Sodium-channel turnover in rabbit cultured Schwann cells

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Radiolabelled saxitoxin has been used as a chemical marker for the voltage-dependent sodium channels expressed in the plasmalemma of rabbit Schwann cells in culture. Proteolytic enzymes destroy this saxitoxin-binding capacity, which gradually reappears with an exponential time constant of about 3.1 days. Exposure of cultured Schwann cells to tunicamycin, an inhibitor of glycosylation, leads to a progressive exponential fall in saxitoxin-binding capacity, again with a time constant of about 3.1 days. The assumption that the steady-state density of Schwann cell sodium channels is maintained by a constant synthesis of channels in the face of a rate of loss from the membrane proportional to the amount of channel already present, leads to the conclusion that these channels have an average lifetime of about 3.1 days. The metabolic consequences of this rapid turnover of Schwann cell sodium channels is discussed.

Introduction

Rabbit Schwann cells in culture express plasmalemmal voltage-dependent sodium channels (Shrager et al. 1985) whose electrophysiological properties are similar to those described for mammalian axolemmal channels (Chiu et al. 1979). Indeed, it has been clear for some years that most of the sodium channels in a mammalian myelinated peripheral nerve trunk are associated with the satellite cell rather than with the axon itself (Ritchie & Rang 1983; Ritchie 1986). Rat astrocytes, satellite cells of the central nervous system, also express such channels in culture. The function of these satellite-cell sodium channels, if any, remains unsettled, as indeed is the question of whether the satellite-cell channels exist normally at all in vivo or only in the abnormal conditions of cell culture (Shrager et al. 1985) or of Wallerian degeneration (Ritchie & Rang 1983). One speculation is that they are indeed present in Schwann cells of the intact animal whence they may ultimately be transferred to (and used by) the axolemma (Shrager et al. 1985; Gray & Ritchie 1986). This local source of sodium channels would supplement (but not replace) the more generally accepted supply by axoplasmic transport from the cell body in conditions where this transport might be insufficient, especially for the more distal parts of the axolemma, which in man may be as far as 1 m from the cell body. The need for such a supplementary mechanism might be particularly acute if the turnover of the channels in the membrane were fast. Unfortunately, little is known of the turnover time of axolemmal sodium channels. The present experiments failed to settle the question of the presence normally in vivo of satellite-cell sodium channels.
channels; but they did provide a measure of the average lifetime of the sodium channels in the cultured Schwann cells, and this might give some indication of their axolemmal lifetime.

Methods

Primary cultures of Schwann cells were made from rabbits 1 day old (killed by exposure to 100% CO₂) by standard methods (see, for example, Shrager et al. 1985). Sciatic nerves were cut into small pieces with fine scissors, dissociated by exposure to a proteolytic enzyme mixture (Dispase, 5 mg ml⁻¹; collagenase, 1 mg ml⁻¹) for 1 h at 37 °C, and then plated in 75 cm² tissue culture flasks coated with collagen. Cytosine arabinoside (15 μM) was present for the first three days to inhibit fibroblast growth. The primary cultures were then left for at least one day for the cells to adhere to the collagen-coated surface in order to separate them from the debris of the dissociation procedure. These adhering Schwann cells proliferate slowly, if at all, during the period of the experiment.

At various times after plating the Schwann cells were harvested and assayed (at about 4 °C) for saxitoxin-binding capacity (Ritchie & Rogart 1977). The saxitoxin, labelled by the method of Ritchie et al. (1976), had a specific radioactivity of 25 Ci mmol⁻¹ and a radiochemical purity of 85%. Cells were exposed to the [³H]-saxitoxin for 30 min in the presence and absence of 8 pM unlabelled saxitoxin. This latter concentration was sufficient to inhibit completely the saturable component of uptake so that the non-specific component of uptake was revealed and the saturable uptake determined by subtraction. The uptake was usually determined at a standard concentration of 2.4 nM; this concentration is more than twice the value of the equilibrium dissociation constant (about 1 nM). The uptake was expressed per milligram protein determined by the Lowry method (Lowry et al. 1951).

Wherever possible, mean values ± the standard errors of the mean are given.

Results

As can be seen in figure 1, one day after setting up the culture very little saxitoxin-binding capacity was present. This binding capacity, however, developed over the next few days to reach a plateau 4–6 days after dissociation. Thereafter, the saxitoxin-binding capacity (expressed per milligram protein) increased relatively little. There was, however, considerable scatter in the experimental results, partly owing to the fact that in spite of the large number of animals used to produce figure 1 only a relatively small amount of cell (20–40 μg protein) was available for each determination. There was also a considerable variation in the satellite cells’ saxitoxin-binding capacity from one group of neonatal animals to the next, the uptake at the standard concentration varying between 150 and 450 fmol saxitoxin per milligram protein (mean value 262 ± 46 fmol mg⁻¹). In each of the six different preparations whose results are combined in figure 1, therefore, the saturable uptakes at the various times were

† 1 Ci ≈ 3 x 10¹⁰ Bq.
Figure 1. The uptake of $[^{3}H]$saxitoxin by cultured Schwann cells at various times after setting up the primary cell culture. At each time-point, triplicate determinations of the saturable uptake at 2.4 nM were made on each of at least three different preparations; each uptake was expressed as a fraction of the value at 6 days and the mean values were obtained (the bars representing ± one standard error of the mean).

One interpretation of figure 1 is that Schwann cell saxitoxin-binding sites do not exist in vivo, but rather develop gradually over a period of 4–6 days after the process of culturing the cells has begun. However, it is also possible that these channels do exist in vivo but lose their ability to bind saxitoxin because of the culturing procedure. This would be consistent with the finding of Lee et al. (1977) that the proteolytic enzyme trypsin abolishes the ability of tetrodotoxin to block the sodium current in Helix neurons without affecting the magnitude of either the inward sodium currents or their reversal potentials. Support for this latter possibility comes from experiments (open circles, figure 2a) in which at 6 days, i.e. after the Schwann cells had virtually fully developed their ability to bind saxitoxin, they were re-exposed to the dissociation procedure: the cells were harvested, exposed to Dispase–collagenase for 1 h at 37 °C, and then replated. Subsequent assay of saxitoxin-binding capacity (expressed as a fraction of that in control cells of the same age from the same culture) showed that immediately after the enzyme treatment the cells had lost much of their ability to bind saxitoxin, which gradually recovered along more or less the same time course as after the original dissociation procedure. A substantial loss of tetrodotoxin-binding capacity on treatment with proteolytic enzymes, such as trypsin, had already been noted by Reed & Raftery (1976).

The fall in the binding on exposure to the single testing concentration of saxitoxin found after Dispase–collagenase treatment seems to reflect a fall in the maximum binding capacity. Thus in an experiment where a large range of con-
Figure 2. (a) The uptake of \([^{3}H]saxitoxin\) by rabbit cultured Schwann cells after being cultured for 6 days and then exposed either to Dispase–collagenase at 37 °C for 1 h and subsequently replated (open circles), or to tunicamycin (filled circles). At each of various times thereafter, triplicate determinations of the saturable uptake at 2.4 nM were made on each of at least two (usually three) different preparations; each uptake was expressed as a fraction of the value in untreated preparation of the same age from the same cell batch and the mean values obtained (the bars representing ± one standard error of the mean). (b) The same data as in (a) plotted semilogarithmically. The filled circles give the mean values for the fractional uptake after tunicamycin treatment. The line drawn is a weighted least-squares fit (constrained to pass through the point 0, 1.0) to these data points. The open circles represent 1.0 minus the fractional uptake after Dispase–collagenase treatment.


centresations were tested (0.5–8 nM, n = 24) so that a complete binding curve could be obtained (as, for example, in Bevan et al. 1985) the maximal saturable binding capacity was 397 ± 28 fmol mg\(^{-1}\) protein; in cells from the same batch that had been treated three days before by Dispase–collagenase (1 h, 37 °C) the value was clearly reduced by more than 60% (to 140 ± 40 fmol mg\(^{-1}\) protein). The values for the equilibrium dissociation constant (1.26 ± 0.23 nM and 1.58 ± 1.06 nM, respectively) were less accurately determined by the particular concentrations used, but they were not significantly different from each other.

If one assumes that the final plateau for saxitoxin-binding capacity represents the balance between a constant rate of recruitment of new sodium channels into the membrane and that channels subsequently leave the membrane randomly, their rate of loss or removal being proportional to the number already present in the membrane, one expects recovery from the Dispase–collagenase treatment toward the final plateau to be an exponential process in which the time constant will give the average lifetime of the sodium channel. This is based on the assumption that the enzyme-modified sodium-channel protein cannot be repaired \textit{in situ} but has to be replaced. On this basis, the development of saxitoxin-binding capacity, both after the original dissociation procedure and after exposure of Schwann cells to the Dispase–collagenase, suggests the same average channel lifetime of about 3 days. This is illustrated in the semilogarithmic plot shown in figure 2b (open circles). A least-squares linear fit to the points (not shown) indi-
cated a time constant of 3.4 days; if the 5 day point is omitted the points lie close to the fit to the tunicamycin-treated cells (see below) where the time constant is 3.1 days.

The rate of cell turnover can also be estimated if the rate of synthesis is inhibited and the rate of disappearance of saxitoxin-binding capacity is determined. Schwann cells 6 days old were therefore exposed to tunicamycin (1 μg ml⁻¹), which inhibits the glycosylation that is required for maintenance of functional sodium channels, at least in neuroblastoma cells (Waechter *et al.* 1983). The medium in control and tunicamycin-treated flasks was changed daily. As figure 2a (filled circles) shows, after little or no delay the saxitoxin-binding capacity of the tunicamycin-treated cells (expressed as a fraction of that in control cells of the same age and batch) fell roughly exponentially. A semilogarithmic plot (filled circles, figure 2b) indicates a time constant of 3.1 days (half-life 2.2 days).

Cycloheximide (100 μg ml⁻¹), as had been previously found in neuroblastoma cells (Waechter *et al.* 1983), was also found to produce a disappearance of saxitoxin-binding capacity of Schwann cells. This, however, was much more rapid than after tunicamycin exposure, the saxitoxin-binding capacity falling to one half in 6.3 ± 0.6 h (n = 4). This rapid loss of saxitoxin-binding capacity seemed to result not from a disappearance of channels from an otherwise normal Schwann cell, but rather because the cycloheximide treatment resulted in severe cell damage. For example, after 8 h the protein content of the cycloheximide-treated cells that were still adhering to the floor of the flask had fallen to 44 ± 7% of that in the control flasks. Many cells were floating in the supernatant of the treated flasks; most of these cells were unable to exclude trypan blue.

**Discussion**

It seems unlikely that the treatments involved affect only the plasmalemmal sodium channels and do not have more general metabolic effects which could speed up the loss of membrane components or hinder recovery. This could account for the somewhat more rapid disappearance of these channels after tunicamycin and their slower recovery after protease treatment. Taken together, both the reappearance of saxitoxin-binding capacity after protease–collagenase treatment and the disappearance on inhibition of glycosylation agree in suggesting that the average lifetime of sodium channel in the Schwann cell plasmalemma is just over 3 days. These times are somewhat longer than the turnover time estimated by Waechter *et al.* (1983) in neuroblastoma cells. They measured the time course of disappearance of saxitoxin-binding capacity in cells grown in the presence of tunicamycin or cycloheximide and found that the loss of saxitoxin-binding capacity was approximately exponential with a half-life of 26 h; this half-life corresponds with an average lifetime of 38 h. In cultured rat neurons a longer half-life (50 h) has been found (Schmidt & Catterall 1986); this corresponds with an average lifetime of 3 days. The channel lifetime in the membrane thus seems to depend on the type of cell studied. However, the important point is that the membrane lifetime in the axolemma may well be only a few days.

A rapid turnover would clearly impose a large metabolic load on the neuron if all
the channels in the axon had to be made in its cell body. For example, a 1 m length of a myelinated peripheral nerve fibre 20 μm in diameter would have about 500 nodes (see Rushton 1951) each with nearly 100000 channels (Chiu 1980; Chiu & Ritchie 1981). This corresponds to about 0.08 fmol channel per cell, i.e. about 25 pg per cell. Because the number of nodes per unit length is inversely proportional to diameter (see Rushton 1951) and the nodal sodium conductance is directly proportional to diameter (see, for example, Chiu & Ritchie 1981), the number of axolemmal sodium channels associated with any single neuron must be independent of the diameter of the peripheral axon. An average lifetime of 3 days would mean that a cell body 50 μm in diameter (wet cell mass 65 ng) would have to replace channels daily to the extent of less than 0.1% of its total cell protein mass (taken as 20% of the wet cell mass). However, a neuron 10 μm in diameter (wet cell mass 0.5 ng) would be required to replace 8% of its cell protein mass as sodium channels daily; this replacement would seemingly present a not inconsiderable metabolic load. In a remyelinating and regenerating myelinated nerve, where there are many more nodes per unit length of nerve and presumably more sodium channels (see Ritchie et al. 1981; Ritchie 1982), the load would be even more extreme.

The above calculation may well overestimate the amount of channel associated with a single neuron, because the number of nodal channels set by Chiu (1980), which was an upper limit, was only 82000. On the other hand, it may be an underestimate because it ignored the presence of any channels in the internodal region. Ritchie & Rogart (1977) estimated (from the statistical uncertainty of their saxitoxin-binding experiments and their calculated total axolemmal area for the whole nerve trunk) that the average internodal channel density could be as high as 25 μm⁻² if all the channels concealed by experimental error were in the axolemma (the possibility that they were in the internodal Schwann cell plasmalemma not being considered at that time). It is interesting that recent experiments of Chiu & Schwarz (1987) do indeed reveal such a density in the internodal membrane of large (18–20 μm diameter) single fibres of the rabbit sciatic nerve demyelinated with lysolecithin; their value of 0.238 μS for a 10 μm length of internode, when referred to the original axolemma, corresponds with a density of about 27 μm⁻², based on a single channel conductance of 20 pS (Shrager et al. 1985) and on an axonal diameter:fibre ratio of 0.7 (see Ritchie & Rogart 1977). The node itself, which was shown to have the same number as a 1000 μm length of demyelinated internode (Chiu & Schwarz 1987), would contain 40000 channels rather than the 100000 value used above; this would significantly reduce, but by no means remove, the metabolic burden. However, if all the extranodal channels were axolemmal, a 1 m length of a single fibre of 20 μm diameter would have nearly an extra 600 pg channel to be supported; for a fibre 5 μm in diameter the corresponding value would be about 150 pg. Maintenance of such a huge extranodal pool would entail a daily production of sodium channels comparable in size to the total protein mass of some of the smaller neurons. However, it remains unclear, and unlikely, that the internodal pool of channels is associated with the axon rather than with the Schwann cell plasmalemma, because the electrophysiological values of membrane capacity measured by Chiu & Schwarz (1987) in
the demyelinated segments were ten times greater than could be accounted for by the axolemma proper. Indeed, they suggested that they may have been recording from a membrane produced by a lyssolecithin-induced fusion of Schwann cell plasmalemma and internodal axolemma, in which case the channels may have come from either source. It is relevant that the Schwann cell, which must contribute 90% of the putative fused membrane, is known to exhibit a saxitoxin-binding density in culture of about 30 μm⁻² (Shrager et al. 1985).

For a non-myelinated fibre the metabolic load might be even greater than for a myelinated fibre. In a typical non-myelinated nerve, the rabbit vagus, the saturable saxitoxin-binding capacity is 110 fmol mg⁻¹ wet nerve (Ritchie et al. 1976); this value, with an extracellular space of 0.67 mg mg⁻¹ wet nerve (Keynes & Ritchie 1965), corresponds with an uptake of 333 fmol mg⁻¹ fibre. But the fibre actually consists of an axon surrounded by its satellite Schwann cell, the latter comprising about 38% of the combined mass (Keynes & Ritchie 1965). If the Schwann cell in vivo binds saxitoxin to the same extent as in the present culture experiments (i.e. about 400 fmol per milligram protein or 80 fmol per milligram wet cell mass, if the cell protein is taken as 20% of the wet cell mass), the true axonal binding would be about 500 fmol mg⁻¹ axon. This means that a 1 m length of a single non-myelinated fibre of average diameter 0.75 μm (Keynes & Ritchie 1965) with an average saxitoxin-binding capacity of 110 fmol mg⁻¹ (wet) would contain over 60 pg channel protein, i.e. over twice as much as the nodal pool in a single myelinated fibre. Again, for a single neuronal cell body to support a protein pool of this size with a turnover time constant of 3 days might present a severe metabolic burden.

These considerations emphasize the potential difficulty faced by a neuron in maintaining an adequate density of sodium channels (particularly in long axons) if their turnover in the axolemma is as rapid as in its satellite cells, where the average lifetime is only about three days. They seem to provide some support, albeit indirect, for the hypothesis that many of the axolemmal sodium channels are derived from their ensheathing Schwann cells. In this scheme the metabolic load would be shared by many hundred Schwann cells, each of which was associated with just a single internode.

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References


