Regulation of Neural Cell Adhesion Molecule Polysialylation: Evidence for Nontranscriptional Control and Sensitivity to an Intracellular Pool of Calcium

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Abstract. The up- and downregulation of polysialic acid–neural cell adhesion molecule (PSA–NCAM) expression on motorneurons during development is associated respectively with target innervation and synaptogenesis, and is regulated at the level of PSA enzymatic biosynthesis involving specific polysialyltransferase activity. The purpose of this study has been to describe the cellular mechanisms by which that regulation might occur. It has been found that developmental regulation of PSA synthesis by ciliary ganglion motorneurons is not reflected in the levels of polysialyltransferase-1 (PST) or sialyltransferase-X (STX) mRNA. On the other hand, PSA synthesis in both the ciliary ganglion and the developing tectum appears to be coupled to the concentration of calcium in intracellular compartments. This study documents a calcium dependence of polysialyltransferase activity in a cell-free assay over the range of 0.1–1 mM, and a rapid sensitivity of new PSA synthesis, as measured in a pulse–chase analysis of tissue explants, to calcium ionophore perturbation of intracellular calcium levels. Moreover, the relevant calcium pool appears to be within a specific intracellular compartment that is sensitive to thapsigargin and does not directly reflect the level of cytosolic calcium. Perturbation of other major second messenger systems, such as cAMP and protein kinase–dependent pathways, did not affect polysialylation in the pulse chase analysis. These results suggest that the shuttling of calcium to different pools within the cell can result in the rapid regulation of PSA synthesis in developing tissues.

The polysialic acid component of neuronal cell adhesion molcule (NCAM)[1] serves as a temporally regulated modulator of a variety of cell interactions during development (for review see reference 38), with effects having been documented for the facilitation of guidance and targeting of axons (42, 43), migration of neuronal (15, 33), and glial (47) precursors, and development of muscle myotubes (9). In addition, the persistent expression of polysialic acid (PSA) in certain regions of the adult nervous system is correlated with the maintenance of plasticity in cell interactions (see reference 41), including the recent demonstration that enzymatic removal of PSA prevents synaptic facilitation in hippocampal circuits (29).

In addition to temporal control of PSA, its expression is also spatially regulated on the cell surface. The examples of topographical regulation are the rapid and selective expression of PSA at the external surface of secondary myotubes as they separate from primary myotubes in the chick embryo limb (9), and the association of PSA with a distinct segment of the axons in the corticospinal tract (7). It has also been observed in the chick ciliary ganglion that PSA is excluded from the synaptic cleft but present at the tips of the growing terminal (Brusés, J.L., and U. Rutishauser, unpublished observations).

From these observations, it would appear that there is a tight regulation of PSA expression that is integral to its biological function. Thus a key issue is raised, namely how the expression of this carbohydrate is regulated both as a part of developmental programs in the embryo or physiological processes in the adult. Previous studies have suggested that the regulation of PSA expression in vivo occurs at the level of the enzymatic activity (5). Furthermore, there is growing evidence that two distinct Golgi-associated polysialyltransferases (polysialyltransferase-1/ST8Sia IV [PST] and sialyltransferase-X/ST8SiaII [STX]), both members of the sialyltransferase family, are each sufficient to add PSA chains to neural cell adhesion molecule (NCAM; references 20, 30, 49). In particular, in vitro transfection of PSA-negative, NCAM-positive cells with PST or STX

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1. *Abbreviations used in this paper*: CG, ciliary ganglion; endo H, endoglycosidase H; endo N, endoneuraminidase N; IC, intracellular compartments; NCAM, neural cell adhesion molecule; PSA, polysialic acid; PST, polysialyltransferase-1; RT, reverse transcriptase; St, embryonic stage; STX, sialyltransferase-X.

cDNA results in the polysialylation of the NCAM (2, 8). Interestingly, STX transcripts are most abundant in the embryonic brain, where overall PSA expression levels are high, and decrease dramatically after birth, whereas PST transcript levels are lower and remain more constant (2).

This study was designed to investigate cellular mechanisms for regulation of PSA in embryonic tissues. It began with analysis of PST and STX expression at specific stages of development of the chick ciliary ganglion (CG) that contains a relatively homogeneous and developmentally synchronized population of PSA-positive motorneurons. These studies indicated that the STX message is much more abundant than PST in the developing CG. However, neither STX or PST is dramatically regulated during CG development, and thus transcriptional control of these enzymes is unlikely to explain the most rapid stage-specific changes in PSA levels in this tissue, namely its downregulation in conjunction with synaptogenesis. Subsequently, we began to look for possible nontranscriptional mechanisms through the use of pharmacological perturbation of PSA synthesis in embryonic brain tissue. A strong dependence on intracellular stores of Ca^{2+} was found that, together with the demonstration that PSA synthetic activity itself is Ca^{2+}-dependent, could provide a mechanism for rapid and localized control of NCAM polysialylation.

Materials and Methods

Cloning of PST from Chicken

To obtain the chicken PST cDNA, a λgT10 bacteriophage cDNA library constructed from embryonic chicken brain was screened with the human PST cDNA. A 1.8-kb cDNA fragment was isolated, subcloned into pGEN plasmid and sequenced by the dideoxy chain termination reaction. The GenBank database was searched to identify homologous sequences by using the BLAST program (1). By comparison with the human and hamster PST, the isolated fragment appeared to be a partial cDNA clone lacking the 3′ end of the coding sequence. Based on the human and hamster sequences, degenerate primers for the 3′ end of the coding sequence were designed (5′-TGTGCTYYAYTGYTTNACRCAYTTNCCNGT-3′) and together with an internal primer (5′-TCTAGCTCCTGTGGTGGAGTTTG-3′) were used to generate by PCR an expected 572-bp fragment that was needed to obtain the complete cDNA coding sequence of the chicken PST. The analysis of the cDNA sequence showed that the largest fragment within one open reading frame was of 1,080 bases predicting a protein of 359 amino acids with a hydrophobic region of 10 amino acids close to the NH_2 terminus and 2 sialyl motifs (these data are available from GenBank/EMBL/DDBJ under accession number AF008194). The nucleotide sequence homology of the chicken PST with the human, hamster, and mouse homologues was 85, 84, and 84% respectively. At the amino acid level the homology with human PST was 93%, and 92% with hamster and mouse PST.

Northern Blot Hybridization

Chick ciliary ganglia from the desired embryonic stage (St) were collected, frozen in liquid nitrogen, and stored at −70°C. Total RNA was isolated by Ultraspect (Biotecx Laboratories, Houston, TX) and ~20 μg of RNA from each stage was electrophoresed in 1.2% agarose gel containing 2.2 M formaldehyde, blotted onto Hybond-N^+ nylon membrane (Amersham Corp., Arlington Heights, IL), and the RNA was immobilized by UV crosslinking. The isolated 1.8-kb PST fragment described above was used as a probe for Northern blot analysis. The human STX insert was isolated by enzyme digestion from a pcDNA1 plasmid and used to detect the chicken STX mRNA. A 507-bp fragment of the chicken NCAM-180 sequence and a 450-bp fragment of the chicken β-actin sequence were generated by RT-PCR from chicken brain RNA, subcloned, sequenced, and used to detect the NCAM and β-actin mRNA, respectively. All cDNA probes were gel purified and isolated by Geneclean II kit or phenol extraction. cDNA fragments were radiolabeled with a [^{32}P]dCTP before use, using the Rediprime kit (Amersham Corp.). The radiolabeled probes were purified by a G-50 spin column (Pharmacia Biotechnology, Inc., Piscataway, NJ). The membranes were prehybridized for 20 min at 65°C in 4 ml QuikHyb Hybridization Solution (Stratagene, La Jolla, CA), the desired probe was then added at a final concentration of 2×10^6 cpm/ml and hybridized in the same solution for 1–2 h at 65°C. The membranes were washed 2 times for 5 min with 2× SSC/0.1% SDS at room temperature, 2 times for 15 min with 2× SSC/0.1% SDS at room temperature, and 2 times for 15 min with 0.2× SSC/0.1% SDS at 55°C. Before reprobing, the membranes were stripped for 30 min at 65°C in 0.1 M Tris-HCl, pH 8.0, 1% SDS, and 50% formamide. Suitable film exposures were carried out on preflashed Hyperfilm-MP (Amersham Corp.), or when higher sensitivity was required, intensifying screens were used at −70°C. Densitometric scans of the autoradiograms were obtained with a Shimadzu CS-930 scanner at a wavelength of 540 nm.

RT-PCR Analysis of STX, PST, and NCAM mRNA Expression

Reverse transcriptase (RT)-PCR was carried out in a single reaction using the SuperScript One-Step system (GIBCO BRL, Gaithersburg, MD) that uses the SuperScript II reverse transcriptase and the Taq DNA polymerase. The sequence of the specific primer pairs (5′ end/3′ end) used for both the reverse transcription and the polymerase chain reaction, were for PST: 5′-CATGCGTTCCGTCAGGAAGAGG-3′/5′-GAGGATCCAAATGCTCATTGCACAACT-3′; for STX: 5′-GCTGGATGCTGGCCGCGCTCACGCTG-3′/5′-GAGGTTCTGGGACACATTCATGGTGC-3′; for NCAM: 5′-AGATCTAGAGGTTTATGTGATAGCTGA-3′/5′-TTCATGCATAGTGTCAGAGTTAGTGGT-3′; and for β-actin: 5′-CTCCGGTATGTGCAAGGC-3′/5′-CTCGGGGCACCTGAACCT-3′. These primer pairs gave the expected product size of 604 bp for PST, 398 bp for STX, 507 bp for NCAM, and 738 bp for β-actin. To perform semiquantitative RT-PCR analysis of STX, PST and NCAM in the developing chick CG, the exponential phase for PCR amplification was determined empirically by varying the number of cycles from 13 to 24 using 500 ng of total RNA from St 34 chick brain as template. The amount of RNA was then varied from 10 ng to 1 μg at 20 cycles for STX, PST, and NCAM, and 16 cycles for β-actin. Approximately 100 ng of total RNA isolated from CG at embryonic stage 34, 37, and 40 was used for each reaction, and the exact amount of RNA used was normalized in preliminary experiments to give an equal β-actin PCR products at each developmental stage. The PCR products were electrophoresed in 1.5% agarose gels, alkaline transferred to positively charged nylon membranes (Hybond N^+; Amersham Corp.) and hybridized in QuikHyb solution (Stratagene) with ^{32}P-labeled probe at 68°C for 2 h, washed in 2× SSC/0.1% SDS at room temperature and in 0.2× SSC/0.1% SDS at 42°C for 30 min. Suitable exposures were obtained using preflashed Hyperfilm-MP (Amersham Corp.) and the autoradiograms were scanned densitometrically using a Shimadzu CS-930 scanner at 540 nm. For Southern blot analysis of the PCR products, cDNA fragments of each transcript were obtained by RT-PCR, subcloned into either pGEM or pCRII TOPO plasmids, and their identity analyzed by sequencing. The inserts were isolated by enzyme digestion, gel purified, and ^{32}P-labeled using the rediprime kit (Amersham Corp.).

Embryos and Materials

White Leghorn fertile chicken eggs were obtained from a local poultry farm and incubated in a forced-draft incubator at 37°C under a humidified atmosphere, until the desired developmental stage according to Hamburger and Hamilton (13) was reached. Treatment with endoglycosidase H (endo H) of *streptomyces plicatus* from recombinant *Escherichia coli* (Boehringer Mannheim Corp., Indianapolis, IN) was carried out at a final concentration of 8 μg/ml (320 mU/ml) for 4 h at 37°C in 0.15 M sodium citrate buffer, pH 5.5, containing 0.1% SDS, 0.01% β-mercaptoethanol, 2 mM EDTA, 250 μg/ml BSA, 1 mM PMSF, 4 U/liter aprotinin (14). Endoneuraminidase N (endo N) was prepared as described (12) and samples treated for 1 h at 37°C. NCAM was purified from E15 embryonic chick brains by immunoaffinity chromatography using Sepharose conjugated with an anti-chicken NCAM monoclonal antibody (5e) that recognizes all NCAM isoforms (48). Human PST cDNA was a kind gift of Dr. Minoru Fukuda (Burnham Institute, La Jolla, CA). Human STX cDNA was provided by Dr. Paul Scheidegger (University of Michigan, Ann Arbor, MI) and Dr. John Lowe (University of Michigan, Ann Arbor, MI). Ionomycin, A23187, thapsigargin, PMA, staurosporine, forskolin, verapamil, bay

K8644, and BAPTA/AM were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Swainsonine was purchased from Boehringer Mannheim Corp. Tissue culture medium was obtained from GIBCO BRL.

Pulse–Chase Experiments

Chicken tectum from embryonic St 34-36 was chopped into tissue cubes of ~300 μm side by passing the tissue through a 297-μm opening nylon mesh. The tissue cubes were incubated in methionine-free culture medium (Dulbecco's modified Eagles medium containing 50 mM Hepes, pH 7.2) for 30 min at 37°C with constant shaking. The tissue was then pulsed in the same medium containing 250 μCi/ml of L[^{35}S]methionine (Amersham, specific activity 1,000 Ci/mmol) at 37°C for varying times with constant agitation, washed with ice-cold culture medium containing 3 mM nonradioactive methionine, and chased for 2 h in the presence of 3 mM nonradioactive methionine at 37°C. Homogenates were done by sonication in 10 vol of extraction buffer (25 mM Hepes, pH 7.2, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM PMSF, and 4.6 U/liter aprotinin) and clarified at 14,000 rpm for 15 min at 4°C in a microcentrifuge. NCAM was immunoprecipitated overnight at 4°C with the 5e monoclonal antibody conjugated to Sepharose beads (CNBr-activated; Pharmacia Biotechnology Inc.). The beads were washed 5 times in 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1% NP-40, 1 mM EDTA; 5 times in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.1% SDS (39), and resuspended in extraction buffer. The sample was divided into equal parts, endo N, which specifically degrades α2,8-linked polysialic acid (12) was added to one of them, and both aliquots incubated for 1 h at 37°C. The immunobeads were resuspended in sample buffer (22) and incubated for 20 min at 70°C to denature the protein. This temperature was chosen instead of boiling the samples to prevent the hydrolysis of polysialic acid. The samples were electrophoresed in a 7.0% SDS–polyacrylamide gel, the gel was fixed for 30 min in propanol/water/acetic acid (25:65:10), incubated in Amplify (Amersham Corp.) for 30 min, and dried. Fluorographs were obtained by suitable exposure of preflashed Hyperfilm-MP (Amersham Corp.) at −70°C with intensifying screens.

Polysialyltransferase Assay

The polysialyltransferase activity was assessed by the method described (32). A polysialyltransferase-containing enriched fraction was prepared from St 34-36 tectum. The tissue was homogenized with Teflon-glass in 10 vol of homogenization buffer (0.32 M sucrose, 10 mM MES, pH 6.0, 1 mM EDTA, 1 mM PMSF, and 4.6 U/liter aprotinin), centrifuged at 1,000 *g* for 10 min, and the supernatant further centrifuged at 17,000 *g* for 30 min. The pellet was resuspended in 2 vol of extraction buffer (1% NP-40, 10 mM MES pH 6.0, 1 mM EDTA, 1 mM PMSF, and 4.6 U/liter aprotinin), stirred for 30 min, and then centrifuged at 17,000 *g* for 30 min. Glycerol was added to the supernatant at a final concentration of 30% and used as the enzyme source for the assay. The reaction solution contained the following components in a total vol of 50 μl: 20 ml of the tectum extract, 10 μg of purified E14 chick brain NCAM, 10 μM CMP-[^{14}C]-*N*-acetylneuraminic acid (286 mCi/mmol; Amersham Corp.), 100 mM MES buffer, pH 6.0, 20 mM $MnCl_2$, and 2.5 mM ATP. After incubation for 3 h at 37°C, the reaction was terminated by addition of EDTA at a final concentration of 50 mM. To determine if the incorporation of [^{14}C]-*N*-acetylneuraminic acid into NCAM was in the form of an α2,8-linked polysialic acid chain, the sample was divided into equal parts, endo N was added to one of them, and both aliquots incubated for 1 h at 37°C. The samples were then spotted onto 2.5-cm Whatmann DE81 paper discs, rinsed 3 times in 20 mM MES buffer, pH 6.0, 250 mM NaCl, once with 95% ethanol, air-dried, and the radioactivity in the discs was measured in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The difference in the amount of counts between control samples and endo N–treated samples was taken as a measure of the amount of sialic acid that had been enzymatically incorporated into α2,8-linked polysialic acid chains. Specific activity was calculated with respect to total protein as determined by the BCA assay (Pierce Chemical Co., Rockford, IL).

Results

Developmental Expression of STX and PST mRNA in the Chick Ciliary Ganglion

The detectability and size of the chicken STX and PST mRNAs was first analyzed by Northern blot analysis using St 34 chicken brain RNA. 20 μg of total RNA were electrophoresed and hybridized with either chicken PST or hSTX probes. Chicken PST mRNA was found as a unique band at 6.5 kb which is similar in size to the human (30), hamster (8), and mouse (49) PST mRNA. STX mRNA was detected as a single band at 3.6 kb that is smaller than the 5.0–5.7-kb mRNAs identified in human (18), rat (25), and mouse (19; Fig. 1 *B*). The relative amounts of expression of STX and PST was compared by measuring the optical density of the bands of equally exposed films. It was found that STX was seven times more abundant than PST in the St 34 chick brain.

To evaluate the pattern of expression of STX and PST mRNA in a developmental context, the chick ciliary ganglion was chosen because it is composed of only two populations of motorneurons which are well synchronized during the process of target innervation. The CG has a very precise pattern of regulation of the expression of PSA (5), making feasible the comparison between the expression of mRNA levels of the enzymes and the synthesis and expression of PSA. STX, PST, and NCAM mRNA were studied by northern blot analysis using total RNA (20 μg) isolated from pooled ganglia from St 33, 37, and 40-41 chicken embryos (Fig. 1 *A*).

By Northern blot analysis STX mRNA was readily detected during the entire period of CG embryonic development. These results show that the expression of STX mRNA remains fairly constant from St 33 to St 41, with NCAM demonstrating a small decrease during this period. Although PST mRNA was not detected in the CG by this method at any of the embryonic developmental stages studied, its presence could be revealed by PCR analysis (Fig. 2 *A*).

To evaluate further the regulation of PST and STX, the relative amounts of these transcripts at different developmental stages were determined by quantitative RT-PCR analysis. The number of cycles and amount of template

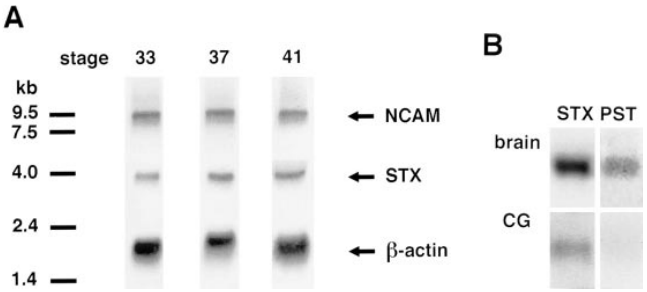

Figure 1. Northern blot analysis of the chicken STX, PST, and NCAM mRNA in CG and brain. (*A*) The developmental changes of STX and NCAM mRNA expression in the chick CG was analyzed by northern blot. Approximately 20 μg of total RNA from the indicated developmental stage were electrophoresed and hybridized sequentially with probes for STX, NCAM, and β-actin mRNAs. (*B*) The presence of PST and STX mRNA in chick brain and CG as analyzed by Northern blots. Approximately 20 μg of total RNA from embryonic stage 34 chicken brain or CG were electrophoresed side by side, and hybridized with probes for PST or for STX. Although PST mRNA was detected in brain, no signal was obtained in CG.

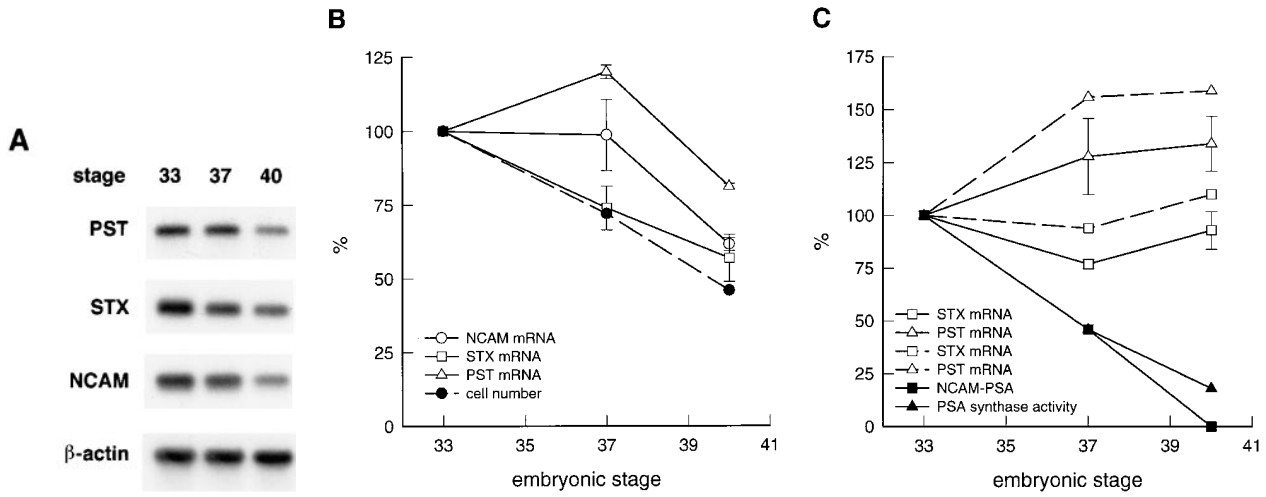

Figure 2. PCR analysis of the developmental expression of STX, PST, and NCAM mRNAs in the chick CG. (*A*) Similar amounts of total RNA (100 ng) were isolated from embryonic stage 34, 37, and 40 chick CG. Specific cDNAs were amplified by PCR using primers for STX, PST, NCAM, and β-actin, with 20 cycles for STX, PST, and NCAM, and 16 cycles for β-actin. The amplified PCR cDNAs were electrophoresed and hybridized with the appropriate probes. As described in Materials and Methods, these conditions had been established as being in the linear range for relative quantitation of the mRNA. (*B*) Quantitative analysis of the PCR products shown in *A*. The autoradiograms were scanned densitometrically and the size of each peak was normalized to β-actin. The plot shows the relative amounts of PST (*open triangles*), STX (*open squares*), and NCAM (*open circles*) transcripts, with the youngest developmental stage studied (St 33) taken as 100%. Each point represents the mean ± SE of three independent reactions. The dashed line represents the developmental changes in neuronal number in the CG caused by naturally occurring cell death (23). (*C*) Normalization of mRNA levels in *B* to cell number and NCAM transcripts. Transcripts for STX (*open squares*) and PST(*open triangles*) were normalized to cell number (*dashed lines*) or the relative levels of NCAM mRNA (*solid lines*). For comparison, the levels of PSA synthase activity (*filled diamonds*) and NCAM *polysialylation (filled triangles*) detected at each developmental stage (5) is also shown. Note that the disappearance of both enzymatic activity and NCAM polysialylation during this developmental period is not accompanied by a corresponding decrease in the levels of STX and PST transcripts.

RNA used was adjusted in preliminary experiments to be within the linear range of amplification. Unique PCR products of the expected size were obtained as analyzed by Southern blots with the selected primers (see methods, Fig. 2 *A*). To quantitate the relative amount of expression of the chicken STX, PST, and NCAM transcript during development, the amounts of the STX, PST, and NCAM product were normalized to the amount of β-actin product detected at each stage. As shown in Fig. 2 *B*, the amount of STX product relative to β-actin declined to ~60% of the levels detected at St 33, whereas PST product decreased to 80%. However, the vast majority of PSA on CG cells is associated with neurons (Brusés, J., unpublished observations), and there is a 50% loss of neurons (but not in overall cell number) in the ganglia over this period, as caused by naturally occurring cell death (23). Therefore, it is appropriate to normalize the levels of PCR product to the number of neurons rather than the amount of β-actin (Fig. 2 *C*). Another normalization strategy would be to evaluate the ratio of transferase to its substrate, that is the level of the NCAM transcript. The result in this case is very similar, as there is a decrease in NCAM product of 61% over this developmental period (Fig. 2 *C*). Together, these two evaluations of PST and STX levels indicate that the level of transcripts for both enzymes does not decrease in parallel with the developmental loss of enzymatic activity or NCAM polysialylation as measured in previous studies (5; Fig. 2 *C*). These results suggested that differential transcription of transferase genes does not underlie changes in PSA in this system, and led to a pharmacological investigation of mechanisms that might directly regulate the PSA biosynthetic process.

Effect of Pharmacological Perturbation of Intracellular Signaling Pathways on NCAM Polysialylation

To investigate the possible role of second messenger pathways in the regulation of NCAM polysialylation, the effect of pharmacological agents known to alter these pathways were tested for their effect on the extent of polysialylation of newly synthesized NCAM in pulse–chase experiments. For this analysis larger amounts of tissue were needed, and the study primarily used St 34-36 embryonic tectum, which produces NCAM with a similarly high level of polysialylation as found in the CG. Small cubes of tectum tissue were incubated for 2 h at 37°C in the presence of the different drugs before a 1-h pulse with [^{35}S]methionine and a 2-h chase. The immunoisolated NCAM was run on SDS-PAGE and fluorographed (Fig. 3). No significant changes in the amount of NCAM polysialylation were produced by agents that (*a*) activate or inactivate protein kinases, such as PMA (1 μM) or staurosporine (10 μM), respectively, (*b*) increase cAMP such as forskolin (1 μM), or (*c*) block or activate calcium channels such as verapamil (10 μM) or bay K8644 (1 μM), respectively. Despite the absence of effects with drugs aimed at calcium channels, a marked decrease in PSA synthesis was observed in tissues treated with the calcium ionophore A23187 (1 μM), indicating that calcium does have a direct role in polysialylation of NCAM. Results similar to those shown for A23187 were

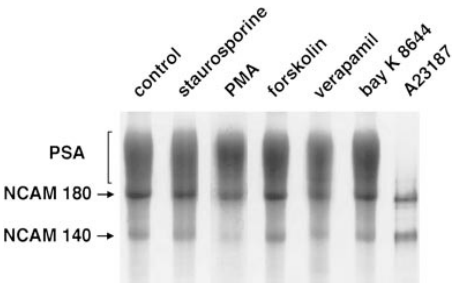

Figure 3. Pharmacological studies of signal transduction pathways on NCAM polysialylation. Fluorograph of the immunoisolated NCAM from tectum treated with the indicated drugs. Tissue fragments (300-μm cubes) from St 34-36 chicken tectum were incubated with 10 μM staurosporine, 1 μM PMA, 10 μM forskolin, 10 μM verapamil, 1 μM bay K 8644, or 1 μM A23187 for 2 h at 37°C, pulse labeled for 1 h with [^{35}S]methionine, and chased for 2 h. NCAM polysialylation was not affected by most of the drug treatments; however, a total block of PSA synthesis was observed with the calcium ionophore A23187.

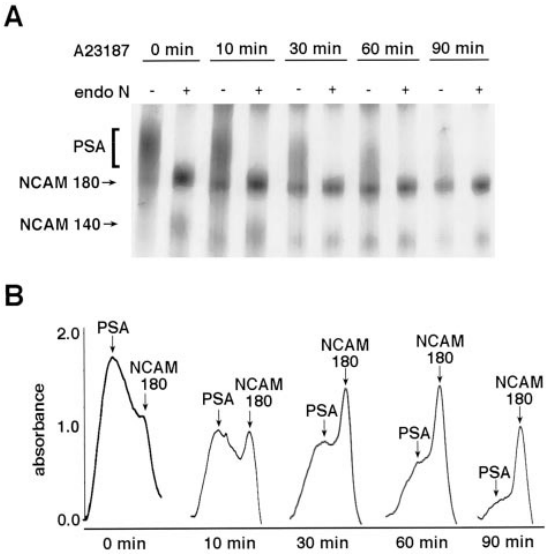

Figure 4. Time course analysis of the effect of calcium ionophore A23187 on NCAM polysialylation. (*A*) Tissue cubes from St 34-36 chicken tectum were incubated with A23187 (1 μM) at 37°C for the indicated time, pulse labeled for 1 h with [^{35}S]methionine and chased for 2 h. After homogenization in extraction buffer, NCAM was immunoprecipitated, endo N–treated (+) and untreated (−) samples were run on SDS-PAGE, and the gel was analyzed by fluorography. (*B*) Densitometric scans of the untreated samples shown in *A*. The peaks corresponding to PSA and nonpolysialylated NCAM are indicated by arrows. Note that NCAM polysialylation starts to decrease after 10 min of treatment with A23187 and has completely ceased after 90 min of incubation.

also obtained with another calcium ionophore, ionomycin (10 μM; data not shown). To confirm the relevance of the most significant pharmacological studies to the CG, dose–response analyses of A23187, forskolin, and phorbol ester were carried out with 15 ganglia per assay, and the results obtained were the same as above with tectum, namely a complete block of polysialylation at ionophore concentrations of 1 μM or higher, and no effect with forskolin and phorbol ester (data not shown).

As a next step, a time course analysis of the A23187 effect on polysialylation was carried out in which tectal tissue (St 34-36) was treated with the drug (1 μM) for varying periods before the [^{35}S]methionine pulse (Fig. 4 *A*). In the untreated sample (0 min), almost all of the newly synthesized NCAM was polysialylated by the end of the chase, that is, it migrated as a polydisperse material above the NCAM-180kD polypeptide band. The presence of polysialylated NCAM in this sample was corroborated by treatment with endo N, which converted the disperse, low mobility material into more distinct NCAM polypeptide bands at 180 and 140 kD. Remarkably, as little as 10 min of incubation with A23187 already induced a decrease in the amount of polysialylation of the NCAM, and this effect gradually increased to a complete blockade of PSA synthesis over a period of 90 min. These results are represented quantitatively in Fig. 4 *B*, in terms of a densitometric scan of the samples (without endo N treatment) at each time point. Note the disappearance of the material running at the position of PSA and the intensification of the NCAM 180-kD band as a function of the length of A23187 treatment.

The Role of Calcium in NCAM Glycosylation

The processing of complex carbohydrates such as PSA involves several successive enzymatic steps that take place in the ER and Golgi (11, 37). Some of these enzymes have been shown to be sensitive to the calcium concentration in their environment, and thus can be affected by calcium ionophores that alter calcium levels in the Golgi and/or ER (21, 40). To evaluate whether the inhibition of NCAM polysialylation caused by calcium ionophores occurred at the level of PSA synthesis, or was a consequence of an interruption in the processing of the carbohydrate core before exit from the ER, control samples and samples treated with A23187 were treated with endo H before the electrophoretic analysis. Endo H cleaves high mannose carbohydrates from proteins and its substrate must contain at least three mannose residues (26). Therefore, the acquisition of resistance to endo H can be used as an indicator that the protein has exited the ER and reached the Golgi. This cleavage can be detected by an increase in the electrophoretic mobility of the glycopolypeptide. As shown in Fig. 5 *A*, both control samples and samples treated with A23187 were resistant to treatment with endo H, that is, no shift in the peptide mobility was detected.

As a control for the efficacy of endo H enzymatic treatment, tectal tissue was incubated for 1 h before the initiation of the pulse with swainsonine (100 μM), a mannosidase II inhibitor that causes an arrest in the processing of high mannose carbohydrate cores (46). Under these conditions a shift in the mobility of the polypeptide was caused by endo H treatment (Fig. 5 *A, arrowhead*). Moreover, treatment with A23187 did not prevent the effect with swainsonine, indicating that it did not affect the processing of the carbohydrate core before reaching the mannosidase II step. These results indicate that NCAM can still exit the ER and reach the Golgi in the presence of the calcium ionophore, and thus suggest that changes in intracellular calcium directly affect the polysialylation of NCAM without

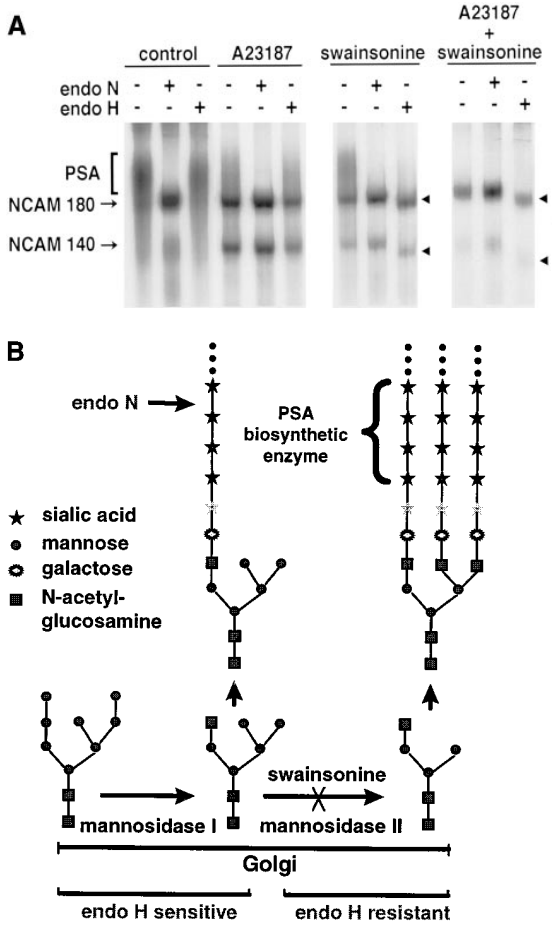

Figure 5. Processing of newly synthesized NCAM in the presence of A23187. (*A*) Stage 34-36 tectum tissue cubes were incubated for 2 h at 37°C in the presence or absence of A23187 (1 μM), swainsonine (100 μM), or both, pulse labeled with [^{35}S]methionine for 1 h and chased for 2 h. After NCAM was immunoprecipitated, each sample was split in three parts, one fraction remained untreated (−) and the others were treated with endo N (+) or endo H (+) for 4 h at 37°C before being run on a 7% SDS-PAGE. In the gel fluorograph NCAM 180- and 140-kD isoforms are indicated by arrows and the polydisperse material running above 200 kD is indicated as PSA. As a positive control for the endo H treatment, the tissue was treated with swainsonine. Under these conditions, a shift in the mobility of the NCAM 180 and 140 isoforms was caused by the endo H treatment (*arrowheads*). Moreover, the incubation of the tissue with A23187 (1 μM) and swainsonine (100 μM) caused the same effect indicating that in the presence of A23187, NCAM is able to complete processing through the mannosidase II step. (*B*) Diagram representing the processing of the N-linked carbohydrate core showing the species that are sensitive and insensitive to endo H in the Golgi.

perturbation of previous steps (11) in the glycosylation process (Fig. 5 *B*).

NCAM Polysialylation Is Affected by Calcium Changes within an Intracellular Compartment

Calcium ionophores such as A23187 or ionomycin have the ability to promote passive transport of calcium across biological membranes in the presence of a concentration gradient (24, 35, 36). In cells exposed to a physiological extracellular calcium concentration (2 mM), the ionophores induce a rapid increase in the cytosolic calcium concentration by transporting ions across the plasma membrane. In addition to this action, calcium ionophores transport calcium across membranes within the cell. This flow is from intracellular calcium stores to the cytosol, as the calcium concentration in intracellular membrane compartments (IC) is higher than in the cytosol. Therefore, calcium ionophores produce two distinct effects inside the cell, a rise in the cytoplasmic calcium concentration, and a depletion of IC.

Both effects of ionophores could potentially affect NCAM polysialylation: the decrease in calcium concentration in the Golgi-associated IC could directly affect the processing and the polysialylation of NCAM, or a rise in cytosolic calcium could activate a second messenger pathway that in turn regulates the polysialylation of NCAM. To examine if IC calcium was involved in the observed arrest of PSA synthesis caused by calcium ionophores, tectal tissue was treated with thapsigargin (1 μM), an inhibitor of the intracellular SERCA (Ca^{2+}-ATP) pump that reloads the IC with calcium (4, 44). As the IC have a constitutive leak of calcium, the inhibition of the calcium pump causes a rapid fall in the concentration of calcium in the IC. After 30, 60, or 90 min of incubation with thapsigargin, polysialylation in the tissue was subjected to a pulse–chase and electrophoretic analysis as described above. The results shown in Fig. 6 indicate a sharp reduction in the amount of PSA added to the NCAM, comparable to that seen with the ionophores, as a consequence of each treatment with thapsigargin. This change is evidenced by a large decrease in the amount of low mobility, polysialylated material. Thus these observations are consistent with a direct effect of calcium on the IC-contained enzymatic process that synthesizes PSA.

Nevertheless, the decrease in the calcium concentration in the IC caused by thapsigargin also results in a transient rise in the cytosolic Ca^{2+} concentration, and therefore the possibility remains that the observed effect of this drug on PSA synthesis is a consequence of a rise in cytosolic Ca^{2+}. To address this issue, tectal tissue was incubated in the presence of BAPTA/AM (75 μM) for 45 min before the addition of thapsigargin or A23187. BAPTA/AM penetrates the cell and then is cleaved to a form that causes chelation of cytosolic Ca^{2+} (27, 45). The coadministration of BAPTA plus thapsigargin or A23187 thus prevents the rise in cytosolic Ca^{2+} concentration induced by either thapsigargin or A23187. As is shown in Fig. 7, treatment with BAPTA not only failed to prevent the effect of thapsigargin or A23187 on NCAM polysialylation, but was also by itself able to strongly suppress the synthesis of PSA. The latter observation is again consistent with a requirement of Ca^{2+} in the IC for polysialylation, reflecting the ability of BAPTA to chelate cytosolic calcium and thereby result in the gradual loss by leakage of Ca^{2+} from the IC.

Finally, to rule out a direct effect of cytosolic calcium on polysialylation and confirm the role of the IC, studies were carried out in which Ca^{2+} was removed from the extracellular medium and the tissue was treated with A23187. Under these conditions the effect of the calcium ionophore is primarily on the IC rather than the cytosol, as there is no gradient concentration between the extracellular fluid and

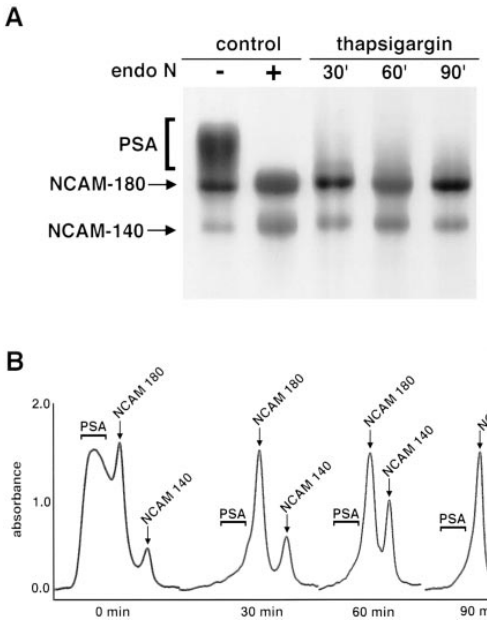

Figure 6. Effect of thapsigargin on NCAM polysialylation. (*A*) Stage 34-36 chick tectum was treated with thapsigargin (1 μM) for the indicated time, NCAM was metabolic labeled with [^{35}S]methionine for 1 h, chased for 2 h, immunopurified, and run on SDS-PAGE. The figure shows the fluorograph of this gel. Thapsigargin (1 μM) caused a marked decrease in the size of the polydisperse PSA-containing material running above 200 kD. (*B*) Densitometric scans of the fluorograph shown in *A*. Zero min represents the scan of the control (endo N untreated) sample. The peaks corresponding to PSA, NCAM-180kD, and NCAM-140kD are indicated.

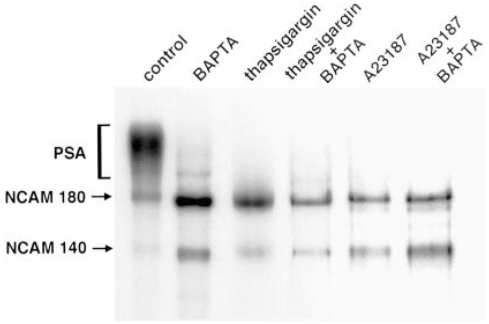

Figure 7. Effect of thapsigargin, A23187, and BAPTA/AM on PSA synthesis. Samples of tectal tissue (St 34-36) were pretreated for 45 min with BAPTA/AM (75 μM) before a 90-min treatment with thapsigargin (1 μM) or A23187 (1 μM). The tissue was pulse labeled with [^{35}S]methionine for 1 h and chased for 2 h. NCAM was immunoisolated, analyzed by SDS-PAGE and the gel fluorographed. Chelation of the intracellular calcium with BAPTA completely prevented polysialylation of NCAM, and the inhibition of PSA synthesis caused by thapsigargin or A23187 was not modified by the addition of BAPTA, indicating that the effect of the calcium ionophore is a consequence of the depletion of calcium of the intracellular stores.

the cytosol. As shown in Fig. 8, the effect of A23187 was not altered by the removal of extracellular Ca^{2+}. Moreover, in this protocol, exposure of the tissue with BAPTA/AM before the treatment with A23187 did not prevent the suppression of NCAM polysialylation caused by the calcium ionophore (Fig. 7). These experimental results provide strong additional support for the hypothesis that the enzymatic process of NCAM polysialylation is dependent on calcium concentration in the IC.

Effect of Calcium Concentration on PSA Biosynthetic Enzyme Activity In Vitro

If NCAM polysialylation is regulated by calcium concentration at the level and site of PSA synthesis, a logical target for the regulation is the polysialyltransferase itself. In fact, our previous studies of polysialyltransferase activity revealed a requirement for divalent cations (32). To test if this enzymatic activity is sensitive to relevant ranges of calcium ion concentration, polysialyltransferase activity was assayed in St 34-36 chick tectum by measuring the incorporation of [^{14}C]CMP sialic acid into α2,8-linked PSA chains on exogenous purified NCAM. As is shown in Fig. 9 a marked increase in PST activity was observed between 0.1 and 1 mM calcium concentration, and the activity decreased when the concentration was >10 mM. This range of optimal calcium concentration is similar to that expected in an intracellular compartment (6, 10). Therefore, it is feasible that changes in the calcium concentration in an intracellular compartment could directly affect the transferase and thus the overall process of NCAM polysialylation.

Discussion

Previous work on the development of ciliary ganglion motorneurons indicated that the up- and downregulation of PSA expression associated with the period of target innervation and synaptogenesis is regulated at the level of PSA enzymatic biosynthesis (5), presumably involving one or more of the recently identified polysialyltransferases PST and STX (2, 8, 20, 25, 30). The purpose of this study was to identify cellular mechanisms by which that regulation occurs, and to identify signaling pathways that might couple the development program with PSA biosynthetic machinery. It was found that that regulation of PSA in the CG appears to reflect a nontranscriptional process, in that it is not obviously reflected in the levels of either PST or STX mRNA. Instead, levels of PSA expression may be related to the independent observations of a calcium dependence of polysialyltransferase activity and a sensitivity of PSA synthesis to calcium levels within an intracellular compartment. The following discussion considers these different parameters first in terms of their individual implications, and then with respect to their possible relationships within the overall process of PSA regulation.

The abundance of STX message in the CG relative to that of PST, suggests that STX is the primary PSA synthetic enzyme in this tissue during the developmental period being considered. However, the efficiency of these transferases on polysialylation of NCAM has not been evaluated in this system, and thus it was important to study transcriptional levels for both enzymes. Fortunately, the results obtained were similar for STX and PST, in that the disappearance of both enzymatic activity and NCAM poly-

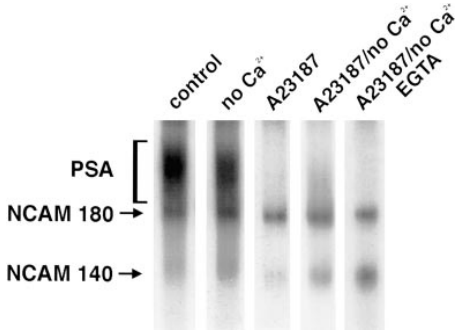

Figure 8. A23187 treatment in the absence of extracellular calcium. Tectal tissue (St 34-36) was incubated for 1 h at 37°C under the indicated conditions: in the presence 2 mM Ca^{2+} (*control*); in calcium-free medium (*no* Ca^{2+}); in the presence of 1 μM A23187 and 2 mM calcium (*A23187*), in the presence of A23187 (1 μM) and in the absence of calcium (*A23187/no* Ca^{2+}), and in the presence of A23187 (1 μM) in the absence of calcium and with 3 mM EGTA (*A23187/no* Ca^{2+} *EGTA*). This was followed by 1-h pulse with [^{35}S]methionine under the same conditions and 2 h chase in the presence of Ca^{2+}. The removal of calcium from the incubation medium did not affect NCAM polysialylation, which was inhibited by the calcium ionophore A23187. The removal of calcium and the addition of EGTA did not modify the effect of A23187, indicating that extracellular calcium is not required for the effect of A23187 on PSA synthesis.

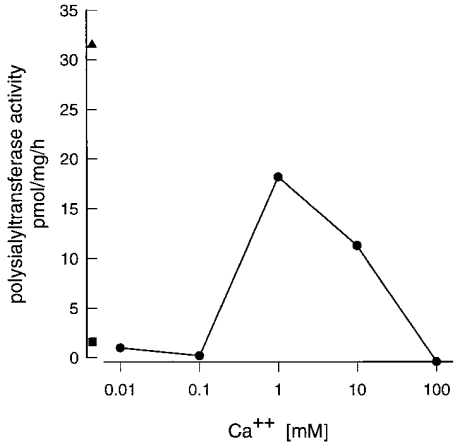

Figure 9. Effect of calcium on polysialyltransferase activity. The effect of calcium concentration on polysialyltransferase activity was investigated using NP-40 extracted membrane proteins from St 34-36 chicken tectum. The PST assay was carried out as described in Materials and Methods. Filled circles indicate the enzymatic activity obtained at the indicated calcium concentration. As control assays, the activity found in the presence of 1 mM EDTA (*square*) and 20 mM Mn^{2+} (*triangle*) are indicated.

sialylation previously found during the period of synaptogenesis (5) was not accompanied by a corresponding decrease in the levels of either the STX or PST transcripts.

As yet, antibodies for detecting these transferase proteins are not available, and thus a possible regulation at the level of translation has not been assessed. On the other hand, pharmacological and biochemical analyses of PSA synthesis have presented two potentially related avenues for nontranscriptional regulation involving intracellular calcium levels, one reflecting the calcium dependence of the cellular biosynthesis of PSA and the other a similar dependence for the transferase activity itself (Fig. 10).

The findings for cellular biosynthesis of PSA are remarkable with respect to the specificity of the pharmacology: (*a*) no other perturbation of major second messenger pathways had any effect of PSA synthesis, indicating that polysialylation is not easily perturbed by a wide spectrum of factors; (*b*) only the final step of PSA synthesis, involving addition of the PSA polymer to the carbohydrate core, was found to be calcium sensitive; and (*c*) the action of thapsigargin indicates a specialized pool of intracellular calcium. However, there is presently no direct evidence that the enzyme actually resides in that particular compartment, except that the likely sites, either late or post-Golgi membrane compartments, would be expected to have thapsigargin-sensitive calcium pumps. In any case, the next step in this analysis will be to localize the enzymes within the cell by immunocytochemistry and then measure calcium levels in these precise compartments.

The finding that transferase activity itself is sensitive to calcium provides a plausible mechanism to account for the pharmacological effects obtained at the cellular level. The in vitro dependence of polysialyltransferase activity on calcium exhibits a sharp differential over concentration ranges (0.1–1 mM) that are physiologically reasonable for a thapsigargin-sensitive compartment. Therefore, it is possible that a physiologically relevant regulation of the concentration of calcium in particular intracellular compartments has the effect of altering the activity of the polysialyltransferase and thus the level of PSA synthesis (Fig. 10). However, it should be noted that the site of calcium dependence during polysialylation has not been determined precisely. The enzyme itself could require calcium as a cofactor, but it is also possible that calcium affects essential properties of the substrate.

Despite the feasibility and simplicity of this proposal, it is also clear that other modes for regulation of PSA expression are available to the cell. In addition to transcriptional, and nontranscriptional control of polysialyltransferase(s), PSA expression at the cell surface can in principle also be affected by differential delivery of presynthesized PSA–NCAM to the cell surface. Intracellular granules containing PSA have been described in pancreatic cells and the calcium-dependent fusion of these granules with the plasma membrane can be induced by depolarization of the membrane (17). Moreover, in oligodendrocyte precursors, NMDA can induce a glutamate receptor-dependent influx of calcium that is proposed to enhance transport of PSA–NCAM to the cell surface (47). A second possible regulatory mechanism could involve changes in the structure of the NCAM substrate to which PSA is attached. However, there is at present no evidence for NCAM isoform effects, in that all major NCAM polypeptides can be polysialylated, albeit with some tissue or cell-type specificity. Moreover, the VASE exon, which appears as PSA is downregulated in some neural tissues, does not appear to affect polysialylation in vitro (31) and in fact is not expressed in the developing CG at all (5). A third possible mechanism would be differential endocytosis of PSA–NCAM, as has been proposed for the apparent

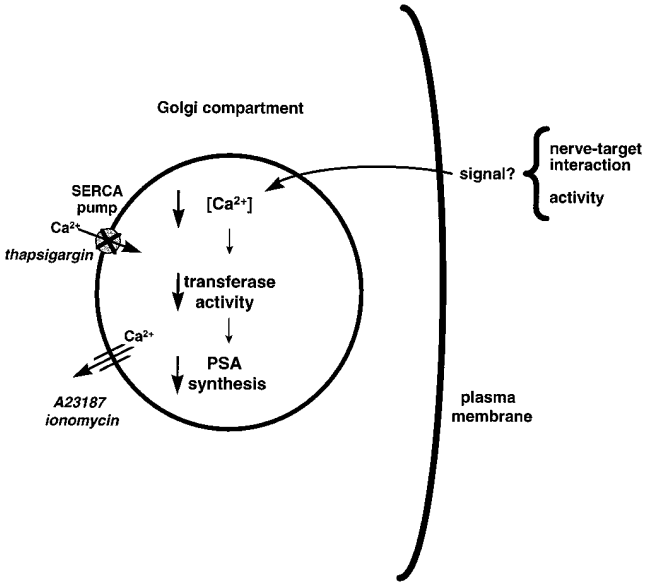

Figure 10. Schematic summary of results and their interpretation at the cellular level. At the left are indicated the primary live cell pharmacological findings of this study, namely that lowering of intracellular stores of Ca^{2+} either by blocking SERCA pumps or introduction of ionophores results in decreased polysialylation. The likely site of this effect, an as yet undefined compartment of the Golgi, contains the transferase that by biochemical analyses has been shown to be sensitive to Ca^{2+} over the 0.1–1 mM range. While the physiological signals that might trigger such changes in Ca^{2+} are not known, two parameters known to affect polysialylation and Ca^{2+} are indicated at right.

NCAM homologue (apCAM) in *Aplysia* (3, 28). In any case, it should be noted that in this study, we are describing an effect of calcium that is clearly distinct from these phenomena, in that we have used a pulse–chase analysis and thus have observed calcium-related perturbations in new synthesis of PSA–NCAM.

Finally, it is interesting to speculate on the overall logistics and strategies associated with regulation of PSA in its biological context. Viewing PSA as a promoter of plasticity in cell interactions, it is reasonable to expect that both extrinsic factors and intrinsic properties of neurons will need to interface with this parameter during the innervation process and synaptogenesis (Fig. 10). One intrinsic property is the spontaneous and characteristic pattern of neuronal activity. Induced patterns of activity are known to affect the expression of surface proteins involved in cell–cell interactions (16), and there is differential expression of PSA on neurons that innervate fast and slow muscle fibers which at least in the adult possess different firing patterns (Rafuse, V., and L. Landmesser, personal communication). With respect to extrinsic factors, it has been observed that contact of motorneurons with muscle cells in vitro causes both a downregulation of polysialyltransferase activity and NCAM polysialylation (5).

A remarkable aspect of these contexts is that they each have a calcium-dependent component and are affected by extracellular signals at a particular time in development. On the one hand, increasing calcium levels in the cytosolic compartment is known to facilitate exocytosis of synaptic vesicles as well as PSA–NCAM. On the other hand, we propose here that high levels of calcium are required in intracellular stores to maintain polysialytransferase activity. Thus one could envision a situation during formation of neuromuscular junctions in which the same or a coordinated set of extracellular signals causes changes in intracellular calcium that enhance exocytosis and also shut down new PSA synthesis. In fact, it has been found that the depolarization-stimulated exocytosis of dense core vesicles involves a rise in intracellular Ca^{2+} that is 46% derived from intracellular compartments (34). It would be naïve and incomplete to assume that regulation of the entire range of PSA-related phenomenology, which includes both neuronal and nonneuronal contexts, is explained solely or even primarily by shuttling of calcium from one cellular compartment to another. Nevertheless, such a working model may serve well as a starting point for investigation of PSA's relationship to overall cell function.

The authors acknowledge the excellent technical assistance of Denice Major.

This work was supported in part by National Institutes of Health grants EY06107, HD18369, and EY11373.

Received for publication 30 June 1997 and in revised form 24 November 1997.

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