Biochimica et Biophysica Acta, 641 (1981) 366—376

BBA 79135

SELECTIVE CHEMICAL MODIFICATION OF PLASMA MEMBRANE ECTOENZYMES

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(Received August 13th, 1980)

Key words: Chemical modification; Aminophospholipid; Ecto-ATPase, (Plasma membrane)

Summary

As part of an investigation of the organization of cell surface macromolecular assemblies, we have treated intact central nervous system cells with chemical probes which react convalently with proteins and aminophospholipids. Selective alterations of the enzymatic activities of ecto-ATPases, ecto-5′-nucleotidases and cholinesterases were obtained under appropriate reaction conditions. The cross-linking reagent, 1,5-difluoro-2,4-dinitrobenzene, was a potent inactivator of ecto-ATPase of C6 glioblastoma, IMR-32 neuroblastoma and of a primary rat astroblast cell line (RB). Ecto-5′-nucleotidase and acetylcholinesterase were less sensitive to difluorodinitrobenzene. 1-Fluoro-2,4-dinitrobenzene at concentrations which inactivated ecto-ATPase had little effect on ecto-5′-nucleotidase. Conversely, 2,4,6-trinitrobenzenesulfonic acid was a potent inactivator of ecto-5′-nucleotidase but had no effect on ecto-ATPase. The difluorodinitrobenzene inactivation of ecto-ATPase and of ecto-5′-nucleotidase as well as the fluorodinitrobenzene inactivation of ecto-ATPase could be prevented by the presence of the appropriate substrates in the reaction medium. In the presence of protecting nucleotide substrates, a decrease in reactivity with proteins and lipids was observed when the isotopic probe fluorodinitro[^{3}H]-benzene was used.

Operational units of the plasma membrane, such as ion pumps or receptors, are rather complex assemblies which depend for proper function on the appropriate composition and localization of their molecular building blocks. Little is

Abbreviations: DFDNB, 1,5-difluoro-2,4-dinitrobenzene; FDNB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SDS, sodium dodecyl sulfate.

known, however, about this organization. By chemical modification of membrane components we have explored some facets of the molecular organization of the membrane surface. Here, we report on the effects of covalently bound chemical probes on the behavior of membrane enzymes of intact, cultured central nervous system cells.

We have investigated the effects of the following reagents: DFDNB, DFNB and TNBS. These reagents react with primary amino groups [1,2] but additions are also formed with sulfhydryl and other functional groups [3—6]. Under appropriate conditions, TNBS does not penetrate the plasma membrane of living cells, but forms a derivative with membrane phosphatidylethanoamine [7]. FDNB reacts with both phosphatidylethanolamine and phosphatidylserine and permeates the plasma membrane [7]. Both reagents react with membrane proteins as well [8]. DFDNB is a bifunctional reagent which can cross-link aminophospholipids to each other or to proteins. Several membrane enzymes [9—12] and receptors [13—15] appear to require a phospholipid microenvironment for optimal activity and selective modification or cross-linking of their components may aid in specifying their composition or organization. Here, we report on the observed interactions of these reagents with membrane ecto-ATPase (EC 3.6.1.3), ecto-5′-nucleotidase (EC 3.1.3.5) and acetylcholinesterase (EC 3.1.1.7). The characteristics of ecto-ATPase and ecto-5′-nucleotidase have been described previously [16,17].

Experimental procedure

Cell cultures

The C6 (rat glioblastoma) and IMR-32 (human neuroblastoma) cell lines were obtained in their thirty-seventh and fifty-first passage, respectively, from the American Type Culture Collection (Rockville, MD). A primary cell line, mostly astroblasts, derived from fetal rat brain was established in this laboratory and designated RB. The RB cell line was started by mincing brains from 20-day-old fetal Sprague-Dawley rats followed by dissociation by repeated pipetting. Cells were plated out in 60 cm diameter plastic culture dishes and grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 6 g/l glucose, gentamicin, polymyxin B and fungizone. After an initial 2 week growth period, RB cells were grown in the same media as the C6 and IMR-32 cells. Cells were grown in Dulbecco's modified Eagle's medium to which 10% fetal calf serum and 50 μg gentamicin/ml of medium had been added. Monolayer cultures were grown on 9.6 cm^2 multiwell plates (Linbro Scientific, Hamden, CT) to confluency at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell monolayers were reacted with reagents in an isoosmotic medium of the following composition: NaCl, 120 mM; KCl, 20 mM; $MgCl_2$, 4 mM; $CaCl_2$, 2 mM; glucose, 5 mM; $NaHCO_3$, 20 mM; Hepes, 20 mM. The pH was adjusted with NaOH to pH 8.1 and the osmolarity was 340 mosM. This solution will be referred to as buffer.

Chemical modifcation

The following general procedure was used for chemical modification prior to enzymatic assay. After removing the growth medium and washing the cells with

2 ml of buffer per well, 1 ml of the appropriate reagent was added. Reagents were freshly prepared by dissolving in methanol and diluting into buffer yielding a 1% methanolic solution. Trays were incubated at 37°C and rotated horizontally at 50 rev./min for 6 s once every 3 min. After a 15 min preincubation period, the reagent solutions were removed and the cells rinsed twice before enzyme assay. The substrate protection experiments were carried out in a similar manner except that 0.5 ml of a nucleotide solution was added 2 min prior to adding 0.5 ml of the modification reagent.

Enzymic assays

Hydrolysis of [γ-^{32}P]ATP was measured according to a modification of the method of Weil-Malherbe and Green [18]. The incubation medium contained 2 mM ATP in buffer and about 0.2 μCi of [γ-^{32}P]ATP/well in a total volume of 0.5 ml. After incubation, $^{32}P_i$ release was measured as follows. 0.2 ml of the mixture was added to 55 μl of 5% $(NH_4)_2MoO_4$ in 4 N H_2SO_4 plus 0.1 M tungstosilicic acid in 0.5 N H_2SO_4 (8 : 2, v/v). The tubes were mixed and 0.5 ml of water-saturated isobutanol added. After vigorous mixing and centrifugation, a 0.2 ml aliquot of the upper phase was withdrawn for scintillation counting. Substrate hydrolysis was usually less than 20% for the 15 min incubation period used. 5′-Nucleotidase was measured similarly except 2 or 5 mM [^{32}P]-AMP was used as substrate. The mean ±S.D. is shown in our figures. C6 cells in passage numbers 40—50 were used for 5′-nucleotidase experiments but cells of higher passage number (110—130) were used for ATPase experiments because we observed an increase in ATPase activity with increasing passage number.

Acetylcholinesterase activity was measured according to the method of Lewis and Eldefrawi [19] by determination of [^{3}H]acetate derived from hydrolyzed [^{3}H]acetylcholine. Cells were incubated for 30 min with 10^{-5} M [^{3}H]acetylcholine in buffer adjusted to pH 7.0. The ecto-cholinesterase activity of C6 and IMR-32 cells was 90—100% inhibited by 10^{-5} M BW284C-51 (1,5-bis(4-allyldimethylammonium phenyl)pentone-3-one) and was not affected by 10^{-4} M tetraisopropylpyrophosphoramide [20]; it therefore was an acetylcholinesterase.

Protein determination

After enzyme assay, the medium was removed and cells solubilized in 2 ml of 0.25% sodium deoxycholate in 1 M NaOH. An aliquot of this digest was taken for protein determination using a modification of the procedure of Lowry et al. [21].

Materials

The reagents DFDNB, FDNB and TNBS (purified grade) were from Pierce Chemical (Rockford, IL); nucleotides were from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium and gentamicin were from Microbiological Associates (Bethesda, MD) and fetal calf serum was from North American Biologicals (Miami, FL). [γ-^{32}P]ATP (2—10 Ci/mmol), [^{32}P]AMP (10—30 Ci/mmol) and fluorodinitro[3,5-^{3}H]benzene (10—25 Ci/mmol) were from New England Nuclear (Boston, MA) and [^{3}H]acetylcholine (328 Ci/mol) was from Amersham Searle (Arlington Heights, IL).

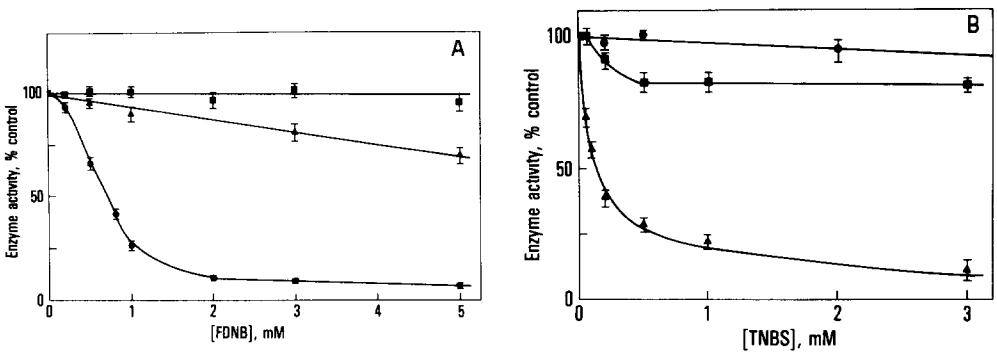

Fig. 1. Reagent-specific inactivation of membrane ectoenzymes by covalent probes. (A) FDNB inactivation, (B) TNBS inactivation. Ecto-ATPase and ecto-5′-nucleotidase control activities were about 8 and 40 nmol P_i released/min per mg cell protein, respectively, and acetylcholinesterase activity was 30 pmol/min per mg cell protein. ●, Ca^{2+}-ATPase; ▲, 5′-nucleotidase; ■, acetylcholinesterase.

Results

Selective chemical modification of cell surface enzymes, expressed by the depression of their catalytic function, could be achieved by two different approaches. (1) By exploiting a definitive selectivity in the interaction between different chemical probes and the respective enzymes and (2) by utilizing the degree of reactivity of a chemical probe at different concentrations.

Two examples of reagent selectivity are shown in Fig. 1A and B. When intact rat glioma (C6) cells were treated with either FDNB or TNBS, marked inactivation of the ecto-ATPase was observed with FDNB (IC_{50}* = 0.7 mM) while ecto-5′-nucleotidase was significantly less sensitive to FDNB. Conversely, TNBS was a potent inactivator of the ecto-5′-nucleotidase (IC_{50} = 0.14 mM) but did not affect ATPase. Neither reagent produced a significant effect on acetylcholinesterase activity. Very similar results were obtained with a human neuroblastoma (IMR-32) and a primary rat brain astroblast cell line (RB) and we consider it probable that the reagent-selective effect is unrelated to cell type. We have also tried the permeant and impermeant imidoesters, methyl acetimidate and isethionyl acetimidate, respectively, but neither inactivated ecto-ATPase after a 30 min treatment with 20 mM reagent.

An example of chemical modification of cell surface enzymes by utilizing concentration-dependent reactivity is illustrated in Fig. 2. The cross-linking reagent DFDNB was shown to be a potent inactivator of ecto-ATPase at low concentrations (IC_{50} = 0.05 mM) while substantially higher concentrations were required to inactivate 5′-nucleotidase (IC_{50} = 0.2 mM) or cholinesterase (IC_{50} = 1—3 mM). This concentration-dependent effect of DFDNB on these three enzymes was found not only with the C6 cell line, but also with the IMR-32 and the RB cells.

Varying reaction conditions other than the concentration affected the results. Ecto-ATPase inactivation by DFDNB varied as a function of concentra-

* IC_{50} is defined as the inhibitor concentration that causes a 50% reduction in enzymatic activity after a 15 min preincubation at 37°C and pH 8.1.

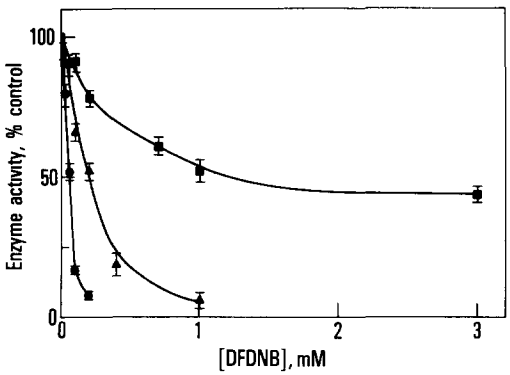

Fig. 2. Concentration-dependent inactivation of membrane ectoenzymes. Enzyme activities were as in Fig. 1. ●, Ca^{2+}-ATPase; ▲, 5′-nucleotidase; ■, acetylcholinesterase.

tion and incubation time. Residual ATPase activity of C6 cells was 65 and 23% of control after 10 min exposure to 0.05 or 0.15 mM DFDNB, respectively. After 30 min, the corresponding values were 27 and 6% of control. Also, inactivation of ATPase by 0.05 mM DFDNB increased sharply as the pH of the reaction medium was raised from 7.7 to 8.3. Changes in temperature from 15 to 22 to 37°C increased ATPase inactivation during a 15 min reaction period with 0.05 mM DFDNB from 23 to 43 to 55%, respectively.

Although the selective modification of the enzymes by chemical probes mitigated against a generalized toxic effect, we measured, as further controls, cell viability and membrane integrity. After incubation of monolayer cultures for 15 min at 37°C with 1 mM FDNB or TNBS or with 0.2 mM DFDNB at pH 8.1, at least 95% of the cells excluded trypan blue. Also, after removal of the reagents and rinsing of the cells with medium, during a subsequent 15 min incubation of the cultures less than 1% of the total lactate dehydrogenase activity of the cells (as determined from culture homogenates) had leaked into the medium.

We have made an estimate of total reaction products formed from the three reagents under conditions representing an approximate IC_{50} for each compound in respect to C6 glioma ecto-ATPase. Cell monolayers were incubated at 37°C with 5.0 mM TNBS, 0.5 mM FDNB and 0.05 mM DFDNB, respectively. After 15 min the reagent mixture was removed, the cultures rinsed twice with buffer and the cells solubilized in 1.0 M NaOH containing 0.25% sodium deoxycholate. The reaction product formed was estimated from the absorbance at 337 or 345 nm. The following amounts of probe were found to be reacted in nmol/mg protein: TNBS, 15.4, FDNB, 57.3 and DFDNB, 15.7. We therefore estimate that the order of reactivity, each differing from the other by an order of magnitude, was DFDNB, FDNB, TNBS. The availability of 2,4-Dinitrofluoro[3,5-^{3}H]benzene made it possible to obtain an estimate of the distribution of the reagent in the cell membrane constituents. Monolayer cultures with an approximate cell density of 20 μg protein/cm^2 were incubated with 1.0 mM [^{3}H]-FDNB for 15 min at 37°C and at a medium pH of 8.1. After removal of the reaction mixture, the monolayers were rinsed twice with buffer and residual radioactivity in the cells was determined. About 2.1 nmol of [^{3}H]FDNB were

TABLE I

AMINOPHOSPHOLIPID LABELLING BY TNBS AND FDNB IN C6 MONOLAYERS

Cells were preincubated for 60 min at 37°C, pH 8.1, rinsed twice and lipids extracted into $ChCl_3/CH_3OH$ (2 : 1, v/v). Phospholipids were separated by two-dimensional TLC according to the method of Rouser et al. [26] and phosphate analysis of spots by using the method of Nelson [27]. PE, phosphatidylethanolamine; PS, phosphatidylserine.

Concentration (mM)	% labelled			
	TNBS		FDNB	
	PE	PS	PE	PS
0.2	1	0	13	8
1.0	2	0	42	19
5.0	11	0	93	78

bound per cm^2 monolayer culture, and 75% of the ^3H could be extracted with $CHCl_3/CH_3OH$ (2 : 1, v/v). This indicated that appreciable interaction with cell lipids had taken place. The residual 25% of the [^3H]FDNB was bound to insoluble proteins. Analysis of the reaction products formed with monofunctional reagents and membrane lipids showed that FDNB reacted with both phosphatidylserine and phosphatidylethanolamine while TNBS formed reaction products with the latter only (Table I). DFDNB reaction products appeared to be distributed equally between protein and lipid fractions. There was some protein-to-protein cross-linking following incubation of monolayer cultures with 10^{-4} M DFDNB for 15 min at pH 8.1 as was shown by polyacrylamide gel electrophoresis of proteins solubilized in 2% SDS. The disappearance of several unidentified protein bands seemed to be matched by an increase in apparent high molecular weight proteins which did not enter the stacking gel. Aminophospholipid reactions with DFDNB were measured following $CHCl_3/CH_3OH$ extraction, acid hydrolysis and ethyl acetate extraction of labelled cultures as described by Marinetti et al. [22]. About 85% of the reaction products were identified as ethanolamine derivatives, some of which appeared in the form of dinitrophenylbisethanolamine.

The inactivation of ecto-ATPase or ecto-5'-nucleotidase could be reduced or prevented by modifying the reaction conditions to include the respective nucleotide substrates. Inosine triphosphate (ITP), an alternate substrate, effectively protected ecto-ATPase from inactivation by DFDNB or FDNB at the respective IC_{50} concentrations of the reagents (Fig. 3A and B). The effectiveness for substrate protection of ecto-ATPases at equal concentrations was ATP > ITP > UTP > ADP or IDP. No protection was afforded by nucleoside monophosphates. Conversely, ecto-5'-nucleotidase was completely protected from DFDNB action by 1 mM UMP (Fig. 4A), but this substrate failed to protect the enzyme from TNBS inactivation (Fig. 4B). This inactivation by TNBS could not be prevented even at UMP or IMP concentrations of 5 mM. While ecto-ATPase protection from the action of DFDNB exhibited a high degree of specificity for the nucleoside triphosphates, ecto-5'-nucleotidase inactivation by DFDNB was prevented not only by mononucleotides but also by di- and trinucleotides.

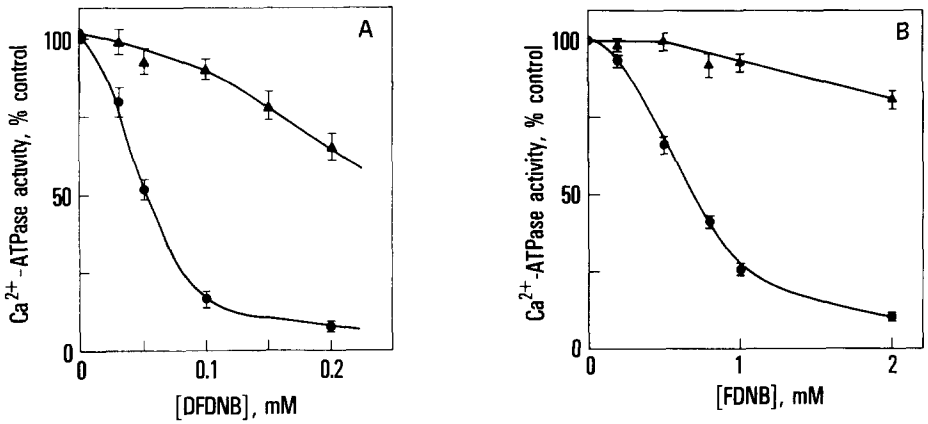

Fig. 3. Protection of Ecto-ATPase from DFDNB and FDNB inactivation. (A) C6 monolayer cultures were preincubated with or without 2 mM ITP at various DFDNB concentrations. Cells were then washed with buffer and ecto-ATPase activity measured. Control activity was 10 nmol P_i released/min per mg cell protein. ●, DFDNB; ▲, DFDNB + ITP. (B) As in A except FDNB was the inactivator. Control activity 6.7 nmol P_i released/min per mg cell protein. ●, FDNB, ▲, FDNB + ITP.

Substrate protection of ecto-ATPases against chemical modification was shown not only by measuring enzyme activity, but also by differential binding of a radioactive probe onto protected as compared to unprotected enzyme. C6 glioma monolayers were reacted with FDNB in the presence of 5 mM ITP under conditions which preserved 94% of the ecto-ATPase activity. The first reaction served to mask the non-specific reaction sites. ITP was chosen rather than ATP in order to avoid side reactions with the adenine amino group. After removal of substrate and excess reagent, the cells were treated with 0.1 mM [^{3}H]FDNB in the presence or absence of ITP (Table II). We found that about 126 nmol of FDNB had reacted per mg cell protein in the ITP-masked condi-

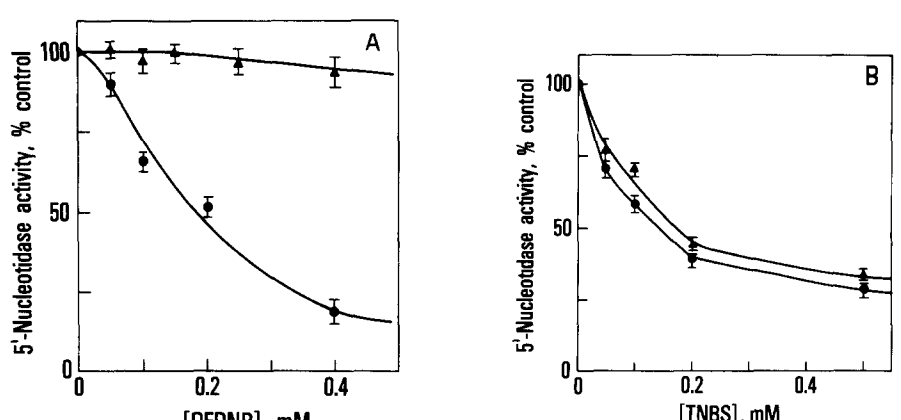

Fig. 4. Protection of ecto-5′-nucleotidase from DFNB and TNBS inactivation. (A) C6 monolayer cultures were preincubated with or without 1 mM UMP at various DFDNB concentrations. Cells were then washed with buffer and ecto-5′-nucleotidase activity was measured. Control activity was 34 nmol P_i released/min per mg cell protein. ●, DFDNB; ▲, DFDNB + UMP. (B) As in A except TNBS was the inhibitor. Control activity was 37 nmol P_i released/min per mg cell protein. ●, TNBS; ▲, TNBS + UMP.

TABLE II

ITP PROTECTION OF MEMBRANE COMPONENTS FROM [^{3}H]FDNB REACTION

C6 monolayer cultures were reacted with 1 mM FDNB for 15 min at 37°C, pH 8.1, in the presence of 5 mM ITP (masking reaction). Cells were rinsed twice and either 5 mM ITP or buffer added, followed by 0.1 mM [^{3}H]FDNB and cells incubated again for 15 min. Lipid values represent radioactivity extracted with $CHCl_3$/CH_3OH (2 : 1, v/v) and protein values the residue after lipid extraction. The cell density was 0.12 mg/cm^2. Data shown represent the average ±S.D. of three experiments.

	Conditions	nmol FDNB incorporated/mg cell protein		
		Total cells	Lipids	Protein
(I) Masking reaction (ATPase protected)	1 mM FDNB + 5 mM ITP	126 ± 7	50 ± 2	76 ± 5
(II) Radioactive labelling reaction				
(A) ATPase unprotected	0.1 mM [^{3}H]FDNB	3.39	1.25 ± 0.16	2.14 ± 0.27
(B) ATPase protected	0.1 mM [^{3}H]FDNB + 5 mM ITP	1.99	0.67 ± 0.07	1.32 ± 0.17

tion. When subjected to the second treatment with chemical probe, another 3.4 nmol/mg protein reacted with the cells in the unprotected condition and 2.0 nmol in the protected condition. Comparison of experiment I and experiment IIB (Table II) indicated that only 1% of the FDNB-reacted sites were critical for the protection of the catalytic properties of ecto-ATPase. A control experiment conducted under identical conditions with IMP did not produce any differences in [^{3}H]FDNB labeling.

Discussion

We have demonstrated that several plasma membrane ectoenzymes could be selectively inactivated by reacting intact cells in culture medium with compounds which form covalent bonds with plasma membrane constituents. For instance, DFDNB which is a highly reactive cross-linking reagent inactivated ecto-ATPase at a concentration of 0.05 mM while ecto-5′-nucleotidase and cholinesterase activities remained unaffected. Conversely, the monofunctional TNBS proved to be a good inactivator of ecto-5′-nucleotidase, but had no effect on ecto-ATPase even at 10-times the IC_{50} for the 5′-nucleotidase. Because these patterns were very similar with C6 rat glioma, human IMR-32 neuroblastoma and rat astroblasts, we suggest that the reactions leading to enzyme inactivation resulted from alterations of the same molecular organization in all cells tested. From our observations it became quite clear, however, that the selectivity was a function of carefully elaborated reaction conditions; drastic increases in reagent concentrations and prolonged incubation times produced indiscriminate inactivation of enzymatic activity.

As an explanation for the observed selectivity we propose that the primary reason was one of differential accessibility of the probes to the ectoenzymes. In addition, it appears probable that differences in molecular configurations and compositions imparted differences in reactivity. When attempts are made to obtain ecto-ATPases or ecto-5′-nucleotidases in soluble form, it is usually observed that the former enzyme cannot readily be separated from the membrane matrix while the latter has been obtained in a highly purified soluble form [23]. Therefore, we assume that the ecto-ATPase site of modification is contained in a more hydrophobic environment which is more accessible to the nonpolar probes FDNB and DFDNB, but relatively inaccessible to the charged TNBS. Conversely, the ecto-5′-nucleotidase resides in a more polar membrane environment where the more hydrophilic TNBS presumably has an advantage of accessibility. This difference between TNBS and FDNB was also illustrated by their reactions with phospholipids. TNBS could react with only a small portion of phosphatidylethanolamine while FDNB reacted with most of the phosphatidylethanolamine and phosphatidylserine under the same conditions. This difference is usually interpreted as being derived from the asymmetric distribution of the phospholipids in the plasma membrane, but it also indicates that FDNB can penetrate into hydrophobic regions of the plasma membrane more readily. The monofunctional FDNB and the cross-linking reagent DFDNB are close structural analogues and it might be expected that their mode of action would be quite similar. There was, however, a 14-fold difference in their IC_{50} values for ecto-ATPase. Although the greater potency of DFDNB may be

simply due to its greater reactivity, we consider it probable that the ability to cross-link membrane components, such as proteins and lipids, played a significant role in this selectivity.

We observed that the inactivation of the ecto-enzymes could be prevented when the respective substrates were present in the reaction medium. We consider that additional strong evidence is that the chemical modification of the enzymes was selective. We suggest that the observed effect was due either to prevention of a chemical modification at or near the active enzyme site or that a critical portion of the enzyme molecule became inaccessible in the presence of the substrate. One hypothesis is that enzyme-phospholipid modification is a significant feature of enzyme inactivation. Phosphatidylserine, for instance, may be an important constituent of ATPase active sites where it could serve to direct substrate binding. It has been found that phosphatidylserine stimulates $(Na^+ + K^+)$-ATPase [9,24] and reactivates erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase after phospholipase A_2 treatment [25]. Also, the relative impotency of TNBS to inactivate ecto-ATPase could be explained by its inability to react with membrane phosphatidylserine.

The principal significance of our findings is that they open two major avenues to further investigations. Selective chemical inactivation of either of the ectoenzymes can be used as a tool for the study of physiologic function of the enzymes. In addition, the chemical probes, since they appear to react with enzyme constituents which are vital to catalytic activity, can be used to investigate the mechanism of action of the enzymes. Furthermore, the covalently bound probes might serve for the isolation, purification and analysis of the molecular constituents of these enzymes which appear to require both protein and lipid moieties for optimal activity.

Acknowledgements

The authors would like to thank Brian T. Adornato and Linda Friedman for their technical assistance. We also thank Dr. E.A. Brown for a computer search of relevant literature.

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