Effects of Benzocaine and Its Isomers on Sodium Permeability and Steady State Sodium Inactivation in the Myelinated Nerve, Obtained by an Improved Dissection Technique

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Abstract. The blocking effects of benzocaine and its isomers (1 mmol/l) on sodium currents in myelinated nerve fibres were tested. As far as the so-called fast sodium inactivation is concerned, benzocaine shifted the h_f-curve in negative direction to a stronger extent than did its isomers, while the potency of the isomers did not differ significantly from each other. The drug-induced reductions of maximum sodium permeability $\bar{P}_{Na}$ were tested at constant test pulses at $h_f = 1$. In this kind of experiments all the three isomers had the same potencies. The findings could not be correlated to the lipid solubilities of the drugs as measured by the corresponding octanol/water partition coefficients. In addition, efforts were undertaken to minimize any noxious pull during the isolation of the axon. Some consequences of the improvements introduced are discussed in terms of the reliability of ionic current measurements in Ranvier nodes.

Key words: Myelinated nerve — Potential clamp — Local anaesthetics — Dissection technique

Introduction

Local anaesthetics block the excitability of nerve membranes by blocking sodium channels (Taylor 1959). This is produced by a decrease of the sodium permeability constant $\bar{P}_{Na}$ (Hille 1966) and, with some compounds (e.g. benzocaine), by a shift of the sodium inactivation curve along the potential axis in negative direction (Hille 1977). Moreover, experiments on the frequency depen-

dent block of sodium channels produced by stereoisomers of local anaesthetics showed a drug-receptor interaction (Yeh 1980).

The aim of the present investigation was to elucidate whether the anaesthetic potencies of benzocaine and its structural isomers on sodium inactivation and on peak sodium permeability could be correlated to the lipid solubilities of the drugs under investigation. Our results did not support the idea that uncharged anaesthetics solvate freely into the lipid part of the membrane.

A preliminary report of some methodical improvements has been published elsewhere (Sommer and Koppenhöfer 1982).

Materials and Methods

Preparation. Experiments were carried out on single myelinated nerve fibres of the frog (*Rana esculenta*). After dissection individual fibres often differ markedly in their appearance. Sometimes they seem to resemble thin rectilinear wires with wide open nodal gaps as has been shown by Derksen (1965) and Stämpfli (1965). In other cases, however, the dissected fibre is clearly bent and often exhibits barely visible Ranvier nodes. The question was whether these differences base on genuine discrepancies in the architecture of myelinated nerve fibres or whether they are artefacts due to inappropriate handling of the preparation, which might more or less be avoided.

It seems likely that in individual preparations with relatively stiff connective tissue, the fibre to be isolated is exposed to stronger teasing and bending forces during dissection than it is the case in preparations of more loosely packed endoneurium. Our impression was that the relatively resistant connective tissue favours the appearance of straight-lined isolated nerve fibres. Moreover, when we teased a nicely bent axon to some extent for a few seconds at will and then released it, the fibre became more or less straight-lined. At the same moment, hardly detectable Ranvier nodes became easily observable. However, when we teased a fibre beyond certain limits for a longer period, the internodes became monoliform (Lubinska and Lukaszewska 1956) and displayed the "beading" phenomenon (Ochs 1965), which is known from short internode intercepts exposed to air in air gap experiments (Sommer 1983). Obviously, beading indicates severe deterioration and fibres of this kind are not suitable for electrophysiological experiments which are concerned with the genuine behaviour of Ranvier nodes in the living animal. If, in fact, the occurrence of straight-lined fibres were due to teasing during the dissection procedure, two questions would arise: 1. what is the genuine appearance of peripheral nerve fibres in the intact animal, and 2. do these changes influence ionic current measurements?

Although Stämpfli and Hille (1976) claimed that "extremely cautious dissection is of paramount importance for electrophysiological work", rectilinear fibres quite often show normal action potentials and acceptable ionic currents. When we tried to follow their suggestion more strictly than we used to do in previous experiments, the percentage of nicely curled fibres increased markedly. Stämpfli (1952) first pointed out that such fibres reflect in what way nerve fibres may be packed in the uninjured nerve. This view is supported by the cross striation of peripheral nerves and their reserve length (Schneider 1952).

The procedure we employed (Sommer and Koppenhöfer 1982; Sommer 1983) follows the instructions described by Stämpfli (1952) and Stämpfli and Hille (1976): the hindpart of a decapitated frog was skinned and a gastrocnemius muscle with its sciatic nerve was freed as carefully as possible up to the hip by splitting the muscle fasciae. The epi- and perineurium of both peroneal and tibial nerves were opened by an incision into the corresponding trunk using microscissors with

highly polished branches (model "Sommer", Gebr. Martin, Tuttlingen, W. Germany) and watchmaker forceps. After pulling away the peroneal nerve and under appropriate illumination, the remaining bundle showed the well-known cross striation (Plate 1, *A*; green arrow) of a peripheral nerve (Fontana 1787; Lehmann 1959) which disappears reversibly upon slight teasing. Splitting the sheath of the tibial trunk (Plate 1, *B*) enabled the latter (1) to be pulled away; direct pulling on the desired branch (3) was avoided by exerting an appropriate counter-pull on branch 2 which goes to the belly of the gastrocnemius (Plate 1, *C*). Branch 3 shows two roots, a sensory bundle (4), ramus cutaneus posterior, and a motor bundle (5) running to the head of the gastrocnemius; branch 3 is still largely sheathed. The following steps differ from the commonly adopted techniques: the scissors used enable desheathing of the mixed branch (3) by separating both bundles up to the region where the branch has already been desheathed (Plate 1, *C*; yellow arrow). Then, if a motor fibre is desired, the sensory bundle (4) is split into two portions, using highly polished dissection needles, in such a manner that the motor bundle spreads out in the form of a fan (made of loosely packed fibres) (Plate 1, *D*) from which a clearly bent axon can easily be isolated. In such a fibre, even with good optics, appropriate darkfield illumination and polarized light, the node of Ranvier is often barely visible (*E*, arrows). This observation again favours the idea that the shape and width of the node can vary according to the tension previously applied to the fibre. For comparison, a typical axon dissected in a conventional manner with conventional tools is shown in Plate 1, *F*; the node (arrows) is clearly visible and the two adjacent internodes appear largely straight-lined. The axon resembles a slightly stretched mammalian fibre as has been shown by Lubinska (1952).

The mean diameter of an isolated frog nerve fibre dissected conventially is about 14 µm (Stämpfli and Hile 1976). Bearing in mind that membrane physiologists always try to use the thickest fibre available, the above dissection procedure yielded a corresponding value of 20.5 ± 0.1 µm (mean $\pm$ S.E.M.; $n = 18$). Therefore, axons dissected conventionally might have been thinned by inadvertent stretch. Hence, we believe that nicely curled isolated axons with narrow nodal gaps rather than straight-lined fibres represent the situation in the intact peripheral nerve.

For the present investigation, nicely curled fibres were mounted as loosely as possible on the recording chamber; largely rectilinear fibres with clearly visible Ranvier nodes were discarded. The node under investigation was superfused continuously by a specific bathing medium.

Measuring device. Ionic current measurements were carried out by means of a commercially available potential clamp device according to Nonner (1969). A simple modification enabled measurement of the absolute membrane potential (Wiese and Duchâteau 1984). Membrane current signals were filtered through a low-pass, fourth-order Bessel filter (-3 db at 80 kHz). No compensation for the influence of the nodal series resistance was employed. The potential clamp system makes use of the so-called air gap of which the inventor (Tasaki 1939) claimed "that in the medullated fibre complete dessication of the medullated region alone does not inflict upon the fibre any noticable change in excitability". Nevertheless, in the air gap methods "the stable (experimental) situation does not last for more than one hour (see Nonner 1969) the axoplasm begins to dry out (see Sommer et al. 1982) which finally results in deterioration of the fibre as a whole. The main advantage of this method, however, is its simplicity" (Derksen 1965).

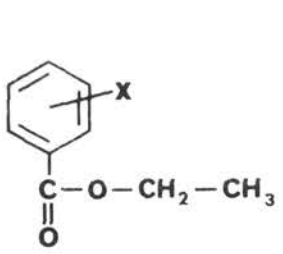
Measuring conditions. Effects of the non-drug-induced slow sodium inactivation were diminished by setting the conventional sodium inactivation variable, h_∞, at about 0.8 at rest. Although no so-cal- "frequency-dependent" blockage of sodium currents by the drugs under investigation was observed, care was taken to keep test pulse intervals as long as at least several seconds.

Nomenclature, Calibration and Statistics. Rectangular pulses of an amplitude U were applied which equalled deviations from the resting potential, V (Dodge and Frankenhaeuser 1959), provided that there was no appreciable resistance in series with the nodal membrane. Absolute values of membrane potential were termed E. Membrane currents were corrected for leakage currents automatically, assuming a potential independent leakage conductance (Dodge and Frankenhaeuser 1958).

Peak sodium currents I_{Na} were calculated from the output voltage of the clamp amplifier and the fibre dimensions, assuming a specific resistance of the axoplasm of 110 Ωcm (Stämpfli and Hille 1976).

The validity of the results was tested by the two-side Welch-test (Sachs 1978). Error probabilities are given as α.

Solutions. The normal bathing medium for the node under investigation was Ringer solution containing (mmol/l): NaCl 112, KCl 2.5, $CaCl_2$ 2.0, tris(hydroxymethyl)-aminomethane HCl-buffer 2.5. Stock solutions of reconsidered content were prepared from benzocaine (Fig. 1, 1) and ethyl *o*-amino benzoate (2) with Ringer solution and they were diluted by Ringer solution to obtain final concentrations of the respective drug in the test solutions of 1 mmol/l. Metacaine (3), however, was added in substance (1 mmol/l) as the highly water-soluble methanesulfonate of ethyl *m*-aminobenzoate (MS-222®, Sandoz AG, Nürnberg, W. Germany); note that the anion has proved to be inert (Sommer et al. 1984). Solutions older than about 10 hours were discarded. Measurements were started 1 min after the application of the respective drug. Recovery was tested in most cases 2 min after removal of the agent. In some experiments, potassium currents were deliberately depressed by adding tetraethylammonium chloride (TEA, 5 mmol/l) to the bathing media (Hille 1967; Koppenhöfer 1967; Schönle and Koppenhöfer 1983). In order to maintain the ionic concentration inside the node at a constant level, the adjacent internodes were cut (Koppenhöfer and Vogel 1969) and immersed in an artificial intracellular fluid (in mmol/l): KCl 108, NaCl 10, "tris-buffer" 2.5. The pH of all solutions employed was set at 7.2 ± 0.1. The temperature was kept at 15 ± 0.5 °C.



	position of —X	
—X		
$-NH_2$	p	(1)
$-NH_2$	o	(2)
$-NH_2 \cdot CH_3SO_3H$	m	(3)

Fig. 1. Structural formulae of three structural isomers of aminobenzoic acid ethylester. 1: benzocaine; 2: ethyl *o*-amino benzoate. 3.: methanesulfonate of ethyl *m*-aminobenzoate (= metacaine).

Results

General results. Following most contemporary users of the conventional Nonner potential clamp device, we kept the sodium inactivation, 1-h_∞, at 0.2 ± 0.01 (mean ± S.E.M.) by an appropriate electrotonus. Assuming the nodal series resistance (Koppenhöfer and Schumann 1979; Schumann 1980; Sigworth 1980; Koppenhöfer et al. 1984) to be negligibly small, and under proper measuring conditions, the reading of the voltmeter in compartment A of the recording chamber (Wiese and Duchâteau 1984) showed a corresponding holding potential $E = -75.0 \pm 0.3$ mV (mean ± S.E.M.; $n = 24$). A slight increase in E to -79.4 ± 0.3 mV during the experiments might have been due to some unavoid-

able deterioration of the fibre,e.g. and unspecific decrease of membrane resistance and/or an increase in the nodal series resistance.

An increase in the mean fibre diameter improves the reliability of membrane current measurements in two ways: first, with thicker fibres, at the high-frequency end a higher accuracy of the experimental data and a higher stability of the recording system against oscillations can be achieved (Steinmetz 1979). Second, in thicker fibres, the low-pass property of the current measuring internode, Z_{ED}, (Schumann et al. 1983) distorts current measurements to a lesser extent than it does in thinner fibres. Assuming the inner/outer diameter ratio to be independent of the dissection technique chosen, the gain of bandwidth can be calculated. Figure 2 shows the frequency dependence of a normalized current measuring internode, given as $|Z_{ED}/R_{ED}|$, according to the calculations of Schumann et al. (1983). Curve A applies to the mean diameter of fibres dissected conventionally (14μm), and curve B to that of fibres dissected using the modified technique (20.5 μm). The increase in bandwidth amounts to about 2.2, as can be seen by the intercept of the curves on the −3 db level (dotted line).

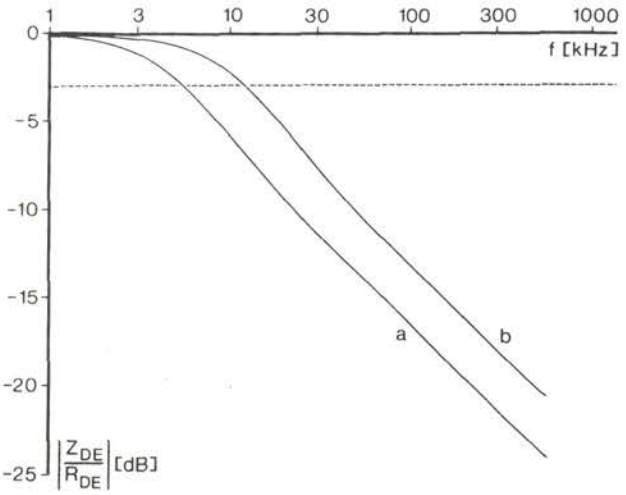


Fig. 2. Calculated absolute value of the impedance Z_{ED} of a standard current measuring internode related to the ohmic component R_{ED} as a function of frequency f. Fibre diameter: 14 μm (*a*) and 20.5 μm (*b*). Specific resistance of the axoplasm: 110 Ωcm. Myelin capacity: 1.3 pF/mm. Length of the internode: 2 mm.

Although the shape of the sodium current-voltage relation for myelinated fibres has been known for some time (Dodge and Frankenhaeuser 1958), to our knowledge, differences in steepness of its two branches as well as discrepancies of the position of its peak value on the potential axis (e.g. Chiu 1980; Franken-

haeuser 1959; Khodorov et al. 1976; Mozhayeva et al. 1977; 1986; Nonner 1969; Schumann 1980; Schwarz and Spielmann 1983; Steinmetz 1979) have been mostly ignored. Obviously, a steep negative branch is the first step for the so-called regenerative artefact which might result from "a high impedance in series with the membrane" (Århem et al. 1973), thus preventing appropriate potential clamp conditions. In our experience, nicely curled and loosely mounted fibres never showed all-or-none responses to close-to-threshold test pulses. Moreover, such fibres tend to exhibit a flatter negative branch of their sodium current-voltage relation than rectilinear axons. Consequently, both branches are often of similar absolute steepness and the peak value of such curves is shifted towards more positive pulses as compared to the position of the peak value in fibres dissected conventionally. In two out of three experiments, the peak value was even very close to the absolute membrane potential $E = 0$ (Fig. 3). The curve resembles the sodium current-voltage relations of the untreated node described by Mozhayeva et al. (1986), Schwarz and Spielmann (1983) and, moreover, the curve presented by Koppenhöfer et al. (1984) for optimum electronic compensation of the influence of the nodal series resistance. We believe that in specific experiments, considerable differences in magnitude of the series resistance have been involved in discrepancies of the sodium-current voltage relations of myelinated nerve fibres as reported by different authors. After all, it would make sense if clearly bent fibres often exhibit a comparably small series resistance.

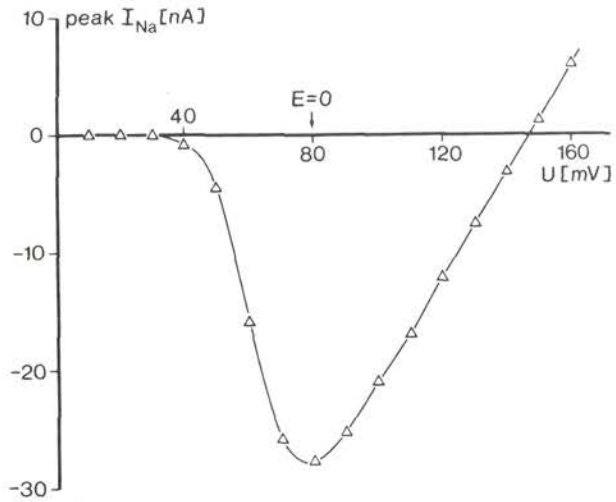


Fig. 3. Peak sodium current-voltage relation measured on a clearly curled axon. *U*: test pulse amplitude. Each test pulse was preceded by a hyperpolarizing prepulse of −40 mV in amplitude and 50 ms in duration. $E = 0$: zero absolute membrane potential. Continuous line: calculated by spline interpolation (Wiese 1985).

Drug effects. The potential dependence of the conventional sodium inactivation variable, h_∞, was measured by peak sodium currents, I_{Na}, elicited by constant test pulses preceded by conditioning prepulses of various amplitudes U and sufficient duration (Fig. 4, inset). The empirical equation

$$h_\infty = \frac{1}{1 - \exp((U - U_h)/k_h)} \tag{1}$$

(Hodgkin and Huxley 1952; Frankenhaeuser 1959) was applied twice to each set of data measured. The first fit to peak sodium currents, I_{Na}, as fractions of I_{Na}, available after a prepulse of $U = -60$ mV, yielded the theoretical maximum value which I_{Na} would attain after very large negative prepulses. After normalizing the experimental values to the theoretical value found by the first fit (measured points in Figs. 4, 5 and 6), the second fit was performed (continuous and interrupted curves, respectively). In Ringer solution (open triangles), the mean values ($\pm$ S.E.M.) of U_h and k_h from $n = 23$ experiments were 8.5 ± 0.4 mV and 6.2 ± 0.1 mV, respectively; this agrees reasonably well with the corresponding data reported by Frankenhaeuser (1959). Upon the application of benzocaine (1), the experimental values (Fig. 4, filled symbols) were shifted reversibly to more negative potentials, $U_h = -15.3 \pm 1.1$ mV ($n = 7$) and the availability of sodium permeability at holding potential, h_0, was reduced from 0.83 ± 0.01 to 0.14 ± 0.02. Moreover, there was a significant decrease ($\alpha < 0.0001$) in steepness of the curve, k_h being 8.1 ± 0.3 mV.

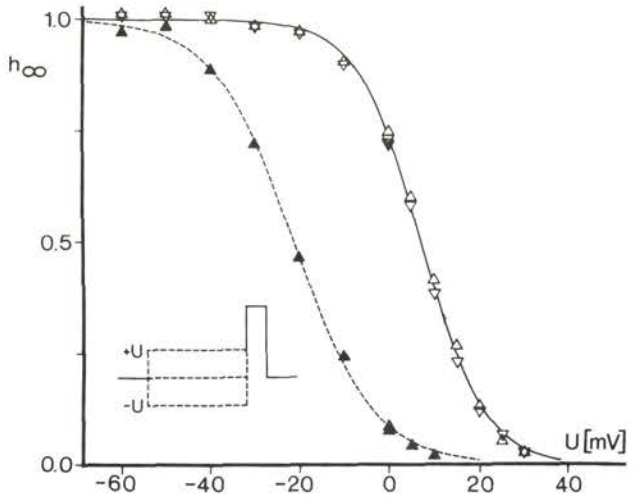
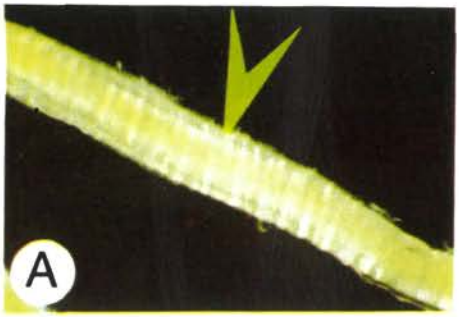
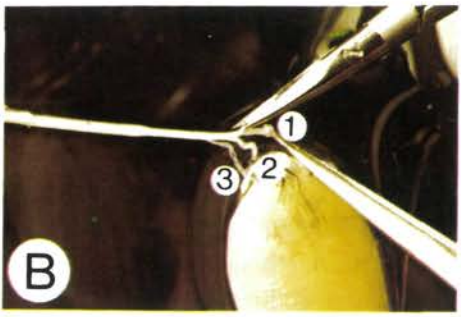


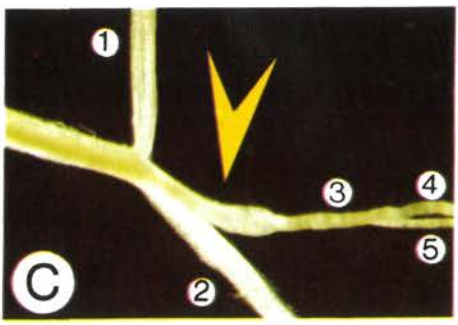
Fig. 4. Sodium inactivation curves in Ringer solution (open symbols) and in the presence of 1 mmol/l benzocaine (filled symbols). Both solutions contained 5 mmol/l TEA. h_∞: normalized peak I_{Na} after 50 ms conditioning prepulses of various amplitudes, U. Test pulse amplitude: 60 mV. The curves were calculated by equation 1. Holding potential: $E = -73$ mV. Inset: pulse program, not drawn to scale. For further details, see text.

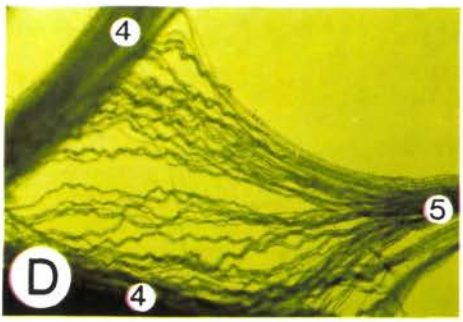
Plate 1. Dissection of a single myelinated nerve fibre of the frog. *A*: Cross striation (arrow) of the sciatic nerve after splitting the nerve sheath and pulling away the peroneal nerve. *B*: Splitting the shearth of the tibial trunk (1). Close to the stump of the peroneal nerve (left side) there is the desheathed portion of the nerve, shown in *A*. *C*: Pulling away the tibial trunk (1) under counter-pull on motor branch (2) of *m. gastrocnemius*. The central portion of the mixed branch starts to spread (arrow). *D*: Splitting the sensory bundle (4) of the mixed branch (3) produces a fan of loosely packed fibres of the motor bundle (5). Note the well-preserved fibres in the centre and the rectilinear marginal fibres, the latter obviously were exposed to intolerable stretch. *E*: A well-preserved and nicely curled fibre from the centre of the fan. Note the barely visible node (arrows). *F*: A rather rectilinear axon dissected conventionally. The node is clearly visible (arrows).
Techniques: *A*: Dark field, polarized light, dissection microscope (Wild, M3). Magnif.: 29×. *B*: Flashlight (Leica R3, Macro-Elmarit). Magnif.: 1.8×. *C*: Dark field. Dissection microscope (Wild, M3). Magnif.: 29×. *D*: Bright field, colour filter. Microscope (Leitz Dialux 22, Leica M3). Magnif.: 73×. *E*: Bright field, colour filter. Microscope (Leitz Dialux 20, Leica M3). Magnif.: 623×. *F*: like in *E*, but with lambda-platelet instead of colour filter.

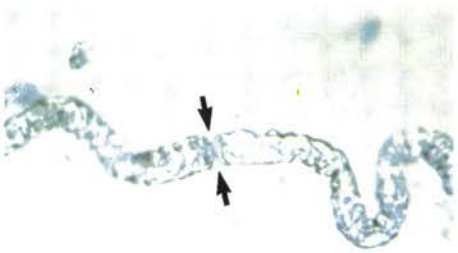
Plate 1

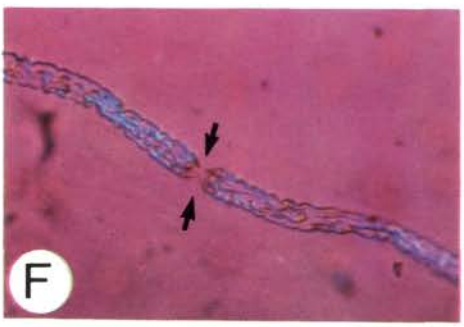












efficient than metacaine (3) or ethyl *o*-amino benzoate (2); the potencies of the latter two isomers did not differ significantly. Maximum sodium permeability $\bar{P}_{Na}$, however, was reduced by the drugs to the same extent. The observed shift and slight decrease in steepness of the inactivation curve by benzocaine has already been documented by Hille (1977), the smaller shift caused by 2 and 3 ask for some comments.

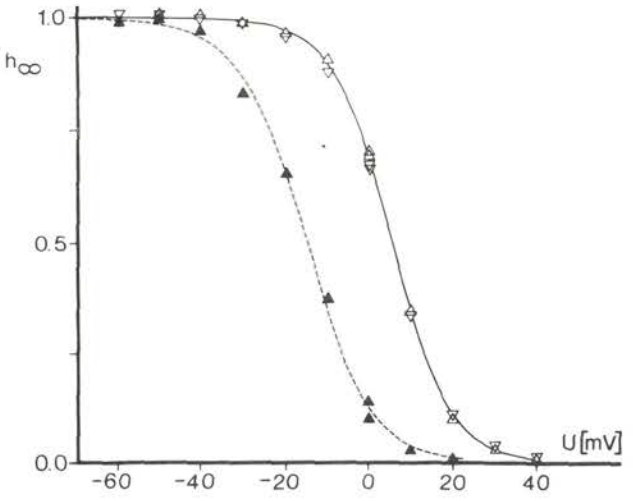


Fig. 6. Sodium inactivation curves in Ringer solution (open symbols) and in the presence of 1 mmol/l metacaine (filled symbols). Both solutions contained 5 mmol/l TEA. Holding potential: $E = -74$ mV. For experimental conditions, see Fig. 4.

For a polarized biomembrane the actual proton activity within the membrane matrix is remarkably reduced compared with the bulk pH of 7 (see Lüllmann and Peters 1979). The pK_a-values of the drugs under investigation, however, are between 2.2 and 3.6 (Cumming 1907; Robinson and Biggs 1957; Froese 1986). Consequently, in the membrane these substances should be uncharged and we doubt that solely a simple binding to a charged so-called "receptor" (Ritchie 1975) as defined by Hollenberg and Cuatrecasas (1979) would play a major role in the action of these local anaesthetics on sodium inactivation. Our view is not necessarily at variance to the finding of Sommer and coworkers (1984) that the dose-response relation of metacaine (3) can be fitted to a Langmuir adsorption isotherm (Ziegler 1987). Thus, the observation that 2 and 3 act on sodium inactivation to a lesser extent than 1 should rather be correlated to other mechanisms, such as different lipid solubilities of the drugs under investigation, bearing in mind that "a relationship between local anesthetic potency and lipid solubility

has been observed so often that it is generally believed that the primary effect of local anesthetics is on the lipid component of the membrane" (Lee 1977). Following the membrane expansion theory (Seeman 1972), the annular transition model (Lee 1976) or the concept of Trudell (1980) of the action of local anaesthetics, and taking into account that both charged and uncharged forms of amine local anaesthetics may be incorporated into lipid membranes (Surewicz and Leyko 1982), one could assume that benzocaine is more liable to incorporate in the lipid matrix of the membrane than 2 or 3, thus increasing sodium inactivation more effectively by disturbing the lipid matrix. In this connexion the octanol/water partition coefficients of 1, 2 and 3 have been measured (Froese 1986). The corresponding figures are 65.9, 345.9 and 47.9, respectively. If, like in other preparations (Casanovas et al. 1985) but at variance to conclusions drawn by Trudell (1980), the octanol/water partition coefficient reflects the solubility of the drugs in the membrane matrix, the observed shifts of sodium inactivation curves would not solely be caused by incorporation of the substances in the lipid phase.

On the other hand, benzocaine and its isomers did not differ in their potencies to block maximum sodium permeability. Unfortunately, this finding can neither be correlated to their octanol/water partition coefficients. Experiments on the blocking effects of derivatives of amino benzoate, however, suggest that a minimum octanol/water partition coefficient is necessary for their blocking potencies to become manifested (Froese 1986).

The nodal series resistance: The dissection technique outlined above offers thicker fibres and remarkably looking sodium current-voltage relations. Thicker current measuring internodes distort the kinetics of current records less than do thinner internodes (Schumann et al. 1983).

The peak sodium current-voltage relations resemble corresponding curves derived under optimum compensation of the current proportional voltage drop across the nodal series resistance (Koppenhöfer et al. 1984). Evidently, a nicely curled fibre exhibits a negligibly small series resistance although the nodal gap of such fibres is barely visible. This can easily be understood because, in teased fibres, the outer gap contour becomes wider while the myelin of the adjacent internodes tends to flow together at the inner gap level, eventually covering the axolemma (Schneider 1952; Sommer unpublished) and thus forming an unphysiological high series resistance. Assuming nicely curled fibres are better preserved than straight-lined axons, we believe that in the living animal the series resistance of the Ranvier nodes might be even smaller than it has been measured by Drouin and Neumcke (1974) and suggested by remarkable differences in steepness of both branches of sodium current-voltage relations reported so far (for references, see pages 213, 214). Moreover, "much remains obscure about the nature of the barrier around the fibre at the node" (Hess and Young 1952), and

we wonder about the low impedance of the dense structures lying in series with the nodal membrane of thick myelinated nerve fibres (Landon and Hall 1976; Berthold 1978; Rydmark 1982; Berthold and Rydmark 1983; Sommer unpublished). Nevertheless, for practical reasons it is more favourable to voltage clamp excitable membranes exhibiting a genuine small series resistance than to compensate for the influence of an intolerably high series resistance using any sophisticated electronic device.

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