Stimulation of cyclic AMP in chondrocyte cultures: effects on sulfated-proteoglycan synthesis

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The effects of $N^6,O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate (DBcAMP), 8-bromoadenosine 3′:5′-cyclic monophosphate (8Br-cAMP), 3′:5′-cyclic monophosphate (cAMP), L-isoproterenol and L-epinephrine on sulfated-proteoglycan synthesis by rabbit articular chondrocytes were compared. DBcAMP and 8Br-cAMP in the presence or absence of 3-isobutyl-1-methylxanthine (IBMX) stimulated sulfated-proteoglycan biosynthesis after 20 h of incubation. cAMP had no significant effect. Both DBcAMP and 8Br-cAMP increased the hydrodynamic size of the newly synthesized proteoglycan monomer (A1D1) relative to control cultures. By contrast, although isoproterenol and epinephrine stimulated total cAMP synthesis, neither stimulated sulfated-proteoglycan synthesis. Whereas intracellular cAMP accumulated after incubation with DBcAMP and 8Br-cAMP, this was not the case with isoproterenol whether IBMX was present or not. Thus, stimulation of sulfated-proteoglycan synthesis by cAMP analogues in chondrocyte cultures appears to be dependent on increased intracellular cAMP accumulation rather than total cAMP biosynthesis.

Cyclic AMP *Chondrocyte* *Proteoglycan*

1. INTRODUCTION

The role 3′:5′-cyclic monophosphate (cAMP) plays in the metabolism of mammalian hyaline cartilage remains undefined. In vitro incubation of embryonic cartilage, adult canine and foetal bovine cartilage as well as foetal rat and rabbit costal chondrocyte cultures with cAMP or cAMP derivatives has been shown to augment sulfated-proteoglycan synthesis [1–3], and hyaluronate synthesis [4]. Somatomedins, generally considered important for the sulfation of glycosaminoglycans, either fail to stimulate adenylate cyclase [5] or may even inhibit adenylate cyclase activity in subcellular membranes of chondrocytes and cartilage derived from chick embryos [6]. Adrenergic hormones epinephrine and isoproterenol have been shown to stimulate cAMP synthesis in cartilage and chondrocyte cultures [7,8]. In this study, $N^6,O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate (DBcAMP), 8-bromoadenosine 3′:5′-cyclic monophosphate (8Br-cAMP), cAMP, L-isoproterenol and L-epinephrine were compared with respect to sulfated-proteoglycan synthesis by rabbit articular chondrocytes in monolayer culture.

Here, we demonstrate that DBcAMP and 8Br-cAMP, but not cAMP itself significantly increased sulfated-proteoglycan synthesis. Stimulation of sulfated-proteoglycan synthesis was not achieved by incubation with isoproterenol or epinephrine, despite stimulation of chondrocyte cAMP synthesis by these hormones.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Monolayer cultures of rabbit articular chondrocytes were established from pooled articular joints of the shoulder, knee and hip of immature

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(1.5–2.0 kg) New Zealand albino rabbits as in [9]. After growth to confluency (7–10 days) in Ham's F12 medium (Gibco, Grand Island, NY) containing 10% foetal bovine serum (KC Biological, Lanexa, KA), penicillin-streptomycin (0.1%), fungizone (1%) and mycostatin (0.1%) (Gibco, Grand Island, NY), the cells were subpassaged by brief treatment with trypsin (15–20 min, 37°C). First passage cultures were established in 60 × 15 mm tissue culture dishes (Falcon, Oxnard, CA) at an initial density of 3×10^5 cells/dish in Dulbecco's Modified Eagle's medium (DMEM) (sulfate-free, $MgCl_2$, 165 mg/ml substituted for $MgSO_4$; Gibco) containing 10% foetal bovine serum, penicillin-streptomycin, fungizone and mycostatin, as above. After addition of serum and streptomycin sulfate, the total inorganic SO_4 content of the medium was 12–13 mg/l [10]. The cultures were maintained at 37°C in an atmosphere of 10% CO_2/90% air. All experiments were conducted on sub-confluent cultures (3 days after establishment of first passage cultures).

Chondrocyte cultures were incubated in DMEM containing 10% foetal bovine serum and supplements as above and each of several compounds, cAMP (1 mM), DBcAMP, sodium salt (1 mM), 8Br-cAMP, sodium salt (1 mM) (Sigma, St. Louis, MO), or medium alone. Some cultures contained 3-isobutyl-1-methylxanthine (IBMX) (100 μM). Several control compounds were also utilized, sodium butyrate (1 mM) for DBcAMP, 8Br-5′-monophosphate (1 mM) for 8Br-cAMP, and guanosine 3′:5′-cyclic monophosphate, sodium salt for cGMP, all 1 mM. Cell cultures were pre-incubated for 30 min with each compound. The cultures were washed with Gey's BSS and then incubated in the absence or presence of each compound for 5 or 20 h containing $Na_2{}^{35}SO_4$ (5 μCi/ml) (New England Nuclear, Boston, MA, 600–800 mCi/mmol).

The medium and cells were separated. Medium was extracted by mixing a volume of medium with an equal volume of 8 M guanidine·HCl/0.1 M sodium acetate (pH 5.8) containing 20 mM Na_2EDTA, 10 mM benzamidine, 0.1 mM 6-aminohexanoic acid and 10 mM phenylmethylsulfonyl fluoride. The cell layer was extracted with 4 M guanidine·HCl/0.1 M sodium acetate (pH 5.8) containing half the concentration of proteinase inhibitors as above. Cell and medium 4 M guanidine·HCl extracts were subjected to CsCl isopycnic density gradient ultracentrifugation under associative conditions [0.5 M guanidine·HCl/0.1 M sodium acetate (pH 5.8), ϱ_o = 1.60 g CsCl/ml] for 45 h at 10°C (g_{av} = 81908). The bottom ¼ fraction of the gradient tube (A1) was then subjected to a second gradient ultracentrifugation this time under dissociative conditions (4 M guanidine·HCl/0.1 M sodium acetate, ϱ_o = 1.50 g CsCl/ml). The 2 most bottom fractions (A1D1 and A1D2) were dialyzed against double-distilled deionized water in dialysis membranes (Spectrapor, Los Angeles, CA) with a molecular mass cutoff of 6 kDa.

2.2. *Measurement of $^{35}SO_4$ incorporation and gel filtration*

The retained $^{35}SO_4$ was measured by liquid scintillation spectrometry in a Packard-Searle model 3255 liquid scintillation system in Aquasol (New England Nuclear) using the ^{14}C channel to detect $^{35}SO_4$. Counting efficiency was 75–80%. A portion of the dialyzed cellular and medium A1D1 and in some cases A1D2 fractions was applied to a column of Sepharose CL-2B (0.8 × 118 cm) and eluted with 4 M guanidine·HCl/0.1 M sodium acetate (pH 5.8) [11], to obtain the average partition coefficient (K_{av}) of the newly synthesized sulfated-proteoglycan monomer (A1D1) and other polydisperse $^{35}SO_4$-containing proteoglycans (A1D2).

2.3. *Stimulation of cAMP synthesis and cAMP determination*

Sub-confluent chondrocyte monolayers were incubated for 30 min with either DBcAMP (1 mM), 8Br-cAMP (1 mM), or L-isoproterenol (Sigma) or L-epinephrine (Sigma) (0–100 μM). The cultures were washed with GBSS and incubated for an additional 0–20 h with or without the cAMP analogues, L-isoproterenol or L-epinephrine in medium with or without IBMX (100 μM). Some cultures were incubated for the same period of time with $^{35}SO_4$ (5 μCi/ml). In some of the isoproterenol experiments, cultures were co-incubated with isoproterenol and the β-antagonist, DL-propranolol (100 μM). Cultures incubated with $^{35}SO_4$ were analyzed for $^{35}SO_4$ incorporation. Intracellular cAMP accumulation and cAMP secreted into the medium was measured by RIA

[12]. DNA content/culture in DBcAMP, 8Br-cAMP, L-isoproterenol, L-epinephrine-treated and control cultures was measured by a modification of the diphenylamine technique [13].

2.4. *Statistical analysis*

Statistical analysis was performed by a two-tailed Student's *t*-test to measure the significance of differences between the means of the sample groups. A $p < 0.05$ was taken as evidence of statistical significance.

3. RESULTS

3.1. *Effect of cAMP, DBcAMP and 8Br-cAMP on proteoglycan synthesis*

DBcAMP and 8Br-cAMP stimulated $^{35}SO_4$ incorporation after 20 h of incubation, but not after 5 h. cAMP failed to increase $^{35}SO_4$ incorporation (table 1). cGMP was without effect (not shown). DBcAMP and 8Br-cAMP stimulated proteoglycan synthesis in the presence (table 1) or absence (table 2) of IBMX, a phosphodiesterase inhibitor, and only if the cAMP analogues were in the medium during the 20 h labeling with $^{35}SO_4$ (table 1). Treatment of chondrocytes with either DBcAMP or 8Br-cAMP did not alter the DNA content of the cultures (control, 5.7 μg DNA/culture; DBcAMP, 5.1; 8Br-cAMP, 5.5; cAMP, 6.3; sodium butyrate, 5.1; 8Br-5′AMP, 5.7, average of 5 pooled cultures in each group). This result differs from previous work [13] which showed that maintenance of chondrocytes in DBcAMP for up to 6 days was markedly cytotoxic. The shorter periods of exposure to the cAMP analogues used in the present study were not cytotoxic. In addition, the cAMP analogues altered slightly the partitioning of incorporated $^{35}SO_4$ between the cellular-pericellular compartment and the medium into which the bulk of the proteoglycan was secreted (table 3).

Table 1

Effect of cAMP and cAMP analogues on $^{35}SO_4$ incorporation by chondrocytes

Group	Incubation time			
	5 h $^{35}SO_4$ (cpm/culture × 10^{-5})[a]		20 h $^{35}SO_4$ (cpm/culture × 10^{-5})[a]	
	(+)	(−)	(+)	(−)
Control	–	4.14 ± 0.20	–	9.09 ± 0.44
cAMP (1 mM)	4.41 ± 0.06	4.09 ± 0.20	11.04 ± 0.90	7.79 ± 0.14
Sodium butyrate (1 mM)	4.18 ± 0.05	3.93 ± 0.09	7.71 ± 0.26	7.71 ± 0.55
DBcAMP (1 mM)	4.47 ± 0.12	4.08 ± 0.09	14.12 ± 1.23[b]	7.76 ± 0.36
8Br-5′AMP (1 mM)	4.22 ± 0.08	4.03 ± 0.24	8.92 ± 0.27	7.92 ± 0.24
8Br-cAMP (1 mM)	4.41 ± 0.09	4.03 ± 0.24	16.71 ± 1.15[b]	8.49 ± 0.40

[a] Mean ± SD ($n = 3$)
[b] $p < 0.05$

First passage articular chondrocytes at sub-confluency (3 days after subculture) were preincubated for 30 min at 37°C with each of several compounds listed above and IBMX (100 μM). Following this preincubation, cells were incubated in the absence (−) or presence (+) of these compounds together with $^{35}SO_4$ (5 μCi/ml) for 5 or 20 h. Medium was extracted by mixing an equal volume of medium with 8 M guanidine·HCl/0.1 M NaAc (pH 5.8) containing 2× proteinase inhibitors [26] and dialyzed against deionized distilled H_2O in membranes with a 6–8 kDa molecular mass cutoff at 4°C for 24 h. The retained $^{35}SO_4$ was counted by liquid scintillation spectrometry in Aquasol. Radioactivity was expressed as cpm/culture, since the DNA content of the cultures was not different at either 5 h or 20 h among the various groups

Table 2

Effect of IBMX on DBcAMP and 8Br-cAMP stimulation of proteoglycan synthesis

Group	IBMX (100 μM)	$^{35}SO_4$ (cpm/culture × 10^{-5})[a]		
		Cellular	Medium	Total
Control	–	1.49 ± 0.04	6.86 ± 0.47	8.35 ± 0.50
Control	+	1.92 ± 0.01	8.22 ± 0.16	10.14 ± 0.17[b]
DBcAMP (1 mM)	–	3.51 ± 0.20	10.52 ± 0.30	14.03 ± 0.48
DBcAMP	+	3.79 ± 0.27	11.81 ± 0.46	15.60 ± 0.69[b]
8Br-cAMP (1 mM)	–	2.54 ± 0.02	8.99 ± 0.04	11.54 ± 0.04
8Br-cAMP	+	3.31 ± 0.26	11.97 ± 0.93	15.29 ± 1.13[b]

[a] Mean ± SD (n = 3)
[b] $p < 0.05$ (control, DBcAMP, 8Br-cAMP without IBMX compared to cultures containing IBMX)

First passage articular chondrocytes at sub-confluency (3 days after subculture) were preincubated for 30 min at 37°C with either IBMX (100 μM), DBcAMP or 8Br-cAMP (1 mM) alone or in combination with IBMX. Following preincubation, the cells were incubated in the presence of DBcAMP or 8Br-cAMP with or without IBMX and $^{35}SO_4$ for 20 h. $^{35}SO_4$ incorporation was measured as given in table 1

Table 3

Distribution of $^{35}SO_4$ in chondrocyte cultures treated with cAMP analogues

Group	$^{35}SO_4$ (cpm/culture × 10^{-5})	
	Cellular[a] (%)	Medium[a] (%)
Control	1.55 ± 0.09 (15.1)	8.71 ± 0.62 (84.9)
Sodium butyrate (1 mM)	1.20 ± 0.08 (15.5)	6.51 ± 0.31 (84.5)
DBcAMP (1 mM)	3.16 ± 0.30 (22.4)	10.96 ± 0.94 (77.6)
8Br-5'AMP (1 mM)	1.43 ± 0.05 (16.0)	7.49 ± 0.23 (84.0)
8Br-cAMP (1 mM)	3.23 ± 0.43 (19.3)	13.48 ± 0.93 (80.7)

[a] Mean ± SD (n = 3)

First passage chondrocyte cultures at sub-confluency were preincubated with various agents for 30 min at 37°C. Cultures were incubated with each agent including $^{35}SO_4$ (5 μCi/ml) for 20 h. Medium was separated from the cellular layer. The medium and cell layer were extracted separately with 4 M guanidine·HCl/0.1 M sodium acetate containing proteinase inhibitors and dialyzed against deionized distilled H_2O with 6–8 kDa molecular mass cutoff at 4°C for 24 h

Neither DBcAMP nor 8Br-cAMP altered the percentage of $^{35}SO_4$ in cellular or medium A1 fractions or A1D1 fractions (A1, medium, control, 46.8%; DBcAMP, 50.3%, 8Br-cAMP, 50.2%; A1D1, cellular, control, 42.9%, DBcAMP, 38.6%, 8Br-cAMP, 41.4%, A1D1, medium, control, 53.4%, DBcAMP, 52.95%, 8Br-cAMP, 57.6%). Chromatography of A1D1 fractions on

Sepharose CL-2B eluted with 4 M guanidine·HCl (fig.1) revealed that both DBcAMP and 8Br-cAMP decreased the K_{av} of the larger of two proteoglycan species of rabbit articular cartilage [14–16]. The smaller proteoglycan (K_{av} 0.80) eluting near the included volume of the column (V_t), and recently reported to be synthesized by articular, but not epiphyseal chondrocytes [17] was unaffected by either DBcAMP or 8Br-cAMP in cellular and medium A1D1 fractions. In addition, 8Br-cAMP shifted slightly the K_{av} of medium fraction A1D2 from 0.5 (control) to 0.42 (not shown).

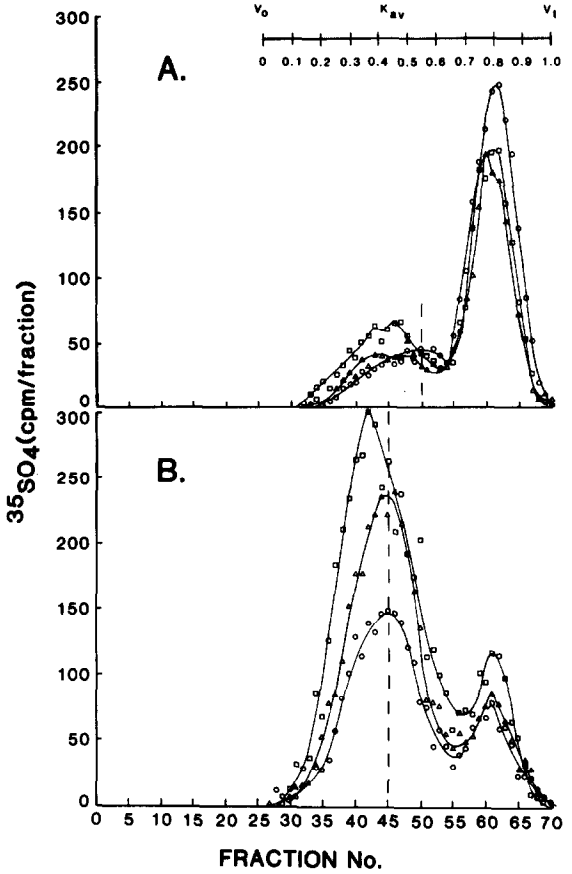

Fig.1. Sepharose CL-2B chromatography elution profiles of $^{35}SO_4$-labeled proteoglycans in the A1D1 fraction in cellular extracts and culture medium of control and DBcAMP or 8Br-cAMP-treated chondrocyte cultures. Sub-confluent cultures were labeled with $Na_2{}^{35}SO_4$ (5 μCi/ml for 20 h) as described in the text. Proteoglycans were extracted with 4 M guanidine·HCl/0.1 M sodium acetate (pH 5.8) containing proteinase inhibitors [26]. After dialysis, the radioactive material was freeze-dried, reconstituted in 4 M guanidine·HCl/0.1 M sodium acetate and layered onto the column. Column fractions of 0.9 ml were collected (flow rate = 10 ml/h). Recovery of radioactivity from the column averaged 98%. The vertical dotted line shows the K_{av} of untreated control cultures. V_o is the void volume; V_t is the total column volume: (A) cellular, (B) medium; (○—○) control, (△—△) DBcAMP, (□—□) 8Br-cAMP.

3.2. *Effect of L-isoproterenol and L-epinephrine on proteoglycan synthesis*

L-Isoproterenol failed to stimulate proteoglycan synthesis. Over a wide range of concentrations (0–100 μM), isoproterenol failed to stimulate $^{35}SO_4$ incorporation after 20 h incubation even when isoproterenol was present in the medium (fig.2). The DNA content (μg/culture) was not altered by L-isoproterenol from concentrations of

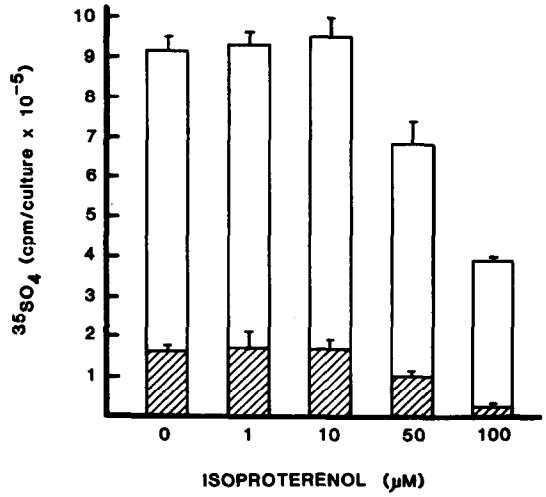

Fig.2. Effect of isoproterenol on $^{35}SO_4$ incorporation by chondrocytes. Sub-confluent cultures were preincubated with varying concentrations of isoproterenol for 30 min at 37°C. Cultures were washed with GBSS and incubated in medium containing isoproterenol (0–100 μM) and $Na_2{}^{35}SO_4$ (5 μCi/ml for 20 h). Cellular and medium compartments were extracted with 4 M guanidine·HCl/0.1 M sodium acetate as described in the text. Data are expressed as cpm/culture × 10^{-5}. Below concentrations of 50 μM, no difference in cell numbers or DNA content (μg/culture) was measured. Control, 2.8 μg/culture; isoproterenol 1–50 μM, 2.8, 100 μM, 0.6 μg. Open area, total $^{35}SO_4$ incorporation; hatched area, cellular $^{35}SO_4$ incorporation. Mean ± SE, n = 3.

1–50 μM (control, 2.8, 1 μM, 2.9, 10 μM, 3.0, 50 μM, 2.8, average of 6 pooled cultures). The DNA content of cultures treated with isoproterenol (100 μM) was significantly diminished (0.6 μg/culture). Thus, reduced incorporation of $^{35}SO_4$ at this concentration of isoproterenol was probably a result of cytotoxic effects of the hormone on the cells.

The presence or absence of IBMX had no statistically significant effect on $^{35}SO_4$ incorporation (control, 8.35 ± 0.50/cpm per culture × 10^{-5}, control + IBMX, 10.14 ± 0.17, isoproterenol, 10^{-5} M, 8.28 ± 0.17, isoproterenol + IBMX, 9.95 ± 0.57, mean ± SD, n = 3). The partitioning of $^{35}SO_4$ between cellular and medium compartments was also unaffected (fig.2).

Incubation with L-epinephrine (0–100 μM) for 20 h also failed to stimulate proteoglycan synthesis. No effect on DNA content/culture was seen at concentrations at or below 50 μM. A cytotoxic appearance of the cells was frequently observed at a concentration of 100 μM (not shown). $^{35}SO_4$ incorporation was markedly inhibited at the higher epinephrine concentrations (100 μM).

3.3. *Effect of DBcAMP, 8Br-cAMP, isoproterenol and epinephrine on cAMP synthesis*

Immunoreactive cAMP accumulated within chondrocytes after treatment with DBcAMP and 8Br-cAMP. The amount of intracellular immunoreactive cAMP found was independent of the presence of IBMX (8Br-cAMP, 282.5 ± 57.6 pmol/culture; 8Br-cAMP + IBMX, 245.6 ± 45.0 mean ± SE, n = 5) suggesting the relative resistance of these analogues to phosphodiesterase. Control culture immunoreactive cAMP was reduced if IBMX was omitted (control 14.6 ± 2.0 pmol/culture; control + IBMX, 26.4 ± 3.8). Treatment of chondrocytes with DBcAMP or 8Br-cAMP for 20 h increased cross-reacting intracellular cAMP levels (control, 44.4 ± 13.8; DBcAMP, 2079 ± 124.7, 8Br-cAMP, 410.6 ± 88.1 pmol/culture, mean ± SE, n = 4). Intracellular cAMP content measured just prior to addition of L-isoproterenol (zero time) was 3 pmol/culture which increased to 6 pmol/culture after 0.5 h. Control cultures showed a smaller rise in intracellular cAMP in the first 0.5 h. However, isoproterenol failed to sustain intracellular cAMP levels over a 20 h period although the hormone did stimulate total cAMP synthesis (fig.3). Omission of IBMX reduced intracellular accumulation of cAMP in response to isoproterenol (isoproterenol, 9.8 ± 1.9; isoproterenol + IBMX, 16.5 ± 5.7, mean ± SE, n = 5). By 5 h, isoproterenol-stimulated cAMP synthesis was maximal, which stayed constant at 20 h. Epinephrine also failed to stimulate increased intracellular levels of cAMP, although total cAMP was increased.

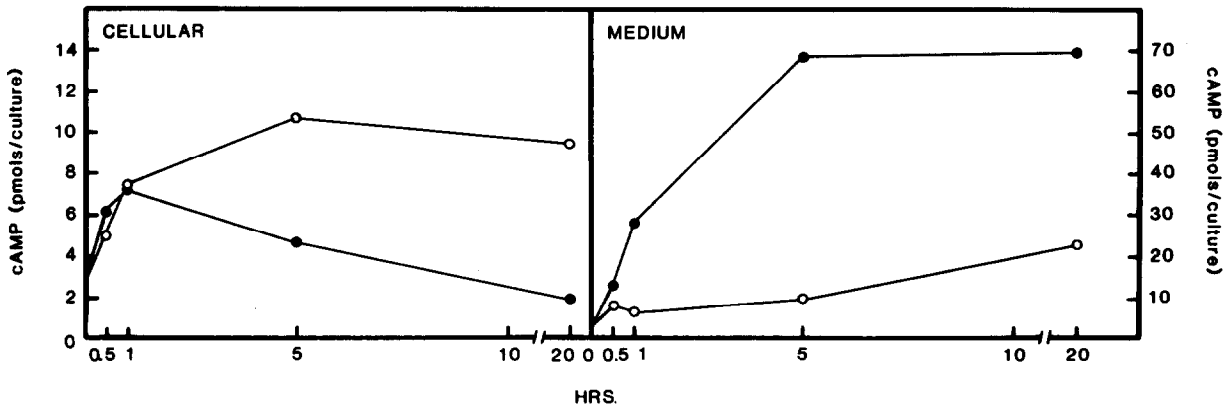

Fig.3. Time course of L-isoproterenol stimulation of chondrocyte cAMP. Chondrocytes were incubated with isoproterenol (10 μM) for the indicated time points. At each time point including zero time, cultures were terminated and intracellular and medium immunoreactive cAMP assayed as described in the text. (■—■) Control + IBMX, (●—●) isoproterenol + IBMX.

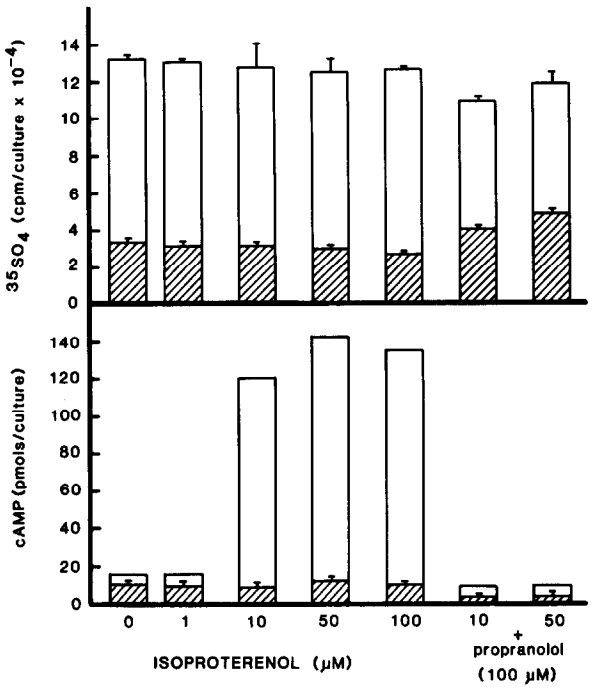

Fig.4. Effect of L-isoproterenol on cAMP and proteoglycan synthesis. Sub-confluent cultures were preincubated with varying concentrations of isoproterenol alone or isoproterenol and propranolol (100 μM) for 30 min at 37°C. Cultures were washed with GBSS and incubated as above in medium containing $Na_2{}^{35}SO_4$ (5 μCi/ml) and IBMX (100 μM) for 5 h. cAMP in cells and medium was measured by RIA. Total and cellular $^{35}SO_4$ incorporation was measured as described in the text. Differences in DNA content (μg/culture) were not detected. (Top) $^{35}SO_4$ incorporation, (bottom) cAMP (pmol/culture). (Open area) Total $^{35}SO_4$ incorporation or cAMP (cellular + medium); (hatched area) cellular $^{35}SO_4$ or cAMP. Mean ± SE, n = 3.

3.4. *Correlation of isoproterenol-stimulated cAMP and proteoglycan synthesis*

Despite stimulation of total cAMP synthesis (cells ± medium), isoproterenol had no effect on sulfated-proteoglycan synthesis after 5 h (fig.4). Propranolol inhibited almost completely the isoproterenol-stimulation of cAMP. No effect on proteoglycan synthesis was seen in isoproterenol-propranolol co-incubates.

4. DISCUSSION

The present experiments have shown that DBcAMP and 8Br-cAMP, agents which cause accumulation of intracellular immunoreactive cAMP, stimulated proteoglycan synthesis by chondrocytes. This result is similar to those in [1] with foetal rat chondrocyte monolayer cultures. In addition, the cAMP analogues decreased the K_{av} of the newly synthesized proteoglycan subunit. This result differs from that obtained by authors in [2]. They reported that although DBcAMP stimulated glycosaminoglycan synthesis by foetal calf chondrocytes, the size of the proteoglycan subunit was unaltered. This occurred despite a relative increase in chondroitin 4,6-disulfate in DBcAMP-treated cultures. In the present study, synthesis of a small proteoglycan was unaffected by DBcAMP or 8Br-cAMP (fig.1). Thus, the larger of two sulfated proteoglycan species found endogenously in rabbit cartilage [14] and synthesized by cartilage organ-explants and chondrocytes [15,16] was specifically affected by treatment with cAMP analogues. The K_{av} of the larger proteoglycan species was, however, larger (0.45) than that found in the A1D1 fraction of chondrocytes grown to high density and containing multiple dense chondroid nodules [16]. 8Br-cAMP increased the hydrodynamic size of sub-confluent chondrocyte newly synthesized A1D1 proteoglycan to that comparable to multi-layered chondrocyte cultures. Isoproterenol and epinephrine, which stimulated total cAMP synthesis, failed to cause intracellular accumulation of cAMP and did not stimulate proteoglycan synthesis.

The effect of 8Br-cAMP and DBcAMP on quantitative proteoglycan synthesis were equivalent despite reports that 8Br-cAMP is more resistant to phosphodiesterases than DBcAMP [18]. In addition, 8Br-cAMP unlike DBcAMP has been shown to bind to protein kinase without intracellular conversion to an active form. When IBMX was excluded from the medium in the presence of 8Br-cAMP or DBcAMP, $^{35}SO_4$ incorporation was reduced. It is uncertain, however, as to how much of the cAMP measured intracellularly is newly synthesized cAMP and how much represents the cAMP analogues which cross-react with the cAMP antibody in the RIA. Approx. 0.006% of the added DBcAMP was found as

either cAMP or cross-reacting DBcAMP in the cell compartment after 1 h. Under basal conditions (no stimulus), however, total cAMP synthesis was greatly reduced if IBMX was excluded and a greater proportion of the cAMP was found extruded into the medium. Other agents able to stimulate cAMP synthesis in chondrocyte cultures (PGE_2, A23187) resulted in intracellular accumulation of cAMP [16]. A23187 which stimulated cAMP 6-fold in 7 min caused marked extrusion of cAMP into the medium as well. Despite the presence of IBMX in the medium of isoproterenol and epinephrine-stimulated chondrocyte cultures, cAMP failed to accumulate intracellularly. A greater amount of cAMP was found in the medium than in control cultures. This release of cAMP into the medium could have come about by a failure of intracellular cAMP to bind to a protein kinase receptor which may increase the secretion of newly synthesized intracellular cAMP into the medium.

Stimulation of proteoglycan synthesis by accumulation of intracellular cAMP is likely related to the known stimulatory effects of cAMP on amino acid transport [19], and total protein and RNA synthesis [20]. Proteoglycan synthesis has been correlated with increased amino acid transport [21] and the G_1 phase of the cell cycle where transport of amino acid analogues is thought to be maximal [1,22]. In addition, intracellular accumulation of cAMP may be responsible for the phosphorylation of newly synthesized proteoglycan, in cartilage [23] and chondrosarcoma cultures [24]. An increase in RNA synthesis is coordinate with cAMP accumulation and the stimulation of poly(A) RNA synthesis occurs by direct nuclear effects of specific cyclic AMP–cytosolic cAMP binding protein complex [25]. The phosphorylation of proteoglycan may play a role in the level of synthesis and the total of newly synthesized proteoglycan secreted into the culture medium. The finding that DBcAMP and 8Br-cAMP stimulation occurred only if it was present in the medium during the entire 20 h labeling with $^{35}SO_4$ suggested that sustained formation of cAMP–protein kinase complexes is required for maximal stimulation of proteoglycan by cAMP in chondrocyte monolayer cultures.

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