INFLUENCE OF CATION CONCENTRATION ON THE SIALIDASE ACTIVITY OF NEURONAL SYNAPTIC MEMBRANES

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Summary:

Low Na^+ ($5X10^{-5}$M) and higher K^+ or divalent cation concentration ($5X10^{-4}$M) fully activate synaptic membrane sialidase towards exogenous gangliosides. As the concentration of Ca^{++} or Zn^{++} is increased above $5X10^{-4}$M, exogenous sialidase activity is inhibited. Increased Na^+ or K^+ concentrations do not give this effect. Activity towards endogenous gangliosides is *not* controlled by external cations. Both endogenous and exogenous activities are optimum over a narrow pH range, 3.9 to 4.5, and activity is low above 6 or below 2. These results suggest that pH and ionic flux may regulate the density of fixed negative charge due to bound sialic acid in the synapse, and influence synaptic transmission, by controlling the separate actions of synaptic membrane sialidase.

Synaptic membranes of mammalian brain have a high content of glycosidically bound sialic acid (1) which may serve to provide a substantial density of fixed negative charge and thereby influence concentrations of mobile positively charged counterions (2). Sialocompounds in the synapse may act as specific binding sites for positively charged neurotransmitters (3). Sialidase (N-acetylneuraminosyl glycohydrolase, (EC 3.2.1.18) occurs in synaptic membranes. It acts at acid pH to release sialic acid, preferentially from di- and trisialogangliosides located in these membranes (4). The end-product, monosialoganglioside, remains membrane-bound. After depleting available endogenous substrate, the sialidase can act upon added submicellar gangliosides (4,5). By decreasing fixed negative charge, enzymatic removal of sialic acid may serve to increase the effective pH (6) which, in turn, inhibits synaptic sialidase.

This study provides preliminary evidence for a relationship between cation concentration and action of synaptic membrane sialidase. We have measured the effect of changes in cation concentration upon synaptic membrane sialidase acting on native and exogenously supplied ganglioside substrates. The cations

studied: Na^+, K^+, and Ca^{++} are involved in nerve impulse transmission (7). Zn^{++}, which has been found in enriched amounts in the hippocampus (8), also was tested.

Methods:

Gangliosides were extracted from gray matter of bovine brain and isolated by partition-dialysis (9), and fractionated as described previously (4). Silica gel G thin layer chromatography plates were obtained from Quantum Ind. Solvents and chemicals were reagent grade and not further purified.

Synaptosomes were isolated from gray matter of bovine brain obtained at slaughter and processed at 4°. The procedure of Eichberg, et al. (10) was modified to maximize the yield of synaptic membrane sialidase without significantly increasing lysosomal or mitochondrial contaminants. The crude "Mitochondrial" fraction (10) was suspended in 0.32M sucrose and layered upon a discontinuous gradient of 0.6, 1.0, and 1.2M sucrose. After centrifugation, the synaptosomes banding at each interface of the 1.0M sucrose layer were collected, pelleted by centrifugation through 0.4M sucrose, and ruptured hypoosmotically. Synaptic membranes were isolated by sucrose gradient centrifugation (11). Characterization of sialidase as a synaptic membrane enzyme and characterization of the subcellular fractions by enzyme markers has been described previously (4). Morphological homogeneity was verified by viewing in an RCA-EMU 4 electron microscope. Samples were fixed in formaldehyde-glutaraldehyde, post-fixed with osmium tetroxide, embedded in Durcupan ACM mixture (12), and stained with lead hydroxide. Total protein (13) and total sialic acid (14) content of subcellular fractions were estimated colorimetrically. Qualitative identification of synaptic membrane ganglioside components was made by thin layer chromatography (15,16).

Endogenous sialidase activity was determined by incubating membrane samples (1 to 2 mg protein) in 1 ml of 0.02M, pH3.9 sodium acetate buffer at 37° for 90 min. Controls were held at 0°. After incubation the samples were cooled in ice and centrifuged at 10,000 χ g for 20 min. at 4°. The supernatants were removed, adjusted to pH7 with NaOH, passed through microcolumns of Dowex 1-χ10 (17,4) and free sialic acid was determined by the thiobarbituric acid procedure (14). The pellets, now essentially devoid of available endogenous substrate, were suspended in 1 ml of 0.02M, pH3.9 sodium acetate buffer containing 400µg of disialo- (75% wt) and trisialo- (25% wt) gangliosides. Samples were incubated for 50 minutes at 37°. Liberated sialic acid was determined in the same manner as for measurement of endogenous activity. These procedures were then used to determine the effect of cations on sialidase activity except that the buffer was the acetate salt of the cation to be studied.

Results and Discussion:

Modification of the isolation procedure for synaptic membrane sialidase resulted in a 30% increase in total yield with no increase in the lysosomal enzyme marker, acid phosphatase, or mitochondrial marker, succinic dehydrogenase.

More myelin fragments and microsomes were expected and could account for an observed decrease in specific activity: 20.9 nanomoles sialic acid

Table I: Endogenous sialic acid liberated from subcellular brain fractions and their sialidase activity with exogenous Di- and Trisialogangliosides and 5'-nucleotidase activity.

Sample[a]	Enzymatically released endogenous sialic acid nmoles/mg. protein	Exogenous sialidase activity nmoles sialic acid/mg. protein/90min.[b]	5'-nucleotidase activity umoles Pi/mg. protein/ 30min.[c]
P_1	4.0	11.9	1.7
P_2	9.0	12.5	2.3
P_3	16.7	3.2	6.9
$0.32\text{-}0.6P_2$	9.6	6.3	5.9
$0.6\text{-}1.0P_2$	8.1	16.3[d]	3.0
$1.0\text{-}1.2P_2$	8.8	15.7[d]	2.0
Pellet P_2	4.7	8.7	1.4

[a] P_1, nuclear fraction; P_2, crude mitochondrial; P_3, microsomal. Decimal numerals indicate sucrose molarities for separation of subfractions by centrifugation.

[b] Mean of duplicates. Standard error did not exceed ± 5%. Samples were not preincubated to deplete endogenous substrate. Incubation was prolonged to 90 minutes and values corrected for endogenous sialic acid release.

[c] Assayed as described elsewhere.[21]

[d] These fractions were selected and combined for study of sialidase activity as described under Methods.

per 90 minutes per milligram membrane protein for the $0.8\text{-}1.2P_2$ fraction in the unmodified procedure, but lower for the 0.6-1.0 and $1.0\text{-}1.2P_2$ fractions, shown in Table I. Application of a more recent procedure used for rat brain (18), was not attempted for the bovine brain material since reported yields of synaptic membrane sialidase appeared lower than expected for intact membranes.

The effect of pH upon activity was similar for both endogenous and

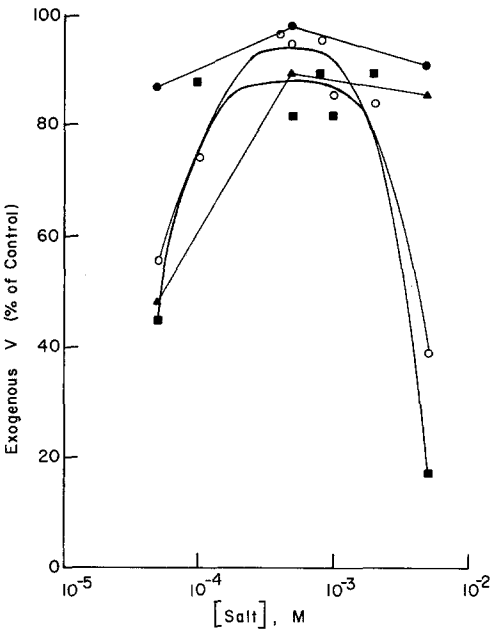

Figure 1. Effect of cations on activity of synaptic membrane sialidase with exogenous di- and trisialoganglioside substrate. Exogenous V (% of control) refers to the ratio of the initial reaction rate of sialidase on added disialo- and trisialoganglioside substrate in the presence of the indicated cation in comparison to that in the presence of 0.02M Na^+, times 100. For procedural details, see Methods. The salt concentration is plotted on a log scale. ■—■, exogenous V obtained in the presence of Zn^{++}; 0——0, Ca^{++}; ▲——▲, K^+; and ●——●, Na^+.

exogenous ganglioside substrate. Activity was optimum between pH3.9 and 4.5. Increase to pH5, or decrease to 3, resulted in a 50% diminution; at pH 6 or 2.2, activity was reduced to about 20%.

No change in endogenous sialidase activity was observed upon change in cation concentration from $5X10^{-5}M$ to $5X10^{-3}M$. In contrast, sialidase activity toward exogenous ganglioside substrate was influenced by specific cations (Fig. 1). Whereas $5X10^{-5}M$ Na^+ was sufficient for complete activity, higher levels of K^+, Ca^{++} or Zn^{++} were necessary to bring the enzyme to full activity. With still higher concentrations of Ca^{++}, or Zn^{++}, there was strong inhibition of activity with exogenous gangliosides. Na^+ or K^+ had no inhibitory effect.

V_{Max} and K_M were determined in the presence of optimum Na^+, and at optimum and at higher, inhibitory, Ca^{++} levels in order to explain inhibition

by supraoptimum concentrations of divalent cations. Di- and trisialogangliosides at concentrations below the critical micelle concentration were test substrates. Reaction time was reduced to 30 min. With Na^+, K_M = 2.7 X 10^{-4} and V_{Max} = 114nmoles sialic acid released/30min/mg synaptic membrane protein. At optimum Ca^{++} concentration, K_M = 10^{-4} and V_{Max} = 114nmoles sialic acid/30min/mg protein. At $5X10^{-3}$M Ca^{++}, K_M increased slightly (no more than 20%) while V_{Max} decreased to 34nmoles sialic acid/30min/mg protein. To ensure that this effect did not result from a cation-induced decrease in the critical micelle concentration of the substrate and an attendant lowering of the concentration of submicellar gangliosides, which appear to be the preferred physical form of exogenous sialoglycolipid substrate for synaptic membrane sialidase (4,5), micellar and submicellar gangliosides (19,20) were measured at a total level of 400µg of gangliosides per milliliter in the presence of increasing concentrations of Ca^{++} and Zn^{++}, and compared with increasing concentrations of Na^+. The micelles were sedimented and collected while in equilibrium with the submicellar forms, and the quantity of aggregated and dispersed sialic acid was estimated (20). Na^+ concentrations of $2X10^{-4}$M to $2X10^{-3}$M had little effect. About 20% of the gangliosides were in sedimentable micellar, form. As Ca^{++} concentration was raised from 10^{-4}M to $2X10^{-3}$M, micelle sialic acid increased from 20 ± 1% to 35 ± 2% of the total. Zn^{++} caused a similar effect. At 10^{-4}M, micellar sialic acid content was 24 ± 1%; at $4X10^{-4}$M, 28 ± 1%, and at $2X10^{-3}$M, 42 ± 2% of the total. The resulting decrease in concentration of submicellar gangliosides may be the reason for the observed (roughly 20%) increase in apparent K_M. Qualitative analysis of the micelles indicated that Zn^{++} but not Ca^{++} caused greater aggregation of trisialo- than disialoganglioside. Less aggregated trisialoganglioside would normally be expected because of its 2/3 lower concentration and higher critical micelle concentration (19). However, these effects of divalent cations upon substrate aggregation were not sufficient to account for the observed diminution in activity of synaptic membrane sialidase.

The effect of cations and pH upon synaptic membrane sialidase suggests the occurrence of a series of events which may have bearing upon synaptic transmission: 1. Activation of synaptic membrane sialidase by a decrease in pH causes sialic acid of presynaptic membrane gangliosides to become partially depleted. 2. Depletion of endogenous membrane gangliosides prepares the membrane enzyme for interaction with extra-membrane substrates. 3. Influx of sodium ion followed by other cations then activates the enzyme towards inter- or postsynaptic substrate. 4. Release of sialic acid lowers a screen of fixed negative charge in the synapse and permits diffusion of positively charged neurotransmitter molecules into the post synaptic membrane. 5. Influx of sufficient calcium ion shuts off extra-membrane sialidase activity, and resialylation can re-establish the screen of negative charge thus regulating neurotransmitter movement. This hypothesis is open to experimental test.

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REFERENCES:

1. Dekirmenjian, H., Brunngraber, E. G., Johnston, N. L., and Larramendi, L. M. H. (1969) Exp. Brain Res. 8, 97-104.
2. Pappas, G. D. and Waxman, S. G. in Pappas, G. D. and Purpura, D. P., Eds. (1972) Structure and Function of Synapses, Raven Press, New York, p. 2.
3. Wesemann, W., Henkel, R., and Marx, R. (1971) Biochemical Pharmacol. 20, 1961-1966.
4. Schengrund, C.-L. and Rosenberg, A. (1970) J. Biol. Chem. 245, 6196-6200.
5. Öhman, R., Rosenberg, A., and Svennerholm, L. (1970) Biochemistry 9, 3774-3782.
6. Conti, F. and Eisenman, G. (1965) Biophys. J. 5, 247-256.
7. Abood, L. G. in Albers, R. W., Siegel, G. J., Katzman, R., and Agranoff, B. W., Eds. (1972) Basic Neurochemistry, Little, Brown & Co., Boston, pp. 41-65.
8. Crawford, I. L. and Connor, J. D. (1972) J. Neurochem. 19, 1451-1458.
9. Folch, J., Arsove, S., and Meath, J. A. (1951) J. Biol. Chem. 191, 819-831.
10. Eichberg, J., Whittaker, V. P., and Dawson, R. M. C. (1964) Biochem. J. 92, 91-100.
11. Whittaker, V. P., Michaelson, I. A., and Kirkland, R. J. A. (1964) Biochem. J. 90, 293-303.
12. Karnovsky, M. J. (1965) J. Cell Biol. 27, 137A-138A.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
14. Warren, L. (1959) J. Biol. Chem. 234, 1971-1975.
15. Wagner, H., Hörhammer, L., and Wolff, P. (1961) Biochem. Z. 334, 175-184.
16. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604-611.
17. Horvat, A. and Touster, O. (1968) J. Biol. Chem. 243, 4380-4390.
18. Morgan, I. G., Wolfe, L. S., Mandel, P., and Gombos, G. (1971) Biochim. Biophys. Acta 241, 737-751.
19. Yohe, H. C. and Rosenberg, A. (1972) Chem. Phys. Lipids 9, 279-294.
20. Lipovac, V., Bigalli, G., and Rosenberg, A. (1971) J. Biol. Chem. 246, 7642-7648.
21. Touster, O., Aronson, N. N., Jr., Dulaney, J. T., and Hendrickson, M. (1970) J. Cell Biol. 47, 604-618.