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Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule

(cell surface modulation/neural development/binding assay/sialic acid-rich glycoprotein)

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ABSTRACT **The neural cell adhesion molecule, N-CAM, is a cell surface glycoprotein found on embryonic and adult neurons and on a variety of ectodermal and mesodermal tissues in very early embryos. During development, it shows local variations in prevalence at the cell surface as well as conversion from an embryonic form (E form) with high sialic acid content to an adult form (A form) with lesser amounts of this sugar. This E→A conversion occurs on different schedules in different brain regions, and it has been hypothesized that both the conversion and the prevalence changes are related to early regulation of pattern formation and connectivity. In order to identify precisely the consequences of these mechanisms of local cell surface modulation of N-CAM, an assay was developed to measure the rate of aggregation either of vesicles reconstituted from lipid and purified N-CAM or of native brain membrane vesicles. In both preparations, aggregation was >95% inhibitable by specific anti-(N-CAM) Fab' fragments. The rates of aggregation of reconstituted N-CAM vesicles and native brain vesicles were found to be inversely related to the sialic acid content of their N-CAM molecules, with full desialylation resulting in about a 4-fold increase in rate over E-form N-CAM. Intermediate rates were obtained both with A-form N-CAM (which contains only one-third of the sialic acid content of E-form N-CAM) and with partially desialylated E-form N-CAM. The rate of coaggregation of reconstituted vesicles containing E-form N-CAM with reconstituted vesicles containing A-form N-CAM was also intermediate, implying that desialylation did not change the nature of (N-CAM)-(N-CAM) binding but only its rate. Even larger alterations in vesicle aggregation rate were seen when the amount of N-CAM per vesicle was altered. A 2-fold increase in the N-CAM-to-lipid ratio of reconstituted vesicles resulted in a >30-fold increase in their rate of aggregation. Moreover, desialylation did not cause a further increase in the rate of aggregation of these already rapidly aggregating vesicles. These results in a model system demonstrate the large range of binding rates that are obtainable by various forms of local surface modulation of N-CAM. They are consistent with the proposal that similar alterations affecting (N-CAM)-mediated cell adhesion *in vivo* may be major factors in pattern formation during development of the nervous system.**

During development, precisely coordinated cell–cell interactions in the nervous system give rise to the various neuroanatomical structures that underlie the ability to receive, transform, and respond to sensory input (1, 2). In certain regions such as the cerebellum, repeating multicellular structures are formed (3). Despite the overall similarity of such structures, the dendritic and axonal arborizations, the connectivity, and the metabolism of each cell are so complex (4) that it is improbable that a completely identical cell occurs elsewhere in the same or another organism.

There are two basic hypotheses concerning the nature of specific cell surface proteins that mediate such modally similar but variant pattern formation in the developing nervous system. The strict chemoaffinity hypothesis (5) suggests that specificity results from the presence of a number of different complementary adhesive proteins on interacting cells; this would require the expression of many different gene products in different portions of a single tissue. On the other hand, the modulation hypothesis (6) proposes that a small number of adhesive proteins can mediate pattern formation, provided that their binding activities are locally modulated by epigenetic means in a dynamic fashion. Potential modulation mechanisms include differential chemical alteration, variation in temporal expression, and variation in prevalence or surface density in different regions of a tissue or organism (7). For the modulation hypothesis to be valid, it is necessary that different binding regimes can occur for the same kind of cell adhesion molecule, leading to graded differences of adhesivity in different regions of a tissue.

The properties of N-CAM appear to fulfill the requirements of the modulation hypothesis on both functional and structural grounds. Anti-(N-CAM) Fab' fragments inhibit pattern formation in histotypic aggregates of retinal cells (8) and in intact neural retinae in organ culture (9). Large changes in the amount of cell surface N-CAM have been observed in early embryonic and neural crest cells (10, 11). Furthermore, the disappearance and reappearance of N-CAM on neural crest cells *in vivo* have been correlated with their migration and their clumping to form ganglia (10). In addition to such prevalence changes, modulation in the amount of sialic acid in N-CAM also has been documented. The embryonic (E) form of N-CAM contains a large amount of sialic acid, some of which is probably in the form of polysialic acid (12). The amount of sialic acid on N-CAM at a given time during development varies among different parts of the nervous system and, in general, decreases as the organism matures (13). This decrease has been found to be delayed in the cerebellum of the mouse neurological mutant staggerer (*sg*) (14). Moreover, binding of N-CAM to N-CAM is not destroyed by neuraminidase treatment which, in fact, appears to increase the ability of soluble N-CAM to bind to retinal cells (15). All of these findings have led to the proposal that there is an inverse relationship between the binding strength of N-CAM and its sialic acid content (7) and to the prediction that the increasing order of binding affinities between the E and adult (A) forms of N-CAM would be: E-E < E-A < A-A.

Aggregation of N-CAM in solution (12) prevents direct measurement of equilibrium binding constants. Because of this,

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Abbreviations: N-CAM, neural cell adhesion molecule; E and A forms of N-CAM, the embryonic and adult tissue forms of N-CAM; P_i/NaCl/KCl, 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, 1.15 g of Na_2HPO_4 per liter (pH 7.4).

and in view of the fact that adhesion *in vivo* is likely to be a kinetically constrained process at least during histogenesis, we devised a kinetic binding assay with lipid vesicles containing N-CAM. We investigated the initial rates of (N-CAM)-mediated aggregation of membrane vesicles prepared directly from brain tissue or of vesicles reconstituted from purified N-CAM and brain lipids. Using this assay, we have compared the adhesive activities of A and E forms of N-CAM, of N-CAMs having different amounts of sialic acid, and of vesicles containing a range of N-CAM concentrations. We find that alterations of both the sialic acid content of N-CAM and of the N-CAM content of the vesicles have large effects on the rates of vesicle aggregation.

MATERIALS AND METHODS

Brain Vesicles. These were prepared as described (12, 13). The vesicles to be used in aggregation experiments were purified by differential centrifugation (supernatant fraction from a 5-min centrifugation at 1,200 rpm) and density equilibrium ultracentrifugation (18 hr at 25,000 rpm on a 14–42% continuous sucrose gradient in P_i/NaCl/KCl [8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 1.15 g of Na_2HPO_4 per liter (pH 7.4)] in a Beckman SW 27 rotor), and their protein concentrations were determined (16). N-CAM content was determined by the ability of vesicles to bind different amounts of ^{125}I-labeled monoclonal anti-(N-CAM) IgG [^{125}I-anti-(N-CAM); clone 1, see ref. 12] during a 15-min incubation at 4°C.

Reconstituted Vesicles. Vesicles were reconstituted from 14-day embryo brain membrane lipids by using purified N-CAM as described (17). In a typical preparation, 20 mg of lipid, 0.5–2.0 mg of N-CAM, and 10^5 cpm (10 μg of protein) of ^{125}I-labeled N-CAM [^{125}I-(N-CAM)] were mixed in detergent solution, the detergent was removed (18), and the vesicles were harvested by ultracentrifugation; 20–50% of the protein became stably associated with the lipid vesicles. To measure N-CAM concentration in reconstituted vesicles, protein and lipid were reseparated by chloroform/methanol extraction, the N-CAM was collected by centrifugation (10 min at 12,000 rpm), and its solution concentration was determined (16).

Vesicles were treated with *Vibrio cholerae* neuraminidase (Calbiochem–Behring) at the indicated concentrations and times in 50 mM NaOAc, pH 5.0/2 mM $CaCl_2$/0.2 mM EDTA, and the released sialic acid was quantitated (19).

Aggregation Experiments. Vesicle aggregation was monitored with a Coulter Counter model ZBI fitted with a 100-μm aperture. To remove large particles before the start of an experiment, brain vesicles and reconstituted vesicles were passed twice through 0.8-μm or 0.4-μm Uni-Pore filters (Bio-Rad). In a typical experiment, up to five 0.5-ml samples of reconstituted vesicles (containing 0.1–1.0 mg of lipid) or brain vesicles (containing 10–100 μg of membrane protein) in P_i/NaCl/KCl containing bovine serum albumin (10 mg/ml) were incubated in scintillation vials at 25°C at 70 rpm. Samples (20 μl) were removed at intervals and diluted to 20 ml with filtered P_i/NaCl/KCl, and the particles larger than 1.5 μm^3 (the Coulter Counter's lowest reliable threshold) were counted. When there were >400 superthreshold particles per ml, the threshold was increased 2-fold, and the sample was recounted; this process was repeated until at the final threshold there were <400 particles per ml. In this way, a histogram was generated describing each sample at each time point. The total volume of particles larger than 1.5 μm^3 was calculated by integrating each histogram; the superthreshold particle volumes were then plotted versus time, and the initial slope of this curve was defined as the initial rate of aggregation. In some cases, particularly at lower particle concentrations, there was a lag before the apparent commencement of aggregation. This was likely due to early aggregation events that did not result in the formation of superthreshold particles; this lag reflecting requisite amplification times was ignored in calculating rates of aggregation. There was a lower limit to the measurement of slow rates of aggregation due to the fact that, as vesicle input was increased to increase the aggregation rate, the background volume of superthreshold particles increased to the point where the increase in particle volume due to aggregation was not discernible. All experiments in this paper were repeated two to four times with <20% variation in the relative rates of aggregation in the comparisons of A-form and E-form vesicles and of neuraminidase-treated and mock-treated vesicles.

RESULTS

A-form N-CAM Is a More Effective Ligand than E-form N-CAM. A and E forms of N-CAM differ strikingly in their relative sialic acid content and in their appearance on NaDodSO$_4$/polyacrylamide gel electrophoresis (13, 14). In order to determine whether there are functional differences between the A and E forms of the molecule, reconstituted vesicles were made containing the A or E form, and their initial rates of aggregation were quantitated and compared. Particular care was taken to use preparations of E- and A-form vesicles containing closely similar ratios of protein to lipid (see below). Rates of aggregation at four different vesicle dilutions were calculated as described from data in Fig. 1 *A* and *B* and were plotted versus vesicle concentration on a log-log scale (Fig. 1*C*). These plots revealed that: (*i*) the rate of aggregation increased with the square of vesicle concentration (A form, slope = 2.04; E form, slope = 2.17), and (*ii*) at a given vesicle concentration, the rate of aggregation for A-form vesicles is about 3.5 times that of E-form vesicles.

The results of several control experiments strongly implied that the observed vesicle aggregation was mediated by the N-CAM molecule. Vesicles made, for example, without N-CAM or N-CAM vesicles treated with trypsin did not aggregate. The aggregation of N-CAM vesicles (containing 5 μg of N-CAM) was >95% inhibited by 20 μg of rabbit anti-(N-CAM) Fab′ fragments, by 130 μg of the soluble Fr2 (15) fragment containing the binding site of N-CAM, or by monoclonal anti-(N-CAM) IgG (clone 1) added in excess (1 mg) in order to make it "functionally monovalent." The temperature dependence of vesicle aggregation, a 2-fold increase for each 14°C increase within the range analyzed (22–45°C), is also consistent with a binding process mediated by protein–protein interactions.

Rate constants indicating the relative aggregation activity of a vesicle preparation can be calculated from the equation $v = k_{agg}a^2$ where k_{agg} is the apparent rate constant, v is the rate of aggregation measured at vesicle concentration a, and the exponent is derived from the concentration dependence of the rate of aggregation shown in Fig. 1*C*, a figure confirmed in all vesicle preparations analyzed. Although formally equivalent to the rate constant of chemical kinetics, k_{agg} should not be considered to be a true rate constant because it is not calculated by extrapolation to ln(vesicle concentration) = 0 and because vesicle aggregation is, at least in part, a polymerization reaction. Nevertheless, this parameter provides a convenient and consistent measure of the relative activity of vesicle preparations at early times and is used throughout the rest of this paper.

The Functional Difference Between E and A Forms of N-CAM Is Due to Sialic Acid Content. In order to test the hypothesis (7) that the difference in apparent rates of N-CAM vesicle aggregation for E and A forms documented above is due to differences in sialic acid content, the N-CAM vesicles were

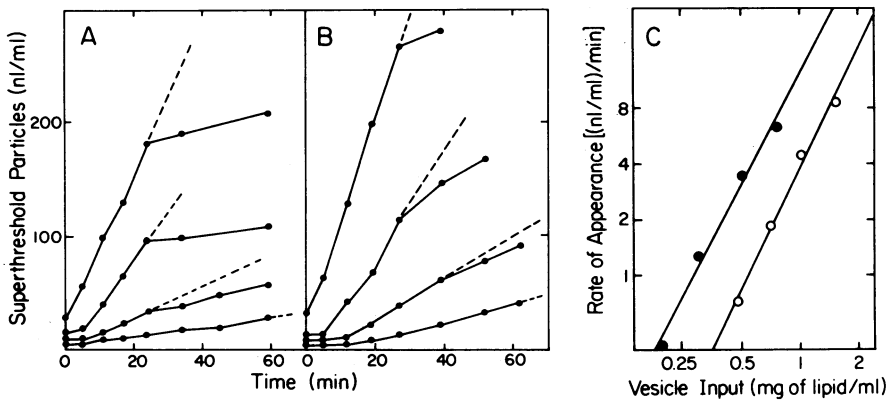

FIG. 1. Aggregation of reconstituted vesicles containing A or E forms of N-CAM. Vesicles were reconstituted, and their aggregation was analyzed as described. The concentration of superthreshold particles is plotted as a function of time for four concentrations of vesicles. Apparent initial rates of aggregation are calculated from the initial slopes (----) of the aggregation curves. (*A*) A-form vesicles (16.3 μg of N-CAM per mg of lipid) aggregated at 0.76, 0.50, 0.30, and 0.20 mg of lipid per ml (curves proceeding from left to right). (*B*) E-form vesicles (16.9 μg of N-CAM per mg of lipid) aggregated at 1.55, 1.03, 0.71, and 0.48 mg of lipid per ml. (*C*) Log-log plot of rate of appearance of superthreshold A-form (●) and E-form (○) particles versus vesicle input.

treated with neuraminidase and their initial rates of aggregation were then examined. Complete removal of sialic acid from E-form vesicles by neuraminidase treatment resulted in a 3.6-fold enhancement in k_{agg} over mock-treated vesicles (Table 1). Partial removal of sialic acid by lesser amounts of neuraminidase revealed a monotonic relationship between the amount of sialic acid removed and the increase in k_{agg}. Only a slight enhancement in k_{agg} (1.2-fold) was seen when fully desialylated vesicles were compared with 70% desialylated vesicles (which had a sialic acid-to-protein ratio similar to that of A-form vesicles). In accord with this observation, neuraminidase treatment of A-form vesicles had almost no effect on their k_{agg} as compared to mock-treated vesicles. It appears, therefore, that removal of sialic acid from E-form vesicles down to the level seen in the A form of N-CAM makes them functionally equivalent to A-form vesicles but that further removal of sialic acid from either species has little effect.

Mock-treated vesicles aggregated 3–10 times as rapidly as did untreated vesicles (Tables 1 and 2). This result in no way jeopardizes the conclusion that neuraminidase treatment enhanced vesicle aggregation inasmuch as, in each case, neuraminidase treatment was always compared to buffer-treated controls. The enhancement of rates for mock-treated vesicles did not appear to be due to the loss of sialic acid because sialic acid release under these conditions was below detection (<5% of the sialic acid in the vesicles). The enhancement also was not due to mild acid hydrolysis of N-CAM inasmuch as a similar preincubation in P_i/NaCl/KCl resulted in a similar enhancement. Finally, it is likely that the enhancement of aggregation by mock treatment is a separate phenomenon from enhancement by removal of sialic acid because 3 hr of mock treatment resulted in a k_{agg} that was only 1.3 times that reached in 1 hr—a figure far below the 3.6-fold difference between 1 hr of neuraminidase treatment and 1 hr of mock treatment. Mock treatment may result in alteration of N-CAM conformation or effective valence or in a change in side-to-side interactions of the molecules in a single vesicle. In any case, the changes induced by mock treatment do not alter the main conclusion of this study.

To rule out the possibility that modification of lipids by mock or neuraminidase treatment of vesicles affected the vesicle aggregation, lipids were reisolated after such treatment and were reincorporated into vesicles with fresh N-CAM. The aggregation of these vesicles was similar to that of vesicles prepared with untreated lipids.

The Apparent Rate Constant Is Highly Dependent on N-CAM Concentration in Vesicles. The k_{agg} of E-form vesicles is highly dependent on the N-CAM-to-lipid ratio, a 2-fold increase in N-CAM concentration being correlated with a >30-

Table 1. Effect of neuraminidase treatment and mock treatment on vesicle aggregation

Vesicles	Neuraminidase treatment	Sialic acid removed, %	k_{agg}, units*
E form	None	0	1.5
	Buffer only	<5	15
	0.025 unit/ml	50	35
	0.1 unit/ml	70	47
	0.4 unit/ml	>95	55
A form	None	0	3.3
	Buffer only	<5	20
	0.4 unit/ml	>95	22

E-form vesicles (14.0 μg of N-CAM per mg of lipid) and A-form vesicles (11.0 μg of N-CAM per mg of lipid) were pelleted in the ultracentrifuge, resuspended in neuraminidase treatment buffer, and filtered through a 0.6-μm filter. Enzyme and buffer were added to a final lipid concentration of 4.2 mg/ml and the indicated amount of neuraminidase, and the suspension was incubated for 1 hr at 37°C. Incubations were then brought to neutral pH, and vesicles were harvested by ultracentrifugation.

* One unit is the rate of aggregation of a sample (measured in nl of superthreshold product per ml per min) divided by the square of the concentration of vesicles in the sample (vesicle concentration was measured in mg of lipid per ml).

Table 2. Effect of N-CAM-to-lipid ratio on vesicle aggregation

N-CAM/lipid, μg/mg	k_{agg}, units*		
	Untreated	Mock-treated	Neuraminidase-treated
11.7	<1	5.7	25
14.0†	1.5	15	55
16.9‡	3.5	ND	ND
18.8	6.4	48	138
28.3	54	175	173

E-form vesicles were mock-treated or neuraminidase-treated (0.4 units/ml) as described in Table 1. ND, not done.

* See Table 1.

† Same preparation as in Table 1.

‡ Same preparation as in Fig. 1.

fold increase in k_{agg} (Table 2). As described above, usually both neuraminidase treatment and mock treatment of E-form vesicles greatly enhanced their k_{agg}. For the most rapidly aggregating vesicles (Table 2), however, enhancement by mock treatment was diminished, and there was no further enhancement by neuraminidase treatment over mock treatment. When the data from Table 2 were plotted on a log-log scale, the slope of the plot for untreated vesicles was 5.2, suggesting a highly nonlinear dependence of k_{agg} on N-CAM concentration within the vesicle. The slopes of the plots for neuraminidase-treated and mock-treated vesicles are somewhat less—3.5 and 3.8, respectively. Furthermore, the plot for neuraminidase-treated vesicles begins to plateau at the highest N-CAM concentration, suggesting that k_{agg} approached a maximal value. The decrements in slope noted after treatment are consistent with possible alterations in the local surface density or interactions of N-CAM molecules (7) in a single vesicle.

Mixed Vesicle Aggregation. When E- and A-form vesicles were mixed at different ratios (but a constant total concentration), the data (Fig. 2) indicated that E- and A-form vesicles coaggregate with an effective k_{agg} intermediate between that of the E- or A-form vesicles alone. The observed rate of aggregation in E/A mixtures was always significantly greater than the sum of the expected rates of aggregation of each respective component with itself (Fig. 2), indicating that E- and A-form vesicles must coaggregate. On the other hand, the rate of aggregation of mixtures was always greater than that of a pure E-form population at the same total concentration and less than that of a pure A-form population. Similar results indicating coaggregation at an intermediate rate were obtained when E-form vesicles and neuraminidase-treated E-form vesicles were mixed.

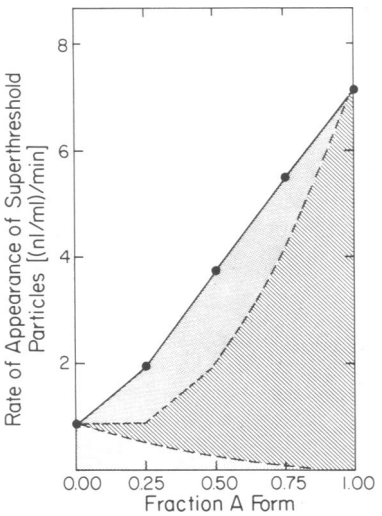

FIG. 2. Mixing experiment. E-form vesicles (14.0 μg of N-CAM per mg of lipid) and A-form vesicles (16.3 μg of N-CAM per mg of lipid) were coincubated at the five indicated proportions with a constant total of 0.8 mg of vesicle lipid per ml in each mixture. The apparent initial rate of aggregation of each mixture is plotted (●) versus the fraction of A form. The calculated contributions of E–E (lightly stippled) and A–A (hatched) interactions in each mixture were obtained by multiplying the rate of aggregation of a pure population of E-form vesicles or A-form vesicles by the square of the relative concentration of each vesicle species in a mixture in order to reflect the second-order dependence of the rates on vesicle input. For example, for the fraction A form = 0.25, the A–A contribution would be $(0.25)^2$ × the apparent rate of aggregation of the pure A-form population, and the E–E contribution would be $(0.75)^2$ × the rate of aggregation of the pure E-form population. The residual aggregation in each mixture (heavily stippled) is attributable to E–A interaction.

Aggregation of Brain Vesicles. The aggregation of membrane vesicles prepared directly from embryonic and adult brain tissue can be analyzed by the same methods used for reconstituted vesicles. These vesicles also aggregate by an (N-CAM)-mediated mechanism (and, thus, can be described by their A-form or E-form N-CAM); rabbit anti-(N-CAM) Fab' fragments inhibited the aggregation of E-form brain vesicles by >95% and A-form brain vesicles by >90%. In experiments with brain vesicles, the strong dependence of k_{agg} upon N-CAM concentration and upon relative sialic acid content that was observed in reconstituted vesicles was confirmed. A-form brain vesicles aggregated only 1.5 times as rapidly as E-form brain vesicles (Table 3), but this is consistent with the fact that A-form brain vesicles contain only about 70% as much N-CAM per total membrane protein. Specifically, E-form brain vesicles containing 100 μg of membrane protein bound 0.73 μg of ^{125}I-anti-(N-CAM) IgG (clone 1), whereas the same amount of A-form brain vesicles bound only 0.50 μg. As with reconstituted vesicles, neuraminidase treatment of E-form brain vesicles greatly enhanced (5.1-fold) their rate of aggregation over mock-treated vesicles, whereas neuraminidase treatment of A-form brain vesicles had little effect (1.2-fold enhancement over mock-treated vesicles).

The effect of N-CAM concentration on brain vesicle aggregation can be analyzed in the absence of differences due to sialic acid content by comparing the aggregation of neuraminidase-treated E-form and neuraminidase-treated A-form brain vesicles. Neuraminidase-treated E-form brain vesicles aggregated 3.6 times as rapidly as neuraminidase-treated A-form brain vesicles. This ratio is consistent with a 3.4th-order dependence of k_{agg} on the respective N-CAM concentrations in the neuraminidase-treated brain vesicles, similar to the dependence observed for neuraminidase-treated reconstituted vesicles. Finally, the large enhancement of k_{agg} caused by mock treatment of reconstituted vesicles did not occur in brain vesicles; this may reflect differences in insertion, arrangement, or the presence of other surface proteins in the "native" vesicles. Despite this difference, the effects on aggregation rates mediated by variations in sialic acid concentration and N-CAM prevalence were similar in the two classes of vesicles.

We previously have observed that incubation of E-form N-CAM solutions resulted in autolytic degradation of the molecule (12). However, proteolysis does not appear to play a role in the enhancement of vesicle aggregation during neuraminidase treatment inasmuch as N-CAM isolated after neuraminidase treatment of E-form brain vesicles (data not shown) contained the same protein components in a similar ratio as did N-CAM that was neuraminidase-treated while bound to an immunoaffinity support (15). These ratios showed no relative in-

Table 3. Aggregation of brain vesicles

Incubation	k_{agg}, units* E form	A form
None	1,340	1,950
Buffer only	1,810	2,200
Neuraminidase	9,230	2,560

E- and A-form brain vesicles were pelleted at 18,000 rpm in the Sorvall centrifuge, resuspended at 4 mg/ml of membrane protein in neuraminidase treatment buffer or neuraminidase treatment buffer containing 1 unit of neuraminidase per ml, filtered through a 0.8-μm filter, incubated 3 hr at 37°C, and then harvested by two rounds of centrifugation in the Sorvall.

* For brain vesicle experiments, one unit is the rate of aggregation of a sample (measured in nl of superthreshold product per ml per min) divided by the square of the concentration of vesicles in the sample (vesicle concentration was measured in mg of membrane protein per ml).

crease in the amount of the characteristic M_r 65,000 autolysis product (12).

DISCUSSION

We have identified two parameters that control the initial rate of vesicle aggregation mediated by N-CAM: the sialic acid content of the N-CAM molecules in the vesicles and the N-CAM content of the vesicles. These effects were apparent both in vesicles prepared directly from brain tissue and vesicles reconstituted from purified N-CAM and lipid. They have salience in understanding the mechanisms of local cell surface modulation (6, 7) by E → A conversion (14) and by alteration in N-CAM prevalence at the cell surface (10, 11).

Total removal of sialic acid from the E form of N-CAM caused up to a 5-fold increase in the rate of vesicle aggregation, whereas partial removal resulted in a monotonic order of aggregation rates that was inversely related to the amount of sialic acid remaining on the N-CAM molecules. Vesicles containing the A form of N-CAM, which *in vivo* contains much less sialic acid than E-form N-CAM, also aggregated at an enhanced rate, and their rate of aggregation was only marginally enhanced further by neuraminidase treatment. These results give strong support to the charge-perturbation binding model for N-CAM (7), which suggests that the high charge density of sialic acid on E-form N-CAM acts to inhibit the formation of (N-CAM)-(N-CAM) bonds. Furthermore, the fact that the adhesive activity of N-CAM can be varied in a graded fashion by a chemical alteration demonstrates the pertinence of the hypothesis that pattern formation in the nervous system arises in part through the local modulation of N-CAM function (7). These results are particularly provocative in light of the fact that the loss of sialic acid from N-CAM during maturation occurs at different rates in different brain regions and is delayed in the cerebellum of the mouse neurological mutant staggerer (14).

Independent of the effects of chemical modulation by alterations in sialic acid content, changes in N-CAM concentration produce large changes in vesicle binding: aggregation rate was found to be increased in proportion to at least the 3.4th power of N-CAM concentration. This result is consistent with the possibility that N-CAM function *in vivo* might be modulated by global temporal changes in, or local variations in, the prevalence of N-CAM. Indeed, neural crest cells *in vivo* have been found to lose N-CAM during migration and to aggregate and differentiate into ganglia concomitant with their reexpression of surface N-CAM (10, 11). Although at present there are no absolutely quantitative data concerning the range of N-CAM concentrations found in different regions of the developing brain *in vivo*, strong relative differences have been found (data not shown).

The studies in this paper all deal with the initial rate of vesicle aggregation. These rates also appear to be correlated with the final extent of the reaction in terms of the observed plateaus in aggregation (see below). In any case, the initial rate of reaction seems a reasonable parameter to use to analyze vesicle aggregation and serves as a model system for analysis of the dynamic interactions occurring during pattern formation in the nervous system. There were two major features of interest in the process of vesicle aggregation: second-order dependence of initial rate of aggregation on vesicle input and the occurrence of a relative plateau in the total volume of aggregated particles after the period of initial rapid aggregation. In chemical kinetics, the order of a reaction is theoretically the same as the number of molecules involved in the basic reaction. In analogy to chemical kinetic theory, our empirical result that the initial rate of vesicle aggregation is proportional to the 2nd power of vesicle input implies that the basic step in early vesicle aggregation involves two particles. This result is intuitively reasonable and suggests that, at least in this respect, the analogy to chemical kinetic analysis is valid. Of course, higher order interactions may play increasingly predominant roles at later times.

After an initial period of vesicle aggregation at a relatively constant rate, the total volume of superthreshold particles in a sample starts to plateau. The height of the plateau is monotonically related (at constant vesicle input) to the k_{agg} of the vesicle preparation, yet was never >20% of the total vesicle input. Factors that may limit the accumulation of superthreshold particles are (*i*) the possibility that some vesicles in a preparation may not be aggregation competent, (*ii*) the possibility that, as the level of aggregation of a sample increases, the occurrence of a back reaction increases, and (*iii*) the likelihood that, as the level of aggregation of a sample increases, reactions between already superthreshold particles are favored, thus reducing the consumption of subthreshold "monomer."

We demonstrated in the present study that the function of N-CAM in an *in vitro* model system is modulated by alterations either in structure or in prevalence that are already known to occur *in vivo*. The results provide a reasonable mechanistic basis for the hypothesis that local surface modulation of N-CAM function (7) plays a major role in pattern formation in embryogenesis and during the development of the nervous system.

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