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Synthesis of sodium channels in the cell bodies of squid giant axons

(membranes/proteins/neurons/microtubules/axotomy)

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ABSTRACT **Giant axons in squid are formed by fusion of axons from many small cell bodies in the giant fiber lobe (GFL) of the stellate ganglion. Somata of GFL cells *in vivo* are inexcitable and do not have measurable sodium current (I_{Na}) when studied with microelectrode or patch-electrode voltage-clamp techniques. If GFL cells are separated from the giant axons and maintained in primary culture, axon-like I_{Na} can be recorded from the somata after several days. Incorporation of Na channels into GFL cell bodies requires protein synthesis, intracellular microtubule-based transport, and the lack of a morphologically defined axon to serve as a sink for channels synthesized in culture.**

Voltage-controlled Na channels are integral membrane glycoproteins that mediate the electrogenic flow of extracellular Na ions into animal cells and subserve the propagation of action potentials used by neurons for rapid signalling via axons (1). Functional aspects of these proteins (2, 3) and primary structure (4, 5) are known in detail; biochemical (1, 6) and higher-order structural (7) characterization are rapidly progressing. Considerably less is known about the biosynthesis, distribution, and degradation of Na channels, and studies of these phenomena have utilized biochemical (8, 9) and morphological (10) approaches.

Voltage-clamp methods provide another excellent probe for detecting functional Na channels because of the high degree of resolution provided (11, 12). Such measurements have been used to map Na channel distribution in skeletal muscle fibers (13, 14) and to assess the mobility of channels in the plasma membrane (15). We have undertaken an electrophysiological approach to begin studying the dynamic features of Na-channel cell biology in the numerous cell bodies in the giant fiber lobe (GFL) of the squid's stellate ganglion, which give rise to the giant axon through fusion of their axonal initial segments (16). This system is morphologically simple, experimentally accessible, and the axon has been the subject of extensive investigation (17).

Although the many small GFL somata must normally synthesize Na-channel proteins for the giant axons (18), they are inexcitable themselves (19). We find that if these cell bodies are dissociated from the giant axons and maintained in primary culture, Na current (I_{Na}) appears within several days. This condition persists for at least 2 weeks and is inhibited by actinomycin D or by demecolcine, a colchicine derivative. Our results suggest that an important step leading to the appearance of operational Na channels in the soma membrane involves intracellular translocation of newly synthesized channels along microtubule pathways.

MATERIALS AND METHODS

Cell bodies (diameter, 20–40 μm) were isolated from the posterior tip of the giant fiber lobe of stellate ganglia (16, 20) in *Loligo opalescens* and *Lolliguncula brevis* (21) and maintained in primary culture at 15°C essentially as described by Bookman and coworkers (22, 23). Excised GFL tips were treated with nonspecific protease (10 mg/ml, type XIV; Sigma) for 45–60 min at room temperature (18°C–22°C) and cultured without mechanical dispersion. Axon-less cell bodies were isolated on the day of electrophysiological study by manually passing the cultured tissue through an air/water interface and collecting the cells, which were thereby released. Normally, ganglia tips were treated with protease on the day of excision (day 0), but enzyme treatment could be carried out at any time during culture with no deleterious effects on the amount or properties of I_{Na} present.

Whole-cell recordings used a tightly sealed single electrode (24) and a List EPC-7 voltage clamp (Medical Systems, Garden City, NY) operating at low gain with series resistance compensation adjusted for 50–70%. Signals were filtered at 10 kHz and digitized at 10 or 50 μsec per point. Linear ionic and capacity currents were removed by subtracting negative going control pulses, which were 1/4th the size of test pulses and delivered from the holding potential (−80 mV). Electrodes were ≤1 MΩ in resistance when filled with the standard internal medium: 100 mM sodium glutamate/300 mM tetramethylammonium glutamate/50 mM NaF/50 mM NaCl/10 mM Na_2-EGTA/25 mM tetraethylammonium chloride/10 mM Hepes. The standard external solution for measuring I_{Na} contained: 470 mM NaCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 10 mM Hepes. CsCl (10 mM) was sometimes included to eliminate small inward currents through K channels. Actinomycin D and demecolcine (*N*-deacetyl-*N*-methyl colchicine) were obtained from Sigma. Experiments were carried out at 10°C–12°C.

RESULTS

Intracellular microelectrode recordings have indicated that GFL cell bodies *in situ* are inexcitable (19) and probably have a much lower Na channel density than that in their daughter giant axons. We have confirmed this finding by using a two (intracellular) microelectrode voltage clamp and intact ganglia (i.e., manually desheathed and with giant axons ligated). Resting potentials (measured with a 3 M KCl-filled microelectrode) ranged from −40 to −55 mV. Inward currents were not observed in eight cells studied at a holding potential of −70 to −80 mV.

A more critical test for I_{Na} is provided by whole cell recordings from enzymatically cleaned cells. Thus, Bookman and coworkers (22, 23) in developing the GFL cell preparation detected inward I_{Na} in isolated somata of only 0.1% the density found in the giant axon (cf. ref. 25 for axon properties). Fig. 1*a* shows records from a freshly isolated (day 0) GFL soma in an experiment for which 110 mM K was

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Abbreviation: GFL, giant fiber lobe.

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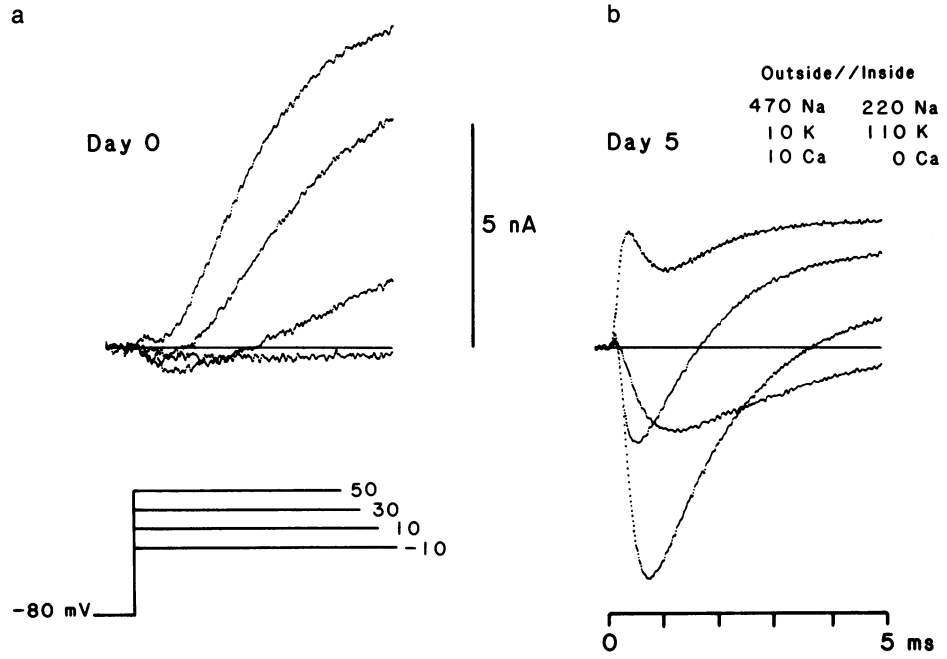

FIG. 1. Changes in currents recorded from cell bodies of GFL neurons (*Loligo*) with time in primary culture. (*a*) Small inward and large outward currents are present on the day of isolation from the stellate ganglion (day 0). When normalized to cell capacitance (165 pF), the largest outward current has an amplitude of 0.04 nA/pF (1 nA/pF approximates 1 mA/cm^2). Voltage steps used are shown below. (*b*) Large transient inward and outward currents are present by day 5 of culture. Maintained outward current for the pulse to +50 mV has an amplitude of 0.09 nA/pF. Same voltage steps used as in *a*. For these experiments, 110 mM K was included in the internal medium (tetramethylammonium was reduced on an equimolar basis) and tetraethylammonium was omitted to reveal outward K currents.

included in the internal medium and tetraethylammonium was omitted. As reported (22, 23), the only apparent currents are small inward currents through Ca channels (see also below) and large outward K currents (I_K). I_K in the GFL soma is similar to that in the giant axon, but it is present at only ≈10% the density found in the axon (26).

In contrast, another cell from the same ganglion that had been cultured for 5 days showed large transient currents (inward and outward) when studied under otherwise identical conditions (Fig. 1*b*). I_K remains basically unaltered in form and amount when normalized to cell capacitance (see legend to Fig. 1 and Table 1). The dramatic change in the pattern of currents between day 0 and day 5 reflects the appearance of Na channels.

Fig. 2 identifies currents on day 0 vs. day 6. The K-free internal medium contained tetraethylammonium, and I_K is therefore absent. Voltage steps used are shown in Fig. 2*a*. Fig. 2 *b–d* shows that the small inward currents present on day 0 are related to Ca and not Na channels: (*i*) Inward currents activate fairly slowly and do not inactivate during a 30-msec pulse (Fig. 2*b*). (*ii*) They are unaffected by a Na-free external medium (Fig. 2*c*). (*iii*) They are abolished by 10 mM $ZnCl_2$ applied externally (Fig. 2*d*). Finally, they are increased in amplitude by raising external Ca and are unaffected by 1 μM tetrodotoxin (not shown).

Nearly all current recorded on day 6 is identifiable as I_{Na} with properties similar to axonal I_{Na}: (*i*) Large inward and outward currents rapidly activate and inactivate during a 5-msec pulse when both internal and external media contain Na (Fig. 2*e*). (*ii*) A Na-free external medium eliminates the transient inward current and increases outward current (Fig. 2*f*). (*iii*) Tetrodotoxin (100 nM) abolishes the transient currents (Fig. 2*g*). The small inward current persisting in tetrodotoxin, like that on day 0, is blocked by Zn (Fig. 2*h*) and is Ca-channel related.

Fig. 3 establishes the time course over which I_{Na} develops in culture. Mean (± SEM) values of Na conductance normalized to cell capacitance (G_{Na}, see legend to Fig. 3) are plotted vs. time in culture (●). Functional Na channels are not detectable on day 0. A small amount of G_{Na} appears after 1 day; by 3 days G_{Na} increases dramatically and then remains elevated for at least 2 weeks. The absolute amount of Na conductance in day 9–13 cells is directly proportional to cell surface area as estimated by capacitance measurements (data not shown), and G_{Na} at this time is 10–15% of that in the giant axon. As indicated in Table 1, potassium conductance (G_K) does not increase during culture, nor does calcium conductance as suggested by Fig. 2.

The exact time course of G_{Na} development in cultured GFL neurons depends on at least two factors, which we have identified. First, the species of squid appears to matter. In GFL cells from *L. brevis*, a warm-water species (21), G_{Na} increases considerably more slowly in culture at 15°C than it does in *L. opalescens* (Fig. 4). Second, mechanical dispersion of cell bodies from *L. opalescens* ganglia on day 0 apparently retards the subsequent development of G_{Na} in culture so that the time course would resemble that of *Lolliguncula*. Both effects may help explain why only very small I_{Na} was previously reported in dispersed GFL cells from *Loligo pealei* (23).

If GFL neurons are cultured in the presence of actinomycin D (50 μg/ml), an inhibitor of mRNA synthesis, the initial appearance of an increased G_{Na} (e.g., on day 3) is unaffected [Fig. 3 (○)], but G_{Na} then rapidly declines over the next several days. This strongly suggests (*i*) that continual synthesis of Na-channel proteins is necessary to support a high G_{Na} and (*ii*) that the initial appearance of G_{Na} in the presence of actinomycin reflects the insertion of channels, which were already synthesized or being synthesized from existent mRNA on day 0. This population of channels then disappears with a time constant of ≈1.2 days. How reliably this can be equated with the degradation rate for Na channels themselves depends on the unknown lifetime of the day 0 mRNA.

Another way to block the appearance of Na channels in cultured GFL cells is to include the colchicine derivative demecolcine in the culture medium (see Table 1). The low

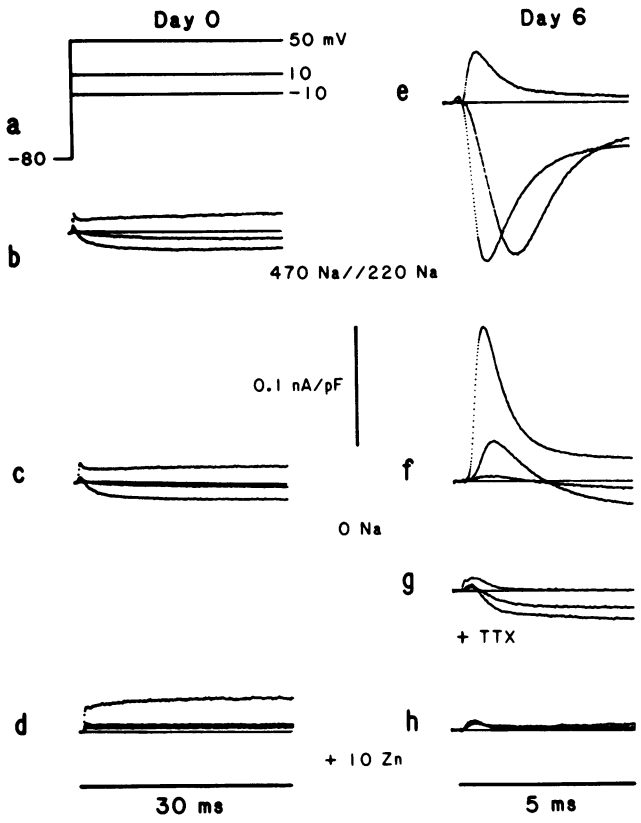

FIG. 2. Identification of Na current in cultured GFL neurons (*Loligo*). (*a*) Voltage steps shown apply to all panels, but note the difference in time scales for the day 0 cell (*b–d*) vs. the day 6 cell (*e–h*). For these experiments, standard internal and external media for measuring I_{Na} were used (see text). (*b* vs. *e*) Recordings made in the presence of internal and external Na reveal the development of large inactivating currents by day 6 of culture. (*c* vs. *f*) A Na-free external medium has no effect on day 0 but eliminates the transient inward current on day 6. (*g*) Tetrodotoxin (100 nM) blocks all transient current on day 6; it has no effect on day 0 (not shown). (*d* vs. *h*) $ZnCl_2$ (10 mM) applied externally abolishes inward current on day 0 and the tetrodotoxin-resistant current on day 6. Current for the pulse to +50 mV in *h* is not shown.

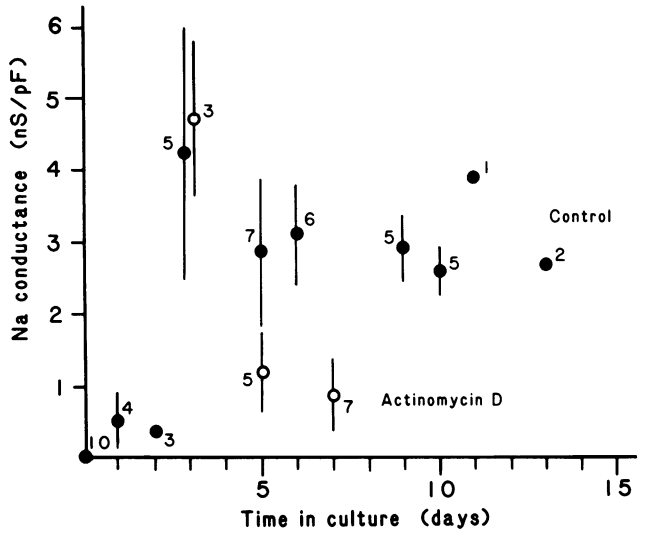

FIG. 3. Time course of G_{Na} development in the somata of cultured GFL neurons (*Loligo*) and the effect of protein synthesis inhibition. Mean values of Na conductance normalized to cell capacitance are plotted vs. time in culture for control conditions (●) and for cells exposed continuously to actinomycin D at 50 μg/ml (≈40 μM) (○). Error bars indicate ±1 SEM; numbers of cells studied are indicated. Standard internal and external media for measuring I_{Na} were used throughout. G_{Na} was estimated as $I_{Na}/(V - V_{Na})$ where V_{Na} is the I_{Na} reversal potential estimated by comparing *I–V* curves recorded with and without inactivating prepulses (50 msec to 0 mV) and/or before and after external application of 0.1–1 μM tetrodotoxin.

effective concentration range of this drug suggests that its site of action involves microtubules and fast axoplasmic transport (27, 28) rather than the channels themselves (29). Thus, intracellular microtubule-based transport of Na channels is probably a prerequisite to insertion in the soma membrane (30, 31).

DISCUSSION

Cell bodies of the giant fiber lobe of the stellate ganglion in squid do not have Na channels in their soma membrane *in vivo*, yet these cells must be the manufacture site of all or most of the Na channels for the giant axons. Although squid axons have been reported to be capable of both receiving newly synthesized proteins from their surrounding Schwann cells (32) and of limited protein synthesis themselves from axonal mRNA (ref. 33, but see also ref. 34), contributions from these alternative sources, if relevant at all to Na-channel trafficking, are probably minor. Results described in this paper indicate that GFL cells appear to be synthesizing Na channels on the day of isolation, continue to do so in culture, and use a colchicine-sensitive step in the incorporation of apparently normal channels into the soma membrane.

If GFL cells have the ability to synthesize and process Na channels, why are no Na channels present in the soma membrane *in vivo*? One possibility is that the ongoing rate of channel production (including mRNA transcription and translation, subsequent channel processing, and insertion into surface membrane) is highly accelerated by axotomy, since cultured GFL cell bodies have been separated from their daughter axons by excision and/or protease treatment on day

Table 1. Levels of G_{Na} and G_K (mean ± 1 SEM) in GFL cells (*Loligo*) grown in primary culture under the conditions indicated

Culture Condition	Days	G_{Na}, nS/pF	G_K, nS/pF
Control	0–2	0.28 ± 0.14 (19)	2.3 ± 1.1 (7)
	3–4	4.2 ± 1.7 (5)	1.6 ± 0.37 (10)
	6	3.1 ± 0.70 (6)	1.2 ± 0.16 (8)
	10	2.6 ± 0.34 (5)	0.90 ± 0.25 (6)
Demecolcine			
50 μg/ml	3	0.14 ± 0.14 (3)	0.57 ± 0.32 (3)
10 μg/ml	4	0.31 ± 0.26 (3)	0.73 ± 0.12 (3)
1 μg/ml	4	1.3 (1)	0.75 (1)
Intact ganglion	4	0.0 ± 0.0 (4)	2.36 ± 0.30 (4)
Control	4	3.1 ± 1.5 (4)	1.0 ± 0.21 (4)
Intact ganglion	7–8	0.17 ± 0.05 (8)	0.35 ± 0.14 (8)

Control conditions and method for determining G_{Na} are described in the text. Demecolcine was added to the control medium on day 0 of culture (1 μg/ml, ≈30 μM). Number of cells studied is indicated in parentheses. G_K (I_K per 100 mV) was estimated from the outward current (I_K) accompanying a pulse to +20 mV from −80 mV recorded within 2 sec after establishing the whole-cell recording mode by rupturing the membrane under the tightly sealed pipette containing the K-free medium used to measure I_{Na}. A single control pulse of −25 mV was scaled and used to remove linear leakage current. Intact ganglia were prepared as described in the text. Day 4 cells received 5 min of protease treatment on day 0 and 30 min on day 4 before study. Day 7–8 cells received no enzyme treatment until the day of study. Control cells for day 4 were cultured in an excised GFL tip in the same dish as the day 4 intact ganglion and received identical protease treatment.

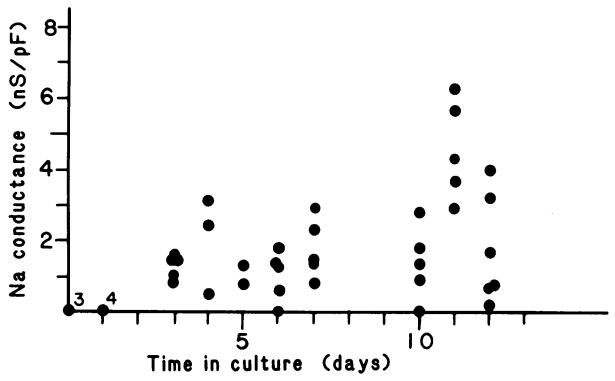

FIG. 4. Development of G_{Na} over time in cultured GFL cells from *Lolliguncula*. Each point represents one cell, except the day 0 (n = 3) and day 1 (n = 4) points.

0 (35). The fact that the initial appearance of Na channels in culture is unaffected by actinomycin 3 days after its application argues against increased mRNA and protein synthesis.

A highly stimulated synthesis rate also is unlikely when one considers what the normal demand for GFL cell-produced Na channels by the giant axons might be. A single GFL cell in culture has ≈200,000 Na channels in steady state (assuming a density of 37 channels per μm^2, ≈15% of that in the axon). Assuming a 1.2-day degradation time constant (Fig. 3), the ongoing synthesis rate in one cell would be ≈7000 channels per hr. These degradation and synthesis rates are in good agreement with those obtained for much smaller neuroblastoma cells in culture ($t_{1/2}$, 26 hr and 1700 saxitoxin receptors per hr, respectively; ref. 8). If 300 GFL cells (sphere diameter, 40 μm) together form one giant axon (diameter, 300 μm; length, 10 cm), then each GFL cell must support the Na-channel population in an axonal area ≈60 times that of its own soma (36). Furthermore, since axonal G_{Na} is 6-fold higher than the maximal G_{Na} expressed in GFL cells, then at steady state *in vivo* a GFL cell would support 360 times the maximum number of Na channels that it can insert into its own soma membrane in culture. If Na channels in the giant axon are degraded as rapidly as in the soma, then the true synthesis rate of which the GFL cell is capable would be 360 times higher than the rate observed in culture, or about 2.5 million channels per hr per cell.

These figures are only meant to be rough indicators, but they do serve to show that GFL cells in culture may be inserting only a small fraction of the Na channels synthesized into their soma membrane. The missing channels are presumably located in intracellular membranous compartments (cf. ref. 9).

Another possibility for the normal absence of Na channels in GFL cells and for their inappropriate appearance in culture is that prior to axotomy all Na channels synthesized are preferentially transported, presumably via microtubule-based axoplasmic transport (28), out of the somata to the giant axons where they are utilized. In consonance with this idea, we find that I_{Na} does not appear in GFL cells cultured in whole intact ganglia in which all giant axons are ligated and no enzymatic treatment (or very little—e.g., 5 min) is used until the day of electrophysiological study (see Table 1). Controls for these experiments utilized an excised GFL tip with the identical protease schedule as the day 4 intact ganglion. These axotomized (but not protease treated) control cells developed I_{Na} in culture normally. The control data also show that protease treatment is neither the cause of the absence of Na channels on day 0 nor the stimulus that triggers Na-channel production in culture.

It is thus possible the GFL cells cultured in intact ganglia that have not been axotomized continue to synthesize Na channels and to transport them along microtubules down their unfused initial axon segments to the large sink provided by the giant axons. In axotomized GFL neurons, which do not regenerate a definable axon under our culture conditions, the established microtubule pathways may simply become misdirected and "mistakenly" transport a fraction of the newly synthesized channels to the soma membrane where they can be inserted and function perfectly well. As discussed above, this fraction may be a small one.

Although this simple hypothesis may qualitatively account for our observations concerning G_{Na} in cultured GFL cells, several quantitative data demand additional explanation. G_{Na} does not increase continuously with time but reaches a limiting value of only ≈15% of that found in the giant axons. A similar relatively low value for G_K exists in normal GFL cell bodies and does not increase in culture. What factors set these limits for each channel type in the soma and axons of these neurons remains an important question. One unconventional possibility worth considering is that Na-channel synthesis by a cultured GFL cell is actually inhibited by the inappropriate development of G_{Na} in the soma membrane following axotomy. Such regulation of channel protein synthesis could be an important factor in normal neuron function.

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