Further Investigations on the *p*-Nitrophenylphosphatase Activity of Intact Ehrlich Ascites Tumor Cells *

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Z. Naturforsch. **33 c**, 227 – 230 (1978) ; received January 23, 1978

Phosphatase Activity, Ehrlich Ascites Tumor Cells, SH Blocking Agents

The substrate specificity and the effects of nucleotides and SH-blocking agents on the *p*-nitrophenylphosphatase activity of intact Ehrlich ascites tumor cells (EAT) cells were studied. DL-β-Glycerophosphate, *o*-phosphoethanolamine, cholinephosphate, glucose-6-phosphate, *o*-carboxyphenylphosphate,, phosphoenolpyruvate and AMP were not attacked by intact cells. ATP > GTP > UTP > PP_i > pNPP were cleaved with decreasing velocity. A stimulation of the cleavage of *p*-NPP by the following nucleotides was observed with decreasing effectivity: ATP > ADP > GTP > UTP; AMP was ineffective. The phosphatase activity was not affected by malate, tartrate and glutathion disulfide. The SH blocking agents diamide and thimerosal were more effective inhibitors of the pNPPase than of the ATPase activity, whereas the hydrolysis of ATP is more affected by the ATP analog adenylylimidodiphosphate. The present data are best compatible with a double headed enzyme: Both active sites interact with ATP, only one is active against *p*-NPP and sensitive against SH-blocking agents.

In previous investigations on the effect of SH-blocking agents on the cell surface of Ehrlich ascites tumor (EAT) cells we have shown [1 – 3], that intact cells exhibit *p*-nitrophenylphosphatase activity, which is impaired by chemical alterations of the plasma membrane. Such alterations were produced by blocking surface SH groups or by treatment of the cells with neuraminidase. To further characterize this phosphatase activity of intact cells, we studied its pH and temperature dependence [4]. The results of these experiments suggested that measurement of the phosphatase activity of intact cells is a convenient method for studying temperature dependent changes of the membrane.

In order to obtain further information on the nature of the *p*-nitrophenylphosphatase activity of intact cells, we have performed additional experiments on the substrate specificity, the influence of nucleotides and the effect of sulfhydryl reagents on the enzyme activity.

The results of these investigations give evidence that the *p*-nitrophenyl phosphatase activity of intact EAT cells is a manifestation of the Na^+, K^+-ATPase of the plasma membrane.

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* Supported by Deutsche Forschungsgemeinschaft (SFB 103).

Materials and Methods

Chemicals

p-Nitrophenylphosphate (*p*-NPP), ATP, ADP, AMP, GTP, UTP, glucose-6-phosphate, DL-β-glycero-phosphate, phosphoenolpyruvate, glutathione, adenylylimidophosphate (AMP-PNP) were from Boehringer, Mannheim. Serum albumin was from Roth, Karlsruhe, morpholinopropanesulfonic acid (MOPS) and thimerosal (ethylmercurithiosalicylate) were from Serva, Heidelberg. *o*-Carboxyphenylphosphate, phosphoethanolamine, cholinephosphate were from Sigma, München. N,N,N′,N′-Tetramethylazoformamide (diamid) was synthesized according to ref. [5]. All other substances were from Merck, Darmstadt. The culture medium described by Schindler [6] was used.

Cell suspensions

Hyperdiploid EAT cells grown in female NMRI mice were used. 1 – 2 ml ascites fluid were mixed with 50 ml culture medium at 37 °C. The cells were separated by centrifugation for 2 min at $500 \times g$ and washed twice with Ringer solution pH 6.9 containing 1% albumin. An aliquot of the cell suspension in Ringer solution corresponding to 4.18×10^7 cells was centrifuged and the cells were suspended in incubation buffer pH 6.9 containing 10 mM MOPS, 5 mM KCl, 2 mM $MgCl_2$ and 140 mM NaCl. This cell suspension was used for further experiments. The number of cells was determined by

turbidity measurements at 578 nm, the number of dead cells was evaluated by the method of Kaltenbach [7].

Measurement of the enzyme activities of intact cells

7 ml incubation buffer pH 6.9 were mixed with 2 ml of the cell suspension described above and incubated for 6 min. 1 ml 10 mM *p*-nitrophenylphosphate was added; after 10 and 20 min 5 ml aliquots were chilled at 0 °C and the cells were separated by centrifugation at 0 °C. The supernatant was again centrifuged and 2 ml were mixed with 100 μl 4 N NaOH. Before reading the extinction at 405 nm against a blank of 1 mM *p*-NPP the samples were clarified by centrifugation.

Influence of activators and inhibitors on the enzyme activity

The effect of ATP, ADP, AMP, GTP, UTP and Pi on the *p*-NPPase activity of the cells was measured by mixing 3 ml incubation buffer, 1 ml cell suspension and 0.5 ml of the respective nucleotide in MOPS buffer. After 6 min at 37 °C, 0.5 ml 10 mM *p*-NPP was added. The supernatant was analyzed for *p*-nitrophenol as described above. Control experiments were performed by mixing 3.5 ml buffer, 1 ml cell suspension and 0.5 ml 10 mM *p*-NPP. This reaction mixture was also employed for studying the substrate specificity; instead of *p*-NPP the reaction was started by 0.5 ml 10 mM DL-β-glycerophosphate, glucose-6-phosphate, *o*-carboxyphenylphosphate, phosphoethanolamine, cholinephosphate, phosphoenolpyruvate, AMP, ADP, ATP, GTP or UTP. In these exeriments the incubation buffer contained 1 mM glutathione. After 10 and 20 min 2 ml samples of the reaction mixture were mixed with 0.5 ml chilled 25% trichloroacetic acid. After one hour at 0 °C the solutions were centrifuged. In the supernatant phosphate was determined according to the method of Eibl and Lands [8]. In control experiments the phosphate concentration in mixtures containing cells without substrates, substrates without cells and pure buffer was also determined. The hydrolysis of ATP was in addition evaluated by way of the luciferin, luciferase system [9].

To test the effect of glutathione on the phosphatase activity of the cells, the rate of hydrolysis of the substrates *p*-NPP, PP_i, ADP and ATP was compared in the presence and absence of glutathione with the same charge of cells.

The inhibition of the enzyme activity by diamide was studied with *p*-NPP, PP_i and ATP as substrates. The reaction mixture contained 3 ml buffer, 0.5 ml 1 mM or 0.1 mM diamide in MOPS buffer, 1 ml cell suspension and 0.5 ml 10 mM substrate. The rate of hydrolysis was followed by phosphate analysis as described above.

The inhibition by thimerosal was investigated with *p*-NPP and ATP als substrates. The conditions of incubation were the same as described for diamide. The final concentration was 10^{-4} mM thimerosal.

The effect of AMP-PNP on the phosphatase activity of the cells was studied with *p*-NPP, ATP and ADP as substrates.

Results and Discussion

Substrate specificity of the phosphatase activity of the intact cells

In the present experiments the phosphatase activity of intact EAT cells was studied in MOPS buffer after washing the cells with Ringer-albumin solution. Using this procedure the number of dead cells did never exceed 2%. Wernstedt [10] has already pointed out the protective effect of albumin on the viability of EAT cells. Our activity measurements, however, were performed in the absence of albumin, because the enzyme activity is impaired by this protein. Since earlier experiments have demonstrated the importance of free SH groups for the activity, the different substrates were tested in the presence of glutathione. The results of these experiments are summarized in Table I.

Because DL-β-glycerophosphate, *o*-phosphoethanolamine, cholinephosphate, glucose-6-phosphate, *o*-carboxyphenylphosphate, phosphoenolpyruvate and

Table I. Rate of hydrolysis of different phosphoric acid esters and nucleotides catalyzed by intact EAT cells at 37 °C and pH 6.9.

Substrate	nM Phosphate 10^7 cells · 20 min	Standard deviation
p-NPP	32	± 1.7
PP_i	143	± 5.2
ADP	276	±23.1
ATP	1633	±11.6
GTP	1543	±10.9
UTP	1533	±11.2

AMP were not attacked by intact cells, they are not listed in the table. The most striking feature of these experiments is the great difference between the rate of hydrolysis of the triphosphates ATP, GTP, UTP on the one side and *p*-NPP, PP_i and ADP on the other side; *p*-NPP for instance is only cleaved with 2% of the rate of hydrolysis of ATP. We did not investigate whether ADP is the real substrate of the phosphatase or the precursor for the synthesis of ATP which is then cleaved by the cells [11]. The rate of hydrolysis of the substrates seems to be the higher the closer the relationship to ATP. This consideration suggests that only one enzyme is responsible for the cleavage of all substrates. Further experiments support this suggestion (see below).

Concerning the cleavage of pyrimidine nucleotides (UTP), Ohishi and Kimura have also observed an UTPase activity at the surface of normal and transformed cells [12], and Robinson [13] reported a CTPase activity of plasma membrane fragments showing Na^+, K^+-ATPase activity; the CTPase activity, however, was only 25% of the ATPase activity. Schoner [14] has shown, that Na^+, K^+-ATPase is also active with other nucleosidtriphosphates than ATP.

Inhibition of the phosphatase activity of intact EAT cells

In order to get further information on the nature of the phosphatase activity of EAT cells, we have studied the effect of some specific phosphatase inhibitors, of a specific ATPase inhibitor and of further SH-blocking agents on the activity.

Malate and tartrate, which are well known phosphatase inhibitors [15] did not affect the phosphatase activity of the cells under the conditions of our experiments. However, we cannot exclude that these dianions do not attain the membrane bound enzyme.

SH-blocking agents, which were not used in our previous investigations [2, 3] were included in the present experiments, because sulfhydryl groups obviously are concerned in the enzymatic activity. Diamide, (N,N,N′,N′-tetramethylazoformamide), a sulfhydryl groups oxidizing reagent, did not impair the viability of the cells up to a concentration of 0.1 mM. Thimerosal was chosen as inhibitor because its effect on Na^+, K^+-ATPase is thoroughly investigated [16]. AMP-PNP was found to be a highly specific inhibitor of ATPases [17, 18]. The influence of these reagents on the hydrolysis of *p*-NPP, PP_i and ATP can be seen from Table II.

Table II. Inhibition of the phosphatase activity of intact EAT cells by diamide, thimerosal and AMP-PNP.

Substrate	Inhibitor				
	Diamide [mM]		Thimerosal [mM]	AMP-PNP [mM]	
	10^{-2}	10^{-1}	10^{-4}	10^{-4}	4×10^{-4}
p-NPP	56.7	67.5	28.9	8.3	16.7 *
ATP	—	5.6	19.4	18.7	29.0 *
PP_i	21.8	34.7	—	—	—

% Inhibition after 10 min of incubation with the inhibitor.
* 20 min incubation.

In the concentrations applied, diamide is the strongest inhibitor of the *p*-NPPase activity; after 20 min the inhibition of the PP_i hydrolysis is 10% and the inhibition of the cleavage of ATP only 7% of the inhibition of the *p*-NPP hydrolysis. Diamide seems to inhibit preferentially the *p*-NPPase activity of the cells. The same tendency is observed with thimerosal (ethylmercurithiosalicylate), but the difference between the inhibition of *p*-NPPase and ATPase activity is less pronounced. Thimerosal is highly toxic for the cells, therefore it is not possible to use higher concentrations than 10^{-4} mM without impairment of their viability. The inhibition of ATPases by the ATP-analogue AMP-PNP is the consequence of a covalent labelling of the active site and not of a reaction with SH groups. This reagent seems therefore especially suited for a differentiation between the ATPase and *p*-NPPase activity of the cells. The results of our experiments revealed, however, that the hydrolysis of *p*-NPP as well as of ATP is inhibited. Though the ATPase activity is more impaired by AMP-PNP than the *p*-NPPase activity, it seems not possible to differentiate between the two activities by inhibition experiments.

Activation of the p-NPPase activity of intact EAT cells by nucleotides

The experiments of this section were stimulated by observations of Rozengurt [19], who found a considerable activation of the *p*-NPPase activity of transformed cells by ATP but not by other nucleotides. We therefore have studied the influence of

Table III. Activation of the *p*-NPPase activity of intact EAT-cells by different nucleotides.

	Concentration of the nucleotide [mM]	Activity * nM phosphate 10^7 cells·20 min	Rel. activity [% of control]
p-NPP	—	25	100
p-NPP +AMP	2	25	100
p-NPP	2	51	205
+ADP	3	63	255
p-NPP	1	30	121
+ATP	2	56	225
	3	64	258
p-NPP +GTP	2	38	155
p-NPP	1	29	116
+UTP	2	37	151

* Without glutathione.

different nucleotides on the phosphatase activity of EAT cells. The results are illustrated in Table III.

In contrast to the results of Rozengurt [19] all nucleotides tested (except AMP) cause an activation of the phosphatase activity, ATP and ADP being the most effective compounds. These findings are in good agreement with reports of other groups on the *p*-NPPase activity of isolated plasma membranes of different tissues [20 – 23].

One enzyme with two active sites may explain the present observations

The results of the present experiments suggest, that two different ATP splitting sites are present on the cell surface, one of which has in addition *p*-NPPase activity and is impaired by SH-blocking agents. The second site, which is much more active with ATP as substrate than the first and does not interact with *p*-NPP, is not affected by SH reagents; both sites are close together. This model [16, 18] is compatible with one enzyme containing two active sites and may explain all experimental results:

SH-blocking agents are more effective inhibitors of the *p*-NPPase than of the ATPase activity, because only the first site with low ATPase and *p*-NPPase activity is impaired by these compounds. Glutathione is a more effective activator of the *p*-NPPase than of the ATPase activity of the cells. Since both sites have ATPase activity, they are both affected by AMP-PNP; but the inhibition of the ATPase is stronger than the inhibition of the *p*-NPPase, as was to be expected.

The activation of the *p*-NPPase of EAT cells by nucleotides may be explained by interaction of the nucleotides with the second "high affinity" site, while *p*-NPP interacts with the first "low affinity" site; nucleotides produce an enhencement of the *p*-NPP hydrolysis by cooperative effects. However, an activation should only be observed at low concentrations of the nucleotides. At high concentrations the *p*-NPPase activity should be inhibited. This was indeed observed by Robinson [18] with a purified ATPase preparation.

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