RESEARCH ARTICLE

Thyroid Gland Ecto-ATPase and Some of Its Kinetic Properties

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Abstract

Plasma membrane of human thyroid gland, apart from the transport Mg-independent HCO_3^-ATPase, has been found to contain also HCO_3^--ATPase of ecto-ATPase type, whose activity is not conditioned by the Mg-ion and with substrate in the form of free ATP (ecto-HCO_3^--ATPase). Activity of such kind is found in both healthy cells and those affected by carcinoma. However, in the latter cells, this characteristic of the enzyme is much higher than the norm. We studied certain kinetic properties of the Mg-independent HCO_3^--ATPase, namely relation of its activity to the quantitative content of the HCO_3^--ions and the substrate (free ATP), as well as the pH of the reaction medium. Experiments showed that properties of ecto-HCO_3^--ATPase are different from those of Mg-HCO_3^--ATPase, which allows for a conclusion that the two enzymes can function independently and are involved in different processes in progress in thyroid gland cells.

Key Words: *Thyroid gland, carcinoma, HCO_3^--ion; Mg-HCO_3^--ATPase, ecto-ATPase*

Introduction

The bicarbonate ion (HCO_3^-) is a chemical component vital for an animal body and is actively involved in the alkali-acid homeostasis of a cell. Together with water molecules, the HCO_3^--ion creates a buffer system to provide for the necessary equilibrium and actively resist any pH factor change in the cell. The process is essential for viability of the cell and its disruption may lead to irreversible damage of the latter. Therefore, quantitative change in the ion content is an important characteristic of a live cell. The factor that is responsible for transportation and amount of the HCO_3-ions is the transportation system known as HCO_3^--ATPase. Activity of the enzyme has been found to exist in various tissues, with a particularly high indicator visible in the cells of so-called secretive tissues, such as mucous membrane in pancreas and stomach, mandibular gland, in kidney, duodenum, etc (1, 2, 3). The enzyme has also been identified in the liver, brain, membrane of erythrocyte cells and other tissues (4, 5). Maximum activity of the enzyme has been recorded in mitochondria and plasma membrane.

Later, a fairly high activity of this enzyme was revealed in the glial cells of the brain. It

was suggested that it is just the HCO_3^--ATPase that participates in the process of intracellular pH regulation maintaining the buffer functions in the glial cells (6). Enzyme activity was not only found in various tissues of adult organisms. It was also detected in embryos, possibly participating in the transportationof HCO_3^--ions to embryonic cells. Several propositions have been made concerning the mechanisms of the HCO_3^--ATPase, but the action and regulation of this enzyme, especially compared with other enzymes connected with ATPase activity, still remains obscure and requires additional information to be properly understood (5).

The HCO_3^--ATPase studied so far in the thyroid belongs to the so-called transport ATPase group (P-type ATPase). However, we know that there is another type of ATPase with properties that are different from the P-type transport ATPase, known as ecto-ATPase (7) and classified as E-type ATPase. Research of this type of ATPase is still at early stages, there are still gaps in the knowledge. As per classification, E-type ATPases encompass ecto-ATPases (hydrolyzing ATP to ADP), ecto-apyrases (transforming ATP and ADP into AMP), and ecto-5-nucleotidases (transforming AMP into adenosine). It has been established that E-type is structurally similar to ATPase, consisting of protein of 50-60KDa molecular mass that is glycolised at various degrees in different cells and is a homo-oligomer with 2-3 monomers (8, 9, 10).

Our research revealed that tissue human thyroid gland contains Mg-dependent HCO_3^-ATPase that shows different activity in case of certain pathologies, e.g. follicular carcinoma, adenoma and thyrotoxicosis (5). However, there are references about Mg-independent HCO_3-ATPase availability in the literature. For example activity of this type of enzyme was found in synaptosomal and mycrosomal fractions of albino rat brain. Therefore, it will be interesting to establish availability of Mg-independent HCO_3^--ATPase at healthy thyroid gland and at pathology, such as follicular carcinoma.

Materials and methods

Subjects

The object of our research was the gland tissue extracted by surgery from patients with various thyroid gland pathologies. To prepare the tissue for research it was washed in 0.9% NaCl solution, cleaned from capillaries, separated into particles and homogenized in sucrose solution (0.32M). In order to maintain the enzymatic activity the process was conducted at a low temperature (0–2°C). The subcellular fractions were separated from the homogenate by differential centrifugation in sucrose gradient (11).

Determination of Mg-dependent HCO_3^--ATPase activity

The activity was evaluated using the difference between active (in the presence of HCO_3^- ions) and passive (when instead of $NaHCO_3$ an identical concentration of NaCl was introduced into the reaction medium) ATPase. The reaction medium contained: $NaHCO_3$ (60 mM); $MgCl_2$ (2.5 mM); ATP (2.5 mM); the protein suspension (500 μg/ml) (in a total volume of 3mL); and buffer solution (40 mM Tris-HCl, pH 7.5). The ATPase activity was determined according to the volume of inorganic phosphorus liberated after hydrolysis of ATP (μmole Pi/mg protein/min).

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Protein concentration was measured with a Protein Assay Kit (Sigma, USA), according to the manufacturer's protocol.

Data analyzes

The data were treated statistically by the Student method. Changes in the velocity and enzyme's characteristics were detected by regressive analysis.

Results

Figure 1 shows influence of HCO_3-ion concentration on Mg-dependent HCO_3-ATPase activity of plasma membrane in the cells of both healthy (N) cells and the cells affected by follicular carcinoma (CR).

As the figure shows, maximal Mg-dependent HCO_3^--ATPase activity in the plasma membrane of a healthy gland is recorded at 120-140 mM of bicarbonate, and only following this concentration it shows a reliable reduction. Such regularity is not characteristic of the HCO_3^--ATPase of CR-diagnosed gland cells. In this case, the activity only reaches its maximum when the concentration of HCO_3^- ion is at 60 mM.

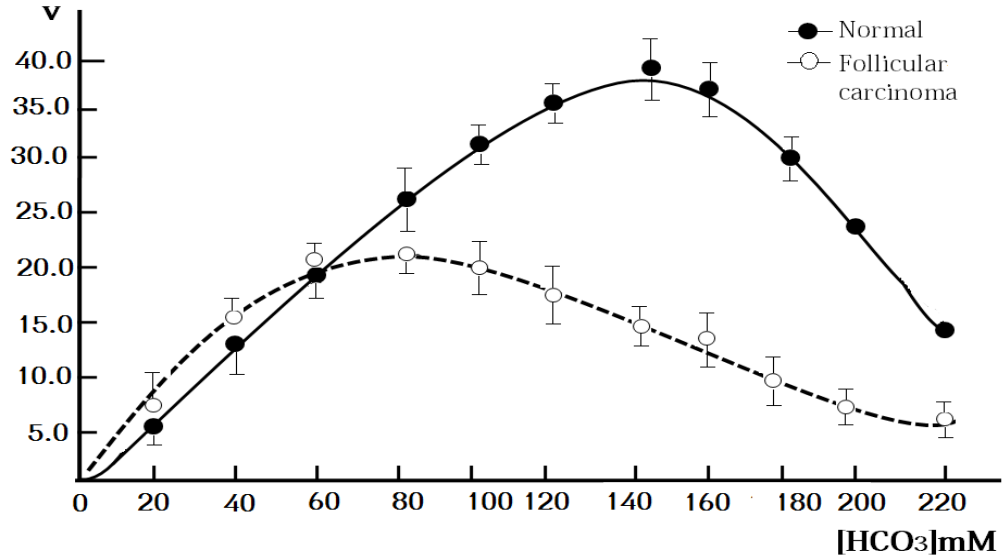

Figure 1. Dependence of HCO_3^{--}ATPase Activity of Plasma Membrane in Thyroid Cells on the Varying Concentration of HCO_3- ions

x axis – enzyme activity in μmol Pi/mg protein/min

y axis – HCO_3^- concentration in the reaction medium

The data on Figure 2 represent change in the activity of healthy and CR Mg-dependent HCO_3-ATPase in the reaction medium under alternating Mg/ATP concentrations. We have found the maximum activity of the enzyme in healthy tissues to occur when MgATP ranges between 2.5 and 3.0 mM. Results are different in case of CR, where enzyme activity raised parallel to MgATP concentration; it practically does not change activity.

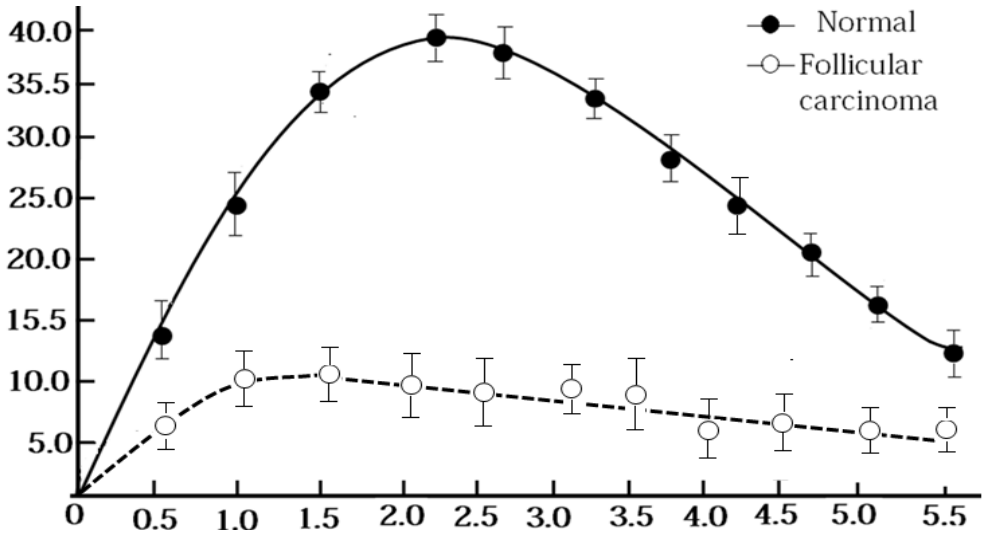

Figure 2. Dependence of HCO_3^-ATPase Activity in Thyroid Gland on the Varying Concentration of Mg/ATP

x axis – enzyme activity in µmol Pi/mg protein/min
y axis – Mg/ATP concentration in reaction medium

Taking into account the results, at the next stage we studied change in the enzyme activity in two scenarios. In the first scenario we observed change in the enzyme activity under constant ATP and alternating Mg concentrations. The second scenario implied constant Mg and alternating ATP concentrations. The results of the experiments are displayed in Figure 3. It is obvious that in healthy cells, rate of ATP (2.5 mM) hydrolysis under alternating concentration of Mg-ions increased on pro-rata bases. After reaching 3 mM and above concentration, enzyme reaction rate was inhibited (Fig 3A). Results were different in the case of CR cells. Higher Mg concentration did not lead to a reliable increase in the activity and it remained practically the same, which may indicate that the reaction rate under the given circumstances does not depend on occurrence of the ions in the reaction medium.

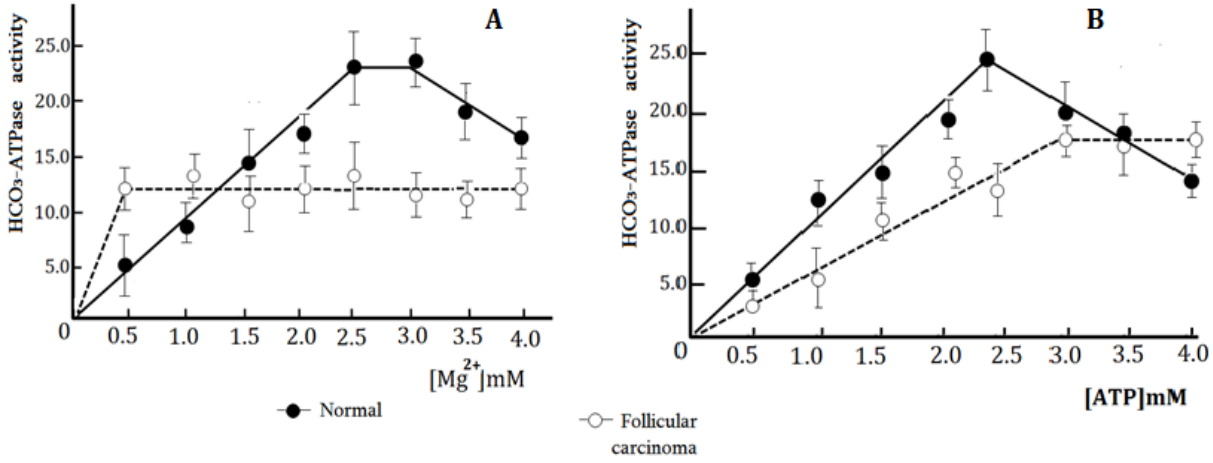

Figure 3. Dependence of Thyroid Gland **HCO_3^-ATPase** Activity on Mg^{2+} (3A) and ATP (3B) Concentrations

x axis – enzyme activity in µmol Pi/mg protein/min
y axis – Mg (A), ATP (B) concentration in reaction medium

Figure 3B shows dependence of enzyme reaction rate on constant Mg and alternating ATP concentrations in the reaction medium. Maximal rate was recorded at 2.5 mM ATP, after which the rate decreased and the curve was almost bell-shaped. In case of gland pathology, maximal reaction rate was also recorded at a 3 mM ATP concentration and its further increase did not practically affect the reaction rate.

After that was studied kinetic parameters of the reactions were observed (Vmax, Km) both in normal and CR cells. At first we established the changes of the enzyme's Vmax and Km under variable Mg-ion and constant ATP concentrations. Figure 4A shows that in healthy cells Vmax reliably rises under constant concentrations of ATP (1.5mM, 3mM, and 4mM). Affinity of the enzyme to Mg-ion also changes (Km1 (-0.5)>Km2 (-0.8)>Km3 (-1.6)).

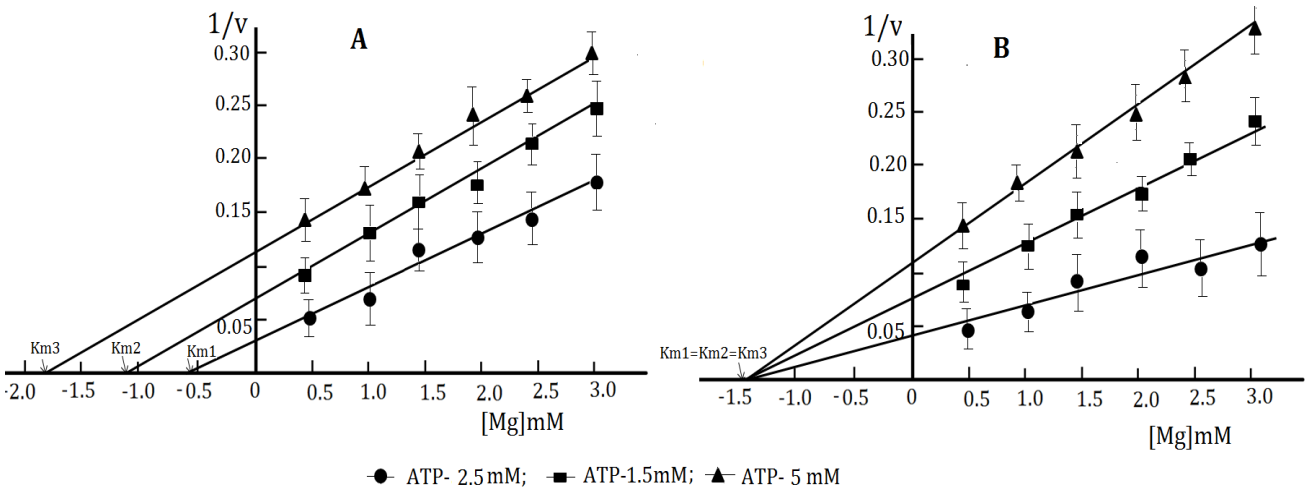

Figure 4. Change in Vmax and Km of Thyroid HCO_3^--ATPase Reaction in Healthy Cells (A) and Cells Affected by Follicular Carcinoma (B) under Alternating Concentration of Mg-ions

x axis – inverted value of reaction rate (1/V)

y axis – concentration of Mg-ions in the reaction medium (mM)

Taking into account the results shown above, we decided to see how different they would be in case of CR (Fig. 4B). Here, we could see that just as in the case of previous data, reaction Vmax rose when ATP concentration was constant (1.5mM, 2.5mM, and 5mM). However, affinity of the enzyme to Mg-ion did not change (Km1=Km2=Km3=-1.35), which was an indicator that the reaction occurred independently from Mg.

The data displayed in Figure 5A show that in healthy cells, HCO_3-ATPase activity under alternating ATP concentrations depends on the content of Mg-ions in the reaction medium. The proof to such a conclusion is both the varying Vmax at three constant Mg-ion concentrations (Mg=1mM, 2.5mM, 4mM) and dissimilar affinity of the enzyme to ATP (Km1 (-0.22)> Km2 (-0.75)>Km3 (-0.5)). The picture is different in case of CR (Fig. 5B). Here, we can see that the reaction rate under alternating ATP concentrations does not depend on the Mg content in the reaction medium ($1/v_1=1/v_2=1/v_3$).

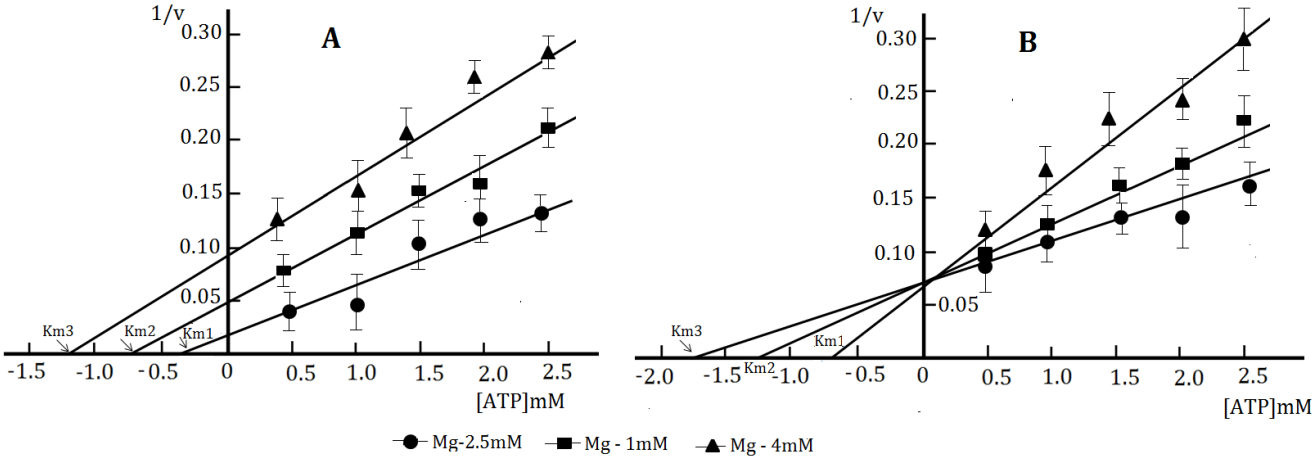

Figure 5. Change in Vmax and Km of Thyroid HCO_3^--ATPase Reaction in Healthy Cells (A) and Cells Affected by Follicular Carcinoma (B) under Alternating Concentration of ATP

x axis – inverted value of reaction rate (1/V)

y axis – concentration of ATP in the reaction medium (mM)

As HCO_3^--ATPase is known to be involved in the intracellular and pH regulation processes, we further studied change in the enzyme activity when the reaction medium had different pH (Figure 6).

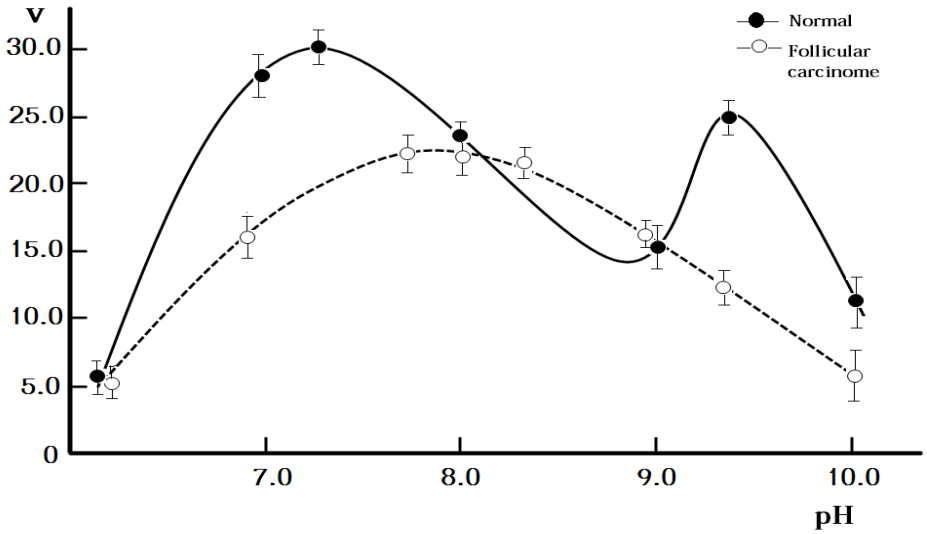

Figure 6. Dependence of **HCO_3^-ATPase** Activity on pH of the Reaction Medium

1 - change in the enzyme activity in healthy cells

2 - change in enzyme activity in cells affected by follicular carcinoma

x axis - enzyme activity (V) in µPi/mg protein/min

y axis - pH in the reaction medium

The difference between HCO_3^--ATPases of healthy and diseased glands is again clearly visible. In the first case, we received two maximal enzyme activities, under pH-7.5 and pH-9.3. As for CR, in this case, the pH, under which the enzyme activity reached its maximum, ranges between 7.5 and 8.5.

The results show that HCO_3^--ATPase displays different properties depending whether it is found in healthy glands or those affected by follicular carcinoma. We, therefore, aimed at finding out what the type of ATPase is. The specific inhibitor employed to classify the ATPase was ARL67156 (6-*N*,*N*-Diethyl-D-*β*-*γ*-dibromo-methylene adenosine triphosphate), which only inhibits ecto-ATPases (Table 1).

Table 1

Effect of ARL67156 on ATPase Activity Powered by HCO_3^--ions in Healthy and Disease-Affected Gland Cells

n=6

Thyroid gland	ARL 67156 (µmol)		%
	0	40	
HCO_3^- -ATPase in healthy cells	22.45 ±1.45	19.93±2.24*	12.3
HCO_3^- - ATPase in cells affected by follicular carcinoma	16.35±2.36	6.06±1.39*	63.0

*p≤0.05

The table shows that existence of ecto-ATPase was proved in both kinds of glands. However, its activity in pathological glands was much higher.

Discussion

As our previous research had shown, various tissues in mammals, and among them human thyroid gland, are characterized by high HCO_3^--ATPase activity (5, 6, 12). It had also been found that under various pathologies, such as adenomas, follicular carcinomas and thyrotoxicosis both activity and kinetic parameters of the enzyme changed. We, therefore, aimed at establishing whether these changes are caused by structural changes in the enzyme or it is an essentially different enzyme activity that takes place.

In the second half of the 20th century, certain enzymes were discovered that had an ability to hydrolyze extracellular nucleotides, including ATP. Initially, questions were asked about existence of a site outside the cell membrane where ATP bonded with the enzyme as scientists had assumed ATP was located and functioned strictly within a cell. However, further research proved that such type of ATPase did exist and it was classified as ecto-ATPase (7, 8, 13, 14, 15). Their characteristics in common were also described, particularly involving activation with 2-valent cations and certain anions; ability to hydrolyze tri-, di- and mono-phosphates; insensitivity to P, F and V type ATPase inhibitors and sensitivity to the specific inhibitor ARL67156 (6-*N*,*N*-Diethyl-D-*β*-*γ*-dibromomethylene adenosine triphosphate) (10, 16). It has also been suggested that this type of ATPase is also involved in regulation of synaptic transportation, functionality of lymphocytes

and the kidney, as well as in the formation of several pathologies, such as epilepsy, tumors, etc. (17, 18, 19).

As Table 1 shows, ARL67156 reduces the enzyme activity in both healthy and CR-affected glands. It is noteworthy that its effect is much higher in pathological tissues than in normal cells (63% and 12.3% respectively), which could point at the existence of both transport and ecto-ATPase in the thyroid gland. However, an ecto-ATP displays a much higher activity under pathology.

The result shows that dependence of HCO_3^--ATPase on the HCO_3-ions in healthy gland cells is different from the same characteristic of the enzyme in CR-affected cells. Analysis of the geometrical shape of the curve in Figure 1 clearly shows a bell-shaped curve of the activity with both - ascending and descending phases. This shape is known to be characteristic of all transport ATPase and to represent a necessary, albeit an insufficient condition for the type (20, 21). Such dependence has been found in carcinoma-affected gland cells, though the affinity of towards HCO_3^--ions is changed.

This assumption is also grounded by dependence of the enzyme on the substrate. MgATP is the known substrate of transport ATPase (21). This makes it different from ecto-ATPase, whose substrate is confined to free, extracellular ATP that, as an important signaling molecule, is characterized by various biological effects (16, 23). As Figure 2 shows, control value of enzyme activity differs from the same indicator in pathological cells. In the case of healthy cells, dependence of enzyme activity on the substrate is clearly of the nature that is characteristic of transport ATPase, with ascending and descending phases. On the other hand, the identified enzyme activity does not show similar dependence in pathological glands (21).

Different characteristics of the enzymes identified in healthy and pathological gland tissues are also apparent by the nature of their dependence on Mg and ATP. Figure 3A clearly shows that HCO_3^--ATPase activity of plasma membrane in glands affected by follicular carcinoma does not depend on occurrence of Mg in the reaction medium. As for, available that Mg-ATPase working at Mg-ion small concentration, when observed increasing of enzyme activity, or the reason of this effect is ecto-ATPase, whereas in condition of Mg ion optimal concentration on reaction area no longer observed an increase effect of enzyme activity. Conversely, on the other hand, ATPase activity in the healthy tissue undoubtedly reflects its dependence on the ion. Another indication involves the enzyme's kinetic parameters (Vmax, Km), which shows variance in the enzyme activity without any change to its affinity to Mg-ions (Fig. 4B), while ATPase activity in the healthy gland is highly dependent on the concentration of this ion (Fig. 3A and 4A).

Literature data show that apart from Mg-dependent ATPase activity, certain tissues have also been found to display non-Mg-dependent ATPase activity (20, 23, 24). Hence, it could be assumed that the highly active ATPase found in the plasma membrane of carcinoma-affected thyroid gland, which is a so-called ecto-ATPase, is also a non-Mg-dependent enzyme.

One indication of such an assumption is the nature of the dependence the enzyme activity displays towards the pH of the reaction medium. pH optimum of ecto-ATPase is known to range between 8.0 and 8.3. This is the case, for example, in the case of ecto-ATPases in the human leukocyte membrane (8). Our data show that the maximum activity in the CR-affected thyroid gland is shown by the enzyme whose pH is between 7.3 and 8.3. The observed relation of HCO_3^-ATPase and pH values in healthy

gland cells are also harmonious with literary data (25).

Thus, as the experiments indicate, plasma membrane of human thyroid gland shows existence of two types of ATPase, whose activities differ according to the functional state of the gland. In particular, healthy glands are characterized by high HCO_3-ATPase activity, HCO_3-ATPase being an Mg-dependent enzyme and classified as P-type transport ATPase due to its properties. Alongside it functions the non-Mg-dependent ecto-HCO_3-ATPase, whose activity in the norm is lower if compared to that of the Mg-dependent HCO_3-ATPase. However, as pathological processes develop, its activity significantly rises. Thus, it can be assumed that in a certain form, it must be involved in the formation and development of the pathology. This subject is still under study.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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