MEMBRANE CHANGES DURING CARTILAGE MATURATION

Increase in 5′-Nucleotidase and Decrease in Adenosine Inhibition of Adenylate Cyclase

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In addition to its role in the propagation of stimuli in excitable tissues, the cell membrane has recently been shown to be involved in the control of growth, proliferation, morphogenesis, and malignant transformation (2, 3, 5, 10, 14, 20, 24, 27, 34, 41, 47, 49). Membrane changes occurring during development (30, 37, 50–52) may play an active role in cytodifferentiation. To examine the validity of this concept in an in vivo system, we studied membrane changes during the maturation of chick epiphyseal cartilage cells. The anatomy of the epiphysis makes it possible to separate cells at different stages of maturation: the proliferative, the growing, and the hypertrophying state. In previous studies, we found differences between the proliferative and the hypertrophying cartilage cells with respect to the reduction in cAMP caused by physiological pressure (42). These differences paralleled the decreasing ability of calcium to inhibit the adenylate cyclase of the respective cell membranes (7). With the emergence of adenosine as a potential intercellular communication molecule (38, 44, 45, 46) we examined the activity of the adenosine generating enzyme 5′-nucleotidase, in proliferative and in hypertrophying cartilage, and the effect of adenosine on the adenylate cyclase of the respective segments.

MATERIALS AND METHODS

Tris-HCl, 5′AMP, β-glycerophosphate, α,β-methylene adenosine diphosphate (AOPCP), mixture of 2′ and 3′AMP, phosphocreatine kinase, creatine phosphate, 3′,5′-cyclic AMP (cAMP), ATP, alumina, dithiothreitol (DTT), sucrose, sodium dodecyl sulfate, Glu-6-P, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2,6-dichlorophenolindophenol, succinate, deoxycholic acid, and cacodylic acid were obtained from Sigma Chemical Company, St. Louis, Missouri. Mercaptoethanol was purchased from Eastman Kodak Co.

Incubated eggs were obtained from Spafas, Inc., Norwich, Conn. AG-50WX4 resin from Bio-Rad Laboratories, Richmond, Calif., α-[^{32}P]ATP, [^{3}H]cAMP, and [^{3}H]5′AMP from New England Nuclear, Boston, Massachusetts, the Aqueous Counting Scintillant (ACS) from Amersham-Searle Corp., Arlington Heights, Ill., and levamisole from Pitman-Moore, Washington Cross, New Jersey.

All membrane preparations were obtained from epiphyseal cartilage dissected from 16-day chick embryos as previously described (7). The proliferative and hypertrophying segments were homogenized for 1 min by a Teflon pestle (3,000 rpm) in 50 mM Tris (pH 7.6) with 3.75 mM mercaptoethanol. The homogenate was centrifuged for 10 min at 15,000 *g* (average). The pellet was suspended in 3 ml of 1 mM Tris (pH 7.6). The supernate was recentrifuged for 15 min at 40,000 *g* (average). This pellet was resuspended in 1 ml of 1 mM Tris and was used for assaying the membrane-associated enzyme activities. All procedures were carried out at 4°C. Protein was determined by the method of Lowry et al. (28). DNA was measured by the method of Burton (11).

5′-Nucleotidase

5′-Nucleotidase was assayed by a modification of the method of Gentry and Olsson (18). The assay mixture contained 100 mM Tris, pH 7.4, 200 μM 5′AMP, 2 mM Mg, 30 mM β-glycerophosphate, 0.25 μCi of [^{3}H]5′AMP and 10–100 μg of protein in a vol of 100 μl. The reaction was carried out at 37°C for 5 min and was stopped by the addition of 200 μl each of 2% $ZnSO_4$ and 1.8% $Ba(OH)_2$. The samples were centrifuged at 2,500 rpm for 5 min, and 250 μl of the supernate were counted in 10 ml of ACS.

Adenylate Cyclase Activity

Adenylate cyclase was assayed according to the method of Salomon et al. (43). The reaction mixture (100 μl) contained 50 U/ml phosphocreatine kinase, 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 20 mM creatine phosphate, 1 mM DTT, 1 mM cAMP, 0.1 mM ATP and 10^6 cpm α-[^{32}P]ATP and 20–120 μg of protein per assay tube. Samples were counted in Bray's solution (8).

Glu-6-Phosphohydrolase Activity

Glu-6-Pase activity was assayed according to the method described by Gunderson and Nordlie (22) on 50–150 μg of protein at 37°C for 10 min. Phosphate was measured according to Ames (1).

Succinic Dehydrogenase Activity

Succinic dehydrogenase activity was assayed according to Green et al. (21) by following the reduction of indophenol spectrophotometrically. The reaction was started by injecting succinate into warm (38°C) reaction mixture which contained 20 μg of protein.

5'-Nucleotidase Cytochemistry

The cartilage cells were isolated by enzyme digestion of the epiphyses segments as described previously (7). 5'-nucleotidase activity was measured on unfixed cells by a procedure modified from Widnell (53). The cells were incubated in freshly filtered media containing 100 mM Tris-acetate buffer (pH 7.5), 1 mM lead nitrate, 2 mM magnesium sulfate, 0.75 mM 5'AMP, and 50 mM levamisole hydrochloride for 30 min at 37°C with mild agitation. The reaction was stopped by sedimenting the cells at 2,000 *g* for 2 min, followed by removal of media, washing of the cells, in cold 0.1 M cacodylate and addition of glutaraldehyde to a final concentration of 6%. Cells were fixed for 10 min at room temperature, then washed with cacodylate buffer and postfixed for 1 h in 1% cacodylate-buffered osmium tetroxide at 4°C. The cells were stained *en bloc* with 0.25% aqueous uranyl acetate, dehydrated through a graded series of acetone, and embedded in Spurr low viscosity media (48). The sections were examined without further counterstaining on a Zeiss EM 10 electron microscope.

RESULTS

Initial experiments were conducted to determine the specificity of 5'-nucleotidase activity and establish appropriate assay conditions for this tissue. As previously proposed (19), β-glycerophosphate was used to inhibit the breakdown of 5'AMP by the ubiquitous alkaline phosphatase (EC 3.1.3.1) (29). Concentrations above 25 mM effectively prevented the breakdown of 5'AMP by "nonspecific" phosphatases. Under these conditions the enzymatic activity was linear with time over a 20-min period and linear with protein concentration between 0.2 and 1.0 mg/ml.

The 5'-nucleotidase activity was susceptible to competitive inhibition by a mixture of 2' and 3'AMP (Fig. 1) (16). The apparent K_i (derived graphically) was 6×10^{-4} M. The apparent K_m for 5'AMP was around 25 μM, a value close to that reported for liver (53). The initial velocity for the enzymatic activity of the whole epiphysis was about 5 nmol/mg protein × min. AOPCP at 25 μM (9) inhibited 60–80% of the activity. The enzymatic activity was pH dependent, rising from 3.2 nmol/mg protein × min at pH 5.5 to 8.2 nmol/mg protein × min at pH 8.0. At 1–2 mM Mg, the activity was about 20% higher than at concentrations below or above this level. The assays were conducted at pH 7.6 and 2 mM Mg.

Having established satisfactory assay conditions, we proceeded to separate a membrane preparation by subcellular fractionation. The enzymatic activities of adenylate cyclase, glu-6-Pase, succinic dehydrogenase, as well as protein and DNA, were measured in all fractions. A 40,000-*g* pellet which showed a threefold increase in the specific activities of 5'-nucleotidase and adenylate cyclase and was free of succinic dehydrogenase and DNA was selected for subsequent studies. On electron microscope examination, this fraction showed vesicular structures characteristic of plasma membrane along with some rough ER but contained no mitochondria nor nuclei. Throughout the above fractionation procedure as well as during more extensive purification on sucrose gradients (not reported here), the increase

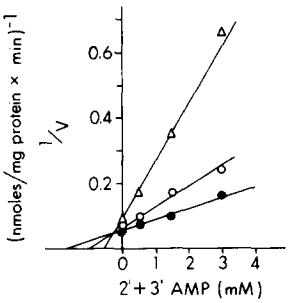

FIGURE 1 Competitive inhibition of nucleotidase activity by 2' and 3'AMP. Plot of the reciprocal of the velocity of 5'AMP hydrolysis as a function of 2' and 3'AMP concentrations. The assay mixture contained 100 mM Tris, 30 mM β-glycerophosphate, and 2 mM Mg at three concentrations of 5'AMP. ($\triangle-\triangle$) 50 μM, ($\circ-\circ$) 100 μM, and ($\bullet-\bullet$) 200 μM 5'AMP.

in specific activity of 5'-nucleotidase paralleled that of adenylate cyclase. From these studies it seemed that, as in other tissues, the 5'-nucleotidase activity was associated primarily with the plasma membrane.

The amount and the distribution of the enzymatic activities were examined as a function of chondrocyte maturation. The results of one among eight exp are presented in Table I. The major observation was a pronounced rise in the 5'-nucleotidase activity of the hypertrophying segment. As seen in Table I, this finding was not due to a procedural artifact since the total recovery and the subcellular distribution of the enzymatic activities among the three segments were similar. The change occurred in the membrane fraction and is most conspicuous when activities per DNA or membrane specific activities are compared (Table II). The 5'-nucleotidase activity increased from 0.68 nmol/μg DNA × min in the proliferative zone to 6.96 nmol/μg DNA × min in hypertrophying zone. The specific activity of the membrane-enriched microsomal fraction increases threefold, from 2.8 to 9.98 nmol/mg protein × min. On the other hand, the adenylate cyclase activity measured in the same samples shows a threefold decrease in specific activity (per protein) in the membrane-enriched fraction.

At the same time, there was a decrease in the inhibitory effect of adenosine on adenylate cyclase. Fig. 2 presents an $^1/_v$ vs. i plot of one of eight exp. The data for the proliferative zone is consistent with noncompetitive inhibition and yields a K_i of about 500 μM; the K_i for the

TABLE I

Distribution and Recovery of 5'-Nucleotidase and Adenylate Cyclase at Three Stages of Cartilage Maturation

		Proliferative		Growing		Hypertrophying	
	Fraction	*mg*	*%*	*mg*	*%*	*mg*	*%*
Protein (mg/288 epiphyses)	Homogenate	12.72	100	15.72	100	19.68	100
	15,000 *g* pellet	3.60	28.3	3.76	26.6	4.40	22.4
	40,000 *g* supernate	7.00	55.0	7.80	49.6	7.40	37.6
	40,000 *g* pellet	1.12	8.8	1.36	8.7	1.40	7.1
	Recovery		92.1		84.9		67.1
		nmol	*%*	*nmol*	*%*	*nmol*	*%*
5'-Nucleotidase (nmol adenosine/ 288 epiphyses/min)	Homogenate	21.8	100	36.4	100	147.4	100
	15,000 *g* pellet	8.3	38.2	11.2	30.6	36.4	24.7
	40,000 *g* supernate	4.3	19.7	10.8	29.9	46.5	31.6
	40,000 *g* pellet	7.5	34.2	7.9	21.6	27.5	18.7
	Recovery		92.1		82.1		75.0
		pmol	*%*	*pmol*	*%*	*pmol*	*%*
Adenylate cyclase (pmol cAMP/288 epiphyses/min)	Homogenate	110.5	100	106.3	100	73.3	100
	15,000 *g* pellet	43.2	39	23.6	22	16.6	23
	40,000 *g* supernate	33.4	30	37.3	35	20.2	28
	40,000 *g* pellet	34.9	31	18.6	18	12.5	17
	Recovery		100		75		68
		μmol	*%*	*μmol*	*%*	*μmol*	*%*
Glu-6-Pase (μmol Pi/ 288 epiphyses/min)	Homogenate	0.71	100	1.01	100	1.11	100
	15,000 *g* pellet	0.12	17	0.17	17	0.24	17
	40,000 *g* supernate	0.54	75	0.69	68	1.17	83
	40,000 *g* pellet	0.07	9	0.21	20	0.39	28
	Recovery		101		105		128
		μg	*%*	*μg*	*%*	*μg*	*%*
DNA (μg/288 epiphyses)	Homogenate	28.8	100	31.1	100	14.0	100
	15,000 *g* pellet	19.6	69.4	21.9	70.4	12.1	86.6
	40,000 *g* supernate	ND*		ND		ND	
	40,000 *g* pellet	ND		ND		ND	

The epiphyses were dissected into three segments; the respective segments were pooled and were processed identically. Assays are described under Materials and Methods.

* Nondetectable.

TABLE II

Changes in 5'-Nucleotidase during Cartilage Maturation

	5'Nucleotidase			Adenylate cyclase		
	Homogenate		Membrane fraction*	Homogenate		Membrane fraction
	nmol/μg DNA/min	*nmol/mg protein/min*	*nmol/mg protein/min*	*pmol/μg DNA/min*	*pmol/mg protein/min*	*pmol/mg protein/min*
Proliferative	0.68 ± 0.17‡	0.90 ± 0.32	2.77 ± 1.33	9.23 ± 1.94	13.57 ± 2.62	38.16 ± 3.07
Growing	1.59 ± 0.37	1.44 ± 0.42	2.97 ± 1.09	0.37 ± 2.08	9.82 ± 1.34	20.97 ± 4.60
Hypertrophying	6.96 ± 2.00	4.33 ± 1.4	9.98 ± 4.05	6.28 ± 0.57	4.50 ± 0.82	10.46 ± 1.68

* Membrane-enriched microsomal fraction.

‡ Means and standard error of the means were computed from eight exp similar to the one described in Table I.

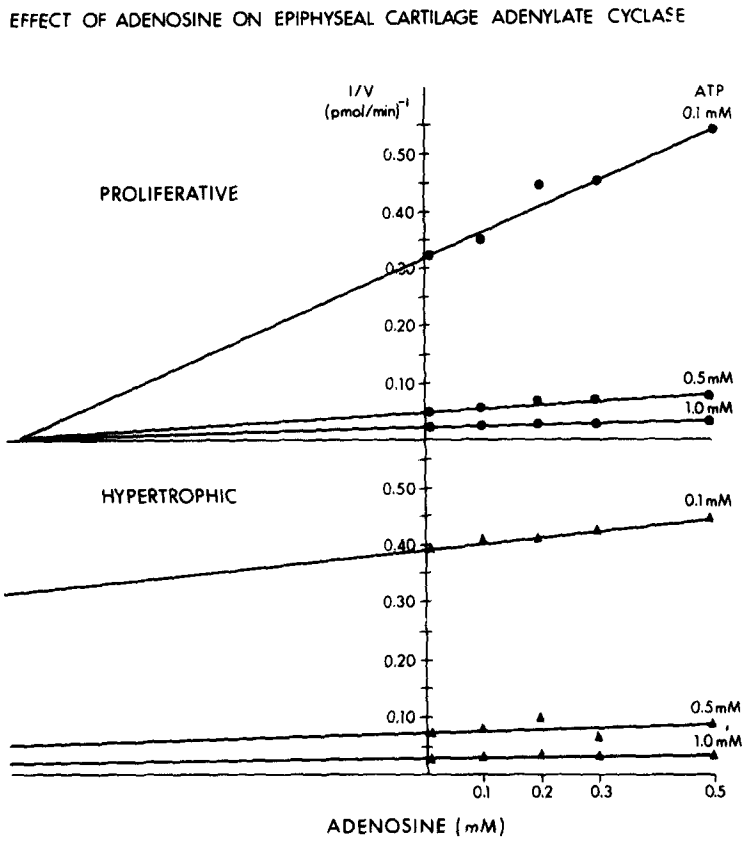

FIGURE 2 Adenosine inhibition of adenylate cyclase activity in proliferative and hypertrophic cartilage membrane preparations. 20 μg of partially purified plasma membrane were assayed as described in Materials and Methods, with the addition of 30 mM β-glycerophosphate, 25 μM AOPCP, 2 mM ethyleneglycolbis[β-aminoethyl·ether]*N,N'*-tetraacetic acid (EGTA), and the indicated concentrations of adenosine. The figure represents a plot of the reciprocal of the enzyme velocity as a function of adenosine concentration.

hypertrophying zone, for the same kinetic model, is above 5 mM.

To confirm the observations on the 5'-nucleotidase increase with cell maturation and obtain independent evidence on the cellular localization of the enzymatic changes, cytochemical studies were conducted on cells isolated from the three segments of the epiphyses. β-glycerophosphate

had to be replaced by a nonphosphate generating inhibitor of alkaline phosphatase. As previously reported in other systems (6), levamisole was found to inhibit uncompetitively the cartilage alkaline phosphatase (Fig. 3). As seen in Table III, 50 mM levamisole inhibited the total 5'AMP phosphohydrolase activity to the same extent as 30 mM β-glycerophosphate. The effect of the two inhibitors at maximum concentrations was nonadditive. AOPCP reduced by 60–70% the β-glycerophosphate and/or levamisole non-inhibited 5'AMP phosphohydrolase activity. Table III also shows the effects of fixation and lead on the 5'-nucleotidase activity of cell homogenates. It can be seen that lead is less inhibitory after fixation, which suggests that both lead and fixation affect the same sites. Cytochemical studies indeed indicated that fixation with concentrations of glutaraldehyde as low as 0.3% for as short as 5 min markedly affected the 5'-nucleotidase located on the cell membrane (53) but had no detectable effect on that localized in the interior of the cell. The cytochemical reactions were therefore carried out on nonfixed cell suspensions in the presence of 50 mM levamisole. Fixation, postfixation, and processing followed.

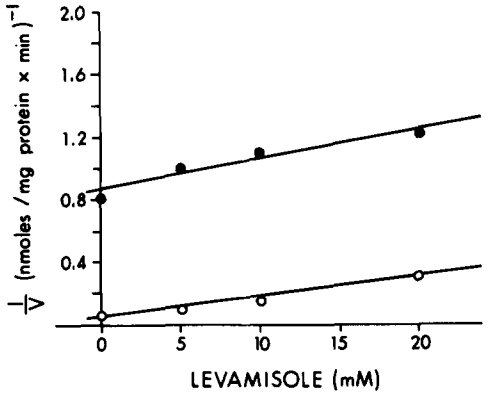

FIGURE 3 Inhibition of 5'-nucleotidase by levamisole. The 5'-nucleotidase assay is described in Materials and Methods. The plot represents the reciprocal of the enzyme velocity as a function of levamisole concentration. The substrate concentrations were (●–●) 50 μM, and (○–○) 200 μM.

Pronounced differences between cells from different epiphyseal segments were observed. Cells from the proliferative zone showed a sparse patchy distribution of reaction product (lead phosphate) along the cell surface and small amounts of reaction product associated with internal vesicles (Fig. 4). Some cells showed lead precipitates associated with nuclear heterochromatin masses. Occasional cells showed no surface reaction product at all. Essentially all cells contained deposits of reaction product within cytoplasmic vesicles (Fig. 4).

Cells from the hypertrophying zone showed significantly more reaction product associated with the cell surface (Fig. 5). Lead precipitates associated with internal vesicles, endoplasmic reticulum, and nuclear heterochromatin were also present. Such distribution is consistent with previous reports on other systems (16, 26, 53) and may represent membrane before exteriorization.

TABLE III

Effect of Lead and Fixation on 5'-Nucleotidase Activity

	Membrane fraction nonfixed	Cell homogenate	
		Nonfixed	Fixed
	nmol/mg protein/min	*nmol/mg protein/min*	
Total 5'AMP phosphohydrolase	42.8	12.8	9.95
30 mM β-glycerophosphate (β-gly-P)	12.3	NI*	NI
50 mM Levamisole (Lev)	10.6	1.53	0.97
30 mM β-gly-P + 50 mM Lev	10.0	NI	NI
30 mM β-gly-P + 50 mM Lev + 25 μM AOPCP	3.4	NI	NI
50 mM Lev + 25 μM AOPCP	3.2	0.47	0.38
1 mM Pb	NI	12.68	7.69
50 mM Lev + 1 mM Pb	NI	0.60	0.59

The 5'-nucleotidase activity was assayed as described under Materials and Methods in nonfixed membranes and in homogenates of fixed and nonfixed cells. Membrane and cell preparation and fixation procedures are described under Materials and Methods. The tissue homogenization procedure described in Materials and Methods was used for the cells.

* Was not investigated.

Cells from the growing zone showed reaction product deposition characteristic of either the proliferative or the hypertrophying cells. Very few cells could be identified having an "intermediate" pattern of reaction product deposition. The transition thus seems to occur suddenly.

The substrate-deleted controls were completely free of lead precipitates. The amount of reaction product was greatly reduced in cells incubated in the presence of AOPCP, and only rarely were nuclear precipitates seen (Fig. 6 and 7).

DISCUSSION

The major findings of this study are a pronounced increase in 5′-nucleotidase activity, a decrease in adenylate cyclase specific activity and a decrease in adenosine inhibition of adenylate cyclase, during epiphyseal chondrocyte maturation. The molecular basis for these changes is not known. The changes may be due to differences in enzyme amount or to the effect of activity-modulating factors. The increase in the specific activity of 5′-nucleotidase and the parallel and equal decrease in the specific activity of adenylate cyclase are consistent with a change in the relative membrane content of the two enzymes through synthesis and degradation. On the other hand, there is a substantial change in the cartilage phospholipid composition during chondrocyte maturation (55), and it is well documented that membrane-bound enzymes are strongly affected by the lipid environment (35, 39, 40). Other factors, such as ions, nucleotides, or regulatory proteins, may also be involved. Molecular characterization of the enzymes and better understanding of membrane turnover (20) will help discriminate between these possibilities.

The physiological significance of the observed changes could be related to the generation of adenosine, the product of 5′-nucleotidase, and its potential role as an extracellular messenger molecule (38, 44, 45, 46). Vasodilation is the best known physiological effect of adenosine (13) which may play a role in the vascularization which accompanies osteogenesis.

Another possible role of adenosine is related to its effect on cAMP, which as a messenger molecule has also been invoked in the control of differentiation (31, 33, 56). We found that, in epiphyseal cartilage, adenosine inhibits the adenylate cyclase at concentrations above 1 μM. In neural tissue, adenosine is stimulatory, acts on a specific site, and has a K_m in the range of 10–100 μM (4, 12, 25, 38, 44, 46). It also increases the cAMP content of platelets (23, 32), thymocytes (57), and bone cells (36). Fat cells release adenosine which, in turn, inhibits adenylate cyclase, reduces cAMP accumulation and inhibits lypolysis (15, 46). If extracellular adenosine were to originate from cAMP breakdown (17, 46), its effect on adenylate cyclase would generate interesting feedback properties. In cells with adenosine-inhibitable adenylate cyclase, such as cartilage and fat cells, cAMP elevations would be spatially and temporally limited, whereas in tissues with adenosine-stimulated cyclase, such as bone, a propagation wave, as seen in development (54), would be generated.

In view of these possibilities, the findings support the working hypotheses that 5′-nucleotidase is part of the membrane enzyme complex which affects the flow of intercellular information (via adenosine and adenylate cyclase). They also support the thesis that membrane changes are a necessary component of cytodifferentiation, providing the link between genetic and epigenetic control of the differentiation process.

SUMMARY

To examine the potential participation of the plasma membrane in differentiation, we studied the enzymatic activities of 5′-nucleotidase and adenylate cyclase as a function of chondrocyte maturation. 16-day-old chick embryo tibiae epiphyses were dissected into proliferative, growing, and hypertrophying zones. Partially purified membrane fractions prepared by differential centrifugation from the respective tissue segments were assayed for enzymatic activity. Cell suspensions from the same segments were examined cytochemically for the presence of 5′-nucleotidase.

The findings show that the 5′-nucleotidase activity of the chick embryo epiphyseal cartilage has the following characteristics: (*a*) it has a K_m of about 25 μM for 5′AMP, and is inhibited by a mixture of 2′ and 3′AMP (apparent K_i about 10^{-4} M) and by AOPCP; (*b*) it is predominantly localized at the cell surface but is also detected in the cytoplasm and in association with nuclear heterochromatin; and (*c*) it increases 10-fold (on a DNA basis) during the maturation of the epiphyseal cartilage cells.

The adenylate cyclase activity has these characteristics: (*a*) it does not change during chondrocyte maturation (on a DNA basis); (*b*) its susceptibility to adenosine inhibition decreases at least 10-fold. The implication of these findings relative

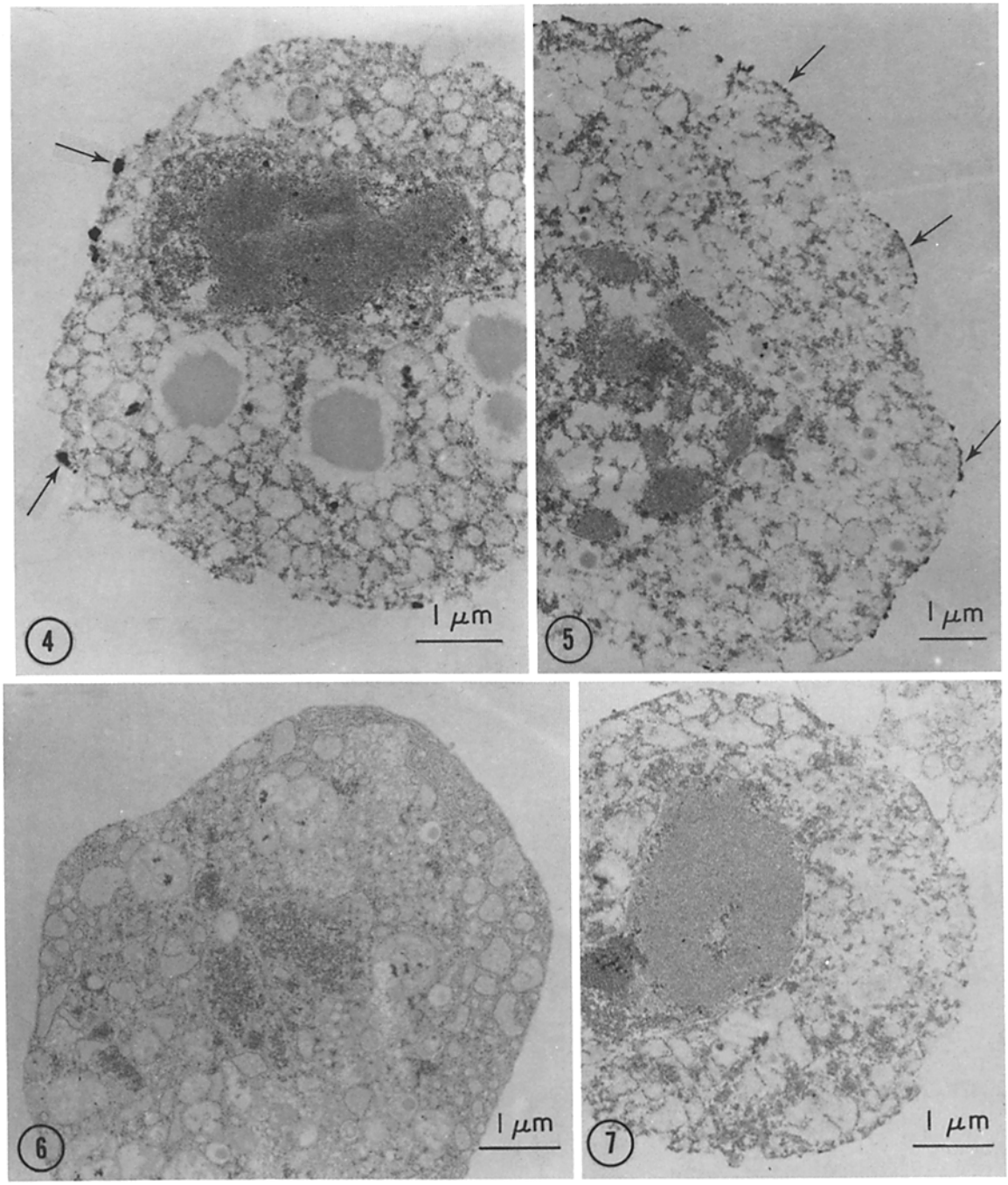

FIGURE 4 Electron micrograph of an unfixed cartilage cell from the proliferative zone. The cell was incubated in 5′-nucleotidase reaction medium. A few areas of reaction product can be seen at the cell surface (arrows). There are scattered reaction product deposits within cytoplasmic vesicles. The section was not counterstained. × 12,000.

FIGURE 5 Electron micrograph of an unfixed cartilage cell from the hypertrophying zone. The cell was incubated in 5′-nucleotidase reaction medium. The plasma membrane shows broad, continuous bands of reaction product (arrows) on its external face. There is very little cytoplasmic reaction product. The section was not counterstained. × 9,500.

FIGURE 6 Electron micrograph of an unfixed cartilage cell from the proliferative zone. The cell was incubated in 5′-nucleotidase reaction medium supplemented with AOPCP (a 5′-nucleotidase inhibitor). No cell surface or internal reaction product deposits are seen. Section is not counterstained. × 12,000.

FIGURE 7 Electron micrograph of an unfixed cartilage cell from the hypertrophying zone. The cells were incubated in 5′-nucleotidase reaction medium supplemented with AOPCP (a 5′-nucleotidase inhibitor). The cell is essentially free of cell surface or internal reaction product deposits. Section is not counterstained. × 12,000.

to a possible role of adenosine in cellular communication is discussed.

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