LOCALIZATION OF THE CATALYTIC SITE OF 5'-NUCLEOTIDASE AT THE INNER SURFACE OF MURINE PLASMOCYTOMA PLASMA MEMBRANES

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1. Introduction

Many 'plasma membrane-marker enzymes' have already been described. The physiologic significance of some of them is still enigmatic, although (Na^+ + K^+)-ATPase or adenylate cyclase, for instance, are well characterized. 5'-Nucleotidase is now generally accepted to be located on the plasma membrane, i.e., to be markedly enriched in this fraction. However, the position of the catalytic site of this enzyme on the outer or inner face of the membrane has not been disputed in terms of cell type and plasma membrane areas. Comparing enzymatic activities of intact cells with that of disrupted cells, some authors have demonstrated that 5'-nucleotidase is an ecto-enzyme in various cells [1,2]. However, Shultz and Thompson [3] noticed an enhanced AMP hydrolysis in cell homogenate when compared to whole cells (at least 8-times). So they considered the hydrolytic site being located at the cytoplasmic face. Using antibodies which inhibited the enzymatic activity assayed in plasma membranes or in purified enzyme, Gurd and Evans [4] conclude that 5'-nucleotidase is located on the outer surface of mouse liver cells. When cytochemical procedures, involving the trapping of inorganic phosphate as lead phosphate, are used to localize this activity, the reaction product was found by many authors to be localized on the external side of plasma membranes both in isolated cell fractions and in situ [5–7]. However, by this method, Benedetti and Delbauffe [8] have shown that 5'-nucleotidase is located on the outer surface of the liver plasma membrane lining the bile spaces while located on the inner surface of liver close junctions. Furthermore, the translocation of 5'-nucleotidase across hepatic membranes in vivo has been reported [9]. Thus the location of the catalytic site on one or the other face of the plasma membrane remains questionable.

Our experimental approach to the problem of 5'-nucleotidase position is as follows:

The enzymatic activity was tested with intact and disrupted cells. With intact cells, only the outer face of the membrane interacts with the substrate; on the other hand, with disrupted cells, the substrate would be exposed to the catalytic sites on both sides of the plasma membrane. Moreover we compared the results obtained with 5'-nucleotidase to the well-located activity of (Na^+ + K^+)-stimulated Mg^{2+}-ATPase [10,11]. Taking advantage of the preparation of inside-out and right side-out plasma membrane vesicles, were able to assay the activity in native and disrupted vesicles.

2. Materials and methods

2.1. *Cells*

MF_2S cells derived from MOPC 173 murine plasmocytoma were adapted to grow as ascites in

Balb/c mice. The cells which were present in the peritoneal fluid were carefully washed in an isotonic buffer and then centrifuged at low speed to remove contaminating red blood cells. The pellet was resuspended in the convenient medium according to the experiment.

Cell-death was followed by trypan blue exclusion at 37°C. Cell viability was used as a measure of plasma membrane integrity [12]. Cellular-lysis was detected under phase contrast microscopy.

2.2. *Plasma membrane purification*

Plasma membranes were isolated from MF_2S cells according to the method of Lelièvre [13,14]. From these membranes, inside-out and right side-out vesicles were separated as already described by Zachowski and Paraf [15].

2.3. *Enzymatic assays*

The incubation medium for 5′-nucleotidase was a 40 mM Tris–HCl, 120 mM NaCl, 5 mM $MgCl_2$, 1 mM Mg–EDTA buffer, pH 7.4 and 5′-AMP (final concentration 1 mM) was used as a substrate [14]. Reaction was initiated by addition of enzyme. At various times, aliquots were taken from the incubation tube to estimate the liberated P_i according to Marsh [16]. Na^+/K^+ ATPase activity was measured by the method of Ottolenghi [17]. Because alkaline phosphatase can hydrolyse monophosphate nucleotides at pH 7.4, this activity was estimated as previously described [13]. All these assays were carried out at 37°C.

3. Results

3.1. *Experiments with cells*

3.1.1. Intact cells

Cells (1×10^7 cells/ml) were kept in suspension at 37°C either in 0.16 M NaCl or in Eagle's medium. At various times, cell-death was checked and an aliquot of 0.1 ml (i.e., 10^6 cells) was transferred to a test tube containing the incubation buffer and enzymatic assays were started by addition of 5′-AMP prewarmed at 37°C.

Under these conditions, specific activity never exceeded 0.18 ± 0.09 μmol P_i/h/mg protein with a cell-death less than 10% for 30 min. Phosphate could be liberated at the cytoplasmic face of the membrane even if 5′-nucleotidase was an ecto-enzyme. However, in the P_i assay, it could be detected, and accounted for the specific activity. Chemicals were used to make the membrane permeable to the substrate. To 0.16 M NaCl and/or to Eagle's medium, were added 40 mM imidazole–HCl buffer, pH 7.4, or 0.1% DOC (final concentration). In these conditions, the percentage of cell-death increased and reached 40–50% within 30 min with imidazole and 100% with DOC. From the results summarized in table 1, it can be seen that the enhancement of specific activity paralleled the cell-surface permeability. We found alkaline phosphatase activity neither with intact nor with permeabilized cells.

3.1.2. Disrupted cells

Cells were resuspended at 4°C in a lytic medium

Table 1
5′-Nucleotidase activity in intact or permeable cells

Incubation medium	Addition	% Cell-death after 30 min incubation	Specific activity (μmol P_i/h/mg protein)
NaCl 0.16 M	None	7	0.18
	Imidazole[a]	40	1.22
	DOC[a]	100	3.05
Eagle's medium	None	6	0.17
	Imidazole[a]	40	1.24
	DOC[a]	100	3.10

[a]For details, see text

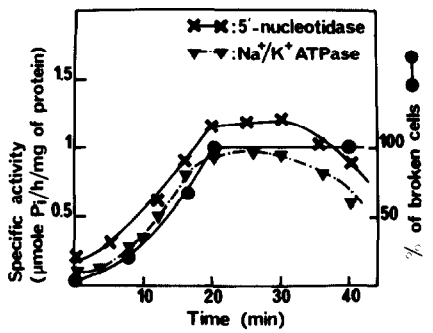

Fig.1. Specific activity of 5′-nucleotidase (X——X) and (Na^+/K^+)-ATPase (▼– · – ·▼) as a function of incubation time in the lytic medium. The percentage of broken cells (●——●) as determined by Trypan Blue exclusion is also plotted.

[13] (2 mM $CaCl_2$, 1 mM $NaHCO_3$, pH 8) at 10^7 cells/ml. At various times the percentage of broken cells and 5′-nucleotidase and (Na^+/K^+)-ATPase specific activities were determined. As seen in fig.1, the specific activity increased with time as cell-breakage increased. The behaviour of 5′-nucleotidase closely paralleled that of (Na^+/K^+)-ATPase the catalytic site of which is known to be located at the inner face of the membrane [10,11]. Again, we failed to detect any alkaline phosphatase activity.

3.2. *Experiments with plasma membrane vesicles*

As previously described [15], 5′-nucleotidase specific activity is about 3-fold higher in inside-out vesicles than in right side-out vesicles. The activity detected in right side-out vesicles led us to hypothetize that they are, at least in part, permeable to 5′-AMP. The difference in activity was abolished by detergents (DOC or Triton X-100) which solubilized the membrane [19] (table 2).

4. Discussion

Our results with intact or disrupted cells and oriented plasma membrane vesicles, led us to conclude that the 5′-nucleotidase catalytic site is located at the inner face of murine plasmocytoma plasma membrane.

When the substrate was supplied extra-cellularly we were able to detect only slight enzymatic activity which we attribute to a small number of damaged cells (<10 %) as seen by Trypan Blue exclusion. This activity does not reflect spontaneous 5′-AMP hydrolysis since substrate hydrolysis in the incubation medium without cells was virtually undetectable. Cells permeabilized by 0.1% DOC exhibited a 15-fold increased activity. These experiments ascertained the lack of penetration of cells by 5′-AMP [2].

Cells incubated in 0.16 M NaCl for more than one hour did not increase enzymatic activity indicating that there was no extracellular release of enzyme.

The 5′-nucleotidase specific activity in MF_2S cells increased as a function of cell-breakage during cell-lysis by hypotonic buffer. This increase closely paralleled that of (Na^+/K^+)-ATPase which is known to be located at the cytoplasmic face; this is another argument for the presence of the 5′-nucleotidase hydrolytic site on this face. Cell lysis did not reveal

Table 2
5′-Nucleotidase activity in inside-out and right side-out vesicles in absence or in presence of detergents

	5′-Nucleotidase activity (μmol P_i liberated/h/mg protein)		
	In absence of detergent	In presence of 0.1% DOC	In presence of 1% Triton X-100
Inside-out vesicles	20.7 ± 1.8	19.5 ± 2.1	20.1 ± 1.9
Right side-out vesicles	6.2 ± 0.8	16.9 ± 1.9	18.5 ± 2.0

Each value represents the mean of results obtained with 5 different preparations of plasma membranes vesicles

the same maximum activity as DOC. This could be explained by inactivation during lysis, sequestration of part of the activity during vesicle formation, or activation by detergents.

Indeed, isolated inside-out vesicles exhibited 3-fold higher 5′-nucleotidase activity than right side-out vesicles. As this discrepancy was abolished by use of detergents which solubilized the membrane, we can conclude that the catalytic site of the enzyme was sequestrated inside of the right side-out vesicles, therefore located at the inner face of the plasma membrane.

One of the most striking problems in studies with 5′-nucleotidase is the hydrolysis of 5′-AMP by non-specific phosphatases, such as alkaline phosphatase. With MF_2S cells, this activity is absent which allows us to assume that the 5′-AMP hydrolysis was due to 5′-nucleotidase hydrolytic activity. Moreover, the failure to detect any alkaline phosphatase cannot be due to the experimental conditions since bacterial alkaline phosphatase in the same conditions could hydrolyse para-nitrophenylphosphate. In the case of ecto-5′-nucleotidase, some authors wondered if the activity they detected was not due to this non-specific phosphatase. De Pierre and Karnovsky [1] ruled out this hypothesis because they inhibited non-specific phosphatases by levamisole. In contrast, Trams and Lauter [2] reported that the 5′-nucleotidase detected could be closely related to the classical alkaline phosphatase at least for certain individual cell-lines.

The relationship between the enzyme response to a specific antibody and the localization of the catalytic site of this enzyme should be interpreted with caution. Gurd and Evans [4] thought that 5′-nucleotidase is an ecto-enzyme since it could be inhibited by antibodies added to vesicles, presumably in the right side-out conformational state. We would like to stress that we were able to inhibit 5′-nucleotidase by effectors acting either on the outer face (concanavalin A) or on the inner face (peptidoglycane) [20]. Thus it seems to us that it is difficult to localize an enzymatic activity by this method. Furthermore, using cytochemical analysis Widnell [6] concluded that 5′-nucleotidase is located at the outer face of plasma membrane since lead phosphate precipitates were present at the external face of microsomal vesicles. This conclusion did not take into account the existence of inside-out vesicles in the microsomal fraction.

Finally, the location of 5′-nucleotidase hydrolytic site on one face or the other of the plasma membrane might be different depending on the cell-line studied.

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