Clustering and Mobility of Voltage-dependent Sodium Channels during Myelination

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In myelinated axons, voltage-dependent sodium channels are segregated at high density at nodes of Ranvier (Rosenbluth, 1976; Waxman and Quick, 1978; Black et al., 1990; Elmer et al., 1990), a distribution that is critical for the saltatory conduction of action potentials (Huxley and Stampfli, 1949). The factors that specifically control the organization and immobilization of sodium channels at nodes are unknown. Recently we have reported that segregation of sodium channels on axons is highly dependent on interactions with active Schwann cells and that continuing axon–glial interactions are necessary to maintain sodium channel distribution during differentiation of myelinated nerve (Joe and Angelides, 1992). The specific recruitment of sodium channels at these early stages of myelination and the conspicuous absence of other axon membrane components suggest that the factors governing sodium channel cluster formation show molecular specificity. However, it is not clear whether these clustered sodium channels originate from a redistribution of preexisting diffusely distributed sodium channels. To determine how Schwann cells might regulate sodium channel distribution during myelination we have examined the lateral mobility of fluorescently labeled sodium channels at defined stages of myelination by fluorescence photobleach recovery using tetramethylrhodamine (TmRhd)-labeled Tityus γ , a sodium channel-specific fluorescent toxin. First, to test whether Schwann cells, in addition to modulating sodium channel distribution, affect the mobility of sodium channels, we cultured dorsal root ganglion neurons in the presence or absence of Schwann cells and monitored sodium channel mobility on cell bodies, axon hillocks, and axons. Even in the absence of Schwann cells, ~80% of the sodium channels were immobile on the time scale of the fluorescence photobleach recovery measurement ($D_L \leq 10^{-12}$ cm²/sec), although the remaining fraction of channels are mobile with diffusion coefficients of $5\text{--}13 \times 10^{-11}$ cm²/sec. Most importantly, in contrast to the effects of Schwann cells on altering the distribution of sodium channels, we found that Schwann cells did not alter the rate of lateral mobility or the mobile fraction of axonal sodium channels. Therefore, although sodium channel distribution depends on Schwann

cell contact, immobilization of sodium channels is independent of Schwann cell contact. These effects appear to be specific for sodium channels because 45% of the succinyl concanavalin-A receptors on the axon are mobile, a fraction that decreases to 25% in the presence of Schwann cells later in development. To determine how sodium channels might be immobilized other than by Schwann cell contact, TmRhd-Tityus γ -labeled dorsal root neurons were treated with 0.5% Triton X-100. Under these conditions, 17% and 22% of sodium channels were extracted from cell bodies and axons, respectively, suggesting that the immobile sodium channels are linked to the cytoskeleton. Consideration of several mechanisms shows that the results are consistent with the possibility that sodium channel clusters arise by the lateral diffusion and trapping or selective exclusion of the small mobile and homogeneously distributed population of sodium channels and/or by localized insertion of newly synthesized channels that preferentially appear on the axon at sites of Schwann cell contact. These changes in sodium channel distribution are subsequently stabilized and maintained by interactions with the subaxolemmal cytoskeleton.

[Key words: sodium channel, myelin, lateral mobility, cytoskeleton]

In myelinated nerve, voltage-dependent sodium channels (NaChs) mediate generation of the action potential by a rapid increase in Na⁺ permeability and saltatory propagation of the action potential along the axon (Huxley and Stampfli, 1949, 1951; Hodgkin and Huxley, 1952). Both electrophysiological and freeze-fracture studies have shown that in myelinated nerve the axon membrane is heterogeneous in its distribution of NaChs, with a density of ~1300/ μ m² at nodes and less than ~100/ μ m² at internodes (Rosenbluth, 1976; Ritchie and Rogart, 1977; Kristol et al., 1978).

Some studies indicate that the structural heterogeneity of developing axons is detectable prior to the formation of mature myelin, and that the differentiation of the axon membrane into nodal and internodal regions occurs as an early event in development (Waxman and Foster, 1980; Wiley-Livingston and Ellisman, 1980). Obviously, the laying down of the myelin membrane profoundly changes the conduction properties of the axon membrane (Smith et al., 1982), but there is much less information on how glial cells in early development might specify NaCh distribution on the axon. The extent to which development and maintenance of the node depend upon glial cell contact has remained an important issue in neurobiology and there has been considerable debate as to what role glial cells play in organizing components of the myelinated axon, although two possibilities have been proposed. Freeze fracture and cytochemical

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stains specific for excitable membrane components have suggested that glial cell contact is necessary for the segregation of NaChs at nodes (Rosenbluth, 1979, 1988; Black et al., 1985; Bigbee and Foster, 1989). For example, in hypomyelinating murine mutants, regions resembling nodal specializations were not observed in freeze-fracture replicas (Schnapp et al., 1976; Rosenbluth, 1979; Rosenbluth and Blakemore, 1984). On the other hand, other studies using similar murine mutants have suggested that NaCh specializations develop independently of glial cell contact (Ellisman, 1979).

Recent work from this laboratory has shown that segregation of NaChs on axons is highly dependent on interactions with Schwann cells (SCs), and that continuing axon–glial interactions are necessary to organize and maintain NaCh distribution during differentiation of the myelinated axon (Joe and Angelides, 1992). The clustering of NaChs by SCs is not a feature of all axonal membrane glycoproteins because concanavalin-A (ConA) receptors and the neural cell adhesion molecule (NCAM) are not clustered. In addition, the formation of NaCh clusters appears to require a specific component of metabolically active SCs because neither latex beads nor membranes prepared from SCs induce channel clustering.

In polarized cells, morphogenesis of the polarized distribution of membrane proteins is determined in part by modulating the lateral mobility and preventing free diffusion of proteins in the membrane (Tsuiji and Ohnishi, 1986; Cowan et al., 1987; Myles et al., 1987; Poo and Young, 1990). During differentiation of the myelinated nerve several factors could contribute to the compartmentalization of NaChs on the axon membrane. One possibility is that direct linkage of NaChs to the axonal cytoskeleton controls and limits free diffusion of NaChs, similar to the linkage between band 3 and ankyrin in erythrocytes (Nigg and Cherry, 1980; Tsuiji and Ohnishi, 1986). Indeed, there is evidence from previous studies that shows that NaChs are linked to the spectrin-based cytoskeleton via ankyrin (Srinivasan et al., 1988), and that this linkage could limit the mobility of NaChs in axon membranes (Wood and Angelides, 1989). Furthermore, immunolight and electron microscopy has shown that in fully myelinated axons, ankyrin is localized at nodes of Ranvier (Kordeli et al., 1990). Alternatively, during development a physical barrier could be established in specific regions of the axon by the subaxolemmal cytoskeletal meshwork, by tightly packed integral membrane proteins, and/or by intimate contact established between the axon and SC. NaChs could move freely within the barrier but may not be able to move outside of it. Examples of such compartmental barriers are the PH-20 protein distributed and maintained to the posterior head region of guinea pig sperm cells (Cowan et al., 1987; Myles et al., 1987) and apical or basolateral membrane proteins that are confined to the apical and basolateral membranes, respectively, by tight junctions of epithelial cells (Diamond, 1977). Recently it has been shown that hippocampal neurons may segregate lipids between compartments by a functional barrier (Kobayashi et al., 1992). A third possibility is that cell–cell contact between closely apposing axon and SC membranes and interactions with components of SC adhesion molecules and/or the extracellular matrix could provide a mechanism to immobilize NaChs (Chow and Poo, 1982).

To understand the cellular mechanisms and factors that differentiate the myelinated axon and how NaCh clusters accumulate and are maintained on axons, in this study we have asked whether the interaction of SCs with axons impose con-

straints on NaCh mobility and in this way contribute to NaCh distribution. To do this, we have measured the lateral mobility of NaChs by fluorescence photobleach recovery (FPR) in cultured dorsal root ganglia (DRGs) in the presence and absence of SCs using a fluorescently labeled NaCh-specific toxin, *Tityus serrulatus* toxin γ (Tityus γ). The results reveal that even in the absence of SCs most NaChs are immobile, although a small population of NaChs (20%) are mobile with diffusion coefficients of $\sim 10^{-10}$ cm²/sec. At all developmental stages the mobility appears independent of SCs. We suggest that lateral diffusion of a small population of mobile NaChs toward a local trap provided by SCs is a possible mechanism for the segregation of NaChs mediated by SCs and that SC contact provides either specific and high-affinity sites to NaChs or a selective barrier to NaCh movement but not to other axonal membrane proteins. Alternatively, we have considered that newly synthesized NaChs may be inserted and immobilized at specific contact sites, a distribution that is maintained later by association with the cytoskeleton.

Materials and Methods

Materials. Minimal essential medium (MEM) was purchased from GIBCO; collagen, from Biomedical Technology, dispase and NGF, from Collaborative Research; fetal bovine serum from Hyclone; rhodamine-conjugated succinyl concanavalin-A (sConA) from Vector; Na¹²⁵I, from Amersham; and tetramethyl rhodamine succinimidyl diester (TmRhd), from Molecular Probes. Fluorescein-conjugated goat anti-rabbit immunoglobulin antibody was obtained from Kirkegaard and Perry. All other reagents were from Sigma. The NCAM antibody was the generous gift of Dr. Karl Pfenninger of the University of Colorado School of Medicine, and the Thy 1.1 antibody was from Dr. Kay Fields, Albert Einstein College of Medicine. Tityus toxin γ was a kind gift from Dr. L. Possani of the National University of Mexico.

Cell culture. Dorsal root ganglion (DRG) neurons and SCs were prepared from day 15 embryos of Sprague–Dawley rats following the procedure of Eldridge et al. (1987) with several modifications. DRGs were removed, digested with 50 U/ml of dispase, and triturated. Dissociated cells were plated on collagen/poly-D-lysine-coated #1 glass coverslips in plating media (MEM containing 23 mM HEPES, 5 gm/liter glucose, 2.2 gm/liter sodium bicarbonate, 10% fetal bovine serum, and 50 ng/ml NGF).

For coculture of DRGs/SCs, plating media was changed into defined media [MEM containing 25 mM HEPES, 5 gm/liter glucose, Bottenstein's N2 supplements (Bottenstein and Sato, 1979), and 50 ng/ml of NGF] within 16–24 hr. Cultures were kept continuously in defined media. Pure cultures of DRGs were prepared by growing cells in plating media and pulse treating three times with 10 μ M 5-fluoro-2'-deoxyuridine and 10 μ M uridine for 1 d at days 1, 4, and 7 from plating.

Immunocytochemistry. The distribution of NaChs and glycoproteins on these neuron–SC cocultures was examined. One-week-old cocultures or pure DRGs were fixed with 95% ethanol/5% acetic acid for 30 min at -20°C , washed with PBS several times, and then incubated with 10% goat serum for 10 min. Cells were then incubated with a 1:40 dilution of mouse monoclonal Thy 1.1 antibody (Fields et al., 1978) and a 1:100 dilution of polyclonal NaCh-specific antibody 7493 (Elmer et al., 1990), overnight at room temperature. Thy 1.1 was visualized with fluorescein-conjugated goat anti-mouse secondary antibody and NaChs were visualized with tetramethylrhodamine-conjugated goat secondary anti-rabbit antibody. For NCAM staining, fixed cells were incubated with a 1:200 dilution of monoclonal NCAM antibody and visualized with fluorescein-conjugated goat anti-mouse antibodies. For the analysis of images to calculate NaCh intensity on axons, cells were double labeled with 7493 and monoclonal ankyrin antibody, and then visualized with rhodamine-conjugated anti-rabbit antibodies and fluorescein-conjugated anti-mouse antibodies, respectively.

Preparation and characterization of NaCh-specific fluorescent toxin probe. Tityus toxin γ is a 62 amino acid NaCh-specific polypeptide toxin that has six lysine residues (Barhanin et al., 1982). Tityus toxin γ was labeled with tetramethylrhodamine succinimidyl ester (TmRhd) using methods described previously (Angelides and Nutter, 1983). Reaction mixtures included Tityus γ and TmRhd at 1:1 mole : mole ratio

in order to minimize modification of the multiple lysine residues. Briefly, 100 μg of Tityus γ (13.7 nmol) was incubated with 7.2 μg of TmRhd (13.7 nmol) in 50 μl of 0.1 M sodium bicarbonate buffer, pH 8.5. After 30 min at room temperature, the reaction was stopped by the addition of 10 μl of 1 M glycine. The labeled toxin was purified using a C_{18} reverse-phase HPLC column eluted with a gradient from 0.1% ammonium acetate in water to 0.1% trifluoroacetic acid in acetonitrile. The optical density of the eluent was monitored at 280 nm. The retention time of native toxin was 46 min and fluorescently derivatized toxins eluted at 47–62 min. These peaks were collected from HPLC and dried under vacuum using a speed-vac (Beckman). Fractions were reconstituted with 50 μl of distilled water and then tested for their ability to compete with the ^{125}I -Tityus γ NaCh binding site on rat brain synaptic membranes (Barhanin et al., 1982).

Thirty micrograms of rat brain synaptosomal membranes were incubated on ice with 1.8 μM of native toxin or varying concentrations of TmRhd-labeled toxins from HPLC fractions in a total volume of 200 μl of binding buffer (20 mM Tris, pH 7.4, 40 mM choline chloride, 5.4 mM KCl, 2.8 mM CaCl_2 , 1.3 mM MgSO_4 , 0.1% BSA) for 30 min. Then 4 nM of ^{125}I -Tityus γ was added for 30 min. Bound toxin was separated from free toxin by rapid filtration on GF/B filters. After washing filters twice with 3 ml of ice-cold binding buffer, ^{125}I -toxin remaining on the filter was measured by gamma counting. Fractions that competed for ^{125}I -toxin binding as did the native toxin were pooled and used for lateral mobility experiments.

Fluorescence photobleach recovery measurements. Cells were pre-washed with phenol red-free MEM containing 9 gm/liter glucose and 2.2 gm/liter sodium bicarbonate for 30 min at 37°C. Cells were labeled with 4 nM TmRhd-Tityus γ for 20 min and washed twice with phenol red-free MEM for 5 min each at 37°C. Control FPR and imaging experiments were performed by first labeling cells with 1 μM unlabeled Tityus γ toxin followed by 4 nM TmRhd-Tityus γ toxin. The nonspecific labeling based upon photon counts obtained under identical optical conditions was <10% of the experimental. Measurement of Schwann cells (bodies) showed that labeling of Schwann cells was also <10% of the fluorescence measured from DRG cell bodies or axons.

Lateral diffusion coefficients (D_L) and mobile fractions (f) of labeled NaChs were measured by the spot photobleaching technique using a Zeiss Universal fluorescence microscope and a 63 $\times$, 1.2 NA water immersion lens placed directly into the culture dish (Angelides et al., 1988). The monitoring argon-ion laser (515 nm, 5 μW) was focused through the microscope to a Gaussian radius of 0.45 μm . The illuminated region was bleached by a 50–200 msec laser pulse (5 μW), bleaching ~70% of the fluorescence. The time course of the fluorescence recovery was followed with the attenuated monitoring beam. The lateral diffusion coefficient and mobile fraction were determined by curve-fitting procedures (Axelrod, 1983) that weight the initial phase of the recovery curve. Diffusion coefficients, D_L (in cm^2/sec), were obtained from $D_L = \omega^2/4T_D$, in which ω is the e^{-2} radius of the beam profile and T_D is the half-time for recovery. The lateral mobility of glycoproteins was measured as above after labeling cells with 30 $\mu\text{g}/\text{ml}$ of rhodamine-conjugated sConA. In a small area such as an axon, measurement of D_L and f are underestimated by approximately 15% (Angelides et al., 1988). Data were analyzed by a Student's t test to distinguish the mean value of D_L or f and the significance of these values in the absence or presence of SCs (Zar, 1984). The standard mean errors are the result of repeated measurements on these cultures. Because there are slight variations in mobilities of fluorescent probes in FPR experiments, due to cell-to-cell variations, a large number of measurements using at least six different culture preparations corresponding to each developmental stage are used in statistical analysis to evaluate differences between developmental stages. The standard mean and deviations were derived from each group and reflect repeated measurements rather than errors in the reliability of the fit. A Student's t test was used to evaluate differences between developmental groups. In some experiments, NaCh mobility was measured in the presence of 5 $\mu\text{g}/\text{ml}$ colchicine or 5 $\mu\text{g}/\text{ml}$ cytochalasin B to disrupt microtubules and actin filaments, respectively (Angelides et al., 1988).

Digital fluorescence imaging. DRG and DRG-SC cocultures stained with antibodies were examined through a 63 $\times$, 1.3 NA oil immersion objective on a Zeiss Axiovert microscope and images were captured in digital format through a 514 $\times$ 514 CCD camera interfaced to a Perceptics image processor. All images were captured within the linear range of the CCD camera with no saturation of the pixels. Images were processed and the integrated fluorescence intensities were calculated within the same image to facilitate comparison of the relative densities. To

compare NaCh intensity on axons with or without clusters, the intensity of each pixel was obtained by following a length of the axon membrane (~14 μm) in noncontact regions (i.e., nonclustered), on regions of axons between clusters (i.e., 14 μm), and at clusters. The intensity of ankyrin that is homogeneously distributed served as an internal control. Background fluorescence intensity was taken at adjacent cell-free regions and subtracted from each of these readings to yield the corrected average fluorescence intensity.

Extraction of soluble sodium channels. To determine whether NaChs are associated with the cytoskeleton, soluble membrane proteins were extracted using the method described by Stya and Axelrod (1983) with minor modifications. One-week-old pure DRGs were labeled with TmRhd-Tityus γ and the photon counts (50 msec dwell time) in a 0.45 μm spot, using the monitoring beam of the laser, were measured on the cell body, axon, and axon hillock. The same cells were then treated with 0.5% Triton X-100 in cytoskeletal protein stabilizing buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl_2 , pH 7.5) (Marsh and Letourneau, 1984) for 3 min and washed twice with the same buffer. Photon counts were remeasured in these regions under identical optical conditions and then directly compared.

Results

Characteristics of sensory neurons in the absence and presence of SCs

To delineate the elements that control the distribution and diffusional rate of NaChs during differentiation of the myelinated axon, we used a myelination-competent culture system of sensory neurons and SCs to carry out the FPR measurements. Compared to intact PNS, the locus and extent of neuronal-glial cell interactions are more easily identified and can be continuously monitored in this culture system and the cell-cell interactions can be controlled at specific developmental stages by the appropriate selection of culture conditions and treatments (Eldridge et al., 1989). In addition, the cultures are accessible and permit testing of specific inhibitors or effectors of NaCh distribution and mobility.

To ascertain the degree of axonal-SC interaction and maturation of axons and SCs during development, DRG and SC cultures used for these studies were examined both morphologically and for the expression of cell surface molecules (Fig. 1). In pure DRG cultures, which were prepared by treating cells with 5-fluoro-2'-deoxyuridine to eliminate proliferating fibroblasts and SCs, all SCs were removed within 10 d, leaving a network of bare axons (Fig. 1A). In these DRGs small axons grow out from cell bodies within a day and within 2 or 3 d axons begin to fasciculate, a stage that coincides with the expression of NCAM (Seilheimer and Schachner, 1988). When SCs are added back to DRG cultures, the rapidly proliferating and mobile SCs contact both single axons and bundles within 2 d (Fig. 1B). SCs at this initial stage are distinguished by a more rounded appearance and do not contact axons tightly. Longitudinal and cross-sectional electron micrographs of 1-week-old cocultures show that a single SC extends amorphous processes and can contact several axons. In some fields these SC processes appear to elongate along the axon surface as well as extend into the axon bundle. As shown (Moos et al., 1988; Seilheimer and Schachner, 1988; Linnemann and Bock, 1989; Wood et al., 1990), these early cell-cell interactions established in the first week involve the expression of cell adhesion molecules such as NCAM and L1 that mediate axon-SC membrane adhesion and elongation of SC processes on axons, respectively.

At 3 weeks axons in pure DRG cultures bundle, although many axons remained as single axons (Fig. 1C), and apart from a more extensive axonal network, these axons do not appear significantly different than those at 1 week. In DRG-SC cocul-

Figure 1. Characteristics of DRGs used for photobleaching studies. DRGs were cultured in the absence (*A*, *C*) or presence (*B*, *D*) of SCs at 1 week (*A*, *C*), 3 weeks (*C*, *D*), and after myelination (*E*, *F*) (*N*, node; *hN*, heminode). Phase micrographs were obtained from living cultures. The inset to *E* is an electron micrograph cross section of the myelinated cultures. Scale bars: *A*–*E*, 25 μ m; inset, 0.5 μ m.

tures, at 3 weeks SCs contact axons much more intimately and tightly (Fig. 1*D*). SCs tightly adhere to axons, elongate along single axons, and assume a more flattened and extended morphology spreading along the axonal membrane. Electron microscopy of these cultures shows that SCs envelop axons and elongate their cytoplasmic extensions further along the axon,

and that single axons are selectively wrapped by SC cytoplasm. In addition, there is a substantial reduction in the gap between axon and SC membranes compared to that at 1 week. However, no SCs appeared to encircle axons more than once at this stage. When this degree of Schwann cell–axon contact had developed, myelination was induced with the addition of ascorbic acid.

Figure 2. Distribution of NaChs and Thy 1.1 on 1-week-old DRGs in the absence (*A*, *B*) or presence (*C*, *D*) of SCs. Cells were double labeled with a mouse monoclonal anti-Thy 1.1 (1:40 dilution) (*B*, *D*) and with an anti-rabbit NaCh-specific polyclonal antibody (1:100 dilution), 7493 (*A*, *C*). NaChs and Thy 1.1 were visualized by rhodamine- and fluorescein-conjugated secondary antibodies, respectively. Fluorescence images were obtained through a 63 $\times$, 1.3 NA objective on a Zeiss Axovert microscope and images were captured in digital format through a 512 $\times$ 512 CCD camera interfaced to a Perceptics Image processor. Micrographs were printed on a Sony color video printer. Scale bar, 20 μ m.

Figure 3. Representative FPR curves of TmRhd-Tityus γ -labeled NaChs in 1-week-old cultures of DRG in the absence ($-SC$) or in the presence ($+SC$) of SCs. The FPR curves were obtained at the cell body (cb) and axon (ax). Locations are indicated. Cells were labeled with 4 nM TmRhd-Tityus γ and then mobility measurements were carried out using a $63\times$, 1.4 NA water immersion objective ($0.45\ \mu\text{m}$ beam radius). Photon counts were measured within 50 msec dwell time. Nonspecific labeling measured in the presence of $1\ \mu\text{M}$ unlabeled Tityus γ toxin was $<10\%$ of the experimental fluorescence. In the FPR experiments where SCs accompanied DRGs, the fluorescence on SC bodies was 50–100 photon counts.

Under phase or bright-field optics, compact myelin segments appeared as thicker birefringent structures (Fig. 1*E*), which was confirmed by electron microscopy (inset, Fig. 1*E*). The average diameter of these myelinated axons is $\sim 1.4\ \mu\text{m}$. In these cultures, SC bodies can still be discerned (SC) and both nodes (N) and heminodes (hN) are found (Fig. 1*E,F*). In our cultures we found more heminodes than nodes; this is reasonable because the formation of a node requires two adjacent SCs on an axon segment. The compact myelin formed by SCs around selected axons and the myelinated axons in these cultures appear indistinguishable from those found *in vivo*.

For the lateral mobility studies here, 1-week-old cultures were used because axons are sufficiently well developed for FPR, and SCs have initiated contact. The extensive contact with axons, even though they have not yet established the one-to-one relationship required for myelination (Webster et al., 1973; Eldridge et al., 1989), had additional effects on NaCh mobility. At later developmental stages, 3-week-old and myelinated cultures were also examined.

Distribution of NaChs and Thy 1.1 on DRG axons in the presence and absence of SCs

To obtain a view of the distribution of the NaCh in these cultures and to provide insights into the origin of the NaChs, DRG–SC cocultures or pure DRGs were labeled with an NaCh-specific antibody, 7493, and anti-Thy 1.1 antibody, which is reported to stain neurons (Fields et al., 1978) (Fig. 2), and digital images were acquired. NaChs remain homogeneously distributed on the axon in the absence of SCs (Fig. 2*A*) but within 1 week NaChs are clustered on axons in the presence of SCs (Fig. 2*C*). The number of NaCh clusters is related to the density of SCs.

In areas of the culture where there are more SCs, NaCh clusters were more abundant. Although SCs have NaChs (Shrager et al., 1985), the anti-NaCh antibody 7493 stains SCs very weakly compared to DRGs ($<10\%$). Therefore, axonal staining can be distinguished from SC bodies or processes (Fig. 2*C*).

This clustering and change in the distribution of NaChs on the axon membrane by SC is not a general feature of all axonal membrane proteins, because the same cultures labeled with neural-specific anti-Thy 1.1 antibody showed that in contrast to NaChs, Thy 1.1 is distributed homogeneously along the axon with no discernible differences in the staining patterns or in the fluorescence intensities between regions on the axon (Fig. 2*B,D*). Both sConA receptors and NCAM also remain homogeneously distributed in the presence of SCs (not shown). Therefore, despite the intimate contact between axon and SC membrane, the SC does not have a general role in organizing all glycoproteins on the axon membrane. These results suggest that NaCh clustering on axons is an NaCh-specific property induced by SCs.

Because the images were obtained in digital format within the linear range of the CCD camera, the average fluorescence intensities per pixel corresponding to the relative density of NaChs could be measured at noncontact and at clustered sites and compared in the same image. At noncontact sites, over a $15\text{--}20\ \mu\text{m}$ distance, the average NaCh fluorescence intensity per pixel was 1670 ± 323 , which compares with 6614 ± 982 at clusters and 1770 ± 528 in regions of the axon between clusters ($\sim 14\ \mu\text{m}$).

As contact between axon and SC becomes more intimate during the next 2–3 weeks in the maturation of these cultures, although the number of NaCh clusters decreased (data not shown), the relative intensity differences between noncontact

A. NaCh

B. Glycoprotein

Figure 4. Comparison of the mobility of NaChs (*A*) and sConA receptors (*B*) on DRGs in the presence (+SC) and the absence (–SC) of SCs during development. Cells at 1 week, 4 weeks, and after myelination were labeled with either TmRhd-Tityus γ or TmRhd-sConA. The fluorescence recovery curves were obtained as in Figure 3 and mobile fractions and diffusion coefficients were determined by curve-fitting procedures (Axelrod, 1983). The number of measurements is shown in each bar; error bars represent SEM.

and contact/clustering sites remained the same. Electron micrographs of these cultures indicate that this stage coincides with the withdrawal of the SC membrane with the multiple axons that is established early on, and with a stage where the SC interacts with fewer axons, ultimately establishing a one-to-one relationship with a selected axon.

Lateral mobility of NaChs during development

Although immunocytochemistry and imaging provide a useful view of the distribution of NaChs and the ultrastructural features that might participate in the segregation of NaChs, how is this topography controlled and maintained by SCs?

A. NaChs **B. glycoproteins**

Figure 5. Statistical comparison of mobilities of NaChs (*A*) and glycoproteins (*B*) in the presence or absence of SCs. Mobile fractions and diffusion coefficients at cell bodies (*cb*) and axons (*ax*) in 1-week-old cultures were compared. Student's *t* tests were used for statistical analysis of two values above and below *p* values.

We have used FPR to examine the role and magnitude of intracellular and/or extracellular constraints to NaCh diffusion and the mechanisms by which NaChs are organized, segregated, and confined to specialized regions of the axon by SCs. In order to investigate whether SCs modulate the axonal distribution of NaChs by regulating NaCh mobility on the axon membrane, we measured the lateral mobility of fluorescently labeled NaChs in cultured DRGs in the presence and the absence of SCs, as in Figure 3.

Even in pure neurons without SCs, we were quite surprised to find that in all regions of the neuron >80% of NaChs were immobile on the time scale of the FPR measurement ($<10^{-12}$ cm²/sec). There was, however, a small mobile fraction (20%) of NaChs on axons, axon hillocks (not shown), and cell bodies with diffusion coefficients of $5\text{--}13 \times 10^{-11}$ cm²/sec (Fig. 4*A*). Measurement of NaCh mobility at NaCh clusters or at uniform sites on the axon showed no discernible differences. Statistical analyses using Student's *t* test show that NaCh mobilities do not change in the presence of SCs (Fig. 5*A*).

Because there are additional changes in NaCh distribution accompanying axon–SC maturation, we measured NaCh mobility in more developed cultures (3–4 weeks), where the number of NaCh clusters had decreased despite the tighter contact between axons and SCs. Even in these cultures, there were no discernible differences between parallel cultures of neurons and neuron–SC cocultures in regions and at times where electron micrographs show more intimate contact with axons and when

single axons are selected to become myelinated (Fig. 4*A*). Thus, at least at the level of the resolution and in the microdomains sampled by FPR, there appear to be no maturational effects of SC contact and axon segregation on NaCh mobility. Even after compact myelin is formed, both the mobile fraction and diffusion coefficients of NaChs are comparable to those measured at 1 week (Fig. 4*A*).

To examine whether these characteristics were similar for all axonal membrane proteins, we measured the mobility of glycoproteins labeled with rhodamine–sConA under the same conditions and at the same developmental stages. In contrast to NaChs, a large fraction of glycoproteins is mobile and the mobile fraction decreases in the presence of SCs (Fig. 4*B*). As shown in Figure 5*B*, the mobile fraction of glycoproteins is altered with SCs in all regions of the axon even in one-week-old cultures. The decreased mobility could be due to the interactions between glycoproteins and cell adhesion molecules and/or extracellular matrix proteins (Jacobson et al., 1987), which are secreted by SCs (Bunge et al., 1986; Eldridge et al., 1989). Measurement of glycoprotein mobility in 3–4-week-old or myelinated cultures showed a significant decrease in the fraction of glycoproteins that were mobile (23–29%; Fig. 4*B*), particularly in the presence of SCs. The decrease in mobile fraction of glycoproteins during development could have its origins in the increased membrane density of membrane proteins (Jacobson et al., 1987) or from the closer apposition of axon and Schwann cell membrane surfaces (Eldridge et al., 1989), which somehow selectively excludes

Table 1. Effect of colchicine and cytochalasin B on mobility of NaChs

	No treatment	Colchicine	Cytochalasin B
A. Mobile fraction (%)			
Cell body	19.5 ± 2.6 ^a	27 ± 2.0	25 ± 0.9
Axon hillock	25.5 ± 2.8	25 ± 1.6	19.5 ± 2.5
Axon	22.3 ± 2.0	26 ± 1.0	18.5 ± 0.8
B. Diffusion coefficient (10E – 11 cm²/sec)			
Cell body	12.8 ± 1.0	18.1 ± 2.6	8.6 ± 2.3
Axon hillock	7.5 ± 1.2	17.1 ± 3.0	13.4 ± 2.5
Axon	7.0 ± 1.0	19.7 ± 3.7	10.9 ± 0.9

^a Standard mean error.

an effect on the mobility of NaChs. These results indicate that the small mobile fraction of NaChs that remains independent of SC contact during development is a property that has specificity for the NaCh protein.

Because SCs did not appear to affect NaCh mobility, we explored whether the immobility of NaChs arose from elements that were expressed in the axon very early in differentiation of the axon. In particular, in view of our previous work implicating the cytoskeleton (Angelides et al., 1988; Srinivasan et al., 1988), we asked whether the cytoskeleton served as a primary force that constrained NaCh mobility on the axon membrane. First, to test whether microtubules or actin filaments were directly involved in immobilizing NaChs in bare axons, neurons were treated with 5 μ g/ml colchicine or 5 μ g/ml cytochalasin B and the lateral mobility of NaChs was measured. Table 1 shows that neither colchicine nor cytochalasin B affects the mobile fraction of channels, although both agents slightly increase the diffusion coefficients, approximately 1.6–2.8-fold, on axons and at axon hillocks. The increase in the rate of lateral mobility in these regions with cytochalasin and colchicine was not a NaCh-specific feature because sConA receptors responded similarly to these agents. Therefore, neither microtubules nor actin filaments appear directly responsible for creating the immobile fraction of NaChs. If the cytoskeleton is involved in immobilizing NaChs, the results of these experiments suggest that it is above the level of actin filaments or microtubules (Srinivasan et al., 1988; Wood and Angelides, 1989).

These experiments, however, cannot unequivocally discriminate whether NaCh immobility is a result of a direct linkage between NaChs and the cytoskeleton or whether the restriction is by a corral-like barrier imposed by the underlying matrix. Bleaching of NaChs in a 0.45 μ m spot, greater than the dimension of the barrier, would also appear immobile (Edidin and Stroynowski, 1991). To ask whether NaChs were confined by a cytoskeletal barrier or directly linked, we determined the fraction of NaChs associated with the detergent-insoluble cytoskeleton by extraction with Triton X-100. Treatment with 0.5% Triton X-100 removes membrane lipids and soluble proteins, but leaves those proteins linked to the cytoskeleton (Marsh and Letourneau, 1984). In cell bodies and axons, 22% and 17% of channels, respectively, were extracted (Table 2). Therefore, it appears that approximately 80% of NaChs are likely to be associated with the cytoskeleton by a direct linkage. Ankyrin has been suggested as the cytoskeleton linker (Angelides et al., 1988; Wood and Angelides, 1989; Srinivasan et al., 1992). In addition, immunocytochemistry of these cultures shows the appearance

Table 2. Fraction of NaChs extracted by 0.5% Triton X-100

	– Triton X-100	+ Triton X-100	% extraction
Cell body	8560 ± 688 ^a	6666 ± 866	22
Axon hillock	2738 ± 246	ND ^b	ND
Axon	731 ± 60	606 ± 33	17

^a Standard mean error.^b Not determined.

of detectable levels of spectrin and ankyrin within a day even without SCs (Joe and Angelides, 1992). This would be consistent with the hypothesis that the axon itself provides constraints on NaChs that limit diffusion NaChs in the membrane.

Discussion

There has been considerable discussion as to what role glial cells play in organizing NaChs on the axon membrane (Smith et al., 1982; Le Beau et al., 1987; Rosenbluth, 1988; Bigbee and Foster, 1989). Several studies have suggested that specialization of the node and the highly restricted distribution of NaChs is determined by glial–axonal contact (Rosenbluth, 1988; Bigbee and Foster, 1989), while other studies have suggested that nodal specializations and clustering of NaChs develop independently of ensheathing cell contact (Smith et al., 1982; Le Beau et al., 1987).

Recently, we have shown that segregation of NaChs on axons is highly dependent on interactions with active SCs and that continuing axon–glial interactions are necessary to organize NaCh distribution during differentiation of the myelinated axon (Joe and Angelides, 1992). Specifically, in the absence of SCs, NaChs are homogeneously distributed along axons, but when SCs accompany or are reintroduced into neuronal cultures, NaCh distribution on the axon dramatically changes from homogeneous to clustered. The change in axonal distribution requires a component of the SC membrane that is expressed upon axon contact and specific for NaChs since other membrane proteins such as Thy 1.1, NCAM, and sConA receptors remain homogeneously distributed. Furthermore, these changes in NaCh distribution are not accompanied by changes in the distribution of ankyrin or spectrin, which remain homogeneously distributed, even though at fully differentiated nodes of Ranvier ankyrin colocalizes with NaChs (Kordeli et al., 1990).

In this work, we have measured the mobility of NaChs to gain insight into whether homogeneously distributed NaChs could redistribute and cluster at specific sites on the axon determined by SC–axon contact by diffusion within the axon membrane. Previous light and electron micrographs have shown the intimate interactions that develop between axon and SC membranes where the SC membrane is closely apposed to and elongates along the axon surface, constantly maintaining its close association with the axon surface (Clark and Bunge, 1989). It is clear that there is a programmed expression of specific adhesion molecules on axons and SCs during this developmental sequence that mediates the axon–SC interaction (Linnemann and Bock, 1989; Wood et al., 1990). During the early stages, when NCAM is expressed, SCs appear to establish contact with many axons and NaCh clusters are abundant. We have observed that the density and number of NaCh clusters, however, decrease as the SC reaches the stage where it commits to a one-to-one

relationship with the axon (E-h Joe and K. J. Angelides, unpublished observations).

Given the extensive interactions between these cells, we asked whether (1) the mobility of NaChs and axonal membrane glycoproteins was modified by SCs, (2) NaCh clusters could originate from a redistribution of preexisting NaChs, and (3) SCs provided constraint or a barrier whereby NaCh clusters were maintained or segregated on the axon.

Surprisingly, the results show that even without SCs NaChs in axons are largely immobile (80%) and that the mobility of NaChs is not modified with SC contact. Moreover, the results suggest that even in the absence of SCs intracellular constraints expressed by the axon itself provide a selective constraint on NaCh movement on the axon membrane. This restrictive force is likely provided by the cytoskeleton since approximately 80% of the NaChs are associated with the detergent-insoluble cytoskeleton, a fraction of NaChs that corresponds to the immobile fraction of NaChs determined by FPR. Neither microtubules nor microfilaments directly regulate NaCh mobility since colchicine and cytochalasin do not alter the mobile fraction of NaChs. These observations are consistent with biochemical evidence showing that NaChs are directly linked to ankyrin and the spectrin-based cytoskeleton (Srinivasan et al., 1988) and that the association of ankyrin and spectrin with NaChs controls NaCh mobility (Wood and Angelides, 1989). Taken together, the FPR and biochemical experiments suggest that even early on in development direct linkages established between NaChs and the ankyrin/spectrin-based cytoskeleton, rather than barriers formed by the subaxolemmal cytoskeletal meshwork, are responsible for immobilizing NaChs. However, the small fraction of mobile NaChs could be regulated by the spectrin-actin (Sheetz et al., 1980) and/or spectrin-microtubule network because the diffusion coefficient of mobile NaChs is increased slightly in the presence of either cytochalasin or colchicine. Spectrin and associated proteins may form corral-like barriers (Edidin et al., 1991) where mobile NaChs move within a spectrin barrier and rarely cross the boundary. Depolymerization of actin or microtubules after cytochalasin or colchicine treatment weakens the spectrin barrier, resulting in increased movement of mobile NaChs. We conclude that even without SCs the subaxolemmal cytoskeleton provides a mechanism by which NaCh mobility is restricted. The intracellular constraints appear strong enough that extensive interactions on the external face of the axon and the remodeling of other axon membrane proteins through SC contact do not influence or alter NaCh mobility.

In contrast to our previous work on the distribution and mobility of NaChs on spinal cord motoneurons, which are also myelinated, we observed that the mobility of NaChs on sensory/DRG neurons did not differ significantly in different regions of the neurons. In spinal cord neurons NaChs are diffusely distributed at low density on the cell body, but segregated at high density at the axon hillock. In addition, on spinal cord motoneurons, NaChs are freely mobile on the cell body but immobilized at the axon hillock. These differences between sensory/DRG neurons and spinal cord motoneurons could be due to the NaCh subtypes expressed by these different neurons (Roy and Narahashi, 1992; Campbell, in press). The intracellular machinery that localizes these NaChs to different parts of the neuron and the differences in density and localization may reflect functional differences in the manner by which the action potential is generated by these fibers (Kandel et al., 1991).

Given that most NaChs are immobilized on the FPR time

scale and linked to the axonal cytoskeleton, how does SC contact modify NaCh distribution and how are the NaCh clusters formed? One possibility is that 20% of the NaChs that are mobile and diffuse are trapped at sites formed by the SCs in a manner similar to the aggregation of ACh receptors (AChRs) at the developing neuromuscular junction of *Xenopus* (Poo and Young, 1990). The clustering and entrapment, however, are selective since Thy 1.1, general glycoproteins, and NCAM, which are laterally mobile on the axon surface, do not accumulate with NaChs at these sites.

If we consider that the existing homogeneously distributed and mobile NaChs measured by FPR are redistributed to clusters upon SC contact, can the preexisting and homogeneously distributed NaChs reach these sites? Analysis of the rates of lateral diffusion of NaChs shows that NaCh movement in the membrane is indeed rapid enough to account for the entrapment of the channels at or near contact sites if the sites of contact serve as an ideal sink or a barrier for channel diffusion. In DRG-SC cocultures, NaCh clusters appear approximately 5 d after reseeding cultures with SCs (Joe and Angelides, 1992). On single axons we have measured that the average distance between NaCh clusters is approximately 14 μm at this stage. An NaCh in random motion with a diffusion coefficient of $\sim 10^{-10} \text{ cm}^2/\text{sec}$ would take approximately 1.5 hr to diffuse a root-mean-square distance of 14 μm ($x^2 = 4Dt$, where x is the distance the molecules move, D is a diffusion coefficient, and t is time). Given these rates it would seem that the rate of lateral diffusion for even 20% of the channels could be consistent with their appearance at cluster sites and would be rapid enough to reach clustering sites even within a day.

Even if the rate is compatible with surface diffusion, trapping, or a selective barrier created by SCs, are there enough NaChs on these axons that redistributed NaChs could reach the densities of NaChs at cluster sites? Measurement of the fluorescence intensity, corresponding to the relative density of NaChs, revealed that NaCh intensity at clusters is about four times higher than that around clusters. If 20% of the NaChs in a 14 μm homogeneous region of the axon were segregated into a 1 μm cluster site, the density of clusters would be four times higher than adjacent membrane. Although we have not excluded the possibility of local incorporation at the cluster site together with a degradation of NaChs in regions between clusters, our results are consistent with the simplest hypothesis that accumulation could result from a redistribution of the small population of preexisting and mobile NaChs.

What could be the molecular basis for NaCh clustering? First of all, the clustering sites must be formed in the presence of SCs and show selectivity. Like that suggested for the clustering of AChRs at the developing neuromuscular junction (Poo and Young, 1990) and lateral migration and recruitment to these sites by agrin (Godfrey et al., 1984), accumulation and trapping of NaChs may arise by binding to specific ligands on the apposing SC membrane, while other axon membrane components that do not have this specificity in binding are excluded from these contact regions. Several reports have shown that cell-cell contact and extracellular interactions may be an important mechanism by which membrane protein distribution is modulated. Chow and Poo (1982) found that in embryonic muscle cells, cell surface lectin receptors underwent rapid redistribution (even at sites remote from the contact sites) that was dependent on the degree of cell contact. Furthermore, there is specificity in the affinity for certain lectins to redistribute at these contact

sites. These studies suggest that specific molecular interactions between components of the contacting surfaces result in redistribution of preexisting, rapidly diffusing surface receptors (Chow and Poo, 1982). The NaCh protein is an unusually high glycosylated protein (30% carbohydrate by weight) (Schmidt and Catterall, 1987; James and Agnew, 1989). Therefore, it is possible that the SC membrane may contain lectin-like molecules specific for polysialic acid or *N*-acetyl- β -D-glucosaminyl oligomers, which are structural features of the NaCh protein (Miller et al., 1983; James and Agnew, 1989) and could serve to trap NaChs. Alternatively, it is possible that NaChs are excluded from specific regions of the axon where SC and axon membranes interact.

How are NaCh clusters produced? It is known that the axon and SCs undergo a programmed expression of adhesion molecules on their surfaces that are important for each developmental stage during myelination (Wood et al., 1990). Such selective adhesion plays an important role in the morphogenesis of the myelinated axon (Rieger et al., 1986). The accumulation of axonal components at sites of SC contact by the binding of axonal membrane receptors with specific ligands on the apposing SC surface could serve as a mechanism for enhancing selective adhesion of the two surfaces and for initiating NaCh cluster formation. Alternatively, localization and immobilization of adhesion molecules targeted to specific regions of the SC membrane could regulate axonal NaCh distribution by the interaction directly with NaChs, an axonal component associated with NaChs (Pollerberg et al., 1987; Persohn et al., 1989), or by creating a selective barrier to NaCh movement into the developing internode. A third possibility is that a specific component in the SC membrane that is expressed only with axonal contact and localized at the site of axonal contact provides an interaction or barrier to mobile NaChs and is sufficient to entrap and seed or exclude the first NaCh aggregates before full SC envelopment occurs. Thus, membrane components on the SC membrane, themselves polarized and immobilized, could provide the localization signals and sites where mobile and diffusing NaChs become entrapped or excluded.

Finally, despite the consistency of our data with a diffusion trap or selective barrier for NaCh segregation, we have not ruled out two other possibilities. First, newly synthesized NaChs could be actively inserted at sites of SC contact that have higher affinity for vesicle fusion. Second, NaChs are inserted into the membrane randomly but turned over more rapidly in regions ensheathed by SCs due to signals transduced only in those axonal regions overlaid by SCs. However, in mobility studies, we did not observe any differences in the mobility of NaChs in regions between clustering sites overlaid by SCs along axons that would likely signal the prerequisite disruption of the interaction between NaChs and the SC membrane and/or NaChs and the cytoskeleton. Moreover, from image analysis, we found that the intensity of NaCh staining at clusters is about four times higher than that at axons without SC contact. Therefore, NaCh clusters should be formed by insertion of NaChs at clustering sites rather than eliminating NaChs from nonclustering sites.

Evidence from experiments performed in cultured chick myotubes shows that AChR clusters are formed by active insertion of new receptors after innervation (Dubinsky et al., 1989). If NaChs were axonally transported in the slow component [0.1–4 mm/day (Vallee and Bloom, 1991)], sorting and accumulation at distal sites could be accomplished and consistent with the rates of appearance of newly synthesized and inserted channels

at clustered sites. In fact, we have observed in micrographs of cultured DRGs, with or without SCs, axon varicosities often stained with NaCh and ankyrin antibodies. Evidence has shown that the varicosities are anterogradely transported membranous organelles associated with kinesin (Hollenbeck and Bray, 1987; Pfister et al., 1989; Hirokawa et al., 1991). These observations provide support for the intriguing idea that these vesicles contain NaChs and ankyrin *en route* to their assembly cluster sites.

In conclusion, we have observed that SCs do not alter the mobility of NaChs on the axon surface yet segregation and localization of NaChs on the axon surface is highly dependent on the interactions with active SCs. The forces that are responsible for the immobilization of NaChs on axons in the absence of SC contact are likely to involve interactions with the subaxolemmal cytoskeleton, while the accumulation of NaChs at high density on the axon in the presence of SCs likely arises from diffusion-mediated entrapment or selective exclusion of a small population of mobile NaChs. It will be important in future studies to identify the nature of the SC–axon components and interactions during development and myelination to gain further insight into the mechanisms and signals by which SCs segregate and maintain NaChs at discrete sites on the axon surface.

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