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Clustered Voltage-Gated Na^+ Channels in *Aplysia* Axons

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Clustering of voltage-gated Na^+ channels is critical for the fast saltatory conduction of action potentials in vertebrate myelinated axons. However, the mechanisms responsible for the generation and maintenance of Na^+ channel clustering are not well understood. In this study we have raised an antibody against the cloned SCAP-1 voltage-gated Na^+ channel of the marine invertebrate *Aplysia californica* and used it to examine Na^+ channel localization in *Aplysia* ganglia and in cultured *Aplysia* sensory neurons. Our results show that there is a large cytoplasmic pool of Na^+ channels in the soma of *Aplysia* neurons. Furthermore, we show that Na^+ channels in *Aplysia* axons are not homogeneously distributed but, rather, are present in distinct clusters. Theoretical considerations indicate that Na^+ channel clustering may enhance action potential conduction. We propose that clustered Na^+ channels may be a fundamental property of many axons, and perhaps of many membranes that conduct Na^+-dependent action potentials.

Key words: Aplysia; *Na^+ channel; sodium channel; ion channel; myelination; demyelination; unmyelinated axon; clustered channels*

The unique properties of neurons are determined by a spatially complex distribution of proteins and organelles. The localization of ion channels is particularly important because they control the voltage responses that regulate action potential initiation and conduction. Therefore, understanding the signaling properties of neurons requires knowledge of both the biophysical properties and the subcellular locations of the constituent ion channels.

Voltage-gated Na^+ channels generate the fast inward current in the neurons of most species (Hille, 1984). Early electrophysiological studies on vertebrate neurons indicated that action potentials were generated at the initial segment of the axon and subsequently propagated by the axon (Coombs et al., 1957a,b). Dendrites were originally believed to be passive structures that lacked detectable Na^+ channel responses (Rall, 1962) but, subsequently, have been shown to conduct and perhaps even initiate action potentials in some neurons (Regehr et al., 1992; Stuart and Sakmann, 1994; Turner et al., 1994; Häusser et al., 1995; Spruston et al., 1995). Recently, dendritic patch-pipette recording of CA1 pyramidal neurons has demonstrated activation of dendritic Na^+ channels after stimulation of Schaffer collaterals (Magee and Johnston, 1995). In addition, evidence for the presence of Na^+ channels in apical dendrites of pyramidal cells has also been obtained using a Na^+ channel antibody (Turner et al., 1994). It is evident that vertebrate Na^+ channels are not confined to the axon but are also present in some dendrites.

Invertebrate neurons have a very different somatodendritic–axonal organization compared to vertebrates (Cohen, 1970; Kandel, 1979). The invertebrate soma usually gives rise to a single process that projects into a dense neuropil where synapses are found. Axons project out from this neuropil. The invertebrate cell body is usually well removed from the sites of synaptic interaction and impulse initiation. Electrophysiological studies indicate that although many invertebrate somata are incapable of supporting action potentials (Goodman and Heitler, 1979; Kuwada, 1981; Gu et al., 1991), some neurons can conduct, and may even initiate spikes, in the cell soma (Alving, 1968).

We have initiated studies on the distribution of the Na^+ channel in *Aplysia californica*. As an experimental system for studies of ion channel localization, *Aplysia* offers a number of advantages. Identified neuronal cell bodies are very large (some in excess of 500 μm) and can be microinjected directly. In addition, ganglia, and even isolated cells, retain their *in vivo* phenotype when cultured (Frazier et al., 1967; Kandel et al., 1967; Dagan and Levitan, 1981; Camardo et al., 1983; Rayport and Schacher, 1986). This suggests that *Aplysia* Na^+ channels are localized correctly in culture. Therefore, the *in vitro* preparation may be a useful model for studying mechanisms controlling Na^+ channel distribution.

In this study we have used a polyclonal antibody directed against the cloned SCAP-1 *Aplysia* Na^+ channel (J. Dyer, W. Johnston, and R. Dunn, unpublished observations) to investigate Na^+ channel distribution in *Aplysia*. In ganglia and in cultured sensory neurons, a large intracellular pool of Na^+ channels was present within the soma. In the axons of ganglia and in the neurites of cultured sensory neurons, Na^+ channels were present in clusters. We suggest that Na^+ channel clustering may be important for propagation of action potentials in this unmyelinated axon and that clustering may represent the general default distribution of Na^+ channels in many axons.

MATERIALS AND METHODS

Production of glutathione S-transferase (GST) protein, SCAP-1-GST fusion protein, and Aplysia *Na^+ channel antibody.* A portion of the cytoplasmic C terminus of the *Aplysia* SCAP-1 voltage-gated Na^+ channel (nucleotides 5241–5906, corresponding to amino acids 1748–1968) (J. Dyer, W. Johnston, and R. Dunn, unpublished observations) was amplified by PCR (30 cycles: 94°C, 30 sec; 50°C, 60 sec; 72°C, 60 sec; using VENT DNA

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polymerase, New England BioLabs, Beverly, MA) and subcloned into pGEM-7Z (Promega, Madison, WI) for DNA sequencing (Sequenase kit, United States Biochemical, Cleveland, OH). The SCAP-1 DNA was then subcloned, in-frame, into the GST fusion protein expression vector, pGEX-3X (Pharmacia Biotech, Piscataway, NJ). Production of GST (for use in immunohistochemistry) or of the full-length SCAP-1 Na^+ channel-GST fusion protein (for immunizing rabbits and also for use in immunohistochemistry) was induced in *Escherichia coli* (DH5α) by 100 μM isopropylthio-β-D-galactoside (IPTG). The GST protein and the SCAP-1-GST fusion protein were purified over a 50% glutathione–agarose bead column, followed by dialysis in PBS, pH 7.3, overnight at 4°C.

Preimmune serum was taken from specific pathogen-free, female New Zealand white rabbits. Rabbits were then injected subcutaneously with ~500 μg (1 ml) of the purified SCAP-1-GST fusion protein emulsified with Freud's complete adjuvant and were boosted at 4 weeks (~250 μg) and 8 weeks (~750 μg) with purified SCAP-1-GST fusion protein emulsified with Freud's incomplete adjuvant. Blood was collected 12 d after the second boost, and the serum IgG was purified over a protein A column and dialyzed in PBS, pH 7.3, overnight at 4°C.

Immunoblotting. To prepare tissue for immunoblotting, pleural, pedal, cerebral, and abdominal ganglia from adult *Aplysia* were desheathed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 3 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 1 mM iodoacetamide, 1 mM 1,10-phenanthroline, 100 μM phenylmethylsulfonyl fluoride and 1 μM pepstatin A. Approximately equal volumes of buffer and desheathed ganglia were then immediately frozen in liquid nitrogen and stored at −80°C. Just before electrophoresis, frozen samples were boiled for 5 min in an equal volume of SDS loading buffer (6% SDS, 10 M urea, 200 mM dithiothreitol, 140 mM Tris, pH 6.8). Duplicate samples of prepared ganglia were electrophoresed on two 6.5% low-porosity SDS-PAGE gels (Doucet et al., 1990). To illustrate the protein spectrum, one of these gels was stained with Coomassie Blue. Proteins on the other gel were transferred to nitrocellulose, which was then blocked overnight at 4°C in blocking solution A [100 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20, 5% bovine serum albumin (BSA), 10% goat serum, and 10% low-fat milk powder]. Protein A-purified SCAP-1 antibody, which was purified further by passing it over an Affigel10 (Bio-Rad, Hercules, CA)-GST affinity column, was then added to the blocked membrane (~5 μg protein/ml, in blocking solution A, overnight at 4°C), followed by goat anti-rabbit horseradish peroxidase (Jackson Immunoresearch, West Grove, PA) at 1:10,000 (in blocking solution A). Detection was by enhanced chemiluminescence (Renaissance, DuPont NEN, Boston, MA).

Cell culture and immunohistochemistry. To confirm specificity, SCAP-1 was subcloned into pHermite, a mammalian expression vector containing the cytomegalovirus (CMV) promoter for gene expression, and transfected into Vero cells using lipofectamine (Gibco, Grand Island, NY). After transfection, cells were grown for 19 hr, plated onto 10 μg/ml poly-L-lysine-coated glass coverslips and allowed to adhere. Cells were then fixed in 4% paraformaldehyde (in PBS, pH 7.4), permeabilized in 100% methanol for 20 min, and blocked for 60 min in PBS containing 3% BSA. Preimmune serum (1:100 in PBS/3% BSA), protein A-purified SCAP-1 antibody (~25 μg protein/ml, in PBS/3% BSA, preincubated overnight at 4°C with 10 μM GST protein), or protein A-purified SCAP-1 antibody blocked by SCAP-1-GST fusion protein [protein A-purified SCAP-1 antibody (~25 μg protein/ml) in PBS/3% BSA, preincubated overnight at 4°C with 10 μM SCAP-1-GST fusion protein] were then added to the coverslips (overnight, 4°C). As an additional control, protein A-purified SCAP-1 antibody (~25 μg protein/ml, in PBS/3% BSA, preincubated overnight at 4°C with 10 μM GST protein) was also added to untransfected Vero cells overnight at 4°C. Binding of the secondary antibody [biotinylated goat anti-rabbit (1:200, Jackson) in PBS/3% BSA] was followed by streptavidin-fluorescein isothiocyanate (FITC; Pierce, Rockford, IL) at 1:100 (in PBS/3% BSA). Immunofluorescence was detected by conventional fluorescence microscopy.

To examine the distribution of the SCAP-1 channel in *Aplysia* nervous tissue, immunohistochemistry was performed on cultured *Aplysia* neurons and on ganglia sections. For cultured neurons, the abdominal ganglia of *Aplysia* were desheathed in artificial seawater (ASW). Sensory cell clusters were dissected out of the ganglia and were then incubated at 37°C for 60 min in protease (10 mg/ml, type IX, Sigma, St. Louis, MO)-containing L15 medium, supplemented for marine species. After washing, cells were dissociated by trituration, plated onto 1 mg/ml poly-L-lysine-coated glass coverslips and grown for 7 d in L15, supplemented for marine species, with 10% *Aplysia* hemolymph added (Schacher and Proshansky, 1983). Cells were fixed in 4% paraformaldehyde (in ASW buffered with 10 mM HEPES, pH 7.5), permeabilized in 100% methanol for 20 min, and blocked in PBS/3% BSA for 60 min. Immunohistochemistry was done as described for Vero cells. Immunofluorescence was detected by conventional fluorescence microscopy. In addition, somata of the sensory neurons stained with the SCAP-1 antibody were also examined by confocal laser scanning microscopy. In addition to these treatments, some coverslips were stained for β-tubulin: monoclonal anti-β-tubulin (1:100, Sigma); biotinylated goat anti-mouse (1:200, Jackson); streptavidin-FITC (1:100, Pierce) or α-mannose residues [biotinylated concanavalin A (Con A; 1:100, Pierce)]; streptavidin-FITC (1:100, Pierce). For ganglia sections, abdominal or pleural/pedal/cerebral ganglia (still within the connective tissue sheath) were removed, pinned onto cardboard, fixed overnight at 4°C in 4% paraformaldehyde (in HEPES-buffered ASW), and then processed for cryostat or paraffin sectioning. Sections (10 μm) were permeabilized in 100% methanol for 20 min, blocked overnight in PBS/3% BSA, and stained for Na^+ channels, β-tubulin, or α-mannose residues, as described above.

RESULTS

Characterization of the antibody

We have cloned the voltage-gated Na^+ channel from the nervous system of *Aplysia californica* (J. Dyer, W. Johnston, and R. Dunn, unpublished observations). This channel, named SCAP-1, shows a high degree of homology to the other cloned voltage-gated Na^+ channels. Like the other voltage-gated Na^+ channels, the protein is large (1996 amino acids) and is predicted to be comprised of four homologous domains, each of which has six predicted transmembrane segments. For preparation of the antibody specific to SCAP-1, the C-terminal 220 amino acid residues of the SCAP-1 channel were produced as a fusion with GST in the expression vector pGEX-3X (Smith and Johnson, 1988). SCAP-1-GST fusion protein was produced in *E. coli*, purified over a glutathione-agarose column, and injected into rabbits. Serum was collected, and serum IgG was purified over a protein-A column.

The specificity of this antibody preparation was tested in two ways. First, proteins prepared from *Aplysia* ganglia were immunoblotted with the antibody (Fig. 1). The antibody recognized one major band of >205 kDa, which is consistent with the size pre-

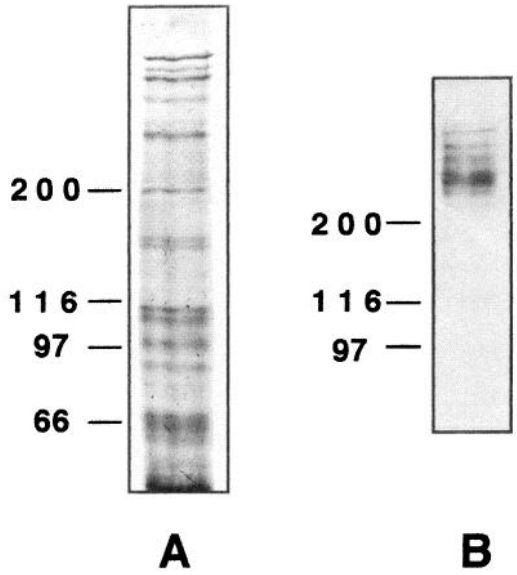

Figure 1. Immunoblot analysis of *Aplysia* ganglia with the SCAP-1 antibody. Duplicate aliquots of ganglia proteins were electrophoresed on two 6.5% low-porosity SDS-PAGE gels. *A*, Gel stained with Coomassie Blue. *B*, Proteins were transferred to nitrocellulose, blocked overnight at 4°C, and immunoblotted with the SCAP-1 antibody. Detection was by enhanced chemiluminescence.

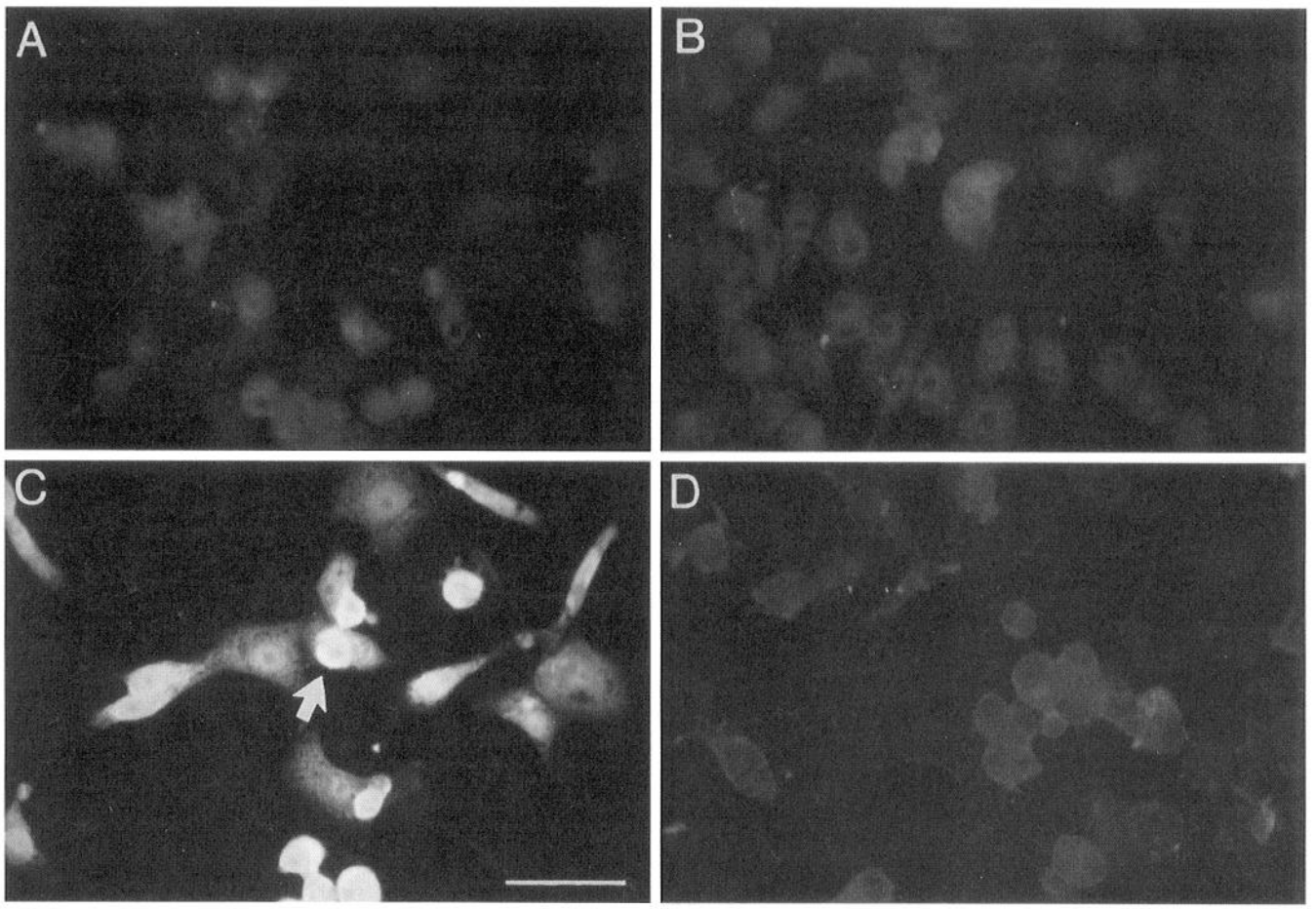

Figure 2. Vero cells transfected with the SCAP-1 Na^+ channel cDNA show SCAP-1 antibody-specific immunostaining. In *A*, *C*, and *D*, cells were transfected with the SCAP-1 Na^+ channel cDNA using lipofectamine. Cells in *B* were not transfected. Cells were fixed and permeabilized. Transfected cells treated with preimmune serum (preimmune negative control) (*A*) show only very pale fluorescence. Untransfected cells treated with the SCAP-1 antibody (preincubated with GST to eliminate any antibody reactivity that might be due to the GST portion of the fusion protein) (*B*) did not stain (untransfected negative control). Transfected cells showed immunofluorescence when treated with the SCAP-1 antibody (preincubated with GST) (*C*). Some cells stained very intensely (*arrow*), whereas others showed paler immunofluorescence. Immunofluorescence of transfected cells was blocked by preincubation of the SCAP-1 antibody with SCAP-1-GST fusion protein (blocked negative control) (*D*). Scale bar, 50 μm.

dicted by the sequence of the cloned channel (nonglycosylated size of 226 kDa) and with the large size of other Na^+ channel α-subunits. In addition, the antibody also recognized several additional, minor bands, also of >205 kDa, which may represent alternatively spliced versions of the protein. This interpretation is compatible with the presence of alternatively spliced Na^+ channels in *Aplysia* ganglia that we have detected by reverse transcription (RT)-PCR (W. Johnston, V. Castellucci, and R. Dunn, unpublished observations).

A second measure of antibody specificity was to determine antibody staining of cultured animal cells that were transiently transfected with an expression vector for the SCAP-1 cDNA. Vero cells were transfected with the SCAP-1 channel subcloned into pHermite, an expression plasmid in which transcription of the SCAP-1 cDNA is from the IE1 promoter of CMV. Figure 2 illustrates the binding of the SCAP-1 antibody to these cells. As can be seen in Figure 2*A*, preimmune serum did not stain transfected cells. Similarly, staining was not observed in untransfected cells (Fig. 2*B*) or in transfected cells treated with antibody that had been preincubated with SCAP-1-GST fusion protein ("blocked" treatment) (Fig. 2*D*). In contrast, when transfected cells were stained with the antibody, we observed very intense staining of some cells, as indicated by Figure 2*C* (*arrow*), and a lower level of fluorescence in other cells. Thus, although the transfected cells displayed varying levels of antigen expression, the antibody appeared entirely specific for cells in the transfected group. These results demonstrate that the SCAP-1 antibody was highly specific for the Na^+ channel when tested in transfected Vero cells.

Na^+ channel distribution in *Aplysia* ganglia

The *Aplysia* CNS is comprised of a series of paired or fused ganglia that are enclosed in a connective tissue sheath (Frazier et al., 1967). Ganglia are joined to each other by commissures and connectives and to the periphery by nerves. Within each ganglion, the monopolar neuronal cell bodies lie on a central neuropil and encompass it. The proximal neuronal processes, most of the synaptic connections, and probably the sites of action potential initiation are contained in the central neuropil (Tauc, 1962).

To examine Na^+ channel localization in the *Aplysia* CNS, we stained tissue sections from the abdominal or pleural/pedal/cerebral ganglia with the SCAP-1 antibody. As shown in Figure 3,

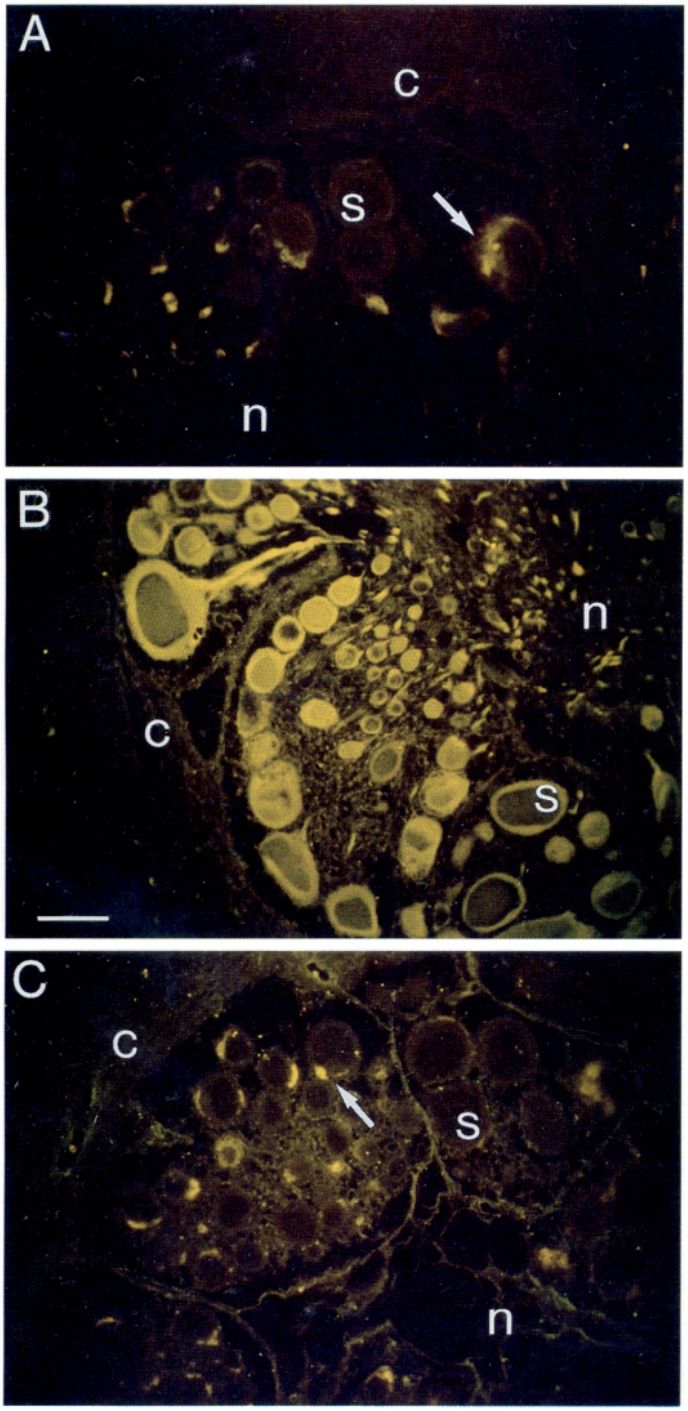

Figure 3. Aplysia ganglia sections treated with the SCAP-1 antibody demonstrate intense cytoplasmic staining of neuronal cell bodies. Paraffin-embedded sections (10 μm) were treated with (*A*) preimmune serum, (*B*) SCAP-1 antibody preincubated with GST, or (*C*) SCAP-1 antibody preincubated with SCAP-1-GST fusion protein. Background autofluorescent granules were present in the soma of some neurons (*arrow* in *A* and *C*). However, intense neuronal staining was seen only when sections were treated with the SCAP-1 antibody (*B*), and this staining was blocked by preincubation of the SCAP-1 antibody with the SCAP-1-GST fusion protein (*C*). In the section treated with the SCAP-1 antibody (*B*), neuronal somata (*s*) showed intense cytoplasmic staining. The neuropil (*n*), which contains the proximal neuronal processes, showed patchy staining. The non-neuronal connective tissue (*c*), which encompasses the ganglia, did not stain. Scale bar, 100 μm.

incubation of sections with either preimmune serum (Fig. 3*A*) or SCAP-1-GST fusion protein-blocked IgG (Fig. 3*C*) showed mainly a background of small, autofluorescent pigment granules in some neuronal cell bodies (*arrow*) (Chalazonitis and Arvanitaki, 1956; Brown and Brown, 1972) and very faint, diffuse staining of cellular and extracellular elements. In contrast, strong Na^+ channel immunofluorescence is observed in sections stained with the SCAP-1 antibody (Fig. 3*B*). Na^+ channels appear as a fairly homogeneous immunoreactive stain within the soma (indicated by *s*) and initial segment of these neurons. The nucleus of these cells appears as an area of weak or no fluorescence that almost fills the center of the cell soma. This pattern of staining suggests that these Na^+ channels are located predominantly intracellularly, rather than at the cell surface. The non-neuronal connective tissue ensheathing the ganglion (indicated by *c*) did not stain with the antibody (Fig. 3*B*), which is consistent with a lack of voltage-gated Na^+ channels in this tissue. In addition, there was no evidence of staining of glial cell bodies, which are often found associated with some neuronal somata. However, because glial cells are very small compared with the neurons and because there is no glial cell-specific marker available, we cannot conclude definitively that glial cells do not stain.

Na^+ channel clusters in *Aplysia* axons

The neuropil (*n*), which contains the proximal neuronal processes, demonstrated antibody-dependent staining (Fig. 3*B*) that was blocked by preincubation of the antibody with the SCAP-1-GST fusion protein (Fig. 3*C*). This staining was intense, but was also patchy, with some areas showing very little staining. In the neuropil, where neuronal processes are arranged in a nonlongitudinal manner, patchy staining could reflect neuronal processes coming in and out of the plane of the section. In addition, patchy staining could result from staining of only some neuronal processes, from foci of high Na^+ channel density, such as sites of action potential initiation, or from clusters of channels along the processes.

To examine the pattern of Na^+ channels in axons, tissue sections containing a nerve/connective (*ne*) were reacted with the SCAP-1 antibody. Axons within the nerve/connective showed intense, patchy staining with the antibody (Fig. 4*B*) that was blocked by preincubation of the antibody with SCAP-1-GST fusion protein (Fig. 4*C*). In these sections, which contain many axonal processes, the patches of Na^+ channel fluorescence occurred along the length of the nerve/connective. This pattern suggests that Na^+ channels occur as clusters in the *Aplysia* axon. Na^+ channel immunoreactivity was restricted to the axons themselves and was not observed in the adjacent connective tissue (*c*).

The sections shown in Figures 3 and 4*A–C* were obtained from paraffin-embedded tissue. To ensure that the clustered pattern of Na^+ channel immunoreactivity in axons was not an artifact of paraffin embedding, we also examined sections that were frozen and then sectioned in a cryostat. Figure 4*D* shows that the pattern was very similar in these cryostat sections; therefore, the patchiness did not result from the embedding process. The SCAP-1 antibody reacts with an intracellular segment of the Na^+ channel, and we were concerned that the clustered nature of the axonal staining could result from inadequate permeabilization. To examine the extent of permeabilization, sections were treated with an antibody to β-tubulin. As shown in Figure 4*E*, the β-tubulin staining shows continuous, fibrous staining of microtubules. Comparison of the pattern for β-tubulin with that observed for the Na^+ channel illus-

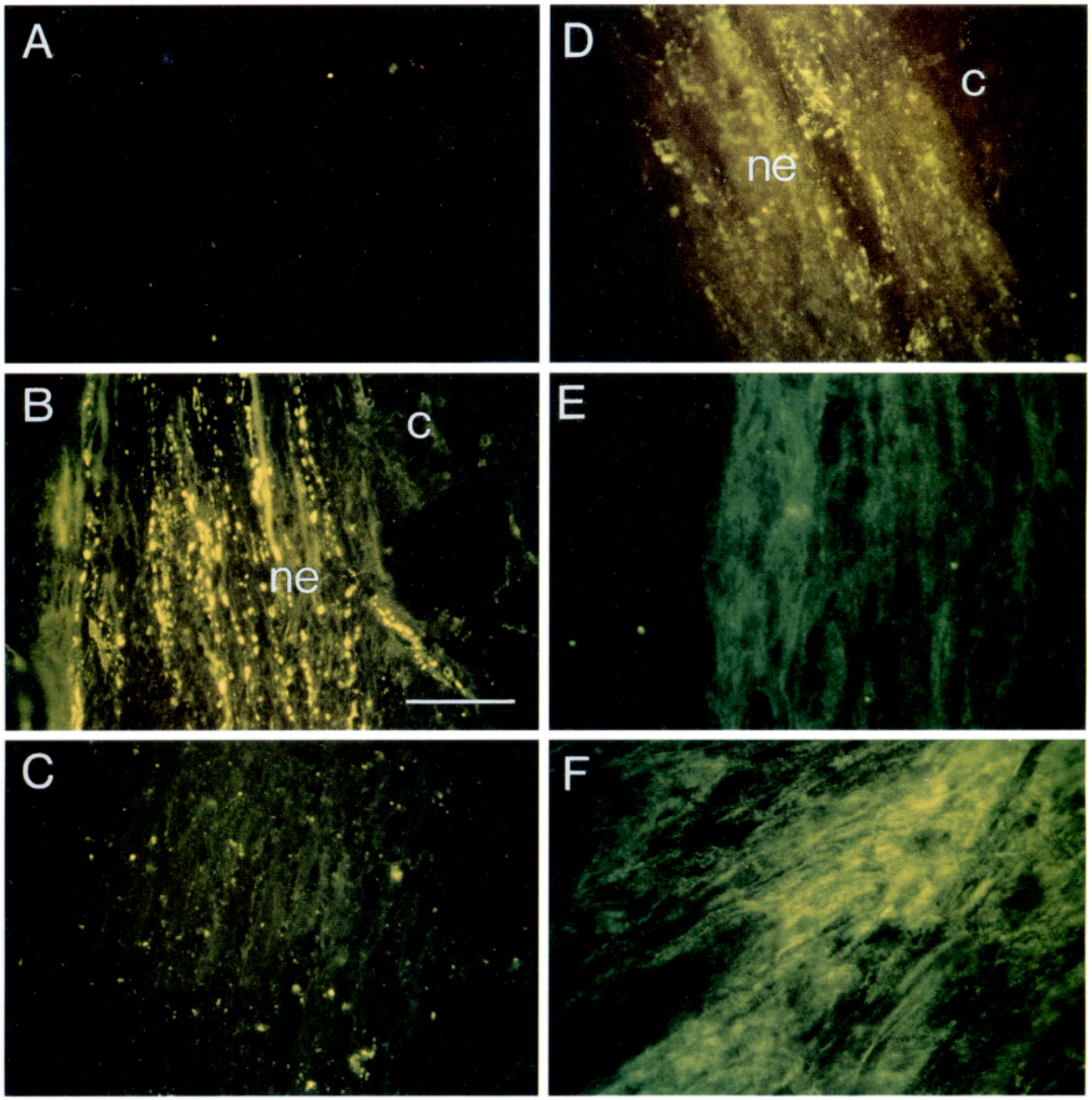

Figure 4. Na^+ channel immunoreactivity in *Aplysia* axons is intense and patchy. Paraffin-embedded (*A–C*) or cryostat (*D–F*) ganglia sections containing nerves/connectives were treated with preimmune serum (*A*), SCAP-1 antibody preincubated with GST (*B*, *D*), SCAP-1 antibody preincubated with SCAP-1-GST fusion protein (*C*), β-tubulin antibody (*E*), or Con A (*F*). Sections treated with preimmune serum (*A*) did not stain. Axons within the nerve/connective (*ne* in *B* and *D*) in sections treated with SCAP-1 antibody (*B*, *D*) showed intense, patchy staining. The connective tissue (*c* in *B* and *D*) encompassing the nerve/connective did not stain. The pattern of staining was very similar in paraffin-embedded (*B*) and cryostat (*D*) sections. Contrary to the patchy staining of Na^+ channels seen in *B* and *D*, staining for the intracellular protein, β-tubulin (*E*), or cell surface glycosylated residues (Con A) (*F*) was homogeneous. Scale bar, 50 μm.

trates clearly that the clustered appearance of these channels is not an artifact of inadequate permeabilization.

To determine whether the patchy pattern of staining observed for the Na^+ channel was a general property of the proteins within the axonal membranes, we used the lectin Con A to stain glycosylated (cell surface) residues. Con A staining is evident as a diffuse, continuous fluorescence on the surface of axons in these sections (Fig. 4*F*). Therefore, the patchy staining of the axons reflects a clustered distribution of Na^+ channels that is not simply a general property of most glycosylated residues on the axon surface.

Na^+ channel clusters in the neurites of cultured *Aplysia* neurons

Aplysia neurons that are cultured *in vitro* maintain normal *in vivo* electrophysiological behavior (Camardo et al., 1983; Rayport and Schacher, 1986). This suggests that Na^+ channel localization is preserved in *Aplysia* neurons in culture; therefore, this system may

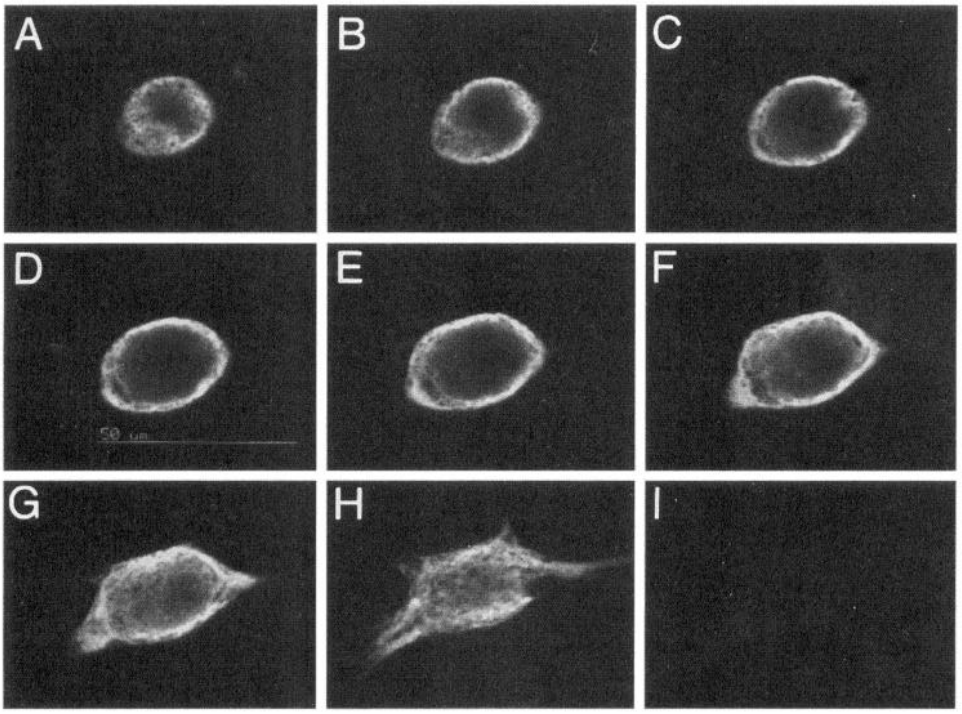

Figure 5. Confocal optical sections show intense cytoplasmic Na^+ channel immunoreactivity in the soma of a cultured sensory neuron. Sensory neurons from the abdominal ganglia of *Aplysia* were cultured for 7 d in L15 supplemented for marine species, with 10% *Aplysia* hemolymph added. After fixation and permeabilization, cells were treated with the SCAP-1 antibody, preincubated with GST. Nine 2 μm sequential optical sections are shown (*A–I*), ending with the section closest to the coverslip on which the cell was grown. Intense staining is seen in the cytoplasm, particularly in the perinuclear region. In *C–E*, the large, unstained nucleus is seen filling much of the center of the cell. Scale bar, 50 μm.

provide a valuable model in which to study Na^+ channel localization. In addition, because it is difficult to identify individual axons in sections of whole nerves/connectives, it may be useful to examine Na^+ channel distribution in cultured neurons, where individual neurites can be traced easily. Therefore, as an adjunct to our studies on Na^+ channel localization in ganglia, we have also examined Na^+ channel localization in cultured *Aplysia* neurons.

Sensory clusters of the abdominal ganglia were removed from adult *Aplysia*. Neuronal cell bodies were dissociated by protease treatment followed by trituration, and individual cells were plated onto poly-L-lysine-coated glass coverslips. Cells were grown for 7 d in L15, supplemented for marine species, with 10% *Aplysia* hemolymph added (Schacher and Proshansky, 1983). Similar to what was seen in ganglia sections (Fig. 3), in cultured cells the soma stained intensely only when antibody was present, and this staining was blocked by preincubation of the antibody with SCAP-1-GST fusion protein (data not shown). In cells treated with the antibody, the intracellular distribution of Na^+ channel immunofluorescence is illustrated in the series of confocal laser scanning images shown in Figure 5. These 2 μm optical sections are parallel to the surface of the slide and proceed from the top of the cell (*A*) to the surface of the microscope slide (*I*). A band of diffuse, uneven Na^+ channel immunofluorescence ~2–10 μm thick can be seen surrounding the nonreactive nucleus. At the bottom of the cell (sections *G* and *H*), the uneven, patchy distribution of Na^+ channel staining is most evident. This distribution is consistent with an endoplasmic reticulum and Golgi localization, which probably reflects active biosynthesis of Na^+ channels in these cultured neurons. In sections *G* and *H*, this staining can be seen to extend into the initial portions of axonal processes that develop on the cultured cells.

Neurites of cultured cells treated with preimmune serum showed only very pale autofluorescence (Fig. 6*A*). With the SCAP-1 antibody, staining was intense and patchy (Fig. 6*B*) and was blocked by preincubation of the antibody with SCAP-1-GST fusion protein (Fig. 6*C*). We also stained cultured cells with an antibody to β-tubulin and found that staining of neurites was homogeneous (Fig. 6*E*). Because the intense patches of Na^+ channel staining were often associated with areas where the neurite appeared to thicken ("varicosity-like" structures) (Fig. 6*B*,*D*, *arrows*), we also stained neurites with Con A, which recognizes cell surface residues and, therefore, should provide a good outline of the neurite. Even where varicosity-like structures were evident (Fig. 6*F*, *arrow*), staining by Con A was fairly homogeneous. Therefore, the intense patches of Na^+ channel staining are not simply a geometric consequence of neurite thickening. These results with sensory neurons suggest that the mechanism that acts to cluster Na^+ channels in *Aplysia* axons is maintained in culture.

DISCUSSION

We have studied the distribution of voltage-gated Na^+ channels in *Aplysia*. To do this, we raised an antibody against the cloned *Aplysia* Na^+ channel α subunit by injecting rabbits with a fusion protein consisting of GST in-frame with 220 amino acids of the C terminus of the cloned SCAP-1 *Aplysia* Na^+ channel. In immunoblots of *Aplysia* ganglia proteins, the antibody recognized one major band and several minor bands of a molecular size greater than 205 kDa. The size of these immunoreactive bands is consistent with the sequence of the cloned SCAP-1 cDNA (J. Dyer, W. Johnston, and R. Dunn, unpublished observations), which predicts a nonglycosylated size of 226 kDa, and with RT-PCR results in our laboratory that suggest the presence of a number of alternatively spliced versions of the SCAP-1 Na^+ channel in *Aplysia* ganglia (W. Johnston, V. Castellucci, and R. Dunn, unpublished observations). The specificity of the antibody was confirmed by staining mammalian cells transfected with an expression vector for the SCAP-1 cDNA. A subpopulation of the transfected cells stained with the antibody at variable intensities. Staining was evident throughout the cytoplasm except for the nucleus. These results show that the antibody recognizes the SCAP-1 polypeptide

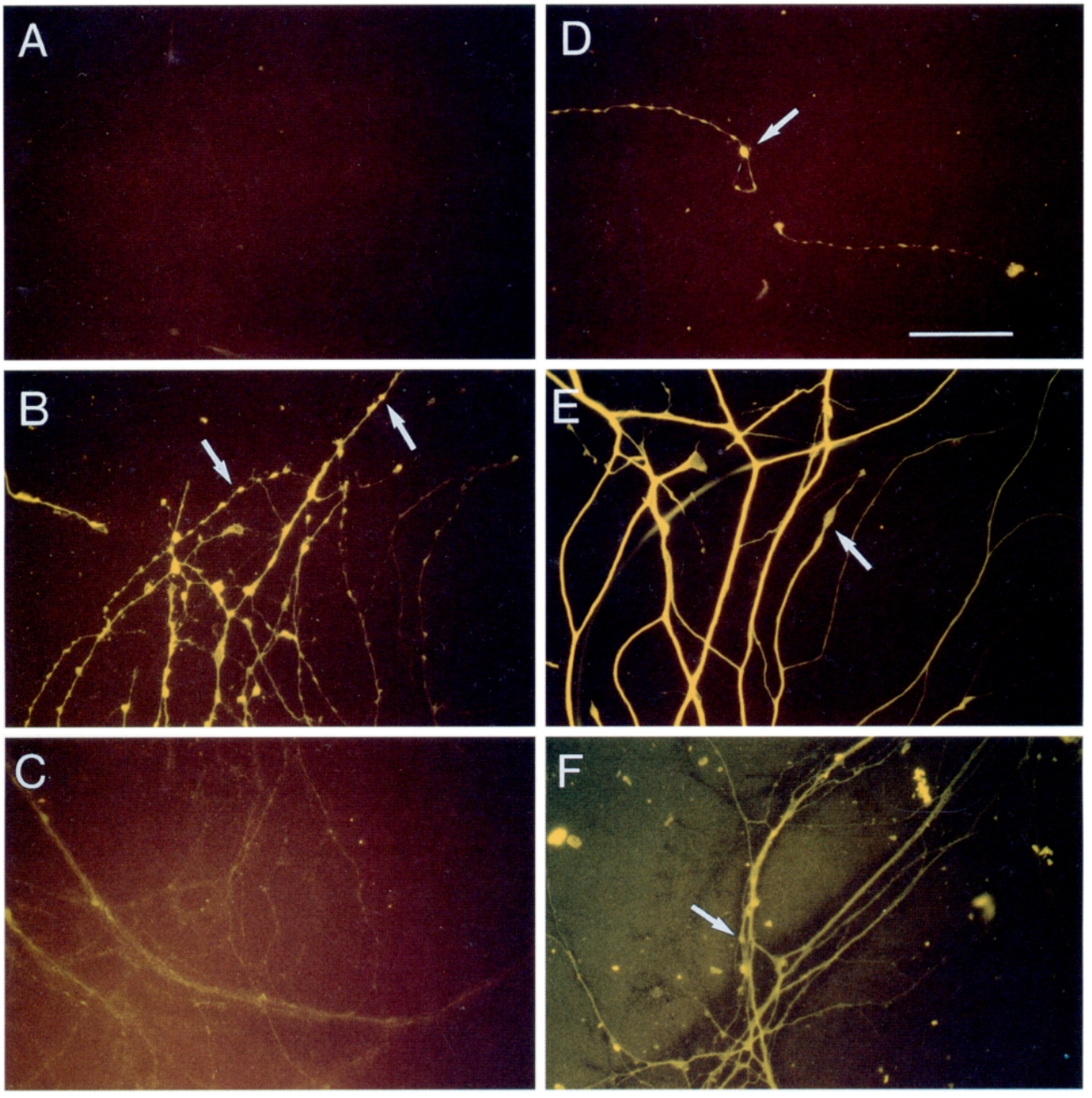

Figure 6. Na^+ channels are clustered in the neurites of cultured *Aplysia* sensory neurons. Sensory neurons were cultured as described in Figure 5 and treated with preimmune serum (*A*), SCAP-1 antibody preincubated with GST (*B*, *D*), SCAP-1 antibody preincubated with SCAP-1-GST fusion protein (*C*), β-tubulin antibody (*E*), or Con A (*F*). Neurites of cells treated with preimmune serum (*A*) or SCAP-1-GST fusion protein-blocked SCAP-1 antibody (*C*) showed only very pale staining. Neurites of cells stained with the SCAP-1 antibody (*B*, *D*) showed intense patchy staining. In some cases, these patches appeared to be associated with areas of neurite thickening (*arrows* in *B* and *D*). Staining for β-tubulin (*E*) or glycosylated residues (Con A) (*F*) was homogeneous even in areas where the neurites appeared to thicken (*arrow* in *E* and *F*). Scale bar, 50 μm.

and that, when reacted with a mammalian cell line, it does not cross-react strongly with other proteins.

Neuronal somata contain a large pool of Na^+ channels

The reactivity of the antibody to native *Aplysia* Na^+ channels was determined by staining sections of *Aplysia* ganglia. Strong signals were observed only in neurons. These signals were blocked by competition with the SCAP-1-GST fusion protein but not with GST alone. Thus, the antibody is specific for the Na^+ channel portion of the fusion protein. In these sections, the neuronal somata showed intense cytoplasmic Na^+ channel immunoreactivity, indicating the presence of a large cytoplasmic pool of Na^+ channels. This is similar to that which has been suggested to occur in the squid stellate ganglion giant fiber lobe (Brismar and Gilly, 1987), in the cockroach mesothoracic ganglion (French et al., 1993), and in developing rat brain (Schmidt et al., 1985). Because biosynthesis of most axonal proteins occurs primarily within the cell body (Hammerschlag et al., 1982; Lodish et al., 1995), a large pool of Na^+ channels may represent an important source of channels for the axon. Confocal microscopy of cultured abdominal ganglion sensory neurons confirmed intense cytoplasmic stain-

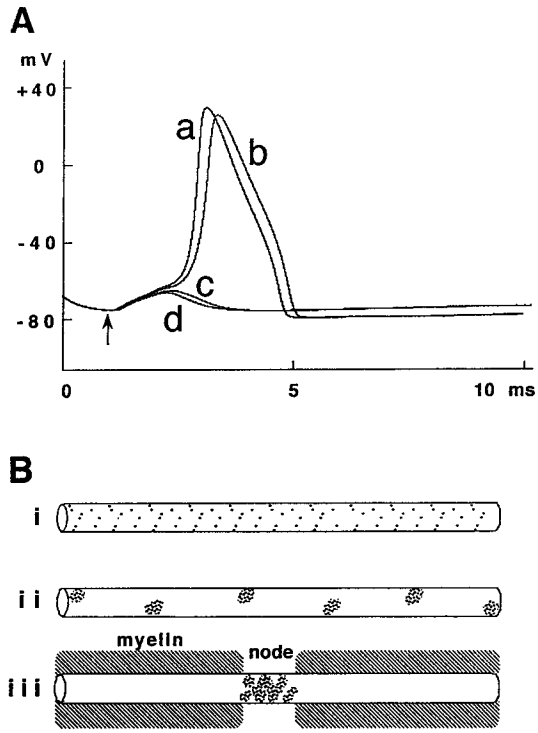

Figure 7. Model predicts that clustering of Na^+ channels decreases the number of channels required for action potential conduction. *A*, Action potential conduction was modeled using Nodus (DeSchutter, 1989). In these traces, a Hodgkin and Huxley delayed rectifier K^+ conductance of 200 mS/cm^2, a leak conductance of 0.3 mS/cm^2, and Hodgkin and Huxley fast Na^+ conductances of 250 mS/cm^2 (*trace a*), 150 mS/cm^2 (*trace b*), 200 mS/cm^2 (*trace c*), and 100 mS/cm^2 (*trace d*) were used. Resting potential was set at −70 mV, membrane capacitance at 1.00 μF/cm^2, membrane resistance at 10.0 k$\Omega \cdot$ cm^2, and cytoplasmic resistance at 100 $\Omega \cdot$ cm. The unitary compartment was a 5-μm-long cylinder of 2 μm diameter, and the total number of compartments was 50. Cluster:intercluster distance was 1:9. *Arrow* indicates the start of a 1-msec-long 0.5 nA current. *Trace a* shows that when Na^+ channels are evenly distributed a Na^+ channel conductance of 250 mS/cm^2 is sufficient for action potential conduction; however, conduction fails if the current is decreased to 200 mS/cm^2 (*trace c*). If, however, the Na^+ channels are clustered, a Na^+ conductance of 150 mS/cm^2 (resulting in a localized conductance of 1250 mS/cm^2 at clusters) is adequate to support action potential conduction (*trace b*). Conduction in the clustered axon does not fail until Na^+ channel conductance drops to 100 mS/cm^2 (resulting in a localized conductance of 833 mS/cm^2 at clusters) (*trace d*). In this simulation, clustering required 40% fewer channels for action potential conduction. *B*, Model of how Na^+ channel clustering decreases the number of channels required for action potential conduction in the unmyelinated axon (*i*, *ii*) and how myelination (*myelin*) may help to exclude these clusters from the internodal region, resulting in widely spaced, higher-density clusters (*nodes*) (*iii*). In *i*, Na^+ channels are not clustered and action potential conduction fails. In *ii*, the same number of Na^+ channels is sufficient to conduct action potentials when they are clustered. In *iii*, myelination excludes the clusters from the internodal region, which facilitates "clustering of the clusters," resulting in a greater distance between clusters (*nodes*) together with a higher density at these areas.

ing, particularly in the perinuclear region. This staining pattern suggests that many of the immunoreactive, cytoplasmic Na^+ channels are located within the biosynthetic compartments (Golgi/endoplasmic reticulum). Although the cytoplasmic pool may also contain channels destined for degradation, the absence of a granular staining pattern suggests that the bulk of the immunoreactive protein is not within a lysosomal compartment.

In most of the neurons visible in the ganglion sections, staining of the initial segment of axon was approximately as bright as staining of the corresponding cell body, suggesting that the initial segment does not have an increased membrane Na^+ channel density relative to the cell body. Part of the explanation for this finding may be that, like the cell body, much of the staining in the initial segment is cytoplasmic. However, there is also electrophysiological evidence that the plasma membrane Na^+ channel density of the initial segment of *Aplysia* axons is not very different from that of the cell body. For example, both the soma and the axon can be excitable, and the threshold of the initial segment, although lower than the soma, is still high relative to the remainder of the axon. Furthermore, the slightly lower threshold of the initial segment (compared with the soma) may reflect its lower capacitance, compared with the soma, rather than a higher density of Na^+ channels. Therefore, it is likely that the initial segment of *Aplysia* axons does not have an increased density of plasma membrane Na^+ channels. This is in contrast to vertebrates, in which a high density of Na^+ channels in the initial segment facilitates action potential initiation. The most likely explanation for this difference between vertebrates and *Aplysia* is that action potentials in *Aplysia* neurons initiate much more distally in the axon (within the neuropil), rather than in the initial segment (Tauc, 1962).

Na^+ channels are clustered in Aplysia *axons*

In the nerves/connectives of ganglia sections, axonal Na^+ channel staining was patchy. A similar pattern was observed in individual neurites extending from cultured neurons. Although these experiments do not allow us to define precisely the distance between clusters, we estimate that in both the ganglia and in the cultured neurons, the distance is on the order of 5–15 μm. This is similar to the distance between Na^+ channel clusters in pyramidal cell dendrites of *Apteronotus* (5–15 μm) (Turner et al., 1994) and between clusters in frog sartorius muscle (10–20 μm) (Almers et al., 1983).

Because our antibody recognizes an intracellular epitope of the Na^+ channel, permeabilization was necessary to allow visualization by immunohistochemistry. Therefore, the Na^+ channel clusters we describe may be localized in the plasma membrane, in the cytoplasm, or in both. A possible role for cytoplasmic Na^+ channel clusters might be as a ready supply of preformed channels. In this case, cytoplasmic Na^+ channel clusters, associated with the cytoskeleton or within transport vesicles, may be concentrated near areas of the plasma membrane where Na^+ channel density or turnover is elevated. Consistent with this, at the nodes of Ranvier there is an increased density of both membrane Na^+ channels and, at least at some nodes, of cytoplasmic channels (Ritchie and Rogart, 1977). In addition to providing a supply of channels destined for insertion into the plasma membrane, cytoplasmic clusters of Na^+ channels could also contain internalized channels destined for degradation.

Na^+ channel clustering may optimize action potential conduction

In addition to a cytoplasmic localization, the clusters of Na^+ channels may be in the plasma membrane. The physiological significance of clustering of Na^+ channels in the membranes of *Aplysia* and other unmyelinated axons is not known. One possibility is that, even in the absence of myelin as an insulator, clustering may be a way of distributing Na^+ channels to speed conduction. In support of this idea, in demyelinated preparations

that conduct action potentials Na^+ channels are not homogeneously distributed along the previously internodal region but, rather, are present in distinct clusters, where they may support slow saltatory conduction (Smith et al., 1982; England et al., 1990). Thus, although myelin is necessary for increasing the speed of saltatory conduction, slower saltatory conduction may still occur in its absence.

Another possibility is that clustering decreases the number of Na^+ channels required for conduction of action potentials. To investigate this possibility, we constructed a preliminary model of the effect of Na^+ channel clustering on action potential conduction (Fig. 7). This model predicts that, across a broad range of K^+ and Na^+ conductances (K^+ channel conductance of 100-1000 mS/cm^2, Na^+ channel conductance of 50–7000 mS/cm^2), 30–60% fewer Na^+ channels are required for action potentials to propagate if the channels are clustered (Fig. 7*A*). Therefore, clustering of Na^+ channels may enable the axon to economize on the number of Na^+ channels required to support action potential conduction.

Na^+ channels are clustered at the nodes of Ranvier in vertebrate myelinated axons (Ritchie and Rogart, 1977; Ellisman and Levinson, 1982). However, the role of glial cells and myelination in Na^+ channel clustering is controversial. Dugandzija-Novakovic et al. (1995) presented evidence that clustering in remyelinating rat sciatic axons requires Schwann cells. However, saltatory conduction preceded remyelination in lysophosphatidyl choline-treated preparations (Smith et al., 1982), and punctate Na^+ channel immunoreactivity developed in the absence of Schwann cells in goldfish nerve demyelinated with doxorubicin (England et al., 1990). Therefore, Na^+ channels may cluster in the absence of myelin and/or glial cells. In addition, although the axons may be atypical, because they do not conduct action potentials over long distances, there is also evidence that Na^+ channels are clustered in a vertebrate unmyelinated axon (retinal nerve fiber layer) (Hildebrand and Waxman, 1983). We now show that Na^+ channels are clustered in the (unmyelinated) axons of an invertebrate ganglion and in the neurites of (glial-free) sensory neurons in culture. Therefore, in the invertebrate *Aplysia*, clustering of Na^+ channels appears to be independent of both myelination and glial cell contact. Based on our results and on the studies discussed above, we hypothesize that in many axons, and perhaps in many membranes that conduct Na^+-dependent action potentials, the default distribution of Na^+ channels is clusters ~5–20 μm apart. In the case of myelinated axons, in addition to increasing membrane resistance, myelination may superimpose a longer intercluster distance (>100 μm) and may help to increase the nodal Na^+ channel density by exclusion of clusters from the internodal region (Fig. 7*B*). When axons are demyelinated, Na^+ channel distribution may revert to a shorter intercluster distance (England et al., 1990) that persists until remyelination occurs.

In summary, in this study we have shown that Na^+ channels in the unmyelinated axons of *Aplysia californica* are present in discrete clusters. We hypothesize that this distribution may be important for optimizing action potential conduction and that Na^+ channel clustering may be a general property of many axons.

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