THE ACTION OF TRYPSIN AND NEURAMINIDASE ON THE SYNAPTIC MEMBRANES OF BRAIN CORTEX

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SUMMARY

The population of intracellular membranes of rat cerebral cortex which exhibited the highest relative specific concentration of bound N-acetylneuraminic acid (NANA) and the highest relative specific activity of acetylcholinesterase (EC 3.1.1.7) (synaptic membranes) was incubated with bacterial neuraminidase and with trypsin under a number of experimental conditions. The electrokinetic profile displayed by the membrane preparation upon zonal density gradient electrophoresis changed as a result of the removal of bound NANA by neuraminidase, while its acetylcholinesterase activity remained unaffected; conversely, the action of trypsin led to the inactivation of acetylcholinesterase before any significant alterations of the electrophoretic mobility of the membranes became apparent. Prolonged incubation alone or in the presence of either neuraminidase or trypsin, resulted in the loss of membrane electrophoretic homogeneity, a circumstance which, most likely, reflects a disaggregation of the structural matrix of the membrane.

INTRODUCTION

Several recent studies of plasma and intracellular membranes of animal cells[1-6] have examined the effects of experimentally induced alterations of membrane structure on selected expressions of membrane function, such as ion transport, enzyme and electrical activity, ligand binding, *etc.* For brain tissue, in addition to the demonstrable differences in composition[7,8] among the notoriously numerous membrane types, different regions of one same membrane, namely the junctional complex *vs.* the extrajunctional portion of the nerve ending membrane, have been shown to be differentially affected by organic solvents[9] and detergents[5,6]. Moreover, it has also become apparent that membranes differ in the relative amounts of bound N-acetyl-neuraminic acid (NANA) associated with each of them and that, in brain cortex, bound NANA is highest in membranes which exhibit the highest acetylcholinesterase (EC 3.1.1.7) activity[8,9].

In the accompanying paper[10] we reported on zonal density gradient electrophoresis as a technique capable of successfully exploiting the characteristic differences in bound NANA content shown to exist among the intracellular membranes of rat

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Abbreviations: NANA, N-acetylneuraminic acid; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone.

cerebral cortex[8]. Thus, the synaptic membrane population rich in acetylcholinesterase could be isolated as a rather homogeneous anodic component. The zonal density gradient electrophoresis technique has recently also been used to examine the electrokinetic properties of rat brain subcellular particles[11].

In the present paper we report effects of trypsin (EC 3.4.4.4) and of bacterial neuraminidase (*N*-acetylneuraminate glycohydrolase; EC 3.2.1.18) on the electrokinetic properties of synaptic membranes as well as on the activity and the structural association of the membrane-bound acetylcholinesterase.

MATERIALS AND METHODS

Animals. Adult Sprague–Dawley rats (200–300 g) of both sexes were used.

Chemicals and enzymes. These were listed in the accompanying paper[10]. L-(Tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin (TPCK-trypsin) and the lima bean trypsin inhibitor were products of Worthington Biochemical, Freehold, N. J. Two preparations of different purity of *Clostridium perfringens* neuraminidase, Types V and VI (Sigma Chemical, St. Louis, Mo.) were used, as specified in detail in the text.

Preparation of synaptic membrane fraction $M_{1.1}$. The homogenization and centrifugation techniques were as described in the accompanying paper[10]. After isolation, Fraction $M_{1.1}$ was pelleted (269 000 × g, 60 min). The pellet was suspended either in Tris buffer (0.05 M, pH 7.2) (for incubation with TPCK-trypsin) or in acetate buffer (0.05 M, pH 5.1) (for incubation with neuraminidase).

Zonal density gradient electrophoresis. The full description of the procedure is given in the accompanying paper[10].

Incubation of fraction $M_{1.1}$ *with TPCK-trypsin and neuraminidase.* Incubations were carried out under a number of different conditions, as specified in the legends. Incubation with TPCK-trypsin, in preparation of electrophoresis, was terminated by the addition of a five-fold weight excess of trypsin inhibitor, while incubation with neuraminidase was stopped by placing the tubes in ice water. Where required, high-speed centrifugation (151 000 × g, 60 min) immediately followed incubation. The pellets, representing the membrane residue, and the supernatant were assayed for acetylcholinesterase and for bound and free NANA, respectively.

Analytical methods. Protein was determined according to LOWRY *et al.*[12] and bound and free NANA according to WARREN[13]. All preparations containing bound NANA were thoroughly dialysed for at least 24 h against several changes of distilled water before hydrolysis was carried out. The absorbance at 549 nm was corrected for the absorbance at 532 nm using the equation proposed by WARREN[13]. It was established that this correction effectively eliminated the contribution of the small amounts of sucrose contaminating the pelleted membranes to color development.

Enzyme assay. Acetylcholinesterase was assayed colorimetrically with acetylthiocholine as substrate[10].

RESULTS

The protein and bound NANA values and the relative specific activities of acetylcholinesterase in the membrane fractions $M_{1.1}$, $M_{1.2}$, $M_{1.3}$ and M_p (ref. 10,

TABLE I

BOUND N-ACETYLNEURAMINIC ACID AND PROTEIN IN SUBFRACTIONS OF PRIMARY FRACTION NEML

NEML: crude fraction containing chiefly membranous elements derived from nerve endings, mitochondria and lysosomes. The centrifugal fractions were isolated as described in METHODS of the accompanying paper[10]. Relative specific concentration: % NANA/% protein; relative specific activity: % acetylcholinesterase/% protein. For a morphological description of closely similar fractions, see ref. 14.

Membrane fraction	Protein mg/g	Protein %	N-Acetylneuraminic acid μg/g	N-Acetylneuraminic acid %	N-Acetylneuraminic acid Rel. spec. concn.	Acetylcholinesterase Rel. spec. act.	NANA (rel. spec. concn.)	Acetylcholinesterase (rel. spec. act.)
$M_{1.1}$	2.11	16.5	52.2	45.1	2.81	1.70	1.65	1.70
$M_{1.2}$	2.66	21.2	24.7	20.6	0.96	0.82	1.17	0.82
$M_{1.3}$	2.48	20.3	14.9	13.6	0.67	0.56	1.19	0.56
M_p	5.86	42.0	22.4	20.7	0.48	0.40	1.19	0.40

TABLE II

INCUBATION OF SYNAPTIC MEMBRANES WITH NEURAMINIDASE: RELEASE OF NANA AND LACK OF EFFECT ON ACETYLCHOLINESTERASE

Fraction $M_{1.1}$ was isolated from approx. 1.8 g of cerebral cortex (2 rats) and was sedimented at 151000 $\times$ g for 60 min. The pellet was suspended in 5 ml of 0.05 M acetate buffer (pH 5.1); 1 ml was withdrawn for assay of acetylcholinesterase, protein and bound-NANA. Acetylcholinesterase, $1A$ (412 nm) units per 5 ml per 5 min; protein, mg per 5 ml; 4.96 and bound-NANA, μg per 5 ml; 48. Type VI neuraminidase (Lot No. 127B-2140, 0.9 mg) was added to the remaining 4 ml and the suspension incubation at the indicated temperature. Aliquots (0.05 ml) were withdrawn periodically for assay of acetylcholinesterase and for separation into the soluble and particulate phase (105000 $\times$ g, 90 min). The pellets were suspended in distilled water and aliquots dialysed (24 h, vs. three changes of distilled water) before the residual, bound-NANA was determined.

Incubation time (min)	Acetylcholinesterase (at 22°) Total* (%)	Acetylcholinesterase (at 22°) Particulate** (%)	Acetylcholinesterase (at 22°) Soluble** (%)	NANA release** (at 22°) (%)	Acetylcholinesterase (at 37°) Total* (%)	Acetylcholinesterase (at 37°) Particulate** (%)	Acetylcholinesterase (at 37°) Soluble** (%)	NANA release** (at 37°) (%)
30	98	100	0	75.5	106	97.2	2.8	89
60	97	99.3	0.7	65.5	112	96.4	3.6	89
120	95	98.4	1.6	69.5	97	95.1	4.9	83

* No loss of enzyme activity occurred upon incubation in the absence of neuraminidase.
** As % of total recovered (soluble + particulate: 100 %).

Figs. 2D–G) are shown in Table I. The bound NANA values fully confirm the analytical findings of LAPETINA, SOTO AND DE ROBERTIS[8] and they also corroborate and account for the observed electrophoretic behavior of Fraction $M_{1.1}$ (ref. 10, Fig. 2D).

As shown in Table II, neuraminidase had no effect on the activity or the structural association of acetylcholinesterase although it caused a rapid and virtually total release of bound NANA. The release was temperature-dependent and tended to be more complete at 37° than at 22°. Incubation of Fraction $M_{1.1}$ with TPCK-trypsin, on the other hand, caused appreciable inactivation of acetylcholinesterase (Table III).

TABLE III

INACTIVATION OF ACETYLCHOLINESTERASE BY TPCK-TRYPSIN

The preparation of Fraction $M_{1.1}$ and the operational aspects of the incubation procedure were as outlined in the legend to Table II. The concentration of TPCK-trypsin was in all cases 0.5 mg/ml.

Incubation time (min)	Percentage*		
	Temp.: 0°	22°	37°
15	—	—	48.1
30	83.6	61.3	—
45	—	—	36.7
60	99.8	64.0	35.2
90	102.3	46.7	31.1
120	95.0	49.4	27.8

* The individual values are expressed as % of suitable controls incubated in the absence of TPCK-trypsin. Mostly, these were not different from values of non-incubated controls.

If TPCK-trypsin was added directly to the enzyme assay system, no loss of activity was observed. Preliminary experiments indicate that, on the contrary, some activation of acetylcholinesterase by TPCK-trypsin may be achieved under suitable conditions (O. Z. SELLINGER AND L. M. NORDRUM, unpublished observations). The effect of exposure of Fraction $M_{1.1}$ to pH 5.1 is shown in Fig. 1, while the effects of incubation of Fraction $M_{1.1}$ in the presence of neuraminidase (Type V, 1 mg liberates approx. 0.15 µg of NANA/min from N-acetylneuraminlactose at pH 5.1 and 37°) are shown in Fig. 2. Incubation at pH 5.1, 22°, 2 h, resulted in the appearance of an additional, cathodic component alongside the characteristic anodic acetylcholinesterase peak (Fig. 2A), while prolonged incubation at 37° led to the obliteration of the anodic peak and to fragmentation. Incubation of the commercial *Electrophorus electricus* acetylcholinesterase with neuraminidase (pH 5.1, 2 h, 22°) caused a decrease in its anodic movement as reflected in the appearance of two cathodic peaks of activity (*cf.* Fig. 4D, ref. 10).

Incubation of Fraction $M_{1.1}$ at pH 7.2, either alone or in the presence of neuraminidase, gave essentially normal electrokinetic profiles (Fig. 3A and B). Incubation with TPCK-trypsin was without visible effects (Fig. 4B), if of short duration (10 min, 37°); prolonging the incubation at 0° or at 37°, resulted in fragmentation of the membranes, a concomitant drop in their electrophoretic mobility and an appreciable loss of acetylcholinesterase activity (Table III).
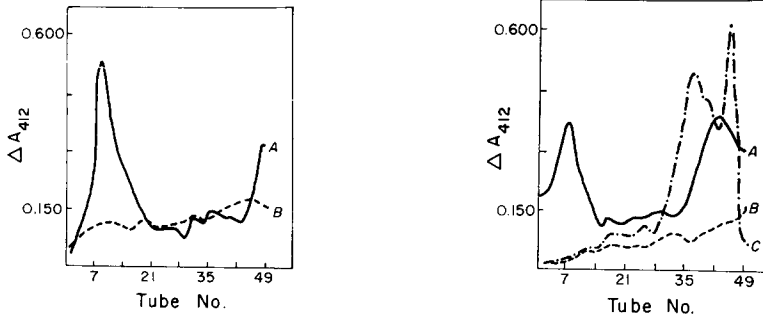
Fig. 1. The effect of incubation at pH 5.1 on the electrophoretic mobility of Fraction $M_{1.1}$. Pelleting of Fraction $M_{1.1}$ was done as described in the legend to Table II. A suspension of the final pellet in 0.05 M acetate buffer (pH 5.1) was incubated for 2 h (A) and 8 h (B) at 37°. Centrifugation (269000 × g, 45 min) yielded a pellet which was suspended in 0.05 M Tris buffer (pH 7.2) in preparation of electrophoresis. In all of the figures, the left side of the panel corresponds to the top of the electrophoresis column (anode), the abscissa refers to tubes in which the effluent was collected through the top of the column and the ordinate to acetylcholinesterase activity expressed as ΔA at 412 nm. The assay conditions were: A, 0.5 ml of sample, 30 min; and B, 0.5 ml of sample, 45 min.

Fig. 2. The effect of neuraminidase on the electrophoretic mobility of Fraction $M_{1.1}$ and of the acetylcholinesterase of E. electricus. For additional details of procedure, see the legend to Table II. A, electrophoretic profile of Fraction $M_{1.1}$ after incubation of 2 ml with 0.5 mg of neuraminidase, Type V for 2 h at 22°; B, as in A, except that incubation was for 13 h; C, E. electricus acetylcholinesterase (2 ml) was incubated in the presence of neuraminidase, Type V (0.25 mg/ml) for 2 h at 22°. The acetylcholinesterase assay conditions were: A and B, 0.5 ml of sample, 30 min; C, 0.005 ml of sample for 25 min.
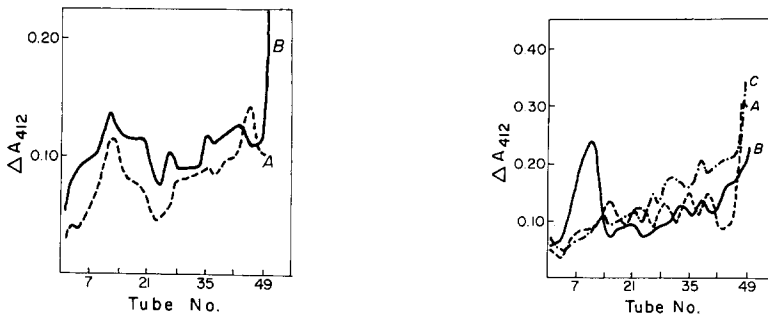

Fig. 3. The effect of incubation at pH 7.2 on the electrophoretic mobility of Fraction $M_{1.1}$. For details of procedure, see the legend to Table II. A, electrophoretic profile of Fraction $M_{1.1}$ after incubation of 1.5 ml (0.05 M Tris buffer) for 2 h at 37°; B, as in A, except that incubation was in the presence of 0.5 mg of neuraminidase, Type V. The acetylcholinesterase assay conditions were: 0.5 ml of sample, 30 min.

Fig. 4. The effect of TPCK-trypsin on the electrophoretic mobility of Fraction $M_{1.1}$. For details of procedure, see the legend to Table II. A, electrophoretic profile of Fraction $M_{1.1}$ after incubation of 1 ml with 0.1 mg of TPCK-trypsin for 2 h at 0°. 0.5 mg of lima bean trypsin inhibitor was added to stop tryptic action; B, as in A, except that incubation was for 10 min at 37°; C, as in A, except that incubation was for 2 h at 37°. The assay conditions were 0.5 ml of sample, 30 min throughout.

DISCUSSION

Although the centrifugal procedures used for the isolation of membrane Fraction $M_{1.1}$[10] differ in some respects from similar previously described procedures[8],

this fraction, in terms of its bound NANA and protein content, appears to be equivalent to the sum of the synaptic membrane[14] fractions M_1 (1.0) and M_1 (1.2) of LAPETINA, SOTO AND DE ROBERTIS[8]. Furthermore, a good analytical agreement may be noted between Fraction $M_{1.1}$ and subfraction A of the crude mitochondrial fraction isolated by SPENCE AND WOLFE[7] and shown by these workers to contain primarily synaptic elements.

Our findings further reveal that the partial removal of the negative charges of Fraction $M_{1.1}$ by neuraminidase (Table II, 2 h incubation, 22°) does not depress the activity or weaken the membrane-association of acetylcholinesterase; on the contrary, enzyme activity was fully retained even in those membranes which had lost their negative charges through neuraminidase action and which upon electrophoresis appeared as a sharp cathodic component (Fig. 2A).

Modulations of the activity of membrane-bound acetylcholinesterase (*E. electricus*) by agents and conditions affecting the electrophoretic mobility of the membrane have recently been described by SILMAN AND KARLIN[15]. It is attractive to speculate as to whether the increments of acetylcholinesterase activity observed by SILMAN AND KARLIN[15] following activation of the enzyme by pH or phosphate ions, may be related to the second electrophoretic peak of *E. electricus* acetylcholinesterase activity which arose in the present experiments following its incubation with neuraminidase (Fig. 2C). This change in the electrophoretic mobility of both Fraction $M_{1.1}$ and the *E. electricus* acetylcholinesterase preparation fully coincides with the observations of SVENSMARK AND KRISTENSEN[16] who noted that treatment of human serum cholinesterase with neuraminidase resulted in a marked elevation of the former enzyme's isoelectric point; the present results also support the role of bound NANA as the primary determinant of membrane surface charges, as proposed by EYLAR *et al.*[17]. They do not, however, agree with the findings of VOS, KURIYAMA AND ROBERTS[11] who reported that 'removal of NANA from brain synaptosomes and synaptic vesicles did not cause alteration of the electrophoretic mobility' of these particles.

The recent observations of SARASWATHI AND BACHHAWAT[18] on the failure of the removal of bound NANA from its tight association with two alkaline phosphatases of ovine brain to alter their activity or kinetics despite marked modifications in the chromatographic behavior of one of them are also germane, for they emphasize that a loss of negative charges through removal of bound NANA need not result in loss of activity on the part of an enzyme, whether membrane-associated or soluble.

The relative proportion of the total bound NANA released by neuraminidase from Fraction $M_{1.1}$ at 37° (Table II) (80–90 %) approximated the values reported by others for the bound NANA released from liver cell plasma membranes (65–75 %)[2] and from the stroma of erythrocytes (100 %)[17], a finding which indicates that most, if not all, of the bound NANA of Fraction $M_{1.1}$ is accessible and/or sensitive to neuraminidase attack under the conditions of the experiments.

Incubation of Fraction $M_{1.1}$ with TPCK-trypsin at 22 and 37°, but not at 0°, resulted in a rapid loss of membrane-bound acetylcholinesterase activity. At 37° the activity was lost before the electrophoretic homogeneity of the preparation was affected, as evidenced by the fact that a 10 min incubation with TPCK-trypsin caused a 50–60 % reduction of acetylcholinesterase activity (Table III) with no significant alteration of the electrophoretic profile of the membranes (Fig. 4B). On the other hand, after 2 h of incubation at 0° (Fig. 4A), electrophoretic homogeneity was virtually

obliterated, yet full acetylcholinesterase activity was retained (Table III). Recently, in somewhat similar experiments, incubation with trypsin at $37°$ has been shown to lead to a considerable inactivation of the Na^+, K^+-activated ATPase of rat brain microsomes[19] at a time when the structure of the membranes bearing the enzyme was only slightly affected. It is likely that in both of these instances tryptic action rapidly involves relatively accessible site(s) of the membrane-associated enzymes and, hence, most probably centers on one or more of their lysine and/or arginine residues[20,21].

REFERENCES

1 D. F. HOELZL WALLACH, in B. D. DAVIS, *The Specificity of Cell Surfaces*, Prentice Hall, Englewood Cliffs, 1967, p. 129.
2 E. L. BENEDETTI AND P. EMMELOT, *J. Cell Sci.*, 2 (1967) 499.
3 J. GALLAI-HATCHARD AND G. M. GRAY, *European J. Biochem.*, 4 (1968) 35.
4 T. PODLESKI AND J. P. CHANGEUX, *Science*, 157 (1967) 1579.
5 S. FISZER AND E. DE ROBERTIS, *Brain Res.*, 5 (1967) 31.
6 E. G. LAPETINA AND E. DE ROBERTIS, *Life Sci.*, 7 (1968) 203.
7 M. W. SPENCE AND L. S. WOLFE, *Can. J. Biochem.*, 45 (1967) 671.
8 E. G. LAPETINA, E. F. SOTO AND E. DE ROBERTIS, *Biochim. Biophys. Acta*, 135 (1967) 33.
9 E. DE ROBERTIS, *Science*, 156 (1967) 907.
10 O. Z. SELLINGER AND R. N. BORENS, *Biochim. Biophys. Acta*, 173 (1969) 176.
11 J. VOS, K. KURIYAMA AND E. ROBERTS, *Brain Res.*, 9 (1968) 224.
12 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND P. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
13 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971.
14 A. R. DE LORES ARNAIZ, M. ALBERICI AND E. DE ROBERTIS, *J. Neurochem.*, 14 (1967) 215.
15 H. I. SILMAN AND A. KARLIN, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1664.
16 O. SVENSMARK AND P. KRISTENSEN, *Biochim. Biophys. Acta*, 67 (1963) 441.
17 E. H. EYLAR, M. A. MADOFF, O. V. BRODY AND J. L. ONCLEY, *J. Biol. Chem.*, 237 (1962) 1992.
18 S. SARASWATHI AND B. K. BACHHAWAT, *Biochem. J.*, 107 (1968) 185.
19 J. SOMOGYI, *Biochim. Biophys. Acta*, 151 (1968) 421.
20 W. LEUZINGER AND A. L. BAKER, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 446.
21 D. H. JEAN AND H. BADER, *Biochem. Biophys. Res. Commun.*, 27 (1967) 650.