The Action of Local Anesthetics on Myelin Structure and Nerve Conduction in Toad Sciatic Nerve

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ABSTRACT X-ray scattering and electrophysiological experiments were performed on toad sciatic nerves in the presence of local anesthetics. In vitro experiments were performed on dissected nerves superfused with Ringer's solutions containing procaine, lidocaine, tetracaine, or dibucaine. In vivo experiments were performed on nerves dissected from animals anesthesized by targeted injections of tetracaine-containing solutions. In all cases the anesthetics were found to have the same effects on the x-ray scattering spectra: the intensity ratio of the even-order to the odd-order reflections increases and the lattice parameter increases. These changes are reversible upon removal of the anesthetic. The magnitude of the structural changes varies with the duration of the superfusion and with the nature and concentration of the anesthetic molecule. A striking quantitative correlation was observed between the structural effects and the potency of the anesthetic. Electron density profiles, which hardly showed any structural alteration of the unit membrane, clearly indicated that the anesthetics have the effect of moving the pairs of membranes apart by increasing the thickness of the cytoplasmic space. Electrophysiological measurements performed on the very samples used in the x-ray scattering experiments showed that the amplitude of the compound action potential is affected earlier than the structure of myelin (as revealed by the x-ray scattering experiments), whereas conduction velocity closely follows the structural alterations.

INTRODUCTION

Despite the tremendous amount of experimental work carried out since the appearence of the first paper concerning local anesthesia (Freud, 1884), the exact mechanisms of action of local anesthetic drugs have not yet been established. This is particularly noticeable at the molecular level. Two general theories have been proposed to explain the molecular basis of the local anesthetic effects: one considers the bulk perturbation of the membrane structure by anesthetics, with its direct consequences for ionic channel function (Seeman, 1972; Lee, 1976); the other proposes that the anesthetic effect on membrane function is due to the binding of the anesthetic molecule to a specific receptor site of the sodium channel at the inner side of the membrane (Strichart, 1975, 1976; Guo et al., 1991). For both local and general anesthetics much evidence has been accumulated to support the specific receptor hypothesis (Hille, 1980, and references therein; Franks and Lieb, 1994, and references therein). However, bulk membrane perturbation still provides part of the explanation for the anesthetic action, and it does not seem very probable that the local anesthetic effect could be accounted for only in terms of a specific receptor site (Lee, 1976, and references therein).

Over the years we have been very much concerned with the structure of myelin, and more specifically with the structural perturbations that accompany events of physiological and pathological interest. To tackle this problem we have developed fast and accurate x-ray scattering techniques that operate on living as well as on dissected nerves while keeping physical, chemical, and electrophysiological parameters under strict control (Mateu and Morán, 1986, and references therein). We have also improved the mathematical analysis of the scattering spectra, with special emphasis on the parameters that characterize structural disorder (Mateu et al., 1996, and references therein).

Some years ago we studied the effects of *n*-pentane, a nonspecific neutral anesthetic, on the structure of myelin in frog and rat sciatic nerves (Padrón et al., 1979, 1980; Mateu and Morán, 1986). We approached the problem using simultaneous x-ray scattering and electrophysiological experiments as a function of concentration and time of exposure to the anesthetics. Morán used the same technique to study the structural and physiological effects of positively charged local anesthetics (Morán, 1984). A more recent analysis of the structural perturbations induced by long exposures to local anesthetics (Mateu et al., 1992) has revealed interesting structural phenomena that, at first sight, did not seem to be relevant to the treatments used in medical practice. Upon closer inspection, nevertheless, the observation by Mateu et al. (1992) that in sciatic nerve the packing disorder is located (at least preferentially) in the cytoplasmic space and that this space is particularly sensitive to temperature (Mateu et al., 1995) seemed to put Morán's early observations in a new perspective and prompted us to look more carefully into his old data.

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As Morán's raw data are no longer available, we have been forced to rely on the analyses carried out in 1986, before the introduction of the new mathematical analysis for the x-ray scattering patterns (Luzzati and Mateu, 1990). We have to stress, though, that the conclusions of the present paper do not hinge upon a sophisticated analysis of the scattering spectra. The strength of Morán's old work was, and still is, the coupling of x-ray scattering experiments with electrophysiological measurements and the extension of the in vitro experiments to the situation prevailing when anesthesia is performed in vivo.

The choice of toad sciatic nerve was dictated by its sturdiness. It has indeed been shown that after dissection these nerves remain electrophysiologically active for several weeks (Edström and Kanje, 1988; Tedeschi and Liuzzi, 1992).

MATERIALS AND METHODS

The animals and the nerves

All of the experiments were performed with wild adult toads (*Bufo marinus*). The sciatic nerves were dissected from pithed animals, desheathed, and mounted on a specimen holder (see below).

The experiments

The sample holder, modified from Rud (1961), consisted of a closed wet chamber provided with three pairs of electrodes, one for nerve stimulation, and two others for recording compound action potential at different positions along the nerve. The chamber was provided with a spreader aimed at superfusing the nerve segment located between the two pairs of recording electrodes, and with two thin mica windows on the path of the x-ray beam (Padrón et al., 1979, 1980). A wet mixture of O_2/CO_2 (95%/5%) that streamed continuously through the chamber preserved an adequate humidity. With this sample holder myelin scattering spectra and compound action potentials could be recorded simultaneously (Padrón and Mateu, 1980). On the other hand, whenever long-lasting x-ray scattering experiments were to be recorded while keeping the amount of anesthetic constant, the nerves were tied off and sealed in a supportive quartz capillary tube after exposure to the anesthetics.

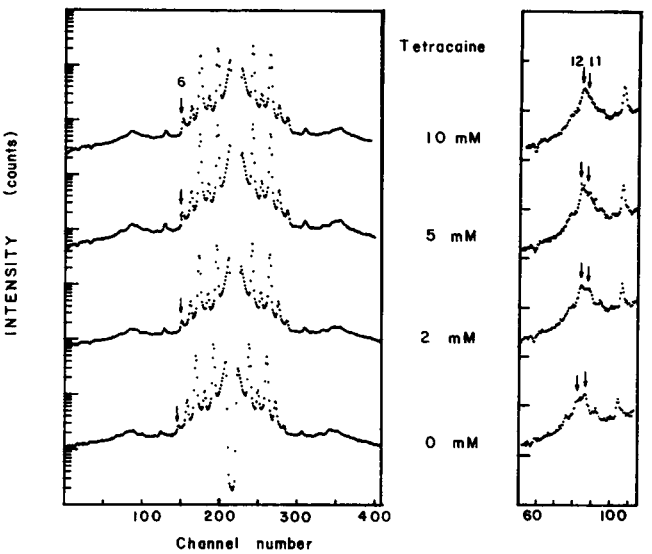

FIGURE 1 (*Left*) Fifteen-minute X-ray scattering spectra of freshly dissected sciatic nerves, superfused with Ringer's solutions containing tetracaine at the concentration marked beside each curve. (*Right*) Amplified high-angle region of the spectra, showing the gradual change of the intensity ratio $I(11)/I(12)$.

Crystallographic analysis

The x-ray beam from an Elliott GX6 rotating anode generator, operated at 30 kV and 30 mA, was linearly focused and monochromatized by a

TABLE 1 Structural data as a function of tetracaine concentration

(mM)	0	1	2	3	4	5	6	7	8	9	10
n	24	4	10	6	6	12	3	4	7	11	10
D (Å)	174.5 ± .3	174.6 ± .8	174.1 ± .5	175.0 ± .6	175.5 ± .9	176.2 ± .3	176.4 ± .8	176.5 ± .6	176.6 ± .5	176.8 ± .4	176.7 ± .8
D_{cyt} (Å)	36.6 ± .3	37.2 ± .3	37.6 ± .2	38.0 ± .2	38.6 ± .2	39.0 ± .3	39.4 ± .3	39.6 ± .3	39.7 ± .3	39.8 ± .2	39.9 ± .2
D_{bil} (Å)	46.4 ± .2	46.2 ± .3	45.8 ± .3	46.0 ± .3	45.9 ± .3	46.0 ± .3	45.9 ± .4	45.9 ± .3	45.8 ± .3	45.9 ± .3	45.8 ± .3
D_{ext} (Å) ± S.E.M.	45.1 ± .3	45.0 ± .3	44.9 ± .3	45.0 ± .3	45.1 ± .4	45.2 ± .3	45.1 ± .3	45.2 ± .3	45.2 ± .3	45.9 ± .3	45.2 ± .3
h	$F^2(h)$ ± SEM										
−1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
+2	65.73 ± .45	66.26 ± .82	66.40 ± .90	67.26 ± .74	67.65 ± .63	68.29 ± .61	68.35 ± .62	68.52 ± .76	68.55 ± .72	68.70 ± .83	68.74 ± .72
−3	10.45 ± .17	9.75 ± .23	8.77 ± .05	7.92 ± .31	7.61 ± .23	7.06 ± .21	6.83 ± .13	6.45 ± .18	6.10 ± .18	6.15 ± .16	6.05 ± .53
−4	93.52 ± .48	95.83 ± .78	97.89 ± .62	98.81 ± .39	100.07 ± .58	101.26 ± .80	101.65 ± .72	102.21 ± .79	102.55 ± .86	102.81 ± .91	103.08 ± .46
+5	14.38 ± .13	13.55 ± 23	12.89 ± .15	12.27 ± .23	11.76 ± .12	11.31 ± .26	11.13 ± .09	10.91 ± .21	10.77 ± .16	10.68 ± .14	10.61 ± .42
+6	1.57 ± .03	2.27 ± .08	2.90 ± .05	2.95 ± .08	3.17 ± .06	3.26 ± .06	3.48 ± .08	3.78 ± .10	3.90 ± .06	3.91 ± .04	3.98 ± .15
+7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
+8	2.25 ± .03	2.33 ± 15	2.35 ± .03	2.65 ± .07	2.56 ± .05	2.72 ± .03	2.71 ± .08	2.79 ± .11	3.02 ± .07	2.96 ± .09	3.08 ± .06
−9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
+10	1.44 ± .02	1.49 ± .04	1.37 ± .02	1.29 ± .04	1.17 ± .05	1.05 ± .02	1.07 ± .06	1.04 ± .07	.98 ± .05	.99 ± .02	1.00 ± .02
−11	3.45 ± .04	2.86 ± .04	2.37 ± .01	1.95 ± .03	2.03 ± .03	1.85 ± .02	1.55 ± .06	1.36 ± .04	1.25 ± .07	1.14 ± .04	1.07 ± .02
−12	1.33 ± .02	1.57 ± .05	1.88 ± .04	2.31 ± .04	2.63 ± .02	2.75 ± .04	3.01 ± .05	3.04 ± .04	2.98 ± .08	2.95 ± .04	3.15 ± .05

The data correspond to 15 minute X-ray scattering experiments performed on sciatic nerves dissected from non-anesthetized animals, soaked for 100 minutes in tetracaine-containing Ringer solutions and then sealed in quartz capillary tubes. The intensities were measured and the parameters D_{cyt}, D_{bil} and D_{ext} determined as described under Methods. n is the number of experiments at each tetracaine concentration. The average values are reported accompanied by their standard error. The signs were used as in Mateu et al (1992, 1995).

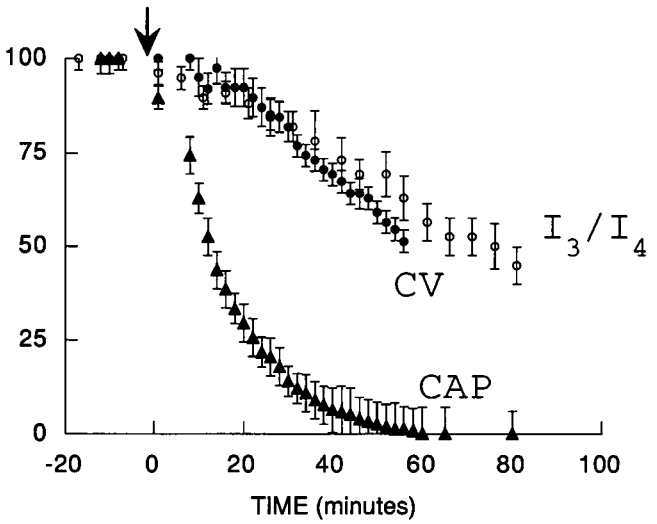

FIGURE 2 Time course of the conduction velocity (●), of the CAP amplitude (▲), and of the intensity ratio $I(3)/I(4)$ (○) determined in sequences of experiments with 2-min spectra recorded from sciatic nerves superfused with Ringer's solution containing 5 mM tetracaine. Percentage changes are shown on the ordinate. Each point is the average of 12 experiments, each performed on a different nerve. The vertical bars show the standard deviations. The onset of the superfusion is marked by the arrow. Note the strong coupling of conduction velocity with the structural changes mirrored by the ratio $I(3)/I(4)$.

Ni-coated bent glass. At the sample, the beam was approximately 1 cm long. The sample-to-detector distance was 225 mm. The spectra were recorded using a one-dimensional position sensitive detector, 80 mm long and 10 mm wide (Gabriel, 1979). The spectra were stored sequentially for later analysis.

The intensity of the reflections was measured by integration of the area under the peaks after subtraction of a background function drawn by hand (the results were not substantially different from those obtained by using the novel algorithms; Luzzati and Mateu, 1990; Mateu et al., 1995). The electron density profiles were computed with the set of signs advocated by Caspar and Kirschner (1971) (and confirmed by Mateu et al., 1995, 1996). The data from different experiments were normalized by setting $\Sigma[F(h)^2] = D$ (Blaurock, 1971, 1982). The thicknesses of the bilayer (D_{bil}) and of the external (D_{ext}) and the cytoplasmic (D_{cyt}) spaces were measured by the distances between the maxima of the electron density profiles (Mateu et al., 1995).

Electrophysiological experiments

Nerve segments approximately 5 cm long were laid on the platinum wire electrodes of the chamber. Stimulation and recording were performed, respectively, at the proximal and the distal ends of the nerve. The analysis of the records was restricted to the conduction velocity (CV) and the maximum amplitude (CAP) of the compound action potential. Stimulation was produced by using a Grass SD9 stimulator at twice the intensity required to produce the maximum CAP amplitude. The signals were amplified with Tektronix AM402 differential amplifiers and stored for later analysis.

RESULTS

In vitro experiments

Superfusion with anesthetic-containing Ringer's solutions has strong effects on the x-ray scattering spectra of the nerve. A few samples of 15-min spectra recorded with variable concentrations of tetracaine are presented in Fig. 1. The spectra are very similar to those previously published by other groups (Caspar and Kirschner, 1971; Blaurock, 1971, 1982) and display some 12 discrete reflections of a one-dimensional lattice, the repeat distance of which is in the range 174–177 Å. The relative intensity of the even

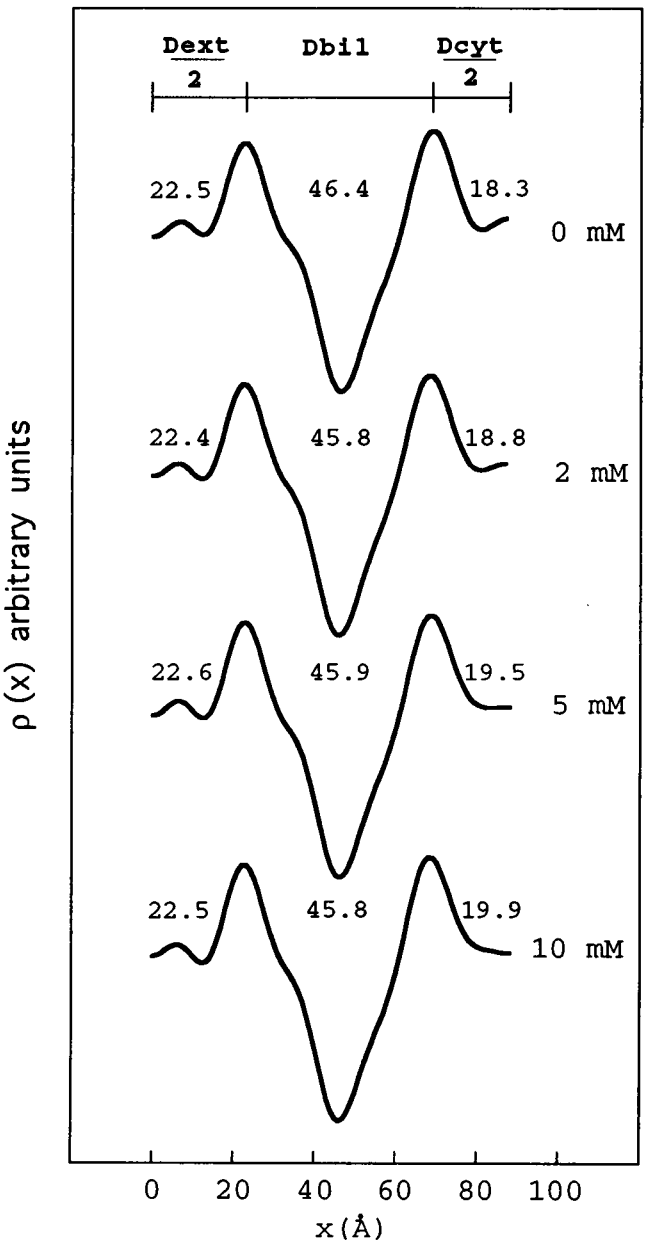

FIGURE 3 Electron density maps of a single myelin membrane plotted at an arbitrary scale, from nerves soaked for 100 min in solutions containing increasing concentrations of tetracaine. The profiles were calculated using the structure factors and phases shown in Table 1. Tetracaine concentrations are indicated for each profile. The measured thicknesses of half the cytoplasmic space ($D_{Cyt}/2$), the bilayer (D_{bil}), and half the external space ($D_{Ext}/2$) are also shown. Note that the myelin membrane structure is practically identical in anesthetized and control nerves (0 mM), that the thickness of the cytoplasmic space increases, and that the thicknesses of the bilayer and external spaces remain constant.

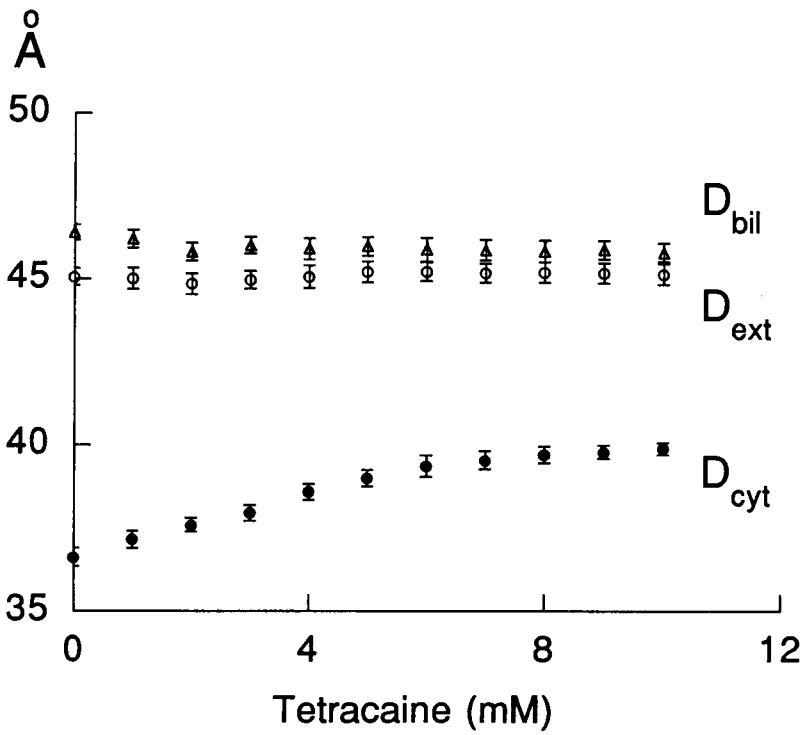

FIGURE 4 D_{bil}, D_{ext}, and D_{cyt} as a function of tetracaine concentration (see Table 1). Note that D_{cyt} steadily increases as tetracaine concentration increases, whereas D_{ext} and D_{bil} remain almost constant.

orders is slightly lower than that previously published for frog sciatic myelin (Caspar and Kirschner, 1971). Eventually (usually beyond 2–3 h of exposure) the spectra give way to a few broad bands characteristic of a disordered distribution of isolated membrane pairs (Mateu et al., 1992). The present work is sharply focused on the intensity ratio of the *h*-even to the *h*-odd reflections, which is particularly sensitive to anesthetics (see Fig. 1 and Table 1).

We have described elsewhere (Mateu et al., 1992) the time course of the effects of fixed concentrations of local anesthetics on the structural parameters, using rat sciatic and optic nerves. We explore in this work the time- and the concentration-dependent effects of anesthetics on toad sciatic nerves (Fig. 1 and Table 1). We also confirm that if the exposure to anesthetics does not exceed approximately 60 min, then the spectra can be reversed to the native form upon superfusion with normal Ringer's solution. Beyond 100 min, some of the structural and the functional perturbations become irreversible.

One novelty of this work is the measurement of the electrophysiological properties of the nerve performed in parallel with the x-ray scattering experiments. This control was established according to a procedure advocated by Padrón and Mateu (1981) (see also Mateu and Morán, 1986). A nerve was mounted in the chamber described under Materials and Methods, and it was kept under continuous superfusion with the anesthetic-containing Ringer's solution. A sequence of 2-min x-ray scattering experiments was then recorded and, at the same time, the compound action potentials were measured. Short exposure spectra are noisy; thus it was convenient to assess the structural perturbations using the intensity ratio of a pair of strong reflections, $I(3)/I(4)$. The time course of the $I(3)/I(4)$ ratio and of the physiological parameters as a function of the exposure to tetracaine is presented in Fig. 2. Anesthetics have an immediate effect on the amplitude of the action potential, which decreases by one-half in ~10 min. In ~1 h the signal fades away altogether. Conduction velocity and the ratio $I(3)/I(4)$ decrease more slowly. Most remarkably, the time courses of these two signals were found to be almost identical.

A more quantitative assessment of the structural effects was sought in the electron density profiles. For this purpose, 15-min x-ray scattering experiments were performed on dissected nerves, which were first soaked for 100 min in tetracaine-containing Ringer's solutions at variable anesthetic concentrations and then sealed in quartz capillary tubes. Several experiments were performed at each tetracaine concentration (see Table 1). The intensity of the reflections was measured (see under Materials and Methods) and averaged; the electron density maps were computed. Some of them are shown in Fig. 3. The thicknesses of the bilayer (D_{bil}) and of the external (D_{ext}) and cytoplasmic (D_{cyt}) spaces were determined as discussed under Materials and Methods. The values of these parameters are reported in Table 1 and plotted in Fig. 4. The most striking result was the steady increase in D and D_{cyt} with increasing tetracaine concentrations, whereas D_{bil} and D_{ext} barely vary.

In vivo experiments and correlations with anesthesia

The main purpose of this work is to investigate the extent to which the structural effects induced in myelin by exposing

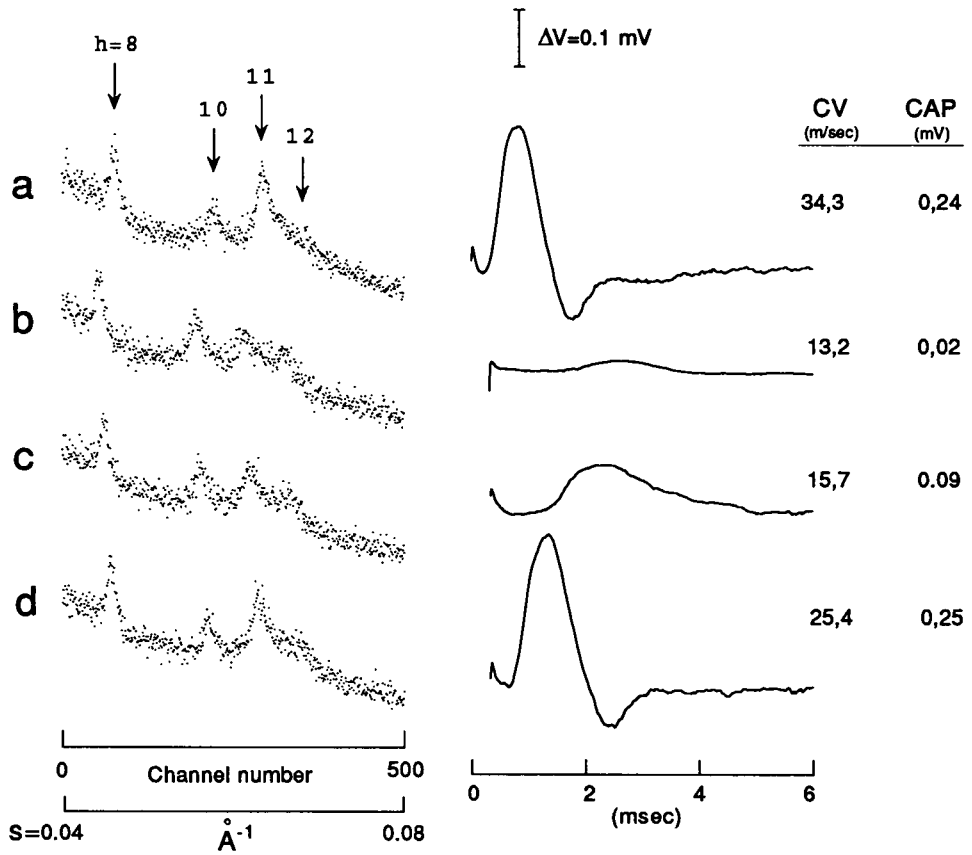

FIGURE 5 Examples of x-ray scattering and electrophysiological experiments. (*Left*) Portion of the 10-min spectra. (*Right*) Records of the compound action potential, with the corresponding values of the amplitude (CAP) and the conduction velocity (CV). *Experiment a*, Freshly dissected, nonanesthesized control nerve superfused in normal Ringer's solution. *Experiment b*, Nerve from the same animal, anesthesized in vivo as discussed in the text, then dissected and superfused with normal Ringer's solution. *Experiment c*, The same nerve, after 120 min of superfusion with normal Ringer's solution. *Experiment d*, The same, after 240 min of superfusion with normal Ringer's solution. Note that in the spectra of the nerves anesthesized in vivo (*Experiment b*) and in vitro (Fig. 2), the effect of tetracaine is to increase the intensity ratios $I(8)/I(11)$, $I(10)/I(11)$, and $I(12)/I(11)$.

a dissected nerve to an anesthetic mirror the effects induced by anesthesia performed in vivo. We have approached this problem in two ways.

One approach involved in vivo observations. One sciatic nerve of each experimental animal was anesthesized by intramuscular injections of a Ringer's solution containing 5 mM tetracaine and 1:200,000 epinephrine. Epinephrine was used to prolong and intensify the action of the anesthetic (Ritchie and Green, 1985). Two injections were given at the vicinity of the nerve at 30-min intervals. These injections do not have irreversible effects, inasmuch as control animals recover motility in a few hours. Immediately after the first injection, the sciatic nerve from the nonanesthesized leg was dissected and mounted on the sample holder; the x-ray scattering and the electrophysiological results of one of these experiments can be observed in Fig. 5 (*Experiment a*). Subsequently, and after the second injection, the nerve from the anesthesized leg was dissected and Experiments b–d in Fig. 5 were performed, the nerve being superfused with normal Ringer's solution. Note that as in Figs. 1, 2, and 6 and in Table 1, the intensity ratio of the even-order over the odd-order reflections increases under the action of the anesthetic. Experiments c and d were aimed at testing reversibility. From the resulting data it thus appears that tetracaine has very similar effects on both the structure of the myelin sheaths and the conduction properties of the nerve when applied in vivo or in vitro.

On the other hand, the physiological significance of the structural perturbations induced in vitro by the anesthetic can be assessed by comparing different anesthetics whose physiological effects are known. For this purpose, the "relative anesthetic potency" is a convenient physiological parameter to use. This parameter is related to the blocking action of these drugs on peripheral nerve; its values have been reported for several local anesthetics (Blaustein and Goldman, 1966). For the comparison we used procaine, lidocaine, tetracaine, and dibucaine. Sciatic nerves were soaked for 100 min in Ringer's solutions containing one of the anesthetics at 5 mM; afterward the nerves were sealed in quartz capillary tubes, 1-h x-ray scattering experiments were performed (Fig. 6), the intensities were measured, the electron density maps were computed, and D_{cyt} was determined. The values of D_{cyt} are plotted as a function of anesthetic potency in Fig. 7. The correlation between the two parameters is striking.

DISCUSSION AND CONCLUSIONS

It is timely to summarize the results of our previous study of the structural effects of local anesthetics on myelin (Mateu et al., 1992), which may well be relevant to the present work, despite the fact that rat nerves rather than toad nerves were used. The 1992 study involved only x-ray scattering and electron microscope experiments, without electrophys-

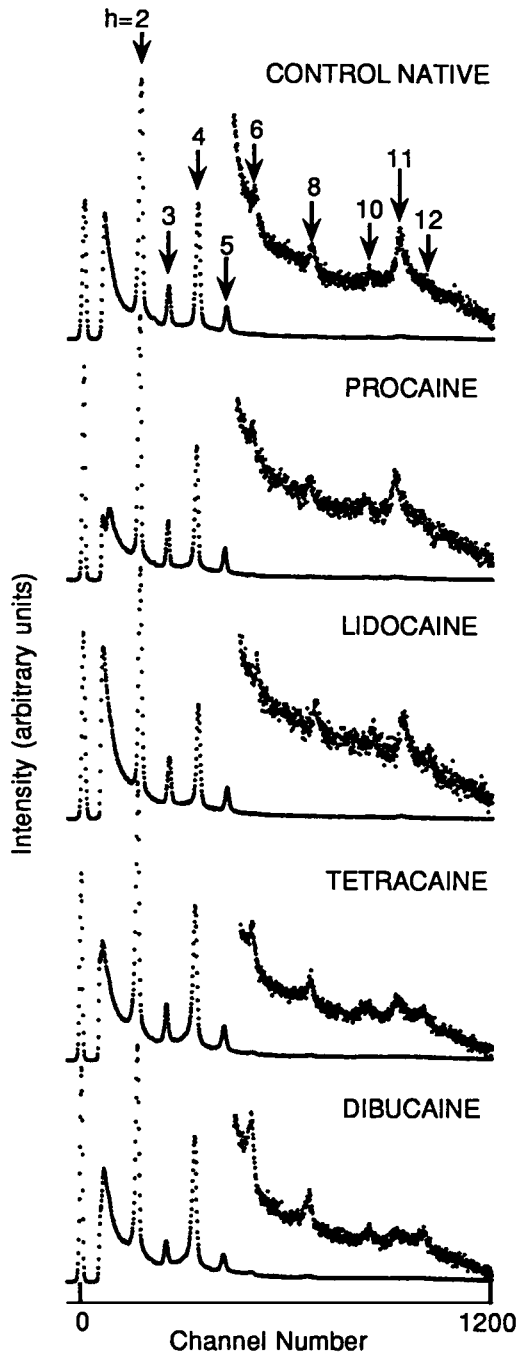

FIGURE 6 One-hour x-ray scattering spectra of sciatic nerves soaked for 100 min in Ringer's solutions containing 5 mM different anesthetics and then sealed in quartz capillary tubes. Note that the intensity ratios $I(6)/I(11)$, $I(8)/I(11)$, $I(10)/I(11)$, and $I(12)/I(11)$ increase in the order procaine, lidocaine, tetracaine, dibucaine.

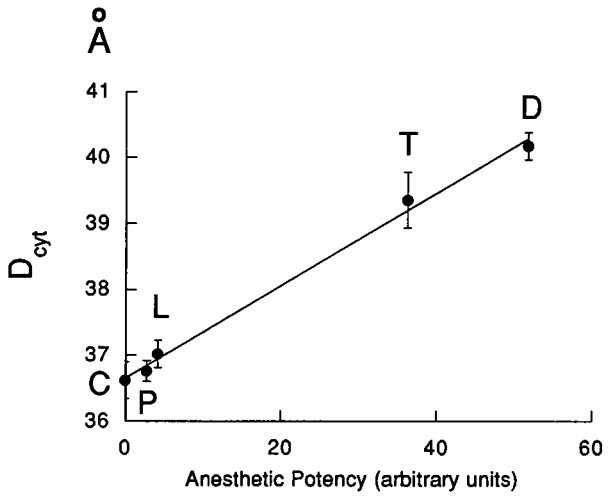

FIGURE 7 Correlation of the structural effects of the anesthetics (as determined by the parameter D_{cyt}) with anesthetic potency (as defined in the text). C, Nonanesthetized control; P, procaine; L, lidocaine; T, tetracaine; D, dibucaine. Note that a very similar linear relationship is obtained if the ratio $I(3)/I(4)$ is plotted instead of D_{cyt}.

iological controls. The x-ray scattering experiments underwent a sophisticated analysis that led to the determination of a variety of structural parameters, many of which have been eluded in the present analysis. We find that over the first 60 min the total amount of myelin (α_{myel}) remains constant, the repeat distance (D) steadily increases (~3 Å in 60 min), the average number of membranes per orderly pack (N) decreases dramatically (one order of magnitude in 60 min), whereas the packing disorder (σ_D) and the fraction of loosely packed membranes (α_{loose}) remain almost constant. Moreover, the analysis of the intensity curves ($i_{motif}(s)$) and the electron microscope observations showed that the anesthetic used, tetracaine, has the effect of first swelling and eventually disrupting the cytoplasmic space of the sheaths.

A distressing limitation of the 1992 study, due mainly to the absence of electrophysiological controls, was the uncertainty as to whether the structural phenomena observed in vitro mirrored the "physiological" effects of anesthesia (namely the effects relevant to the clinical practice) or rather were the consequence of a toxic accumulation.

In the present work the analysis of the scattering spectra was focused on the intensity ratio of the even-order over the odd-order reflections. This ratio mirrors the symmetry of the structure: the ratio decreases as the thicknesses of the external and the cytoplasmic space tend to match. The intensity ratio invariably decreases under the action of the anesthetics (Figs. 1, 2, 6). This observation, coupled with the increase in the repeat distance (Table 1) tells, in keeping with the 1992 observations, that the anesthetics have the effect of increasing the thickness of the cytoplasmic space. The swelling can be assessed qualitatively by the ratio $I(3)/I(4)$ (Fig. 2), or it can be quantified by using the electron density profiles (Fig. 3 and Table 1).

The main conclusion of this work is that both in vivo and in vitro, local anesthetics have the effect of swelling the cytoplasmic space of the myelin sheaths. This conclusion rests on three lines of argument. First, the electrophysiological experiments show that upon exposure to tetracaine, the time course of conduction velocity is almost identical to that of the ratio $I(3)/I(4)$ (see Fig. 2). Second, the x-ray scattering and the electrophysiological signals recorded with

nerves dissected from animals anesthesized in vivo are very similar to those observed when the anesthetic is applied in vitro (Figs. 1 and 5). Third, experiments performed with four different local anesthetics show that the swelling of the cytoplasmic space, a structural parameter, is strongly correlated with a physiological parameter, the "anesthetic potency" (Fig. 7).

Finally, it is worthwhile to point out the striking fact that local anesthetics and *n*-alkanes (Mateu and Morán, 1986), which both slow down the propagation of action potential, still seem to have opposite effects on the structure of myelin: *n*-alkanes decrease (Mateu and Morán, 1986) whereas local anesthetics increase (this work) the thickness of the cytoplasmic space. For the time being, the molecular mecanisms underlying these phenomena are elusive.

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