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# STIMULATION OF PALATAL GLYCOSAMINOGLYCAN SYNTHESIS BY CYCLIC AMP

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Intracellular levels of cyclic AMP in primary cultures of mouse embryo palate mesenchyme cells were elevated by exogenous administration of dibutyryl cAMP, 8Br-cAMP, prostaglandin $E_2$ or prostacyclin. Glycosaminoglycan synthesis was stimulated in a dose-dependent manner. Qualitative analysis by DEAE anion exchange chromatography and sensitivity to hyaluronidase digestion indicated preferential stimulation of hyaluronic acid synthesis. Cyclic AMP may thus play a role in regulating the synthesis of palatal glycosaminoglycans known to be requisite for normal development of the palate.

## INTRODUCTION

Cyclic AMP plays a critical role in normal growth and differentiation of the developing oro-facial region and appears to be specifically required for proper modulation of palatal differentiation (1-3). A positive correlation exists between teratogenic agents or mutant genes known to adversely affect palatal development and alterations in levels of palatal cAMP (4-8).

During development, the mammalian palatal processes undergo a series of morphogenetic movements which brings them to a position between the tongue and nasal septum. Synthesis of palatal extracellular glycosaminoglycans has been implicated as playing a role in this movement (9-11). Moreover, transient increases of palatal cAMP attain maximal values corresponding to the gestational period of maximal palatal glycoconjugate synthesis (2, 3, 10). Elevated levels of intracellular cAMP have been correlated, in other systems, with modulation of synthesis of extracellular glycosaminoglycans (12-17), and have been shown

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to precociously increase glycoconjugate synthesis in immature palatal tissue (1). Cyclic AMP may therefore play a key role in regulating the synthesis of extracellular matrix components requisite for palatal differentiation. In this study we investigated the quantity and quality of extracellular glycosaminoglycans synthesized by palatal mesenchymal cells in response to elevated levels of cAMP.

## MATERIALS AND METHODS

Mature male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were mated overnight, and the presence of a vaginal plug the following morning (day 0 of gestation) was considered evidence of mating. Embryos from day 13 pregnant mice were dissected from uteri in sterile phosphate buffered saline (PBS). Extraembryonic membranes were removed and embryos washed in PBS. Maxillary processes dissected from the embryos were pooled, minced, and then dissociated with 0.25% trypsin (GIBCO 1:250), 0.1% EDTA in Ca-Mg-free PBS for 10 min at 37°C with constant agitation. Trypsin was then inhibited by adding cold (4°C) Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). Primary cultures were initiated by seeding cells into 35-mm tissue culture dishes (Falcon) at a density of $9.4 \times 10^4$ cells/$cm^2$. Cells were grown in MEM with Earle's salts containing 25 mM HEPES buffer, 10% FBS, 4 mM glutamine, streptomycin (100 ug/ml), and penicillin (100 units/ml) at 37°C in an atmosphere of 5% $CO_2$/95% air.

Primary cultures of fetal palatal mesenchyme cells were grown to, and stimulated at, near confluency with one of a variety of compounds (see table 1) known to elevate intracellular cAMP levels. Cells were then labeled, in the presence of these compounds, for 6h (rate of incorporation linear for this period of time) with 2 uCi/ml of D-[6-$^3$H]glucosamine HCl (Amersham; 22 Ci/mmol). At the end of the labeling period, cell monolayers were washed, washes combined with medium and this fraction heat inactivated and pronase (1mg/ml) digested at 55°C for 18h. Residual protein was precipitated with trichloroacetic acid (TCA) at 4°C and centrifuged. The TCA supernatant was exhaustively dialyzed and either cetylpyridinium chloride (CPC) precipitated to determine total glycosaminoglycans (GAG) or subjected to DEAE chromatography for qualitative analysis of palatal GAG's.

For quantitative estimation, carrier hyaluronic acid (50 ug/ml) and chondroitin sulfate (50 ug/ml) were added to the dialysis retentates and GAG's precipitated by incubation with 0.2% CPC at 37°C for 1 hr. CPC precipitable material (GAG's) was collected by centrifugation, dissolved in methanol and counted in a scintillation spectrometer.

For qualitative analysis, dialysis retentates were chromatographed at room temperature on a diethylaminoethyl (DEAE)-Sephacel column (15 x 60 mm) equilibrated with 0.05 M Tris pH 7.2 and eluted with a linear gradient of 0.0 to 1.0 NaCl in 0.05 M Tris pH 7.2. Fractions were counted for radioactivity, assayed for hexuronic acid (18), pooled according to eluted peaks and lyophilized. Labeled GAG's were identified by selective digestion with testicular hyaluronidase (19), nitrous acid (20) and fungal hyaluronidase (21). Labeled GAG's, before and after digestion, were chromatographed on a Sephadex G-75 column equilibrated with 0.05 M Tris pH 7.2 to determine digestion of large molecular weight GAG's.

Cell monolayers were scraped from dishes and sonicated in ice-cold 10% TCA. The acid-soluble fraction was extracted with water-saturated ether, lyophilized to dryness, resuspended in 0.05 M acetate buffer, pH 6.2, and levels of cyclic AMP determined by radioimmunoassay. The acid insoluble fraction was hydrolyzed

Table 1. Synthesis of Glycosaminoglycans by Palatal Mesenchyme in vitro

| Treatment | Palatal Glycosaminoglycan Synthesis cpm/mg protein ± SEM *** | % Change |
|---|---|---|
| Control | 14,946±560 | --- |
| *0.5 mM dBcAMP | 23,633±2,037 | +58% |
| *1.0 mM dBcAMP | 34,689±1,853 | +132% |
| *0.5 mM 8BrcAMP | 24,774±2,134 | +65% |
| *1.0 mM 8BrcAMP | 31,298±1,687 | +109% |
| *1.0 mM 8BrcAMP +1.0 mM theophylline | 36,585±1,461 | +144% |
| *0.1 mM MIX | 18,200±2,884 | +22% |
| *0.5 mM MIX | 17,269±2,356 | +16% |
| *$10^{-9}$M $PGE_2$ | 20,687±1,840 | +38% |
| *$10^{-7}$M $PGE_2$ | 24,041±2,970 | +61% |
| *$10^{-5}$M $PGE_2$ | 22,967±757 | +54% |
| **$10^{-8}$M $PGI_2$ | 30,503±770 | +104% |
| **$10^{-6}$M $PGI_2$ | 28,981±113 | +94% |

*Cells were stimulated for 24 hours and labeled, in the presence of this compound, for 6 h with 2 uCi D-[6-$^3$H]glucosamine HCl.

**Cells were stimulated for 5 minutes and labeled, in the presence of $PGI_2$, for 6 h with 2 uCi D-[6-$^3$H]glucosamine HCl.

***GAG's quantitated by measuring incorporation of $^3$H-glucosamine into pronase digested, non-dialysable, CPC precipitable material. CPM represent the average in each case of no less than four determinations.

in 10% TCA at 90°C for 20 min, centrifuged and DNA in the supernatants determined spectrofluorometrically (22). The TCA pellet was dissolved in IN NaOH and protein determined according to the procedure of Lowry et al (23).

## RESULTS

C57BL/6J palatal mesenchymal cells were grown to subconfluency and intracellular levels of cAMP elevated by exogenous administration of either $N^6$,2'-O-dibutyryl-adenosine-3':5'-monophosphate, cyclic (dBcAMP), 8-bromoadenosine-3':5'-monophosphoric acid, cyclic (8 BrcAMP), theophylline, prostaglandin $E_2$ ($PGE_2$) or prostacyclin ($PGI_2$). When intracellular cAMP was elevated by addition of 0.5-1.0 mM dBcAMP or 8BrcAMP, palatal GAG synthesis was stimulated, in a dose-dependent manner, by 58-132% (Table 1). Treatment of

cells with 1.0 mM 8BrcAMP in the presence of 1.0 mM of the phosphodiesterase inhibitor theophylline resulted in an enhancement of GAG synthesis over that seen in the presence of 8BrcAMP only. Addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX) also resulted in a significant ($p<.05$) stimulation of GAG synthesis (Table 1).

Palatal mesenchyme cells are capable of synthesizing several prostaglandins with $PGE_2$ and $PGI_2$ being in predominance (24, 25). Moreover, these cells respond to prostaglandin stimulation with dose-dependent increases in intracellular levels of cAMP (26). Stimulation of these cells with doses of $PGE_2$ ($10^{-9}$M-$10^{-5}$M) that cause a 1.5-10 fold increase of intracellular cAMP resulted in a marked enhancement of GAG synthesis (Table 1). Prostacyclin ($PGI_2$), at doses that stimulate palatal cAMP 50-90 fold ($10^{-8}$M and $10^{-6}$M respectively), stimulated GAG synthesis to an even greater extent (Table 1). There exists therefore a dose-dependent relationship between the ability of a compound to elevate intracellular levels of cAMP and stimulation of palatal GAG synthesis. Since the cAMP response to $PGI_2$ is maximal by 5 min (26), a transient elevation of intracellular cAMP appears to be adequate to result in a significant stimulation of GAG synthesis.

To determine whether the stimulation of palatal GAG synthesis elicited by cAMP is specific, the effects of non-cyclic adenosine nucleotides and nonadenosine cyclic nucleotides were compared. Adenosine, 5'AMP, ADP and dibutyryl cyclic GMP (all 1 mM) had no significant effect on palatal GAG synthesis.

Qualitative analysis of cAMP stimulated palatal GAG's by DEAE anion exchange chromatography indicated that treatment of cells with either 1.0 mM 8BrcAMP or 1.0 mM dBcAMP resulted in a preferential increase of hyaluronic acid (HA)(Table II). Treatment with 1.0 mM 8BrcAMP stimulated palatal HA synthesis by almost 3 fold while 1.0 mM dBcAMP resulted in a greater than 5 fold stimulation of HA and a significant increase in chondroitin sulfate synthesis as well (Table 2). Material, identified as HA and eluting off a DEAE column at 0.24 M NaCl concentration, co-chromatographed with authentic HA, contained uronic acid and was sensitive to fungal hyaluronidase. Labeled material eluted off a Sephadex G-75 column as smaller molecular weight material after digestion with fungal

Table 2. DEAE Separation of Palatal Glycosaminoglycans
Palatal Glycosaminoglycan Synthesis - CPM/ug DNA*

| Treatment | Hyaluronic Acid | Heparin | Chondroitin Sulfate |
|---|---|---|---|
| Control | 74 | 97 | 370 |
| 1.0 mM 8BrcAMP | 212** | 108 | 302 |
| 1.0 mM dBcAMP | 375** | 102 | 506** |

*values represent radioactivity (cpm/ugDNA) in eluted DEAE peaks (identified as GAG's) averaged from three separations for each treatment.

**values represent significant ($p<.01$) increases when compared to control.

hyaluronidase. Labeled chondroitin sulfate and heparin-like macromolecules synthesized by palatal mesenchyme were also identified by their ability to co-chromatograph with authentic chondroitin sulfate and heparin respectively, their uronic acid content and their sensitivity to testicular hyaluronidase and nitrous acid digestion respectively. Sensitivity to nitrous acid digestion indicated that what was identified as heparin-like material contained N-unsubstituted glucosamine residues. This material may consist of both heparin and heparin sulphate.

## DISCUSSION

Cyclic AMP has been implicated as an important modulator of cell differentiation in many developing tissues (27-29) including the developing secondary palate (1-4, 30). Normal development of the mammalian palate is dependent on proper temporal (1-3, 5) and quantitative (6, 7) synthesis of cAMP. A transient increase in palatal cAMP (2, 5), immunohistochemically localized in part to mesenchymal plasma membranes (3), correlates temporally with the period of maximal palatal glycoconjugate synthesis (10). Moreover, exogenous dibutyryl cAMP can induce precocious glyconjugate synthesis in immature palatal tissue *in vitro* (1). Since palatal extracellular glycosaminoglycans have been frequently cited as playing a critical role in morphogenetic movement of the developing palate (10, 11, 31), these data suggest a role for cAMP in regulating the synthesis of palatal glycoconjugates.

Several prostaglandins are known to stimulate cAMP accumulation in a variety of cells and tissues (32) including the intact palate (33) and palatal mesenchyme cells *in vitro* (26). Two of these prostaglandins, $PGE_2$ and $PGI_2$, known to be synthesized by palate mesenchyme *in vitro* (24, 25) were used in the present study to monitor their effect on glycosaminoglycan synthesis.

Cells were stimulated under conditions of well established cell-cell contact since cells capable of synthesizing hyaluronic acid generally do so only during exponential growth (34, 35) and demonstrate increased responsiveness to hormones in this subconfluent state (36). Moreover, hormone-mediated activation of adenylate cyclase is inversely correlated with cell-density in many cell types (37, 38).

We have shown that murine palatal mesenchymal cells have the ability to produce hyaluronic acid, chondroitin sulfate and heparin-like macromolecules, *in vitro*. The synthesis of extracellular glycosaminoglycans was stimulated by treatment with exogenous dibutyryl cAMP, 8BrcAMP, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, prostaglandin $E_2$ or prostacyclin. Qualitative analysis of palatal glycosaminoglycans demonstrated that addition of exogenous dbcAMP or 8BrcAMP preferentially stimulated hyaluronic acid synthesis.

The mechanism by which cAMP acts to stimulate hyaluronic acid production is unknown. Difficulty in determining the level at which cAMP acts to elicit this cellular response is due in part to its pleiotropic effect on cellular metabolism. Elevated levels of intracellular cAMP have however been suggested as being able to elevate levels of hyaluronic acid synthetase (13) as well as enhance synthesis of extracellular hyaluronic acid by an established line of rat fibroblasts (14, 39, 40) and articular cartilage (17) in culture. The enhancement by theophylline of the cAMP effect on GAG synthesis, shown in the present study, has also been observed in cultures of articular cartilage (17).

Since normal development of the palate requires proper synthesis of glycosaminoglycans (10, 11) and proper temporal and qualitative synthesis of cAMP (5-7), our data suggest that this second messenger may regulate the synthesis of palatal extracellular glycosaminoglycans during craniofacial ontogenesis.

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