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INORGANIC PYROPHOSPHATASE IN CULTURED CELLS

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SUMMARY

1 Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3 6 1 1) activity of cultured cells of KB (Eagle)-human carcinoma of nasopharynx was investigated Enzyme activity of intact, whole cells was compared with that obtained from sonicated preparations The enzyme was not Mg^{2+} dependent and displayed Michaelis–Menten type kinetics only in the presence of 2 mM EDTA Some of the activity was associated with the plasma membrane and appeared to function as an ecto-enzyme Partial inhibition by equimolar concentrations of adenine nucleotides and NADH indicated that substrate specificity was low

2 Inorganic pyrophosphatase of L-929 (Earle) mouse fibroblasts by contrast, was dependent on Mg^{2+} for activation, and no activity was present in 2 mM EDTA The pyrophosphatase of mouse fibroblasts depended specifically on Mg^{2+} since other cations were ineffective It was found, however, that Ca^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} inhibited the enzyme in KB cells

3 In other cell lines investigated, the inorganic pyrophosphatase had the characteristics of the Mg^{2+} stimulated enzyme of mouse fibroblasts In all the cell lines studied so far, the total cellular enzyme activity associated with the external aspect of the plasma membrane ranged from 31 to 100%

INTRODUCTION

We reported recently that two of the classical plasma membrane marker enzymes, Mg^{2+}-ATPase (EC 3 6 1 3) and 5′-nucleotidase (EC 3 1 3 5) functioned as ecto-enzymes [1] We further suggested that this seemingly peculiar sidedness of the two enzymes should be characteristic of all eukaryotic cells A recent communication by DePierre and Karnovsky [2] may lend support to this contention We also explored another possibility, namely, that the hydrolysis of ATP by intact cells was catalyzed by an ecto-ATP-pyrophosphohydrolase (EC 3 6 1 8) When [γ-^{32}P]ATP was used as a substrate and PP_i was added as a trap, we noted the rapid disappearance of PP_i from the system This communication deals with some of the properties of the inorganic pyrophosphatases and ecto-pyrophosphatases

Since our studies indicated the presence of two different types of pyrophosphatases with respect to Mg^{2+} activation we made a more detailed analysis of ionic

requirements Particular emphasis was placed on two cell lines KB (Eagle) human carcinoma of the nasopharynx and L-929 (Earle) mouse fibroblast The extent to which pyrophosphatase in any particular case functioned as an ecto-enzyme was assessed by comparing intact with sonicated cell preparations

MATERIALS AND METHODS

Cell cultures

Experiments were performed principally with suspensions of KB (Eagle)-human carcinoma of nasopharynx and L-929 (Earle)-mouse fibroblasts and HeLa (Gey)-human carcinoma of cervix obtained from Microbiological Associates, Bethesda, Md Occasionally other cultured cell lines were employed and the details are described were necessary Dulbecco's modified Eagles medium and fetal calf serum were obtained from Microbiological Associates Minimum essential medium (Eagle) with Hanks balanced salts solution, penicillin–streptomycin, Fungizone, and Polymixin-B were obtained from Grand Island Biological Co All cultures were maintained in the Eagle–Hanks medium containing 10% fetal calf serum with 50 units penicillin, 50 μg streptomycin, 250 μg fungizone, and 100 units Polymixin-B per ml media

Cell and sonicate preparation

Cell suspensions were immediately centrifuged at 1000 rev /min ($200 \times g$) for 2 min, resuspended in the Eagle–Hanks medium and incubated for 60–90 min at 37 °C with gentle agitation After incubation the cells were washed 3 times with 0 85% (w/v) NaCl (isotonic saline) and kept at +1–2 °C prior to use Cells were counted using a standard hemocytometer Samples of cell suspensions were sonicated using a microtip on a Sonifier Model L575 The sample was kept in ice and sonicated at a resonating frequency 5–10 times in one second bursts for complete breakage

Chemicals

Buffers were prepared using 10 mM Tris–HCl buffer in isotonic saline adjusted to specified pH with sodium acetate–acetic acid or 2-amino-2-methyl-1-propanol for the desired pH All chemicals were reagent grade

Enzyme assays

Assay for inorganic pyrophosphatase was performed in 200-μl vol consisting of 60 mM Tris–HCl buffer (pH 7 4), 87 mM NaCl, 5 mM PP_i, and other reagents as noted in the results Solutions were kept on ice prior to use After the addition of enzyme to the mixture, samples were placed in a 37 °C water bath for incubation Incubation times were 20 min unless indicated otherwise The reactions were stopped by placing the samples in water at 0 °C and adding 0 1 ml of a 1 1 (v/v) mixture of 0 1 N silicotungstic acid in 0 25 M H_2SO_4 and 5% ammonium molybdate in 2 M H_2SO_4 The ammonium molybdate–phosphate complex was extracted into 1 0 ml of water-saturated isobutanol The amount of phosphate released was determined after a modified method of Berenblum and Chain [3] The amount of phosphate released was also determined by using $^{32}PP_i$ (ICN, Isotope and Nuclear Division) as the substrate, and assaying labeled orthophosphate–ammonium molybdate present in the extraction mixture by scintillation counting All samples were determined in duplicate

with appropriate blanks to control for non-enzymatic hydrolysis of PP_i, endogenous P_i or PP_i extracted by the ammonium molybdate, and background contamination About 1 6% of PP_i was non-enzymatic hydrolyzed or complexed using the above silicotungstic ammonium molybdate extraction procedure It was noted that if the silicotungstic acid was not used during the extraction of P_i by ammonium molybdate, that the ammonium molybdate–phosphate complex was less efficiently extracted, probably by binding of either P_i or the complex to available protein Experiments were performed in which both colorimetric and tracer isotope methods were used, and agreement between methods was within 5%

RESULTS

Ion requirements

The optimal pH values for pyrophosphatase of intact KB cells and L-929 fibroblasts were at 8 0 and 7 5, respectively (Fig 1) Alterations in cation composition

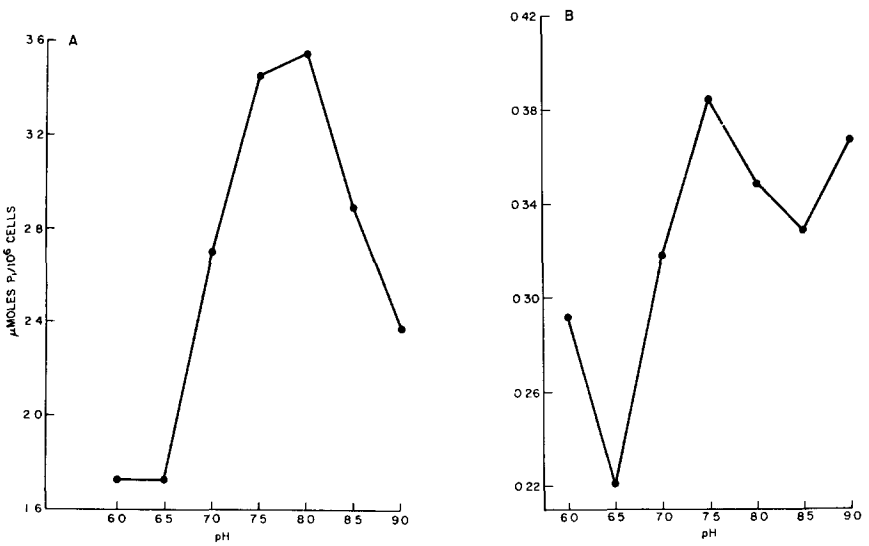

Fig 1 Effect of pH on pyrophosphatase activity of cultured cells Incubation media as described in Materials and Methods Pyrophosphate concentration 5 mM (A) KB cells Mg^{2+} concentration 5 mM (B) L-929 mouse fibroblasts Mg^{2+} concentration 3 2 mM, Ca^{2+} concentration 1 1 mM

of the incubation media did not affect the pattern of the pH curves although specific enzyme activity could be altered markedly In the case of the L-929 cell it appeared as if secondary maxima would lie beyond pH 7 and 9, but in order to avoid permeability problems the range of observations was not extended

The effects of Na^+, K^+, Ca^{2+} and Mg^{2+} were tested individually, in combinations and at various concentrations on cultured intact HeLa, KB and L-929 cells It was found that the pyrophosphatase activity of the KB cell line was unique when compared with a variety of other cell lines Most of its pyrophosphatase activity appeared to be unaffected by either the concentration of Mg^{2+} in the medium or by the addition of EDTA In contrast, using L-929 mouse fibroblasts, addition of 5 mM

Mg^{2+} to the Tris buffered saline medium was found to produce an approximate 20-fold increase in pyrophosphatase activity In the L cells significant pyrophosphatase was not observed when Mg^{2+} concentrations below 1 mM were used (Fig 2) The Mg^{2+} activation pattern of the L cells could be observed also with HeLa and human hepatocyte (Chang) cells (Fig 2), as well as neonatal syrian hamster astrocytes and mouse neuroblastoma N-18 cells One of the tested cell lines (H Ep No 2 (Toolan)-human epidermoid carcinoma of the larynx) displayed a partial Mg^{2+} dependence, about 40% of the total pyrophosphatase activity (at 5 mM Mg^{2+}) remained in the absence of this cation or after addition of 2 mM EDTA

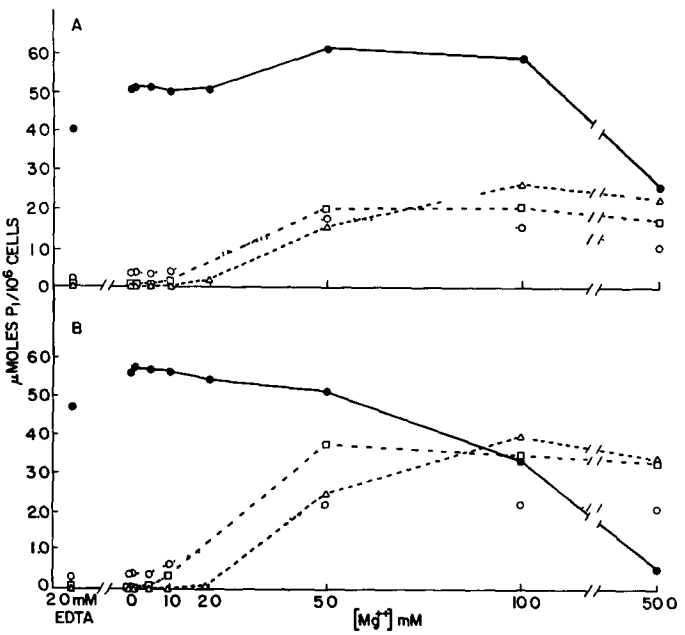

Fig 2 Pyrophosphatase activity of different cultured cell lines Incubation media as described in Materials and Methods, except containing either 2 mM EDTA or Mg^{2+} concentrations ranging from 0 to 50 mM △---△, L-929 mouse fibroblasts, □ - - □, HeLa, ○ ○, Chang, ●—●, KB (A) Whole cells (B) Sonicate

A further distinction between the Mg^{2+} independent and the Mg^{2+}-activated pyrophosphatase was evident in their respective responses to the addition of Ca^{2+} The Mg^{2+}-activated enzyme was strongly inhibited when the two divalent cations were present simultaneously (5 mM Mg^{2+} + 2 mM Ca^{2+}) Ca^{2+} alone at concentrations ranging from 1 to 4 mM failed to activate this enzyme On the other hand, 4 mM Ca^{2+} was only a weak inhibitor of the Mg^{2+}-independent pyrophosphatase when the latter cation was not present In the presence of 5 mM Mg^{2+} the KB cell pyrophosphatase was about 80% inhibited by 2 mM Ca^{2+}

The Mg^{2+}-activated pyrophosphatase was only weakly stimulated when Zn^{2+}, Co^{2+}, or Mn^{2+} were substituted for Mg^{2+} These same cations, by contrast, inhibited the Mg^{2+}-independent pyrophosphatase to some extent For instance, 4 mM Zn^{2+} produced about 50% inhibition in KB cells (0 mM Mg^{2+}) The pyrophosphatase(s)

were not affected by any changes in monovalent cation concentration (Na^+ = 0–87 mM, K^+ = 0–40 mM)

Previous data in the literature had indicated that the actual substrate for inorganic pyrophosphatase was the magnesium complex of PP_i We have found that activity was optimal with a Mg^{2+}/PP_i ratio of unity

Kinetic data

The apparent Michaelis constant (K_m) in intact L-929 cells was found to be 1 10^{-4} M A more detailed study was made with the KB cells (Mg^{2+}-independent pyrophosphatase) It was found that regardless of the experimental conditions, the V observed in sonicated cell-free KB preparations was 10–15% greater than in intact cells When the Mg^{2+} concentrations were varied (0–5 mM) or when EDTA was present, the apparent K_m values for PP_i in intact or sonicated KB cells remained within a range of 0 25–1 67 mM Satisfactory approximation to Michaelis–Menten type kinetics was only obtained, however, if the KB cells were incubated in the presence of 2 mM EDTA

Effects of nucleotides on Mg^{2+} independent pyrophosphatase

The KB cell pyrophosphatase was partially inhibited by ATP, ADP and AMP When these nucleotides were present at concentrations equimolar to PP_i, inhibition of PP_i hydrolysis was about 50% (30–70%) Of the adenine nucleotides tested, ADP

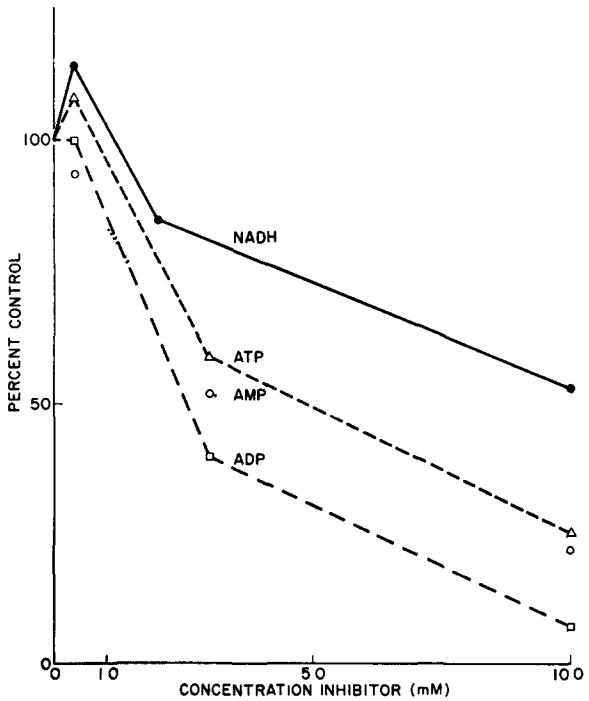

Fig 3 Effect of nucleotides and NADH on pyrophosphatase activity of intact KB cells Mg^{2+} concentration 2 mM Incubation media as described in Materials and Methods

was the most inhibitory Both ATP and NADH consistently produced a small increase in enzyme activity at low (0 4 mM) concentrations (Fig 3)

Comparison of enzymatic activity of intact versus disrupted cells

We have previously discussed the problems associated with assays of presumptive ecto-enzyme activity [1] and we had come to the conclusion that the cultured cells were essentially impermeable to exogenous hydrophilic phosphates Since intracellular phosphate pools became fairly rapidly labeled by superfusion with $^{32}P_i$, permeation of PP_i could not be excluded We were unable, however, to devise a rigorous test for the exclusion of permeation or rates of uptake of PP_i By our estimates, though, it was unlikely that the observed velocity of PP_i hydrolysis by intact cells was compatible with a mechanism which required inward and outward diffusion of substrate and product

The comparison of pyrophosphatase activity in intact with sonicated cells showed that intact cells displayed substantial enzymatic activity Increments observed when the cells were disrupted by brief sonication varied from cell line to cell line (Table I) Comparison of the ecto-enzyme(s) with total cellular pyrophosphatase

TABLE I

COMPARISON OF PYROPHOSPHATASE ACTIVITY OF DIFFERENT CELL LINES

Activity is given as μmoles P_i released per 10^6 cells per 20 min incubation at 37 °C Mg^{2+}, when present, 5 mM

Cell line (origin)	Whole cells	Sonicated cells
KB (carcinoma of nasopharynx) (human)		
Mg^{2+}	6 1	5 1
no Mg^{2+}	5 0	5 5
L-929 (fibroblasts) (mouse)		
Mg^{2+}	1 6	2 5
no Mg^{2+}	0 01	0 02
HeLa (carcinoma of cervix) (human)		
Mg^{2+}	2 0	3 8
no Mg^{2+}	0 08	0 07
Hepatocyte (Chang) (human)		
Mg^{2+}	1 8	2 2
no Mg^{2+}	0 34	0 38
H Ep No 2 (carcinoma of larynx) (human)		
Mg^{2+}	1 7	4 7
no Mg^{2+}	0 92	0 81
N-18 (neuroblastoma) (mouse)		
Mg^{2+}	1 9	5 8
no Mg^{2+}	0 02	0
MA-331 (embryo intestine) (human)		
Mg^{2+}	1 4	4 5
no Mg^{2+}	0 07	0 02

activity indicated that differences in properties (kinetics, inhibitors, cation requirements, etc) were minor A brief summary of our findings with the different cell lines is given in Table II

TABLE II

PROPERTIES OF PYROPHOSPHATASE IN CULTURED CELLS

	KB cells	Mouse fibroblasts	HeLa cells and Chang liver cells
Dependence on Mg^{2+} for activation	No	Yes	Yes
Activity in presence of EDTA	Yes	None	Slight
Kinetics in EDTA	Michaelis–Menten	—	
Effects of Ca^{2+} in presence of Mg^{2+}	Inhibitory	Inhibitory	Inhibitory (HeLa)
Effects of other divalent cations	Inhibitory	Slight activation	
Difference in activity between sonicated and whole cells	Sonicate 10–20% greater than whole	Sonicate 30–40% greater than whole	Sonicate 40–50% greater than whole

DISCUSSION

In the KB cell culturs we have found a pyrophosphatase which seemingly requires no cations for activation, and it was evident that PP_i itself (rather than a metal ion complex) was the substrate in agreement with pyrophosphatase activities as reported from other sources [4–7] With this particular enzyme preparation we observed also a small stimulation by Mg^{2+}, if this is viewed in context with residual activities observed in other cell lines when EDTA was present and/or divalent cations had been omitted, the conclusion can be drawn that both types of pyrophosphatase may occur simultaneously in the same cell or organelle [8, 9]

The partial inhibition by adenine nucleotides in the KB cell pyrophosphatase is reminiscent of the inhibition pattern observed in rat liver [9] The slight stimulatory effect on enzyme activity we observed at low concentrations of ATP and NADH had been noted previously when ATP was used in low concentrations [10]

Both the non-Mg^{2+}-dependent pyrophosphatase of KB cells and the Mg^{2+}-dependent pyrophosphatase of mouse fibroblasts and HeLa cells showed marked inhibition by Ca^{2+} in the presence of Mg^{2+} This also was previously noted in crystalline yeast pyrophosphatase by Kunitz [11], and is in agreement with the generally assigned inhibitory role of Ca^{2+}

Because we found that the *p*-nitrophenylphosphatase activity of intact and sonicated KB cells at pH 7 4 was much greater than that of HeLa cells and mouse fibroblasts [1], our studies do not distinguish the pyrophosphatase activity of these cells from an alkaline phosphatase It is relevant that Cox et al [12], and Herz and Kaplan [13] previously described pyrophosphatase activity in HeLa and other cultured cells and concluded that pyrophosphatase and alkaline phosphatase activities were associated with a single protein

We have commented already on the experimental problems which prevented a rigorous treatment of the assignment of "sidedness" of pyrophosphatase in our cultured cell lines According to the data presented in Table I, only in the case of the

Mg^{2+}-independent KB cell pyrophosphatase does it appear as if the enzyme was predominantly an ecto-enzyme Some ecto-enzyme activity was observed with all studied cell types, this would place inorganic pyrophosphatase into the same category as *p*-nitrophenylphosphatase, leucyl-β-naphthylamidase and phosphodiesterase I, which we have classified as facultative ecto-enzymes, while 5′-nucleotidase and Mg^{2+}-ATPase are obligatory ecto-enzymes [1] (Na^+, K^+)-ATPase and adenylcyclase and probably nucleotide pyrophosphatase were found to function principally on the cytoplasmic side of the plasma membrane

Recent studies in our laboratory have led us to conclude that an interrelationship exists between several of the phosphoesterhydrolases which characteristically are associated with plasma membranes Inorganic pyrophosphatase, however, does not seem to play a role in the proposed scheme and no reason for the presence of pyrophosphatase in the plasma membrane has been advanced so far

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