THE EFFECT OF SH-BLOCKING AGENTS ON THE *p*-NITROPHENYLPHOSPHATASE ACTIVITY OF INTACT EHRLICH ASCITES TUMOR CELLS

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1. Introduction

It is generally accepted that the cell surface is a site of different enzymatic activities such as glycosyltransferases Na^+-K^+ ATPase and 5′ nucleosidases [1]. Nearly all studies on cell surface enzymes have been performed with isolated membranes. Within the scope of our studies on enzyme activities of the cell surface and their relation to functional groups of the outer cell membrane, we have investigated the *p*-nitrophenylphosphatase activity of intact Ehrlich Ascites tumor cells and the effect of chemical alterations of the cell surface on this activity. Chemical modification of the cell surface was performed by the action of neuraminidase and SH blocking agents. In addition to the phosphatase activity the intracellular non protein SH level and the number of surface SH groups after incubation of the cells with different SH reagents were measured. Studies on *p*-nitrophenylphosphatase activity of intact cells have not been published as yet.

2. Materials and methods

N-Ethylmaleimide, iodoacetic acid, *p*-nitrophenylphosphate, (pNPP), and buffer substances were purchased from Merck, Darmstadt. 5.5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was from Serva, Heidelberg. Iodoacetic acid amide, *p*-chloromercuribenzoic acid was from Fluka, Buchs, Switzerland, *p*-chloromercuribenzenesulfonic acid was from Sigma, St. Louis, USA. 6,6′-Carboxypyridinedisulfide (CPDS) was synthesized in our laboratory by Dr E. Schaich, *N*-(4-Carboxyphenyl) -maleimide was synthesized by Dr G. Niebch, neuraminidase was from the Behring Werke, Marburg.

2.1. *Cell suspensions*

Hyperdiploid Ehrlich Ascites tumor (EAT) cells were used for all experiments. The cells cultured in female NMRI mice were harvested eight days after intraperitoneal inoculation. In some experiments in vitro cultured cells after the first passage were employed. Further details of the in vitro cultivation are described in loc. cit. [2] and [3].

Vitality of the cells was tested by staining with nigrosin according to Kaltenbach [4].

2.2. *Chemical and enzymatic tests*

The number of SH groups on the cell surface was determined with CPDS by a modified procedure of Grassetti et al. [5]. The cells were incubated with CPDS in Hanks Balanced Salt Solution (HBSS) and the absorption of the supernatant was measured at 344 nm against an appropriate blank. Cells treated with SH blocking agents were washed three times and thereafter incubated with CPDS.

Determination of non-protein thiol content (acid soluble SH content) was accomplished with DTNB [6] after three times washing the cells in HBSS and disruption with ultrasonics. The proteins were precipitated with perchloric acid, and the supernatant used for the SH determination. Measurement of *p*-nitrophenylphosphatase activity of intact cells was performed in 4.5 ml incubation buffer according to Bonting [7] (CN^- omitted) containing 5×10^6 cells previously washed two times with the same buffer. If measurements were carried out in the presence of inhibitors the concentration was 0.1 mM if not otherwise stated. The reaction was started by 0.5 ml 12 mM *p*-nitrophenylphosphate dissolved in the same medium. Aliquots of the incubation mixture were

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taken after 5, 15 and 30 min. The cells were quickly removed by centrifugation and the supernatant was analysed for *p*-nitrophenol by mixing 1 ml with 50 μl 5 N sodium hydroxid. The absorption was measured at 405 nm against an appropriate sample without cells. *p*-Nitrophenylphosphatase activity of the cell supernatant was controlled after incubation of EAT cells under identical conditions, centrifugation (500 *g*, 2 min) and addition of *p*-nitrophenylphosphate to cell-free solution.

3. Results and discussion

3.1. *The p-nitrophenylphosphatase activity of intact EAT cells*

Incubation of EAT cells with *p*-nitrophenylphosphate results in a linear time dependent liberation of *p*-nitrophenol as it is shown in fig.1.

It is generally accepted that phosphoric acid esters do not penetrate the cells under the conditions of our experiments. Therefore it seems less probable that the substrate pNPP enters the cells and is hydrolysed with subsequent release of *p*-nitrophenol into the incubation buffer. The pNPPase activity in the supernatant of the cells did not exceed 15% of the activity of the complete incubation mixture. Further separation of the supernatant from cell particles and membrane fragments by filtration through glass fiber filters reduces the pNPPase activity of the filtrate to 2–3% of the total activity. The results of these experiments suggest that the hydrolysis of pNPP is brought about first of all by an enzyme localized in the plasma membrane of the intact EAT cells.

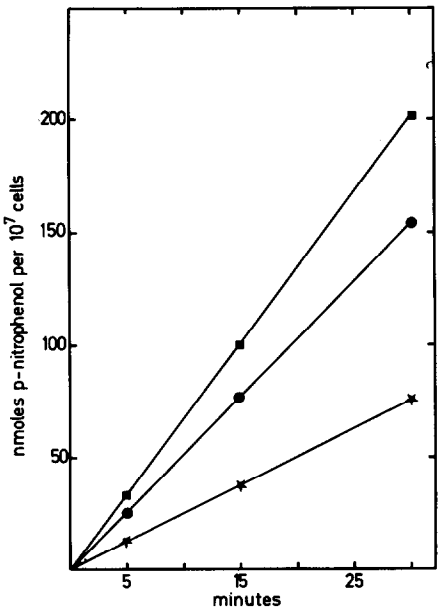

Fig.1. *p*-Nitrophenylphosphatase activity of intact EAT cells at 37°C, pH 7.0. (•) Native cells; (⋆) In the presence of 0.1 mM *N*-ethylmaleimide; (▪) After treatment of the cells with neuraminidase.

This assumption is supported by the data of Forte and Heinz [8] who detected a pNPP hydrolysing enzymatic activity in purified Ascites tumor plasma membranes. The authors suppose the pNPPase activity to be a part of the (Na^+-K^+)-stimulated ATPAse of the outer membrane. In experiments with erythrocyte membranes Nakao and Nagano [9] came to the same conclusion. In accordance with their results we found an activation of the pNPPase of 75% on incubation of the cells with 1.2 mM pNPP and the same concentration of ATP. Under the conditions chosen it was not possible to demonstrate the K^+ dependence of the pNPPase activity of the living cells since the number of dead cells rose rapidly in K^+-free incubation buffer, thus increasing the pNPPase activity from lysed cells.

3.2. *Effect of chemical alterations of the cell surface on the p-nitrophenylphosphatase activity of intact cells*

The pNPPase activity is markedly influenced by chemical alterations of the cell surface. After incubation of the cells with neuraminidase for 30 min the neuraminic acid was completely removed from the cell surface. The amount of 0.49 μmol neuraminic acid/10^9 cells (see Warren [10]) found in our experiments is in good agreement with the value of 0.42 μmol/10^9 cells of Hoelzl-Wallach et al. [11]. The removal of neuraminic acid is accompanied by an enhanced pNPPase activity of the cells. We suggest that the loss of negatively charged neuraminic acid residues causes the enzyme to be more succeptible to the substrate.

Since it is well known that ATPases are influenced by SH blockers [12] it was of interest to study their effect on the pNPPase activity of the living cells. The results of these investigations are summarized in table 1.

The different inhibitory effect of the SH blocking

Table 1
Effect of 10^{-4} M SH blocking agents on the *p*-nitrophenylphosphatase activity of intact EAT cells in Bonting buffer pH 7. The number of dead cells was not significantly affected by the inhibitors under the conditions chosen

Inhibitor	nmol pNp / 10^7 cells × 30 min		% inhibition
Controls	153 ± 27	(40)[a]	0
Iodoacetic acid	114 ± 10	(12)	25
Iodoacetic acidamide	113 ± 5	(6)	25
N-Ethylmaleimide	75 ± 8	(12)	50
N-Carboxyphenylmaleimide	86 ± 9	(12)	43
p-Chloromercuribenzoic acid	37 ± 10	(6)	75
p-Chloromercuribenzene-sulfonic acid	33 ± 5	(12)	78
Carboxypyridinedisulfide	95 ± 9	(6)	37

[a] number of experiments

agents on the pNPPase do not merely reflect their reactivity against SH compounds such as cysteine or glutathione. Sterical factors and the state of charge of the inhibitor seem to be important for the degree of inhibition of the enzyme. The inhibition of the phosphatase activity of intact EAT cells by SH blocking agents does not prove that SH groups are constituents of the activity site of the enzyme. SH groups however, may be important for the enzyme activity by a mechanism concerned with maintaining the structural features of the enzyme in the membrane.

The observed influence of SH blockers on the pNPPase activity was now to be compared with their possible effect on the SH groups of the cell surface and the non-protein SH content (acid-soluble thiol content) of the cells. The results are summarized in table 2. These values reflect the extent of penetration and the reactivity of the inhibitors tested.

Comparing table 1 and 2 no correlation between the inhibition of the pNPPase and the decrease of the non-protein SH level is detectable. This should be expected if the enzyme is located inside the cell.

Table 2
Non-protein SH-level of EAT/cells after incubation with different SH blocking agents and effect of the reagents on the number of surface SH groups of intact cells

SH blocking agents (0.8 mM)	Time of incubation [min]	% Non-protein SH of controls (8 experiments in each case)	% Surface SH of controls
Iodoacetic acid	5	86 ± 2	68 ± 4
Iodoacetic acid amide	5	64 ± 3	60 ± 5
N-Ethylmaleimide	5	10 ± 1	30 ± 4
N-Carboxyphenylmaleimide	5	60 ± 3	70 ± 7
p-Chloromercuribenzoic-acid[a]	5	92 ± 7	98 ± 10
p-Chloromercuribenzene-sulfonic acid[a]	5	100 ± 2	102 ± 10

[a] Concentration 0.08 mM

From the data of table 2 it can be concluded that *p*-chloromercuribenzoic acid and *p*-chloromercuribenzene sulfonic acid do not penetrate the cells in the range of concentration tested. The concentration of the mercurials had to be chosen much lower than for the other compounds in order to avoid an increase in the number of dead cells.

Table 2 also illustrates the effect of the thiol reagents on the number of surface SH groups of intact EAT cells which was determinated with CPDS [13]. The mercury compounds have no influence on the number of surface SH groups, they affect however the pNPPase activity, pCMBS being the strongest inhibitor. The elucidation of this unexpected result in connection with further characterisation of the pNPPase on the surface of intact EAT cells is still under investigation.

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