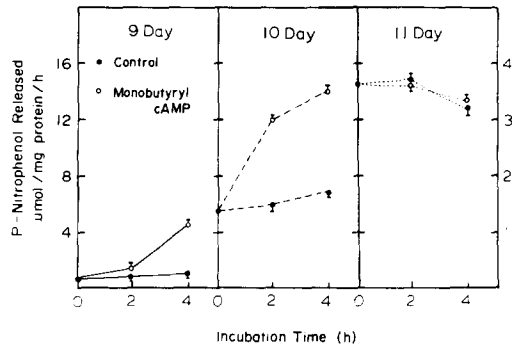

FIGURE 3 Stimulation of alkaline phosphatase activity in vitro in pelvic cartilage from 9-, 10-, and 11-d chick embryos. Pelvic cartilages from 9-, 10-, and 11-d embryos were incubated with (○) and without (●) N^6-monobutyryl cyclic AMP (0.5 mM). After 2 and 4 h of incubation, alkaline phosphatase activity was determined as described in Materials and Methods. Each point represents the mean of three determinations (± SEM).

Effect of Embryo Age on Cyclic AMP Stimulation of Alkaline Phosphatase Activity

Pelvic cartilages from 9-, 10- and 11-d embryos were incubated in vitro with BtcAMP. Fig. 3 illustrates the results of such a study with 0.5 mM BtcAMP. Basal alkaline phosphatase activity was very low in pelvic cartilage from 9-d embryos and was markedly and progressively greater in cartilage from 10- and 11-d embryos. In vitro incubation in medium alone had no significant effect on pelvic cartilage alkaline phosphatase activity. In vitro stimulation of pelvic cartilage alkaline phosphatase activity by BtcAMP depended on the stage of embryonic development. Cartilages from 9-d embryos showed striking stimulation of alkaline phosphatase activity by BtcAMP (220% increase above control at 4 h). Cartilages from 10-d embryos were stimulated 85% above controls, but cartilages from 11-d embryos showed no stimulation of alkaline phosphatase activity by BtcAMP at 2 or 4 h. Similar results were obtained when MIX was used as the stimulating agent (data not shown). It was noted that the maximum alkaline phosphatase activity achieved in cartilage from 10-d embryos by incubation with BtcAMP equalled the basal level that occurs in the unresponsive cartilages from 11-d embryos.

These data suggested that immature chondrocytes, which contain little or no alkaline phosphatase activity, respond to cyclic AMP by inducing alkaline phosphatase activity whereas mature chondrocytes, which contain high levels of alkaline phosphatase activity, are already maximally stimulated and cannot respond any further to the cyclic AMP stimulation. Histochemical staining of the embryonic pelvic cartilage for alkaline phosphatase shows that alkaline phosphatase first appears in the center at day 9, increases in intensity, and spreads centrifugally so that by day 11 the central 65% of the cartilage stains heavily for alkaline phosphatase (Fig. 4). To further test the hypothesis that cyclic AMP stimulates alkaline phosphatase activity in immature chondrocytes and that mature chondrocytes are already maximally stimulated, pelvic leaflets from 11-d chick embryos were cut with a razor blade into end and center sections using the histochemical stain for

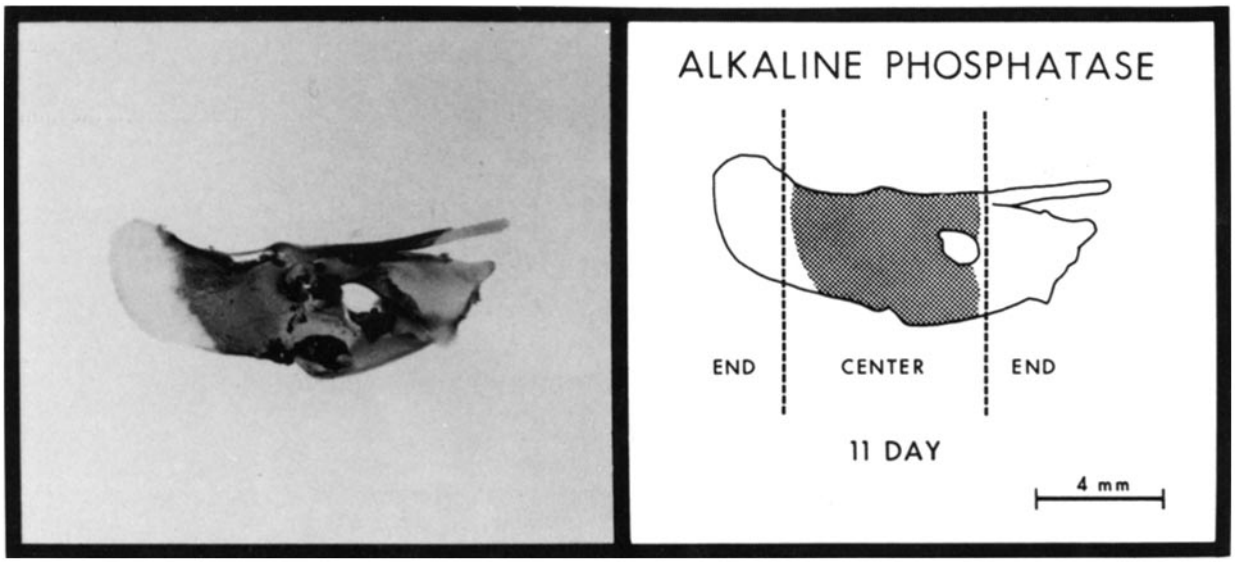

FIGURE 4 Photograph of a pelvic cartilage from 11-d-old chick embryo stained for alkaline phosphatase activity as described in Materials and Methods (left panel). Using the stained leaflet as a template, the ends were separated from the center sections of pelvic cartilage leaflets that had not been stained (right panel). The ends and center sections were used in the in vitro incubation experiments as described in the text.

alkaline phosphatase of the 11-d pelvic leaflet, which is remarkably constant, as the template (Fig. 4). The ends containing immature chondrocytes (little or no stainable alkaline phosphatase) from four cartilages were pooled and incubated in medium alone, medium containing BtcAMP, and medium containing MIX for 4 h, after which cartilage alkaline phosphatase activity was measured. The centers containing mature chondrocytes (stainable alkaline phosphatase) were incubated and processed similarly. The results of four such experiments are shown in Table 1. Alkaline phosphatase activity was significantly increased in the end sections incubated with BtcAMP. As expected, the alkaline phosphatase activity of the control end sections was only 11% that of the center sections. BtcAMP had no effect on alkaline phosphatase activity in the center sections.

TABLE I

Differential In Vitro Stimulation of Alkaline Phosphatase Activity by Cyclic AMP in Center and End Sections of Pelvic Cartilage from 11-d Chick Embryos

Experiment		Ends	Center
1	Control	4.2	35.8
	BtcAMP	8.9	39.2
2	Control	3.6	26.3
	BtcAMP	12.4	18.4
3	Control	3.1	27.7
	BtcAMP	4.0	31.2
4	Control	3.8	36.5
	BtcAMP	13.2	48.8
	MIX	12.6	21.0
Mean	Control	3.6 ± 0.2	31.6 ± 2.6
	BtcAMP	9.6 ± 2.0*	34.4 ± 6.4

The ends and center sections of pelvic cartilage were isolated from 11-d-old chick embryos and incubated in vitro for 4 h with and without 0.5 mM BtcAMP in four separate experiments. In Experiment 4, the effect of 0.25 mM MIX added to the medium was studied. Alkaline phosphatase activity was determined on the ends and the center. Specific activity is expressed as μmoles of p-nitrophenol released/mg protein/h.

* $P < 0.05$ vs. control.

Mechanism by which Cyclic AMP Stimulates Alkaline Phosphatase Activity

The possibility that cycle AMP might induce enzyme or cofactor synthesis was indirectly tested by incubation of the end sections of pelvic leaflets from 11-d embryos in medium or medium containing BtcAMP in the presence and absence of actinomycin D for 4 h followed by measurement of cartilage alkaline phosphatase activity. Preliminary experiments with various concentrations of actinomycin D showed that 0.5 μM inhibited [^{3}H]uridine incorporation into total RNA by 81% during a 4-h incubation and that little additional effect was obtained with threefold greater concentrations. Accordingly, this concentration was used in the experiments and, as shown in Table 2, actinomycin D had no effect on control levels of alkaline phosphatase activity but inhibited BtcAMP-stimulated alkaline phosphatase activity by 75%. In a similar experiment, cycloheximide (100 μM) inhibited BtcAMP-stimulated alkaline phosphatase activity by 90%. Thus, in vitro BtcAMP stimulation of pelvic cartilage alkaline phosphatase activity is inhibited by metabolites known to block DNA-dependent RNA synthesis and protein synthesis.

TABLE II

Actinomyosin D and Cycloheximide Effects on BtcAMP Stimulation of Alkaline Phosphatase Activity

Control	4.9 ± 0.04	BtcAMP	9.7 ± 0.2
Control plus Actinomycin D	5.0 ± 0.2	BtcAMP plus Actinomycin D	6.2 ± 0.3
Control plus Cycloheximide	4.7 ± 0.2	BtcAMP plus Cycloheximide	5.3 ± 0.2

End sections from pelvic cartilage of 11-d embryos were incubated in vitro for 4 h with medium alone (control), medium plus actinomycin D (0.5 μM), medium plus cycloheximide (100 μM), medium and BtcAMP (0.5 mM), medium and BtcAMP plus actinomycin D, medium and BtcAMP plus cycloheximide. Each value represents the mean (± SEM) of three determinations. Specific activity is expressed as μmoles of p-nitrophenol released/mg protein/h.

DISCUSSION

In ovo, cyclic AMP levels in the pelvic cartilage increase dramatically as the embryo develops (0.1 pmol/mg wet weight at 8 d to 1.3 pmol/mg wet weight at 10 d). Alkaline phosphatase activity likewise increases as the embryonic pelvic cartilage matures *in ovo* but its levels are virtually undetectable until day 9 and its maximal activity occurs between days 10 and 11 (Burch and Lebovitz. Manuscript submitted for publication). The temporal relationship between the rise in cyclic AMP levels followed by the increase in alkaline phosphatase activity raises the question as to whether the two phenomena are causally related.

To study this possibility, we incubated embryonic chick pelvic cartilage in vitro with BtcAMP or MIX and determined alkaline phosphatase activity. Either exogenously added BtcAMP or endogenously elevated cyclic AMP levels stimulate alkaline phosphatase activity in immature chondrocytes (no alkaline phosphatase staining) but have no effect on mature (alkaline phosphatase staining) chondrocytes. Thus, it is relatively easy to demonstrate both time-dependent (Fig. 1) and dose-related BtcAMP stimulation of alkaline phosphatase activity in vitro in 9- and 10-d pelvic cartilages (Fig. 2). Since the alkaline phosphatase activity of the center of 11-d pelvic cartilage is eight times that of the immature periphery, it is obvious that the threefold rise in alkaline phosphatase activity observed by incubating the ends with BtcAMP (Table I) is totally obscured during similar incubations using intact 11-d cartilage (Fig. 3). The lack of any in vitro stimulation of BtcAMP on alkaline phosphatase activity in the center sections of 11-d cartilage and the maximum in vitro stimulation of 10-d cartilages to the basal level of 11-d cartilage raise the interesting speculation that cyclic AMP stimulation converts the immature chondrocyte into a mature chondrocyte that is making alkaline phosphatase at relatively fixed rate and is no longer responsive to cyclic AMP.

The potential mechanisms that might be involved in cyclic AMP stimulation of alkaline phosphatase activity in embryonic pelvic cartilage include: (*a*) activation of a preformed enzyme such as cyclic AMP-dependent phosphorylation of an inactive enzyme; (*b*) activation by stimulation of an intermediary protein that changes the kinetic properties of the enzyme; and (*c*) *de novo* synthesis of the enzyme. Definitive proof that cyclic AMP is inducing *de novo* alkaline phosphatase activity through activating gene transcription requires demonstration that the number of mRNA molecules coding for alkaline phosphatase is increased and that the actual quantity of alkaline phosphatase protein is increased rather than just enzyme activity. Neither of these pieces of data is yet available for the embryonic pelvic cartilage. Our previous observations that cyclic AMP stimulation of embryonic chick pelvic cartilage in vitro causes a 54% increase in total mRNA synthesis (3), and that a cyclic

AMP-cytosolic cyclic AMP binding protein complex directly stimulates poly (A) RNA synthesis by isolated cartilage nuclei, and the present finding that addition of actinomycin D to the pelvic cartilage incubation inhibits cyclic AMP-mediated increases in alkaline phosphatase activity by 75% are consistent with the hypothesis that cyclic AMP induction of alkaline phosphatase activity in embryonic chick pelvic cartilage occurs through activation of gene transcription.

Studies demonstrating the induction of alkaline phosphatase activity by cyclic AMP have been reported in rat liver (10) and in several tumor cell lines (11, 12). In those studies as in our present study, actinomycin D or cycloheximide inhibited cyclic AMP-stimulated increases in alkaline phosphatase activity. Direct evidence that cyclic AMP stimulates *de novo* synthesis of alkaline phosphatase protein has been shown for a choriocarcinoma cell line (13). Other factors that have been reported to increase alkaline phosphatase activity include hydrocortisone which increases alkaline phosphatase activity in HeLa cells by a change in the kinetic properties of the enzyme without a change in the total amount of the specific protein (14), and sodium butyrate which also increases alkaline phosphatase activity in HeLa cells (15). Sodium butyrate (1 mM) had no effect on alkaline phosphatase activity in vitro in 10-d chick embryo pelvic cartilage (data not shown).

The in vitro data presented here, which demonstrate that exogenously added butyrylated cyclic AMP derivatives or increases in endogenous cyclic AMP increase alkaline phosphatase activity in embryonic pelvic cartilage which has low or absent basal activity support the *in ovo* temporal data implicating a causal relationship between rising cyclic AMP levels and increasing alkaline phosphatase activity. Although alkaline phosphatase activity is a marker for hypertrophied or mature cartilage, its actual function and relationship to calcification remains controversial. In any event the data suggest that cyclic AMP is a mediator of alkaline phosphatase activity induction and perhaps other cartilage maturation processes.

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