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Blockade of Sodium Channels and Transmitter Uptake Systems in Synaptosomes by Local Anesthetics: Lipid-Mediated or Direct Influence?

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Abstract. Procaine, trimecaine and tetracaine inhibit the active transport of norepinephrine and choline in rat brain synaptosomes with half maximal effects at $10^{-4} - 6.10^{-5}$ mol/l. Depolarization with veratrine (0.1 mg/ml) or 50 mmol/l KCl resulted in neurotransmitter release. Tetrodotoxin (5.10^{-7} mol/l) or charged tertiary amines (10^{-4} mol/l) prevented the veratrine effect but not that of KCl. Microviscosity of synaptic membrane lipid bilayer, evaluated by mobilities of doxylstearate spin labels, was decreased at tetracaine concentrations exceeding 10^{-3} mol/l. It is suggested that the uptake systems for neurotransmitters and sodium channels are blocked through direct action of anesthetics or owing to changes in the annular lipids.

Key words: Neurotransmitter uptake — Sodium channels — Anesthesia

Introduction

It is well established that anesthetics induce multiple effects in excitable and nonexcitable membranes (Seeman 1972). The central question in the current hypotheses on anesthesia mechanisms is to choose between nonspecific (through disordering of membrane lipid phase) and direct influence of anesthetics on the functional systems responsible for nerve and synaptic transmission. In particular, for local anesthesia it is still unknown whether anesthetics interact directly with the sodium channels (Strichartz 1976) or the action potential blockade is caused by "fluidization" of the lipid bilayer (Trudell 1973).

One traditional approach to this problem is to compare the channel blocking concentrations of anesthetic with those effective in increasing the lipid fluidity in natural and model membranes. However, there is considerable controversy on this subject. The "fluidization" of lipids under the influence of anesthetics is well documented (for review see Kaufman 1977). Nevertheless, it is still not quite clear whether the concentrations inducing microviscosity change and those block-

ing the conduction of potential are in fact identical. Some authors could not detect a decrease of the microviscosity at low concentrations of anesthetics (Boggs et al. 1976), whereas others, on the contrary, observed even increased ordering of the bilayer (Rosenberg 1979). It may be noted also that drug sensitivity of the lipid core depends strongly on its chemical composition (Rosenberg 1979), including the presence of proteins (Lenaz et al. 1979). Furthermore, the term "nerve blocking concentration" itself is rather vague because this value varies by 1—2 orders of magnitude in different types of nerve fibres (Seeman 1972). Therefore we thought it reasonable to use brain synaptosomes as a model of peripheral fiber and nerve terminal. Such a model makes it possible to determine the concentration region at which anesthetic affects the sodium channels and neurotransmitter uptake systems and to find out whether concomitant changes in the membrane fluidity do take place. The state of sodium channels was tested indirectly by veratrine-stimulated release of transmitters (Blaustein 1975).

In the presented work it was established that the system of neurotransmitter uptake and sodium channels of synaptosomes is blocked at low concentrations of tertiary amines, which fail to initiate marked changes in the lipid bilayer of synaptic membranes.

Material and Methods

Material

The local anesthetics used were procaine, tetracaine, trimecaine. Tetrodotoxin was obtained from Sankyo Co., Japan; veratrine from Sigma Chemical Co. Radiochemicals were: d,l-^{14}C-norepinephrine (62 mCi/mmol) and [methyl-^{14}C] choline chloride (58 mCi/mmol) (Amersham, England). All the usual chemicals were of a reagent grade.

Preparation Procedures

Synaptic membranes were isolated from brain hemispheres of albino rats (wt 150—200 g) according to Jones et al. (1974). The sediment was resuspended in 0.32 mol/l sucrose with 40 mmol/l Tris buffer, pH 7.4.

For the experiments on uptake and release of neurotransmitters the crude synaptosomal fraction (P_2) was obtained. After initial homogenization in 9 volumes of 0.32 mol/l sucrose the homogenate was centrifuged at 900 g for 10 min followed by sedimentation of the supernatant at 12 000 g for 20 min. The pellet was resuspended in the media for measurements of uptake or release (see below).

Lipids were extracted from synaptosomal membranes according to the method of Folch et al. (1957). Liposomes were prepared by sonication under nitrogen flow.

ESR-Studies

For evalution of the lipid bilayer state two spin labels were used: N-oxyl-4′-4′-dimethyloxazolidine derivatives of 5-ketostearic (NS5) or 16-ketostearic (NS16) acids (Syva). They probe in the hydrophilic

and hydrophobic regions of the bilayer lipid, respectively. The spin labels (38 μg per 1 μl of ethanol) were added to 1 ml of membrane suspension in 0.32 mol/l sucrose, 40 mmol/l Tris-HCl buffer, pH 7.4, containing about 20 mg of protein or 10 mg of lipid. The final concentration of the label in suspension was calculated to be 1×10^{-4} mol/l or 0.4% by weight of lipids under the assumption of 90% label incorporation into the membranes (Rosenberg 1979). The volume ratio of buffer/membrane pellet was 1:1. Incorporation was carried out at 37 °C by shaking. At the dilution used (1:1000) ethanol did not affect parameters of ESR spectra of labels in membranes and liposomes.

ESR spectra were recorded at room temperature using a Varian E104A spectrometer (field modulation amplitude 2G; time constant 0.25). The control with potassium ferric cianide showed that incorporation of the label was complete. Mobility of label NS16 was estimated with the rotational correlation time τ_c from the following equation for rapid isotropic rotation:

$$\tau_c = 2 \times 10^{-8} (\sqrt{h_0/h_{+1}} - 1)\, \Delta H_0 \qquad \text{(Likhtenstein 1974)}$$

where ΔH_0 is the half width of the medium field line and h_0 and h_{+1} are the heights of the medium and low field lines, respectively (Fig. 2). Because in our conditions spin label motion was not purely isotropic (Fig. 2), values of τ_c obtained must be considered approximate.

In the case of label NS5 the order parameter S was taken as a measure of the probe ordering using the equation for anisotropic motion

$$S = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - 1/2(T_{xx} + T_{yy})} \cdot \frac{a}{a'} \qquad \text{(Gaffney 1976)}$$

where $T_{zz} = 30.8$G; $T_{xx} = T_{yy} = 5.8$G; $a = 1/3(T_{zz} + T_{yy} + T_{xx})$; $a' = 1/3(2T'_{\perp} + T'_{\parallel})$, $T'_{\parallel}$ and $T'_{\perp}$ being determined visually from the spectra (Fig. 2).

Measurements of Uptake and Release of Neurotransmitters

For the uptake studies (White 1977; McClure et al. 1980) the P_2 fraction (1.9 ml, 1.0—1.2 mg protein) was preincubated for 25 minutes at 35 °C in the following medium (all the concentrations in mmol/l): NaCl 132, KCl 5; $MgCl_2$ 1.3; NaH_2PO_4 1.2; Tris-maleate buffer 20, pH 7.4; glucose 10; EGTA 0.2; iproniazid 0.1 (for norepinephrine uptake). The ^{14}C-norepinephrine in 1 mmol/l ascorbic acid solution or ^{14}C-choline (0.1 ml), were diluted 20-fold upon addition to final concentrations of 5×10^{-7} mol/l or 3×10^{-6} mol/l, respectively. After 10 min or further incubation the suspensions were cooled on ice and immediately vacuum filtered on microfiber glass filters GF/F(Whattman). The filters were washed 3 times with cold incubation medium. The experiments with anesthetics were carried out in the same succession, the corresponding substance being added at the 15th minute of preincubation. The active transport of norepinephrine was determined as a cocaine-sensitive part of the total uptake (with 2 mmol/l of cocaine the uptake was inhibited by more than 70%). Ouabain at a concentration of 2—4 mmol/l completely suppressed active transport. Kinetic parameters of transport ($K_m = 2 \times 10^{-7}$ mol/l and $V_{max} = 3.5 \times 10^{-11}$ mol/l.mg protein.min) were in accordance with literature data (White 1977). Nonspecific absorption of choline evaluated at 0 °C did not exceed 15% of the total choline uptake.

The studies on neurotransmitter release were performed in the following way. The P_2 fraction (0.5—0.6 mg protein/ml) was preincubated for 5 min at 37 °C in the medium described above, from which EGTA was excluded and 1.2 mmol/l Ca was added. Then ^{14}C-norepinephrine or ^{14}C-choline was introduced and incubation proceeded for the subsequent 10 min, uptake being terminated by cooling on ice. Some portions of cooled suspension were heated to 37 °C. At 3 min the anesthetic of corresponding

concentration or tetrodotoxin (5×10^{-7} mol/l) was added and after 5 min KCl or veratrine was introduced up to final concentrations of 50 mmol/l or 0.1 mg/ml respectively. After 10 min the samples were rapidly vacuum filtered. The filters were washed 3 times with the cold medium. The values of norepinephrine or acetylcholine + choline release upon depolarization calculated as a decrease of radioactivity of pellets on the filters were 40% or 25% for veratrine and 30% or 45% for KCl, respectively. The same procedures but without veratrine or KCl were used for measurements of the spontaneous neurotransmitter release. Filters were dried in the air (at 70°C) and placed into the scintillation liquid PPO + POPOP in toluene. The radioactivity was determined in a Mark III scintillation counter using program 10 for heterogeneous systems.

Protein was assayed according to Lowry et al. (1951).

Every experimental point is a mean ± S.E.M. from at least two independent experiments with eight measurements in each. The levels of significance were calculated by Student's t-test.

Results and Discussion

Trimecaine, procaine and tetracaine are effective inhibitors of norepinephrine (A) and choline (B) uptake (Fig. 1). The sensitivity of both transport systems to each of the local anesthetics is quite similar. So, the concentrations of half maximum inhibition with procaine are 1.4×10^{-4} mol/l for adrenergic and 2.2×10^{-4} mol/l for cholinergic synaptosomes. For the most potent agent tetracaine these concentrations are 7.4×10^{-5} mol/l and 5.6×10^{-5} mol/l, respectively. Because the net uptake measured is an effective value determined by the true active transport and spontaneous release, checks were made of the influence of the anesthetics on spontaneous release or leakage of neurotransmitters.

At concentrations causing 50% inhibition of neurotransmitter uptake neither of the anesthetics used (tetracaine, procaine and trimecaine) induced spontaneous leakage. As seen from figure 1 the tetracaine-induced leakage begins at concentrations, at which uptake is already blocked up to 80%, while spontaneous release of acetylcholine + choline was not significantly affected by anesthetic concentrations to 10^{-3} mol/l. This implied that the observed inhibition of uptake is related to the blockade of active transport through interaction with some sites on presynaptic membrane rather than through its depolarization with subsequent release or leakage of neurotransmitters.

It is well established that local anesthesia manifested as suppression of the action potential results from the sodium channel blockade. The model of the action potential on synaptosomes can be obtained by their depolarization with veratrine, which increases the sodium permeability at rest, or less adequately with high K^+ concentrations, which decrease the potassium equilibrium potential (Blaustein 1975; Li and White 1977).

We studied the effect of synaptosome pretreatment with procaine, trimecaine and tetracaine on the depolarization-induced release of transmitters. Table 1 shows that the specific inhibitor of sodium channels tetrodotoxin (5×10^{-7} mol/l) decreases the veratrine-mediated release of norepinephrine and acetylcholine +

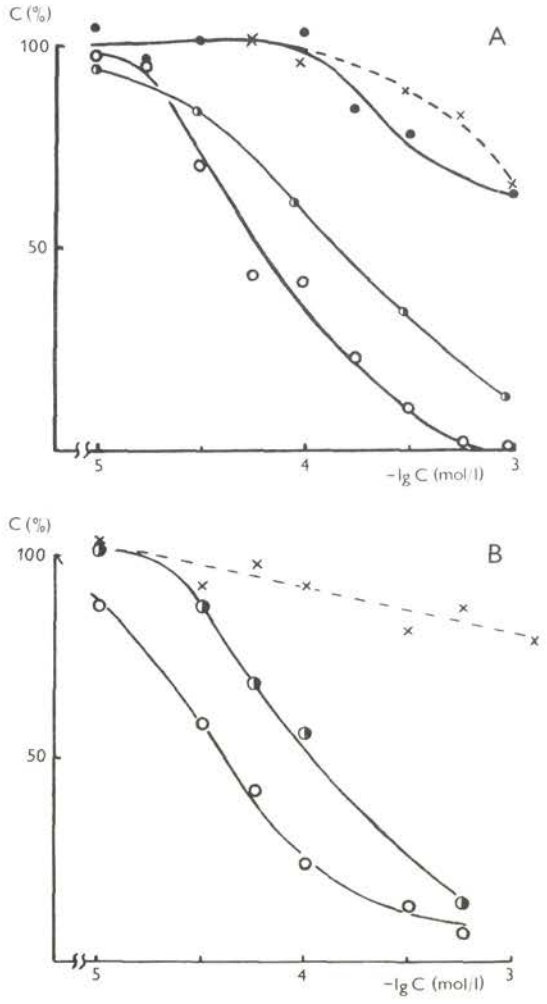

Fig. 1. Effect of trimecaine (●), procaine (◑) and tetracaine (○) on the uptake systems of norepinephrine (A) and choline (B) in rat brain synaptosomes. Dotted lines indicate spontaneous leakage of synaptosomes preloaded with neurotransmitters upon addition of tetracaine (×). Horizontal axis is the negative logarithm of anesthetic concentration. Vertical axis is the per cent content of labeled neurotransmitters in synaptosomes relatively to the control measured in the absence of anesthetics.

choline by 80—90%. Thus in our experimental conditions veratrine opens the sodium channels and initiates the transmitter release through specific depolarization. Anesthetics at a concentration of 10^{-4} mol/l, when ineffective in inducing spontaneous efflux, are able to block veratrine — but not KCl

Table 1. Effects of tetrodotoxin[1] and local anesthetics[2] on release of labeled neurotransmitters from synaptosomes after depolarization with veratrine (0.1 mg/ml) or KCl (50 mmol/l)

Neurotransmitter	Depolarizing agent	Inhibition of neurotransmitter release, per cent			
		tetrodotoxin	tetracaine	procaine	trimecaine
Norepinephrine	1. Veratrine	77±3	57±4	25±5	25±5
	2. KCl	0	0	—	—
Acetylcholine + choline	1. Veratrine	94±2	63±3	39±3	27±4
	2. KCl	0	0	—	—

[1] tetrodotoxin, 5×10^{-7} mol/l
[2] local anesthetics 1×10^{-4} mol/l

-mediated release in a manner similar to that of tetrodotoxin. For example, tetracaine decreases veratrine-induced efflux of both neurotransmitters by about 60%, while failing to modify the release that resulted from synaptosome depolarization in KCl solution (see Table). Such selective effect of tetrodotoxin and anesthetics on veratrine inducement of efflux is quite understandable because for transmitter release it is sufficient to reach a certain level of depolarization which in case of 50 mmol/l KCl but not in that of veratrine is realized without participation of sodium channels. On the other hand veratrine depolarization is due to influx of sodium through its open channels. Hence, experiments with both depolarizing agents show that anesthetics block specifically the sodium channels of synaptosomes thus preventing veratrine depolarization and, consequently transmitter release.

It seems probable that a correlation exists between the pharmacological activity of cationic anesthetics and their blocking efficiency because less potent procaine and trimecaine decrease the veratrine effect by 25—40% only.

In order to answer the question of involvement of the lipid bilayer in blockade by anesthetics of synaptic transmission (inhibition both of uptake and of sodium channels), microviscosity of the lipids in the synaptic membranes was measured with spin labels. In Fig. 2 the mobility parameter τ_c of label NS16 (A) and the order parameter S of label NS5 (B) in the membranes and liposomes are plotted against the tetracaine concentrations. It can be seen that tetracaine decreases the microviscosity of the hydrophobic core (label NS16) of the synaptic membranes but is completely ineffective for liposomes prepared from extracted lipid.

Insensitivity of label NS16 mobility to tetracaine in liposomes in comparison with the distinct effect of the anesthetic in membranes supports the hypothesis of

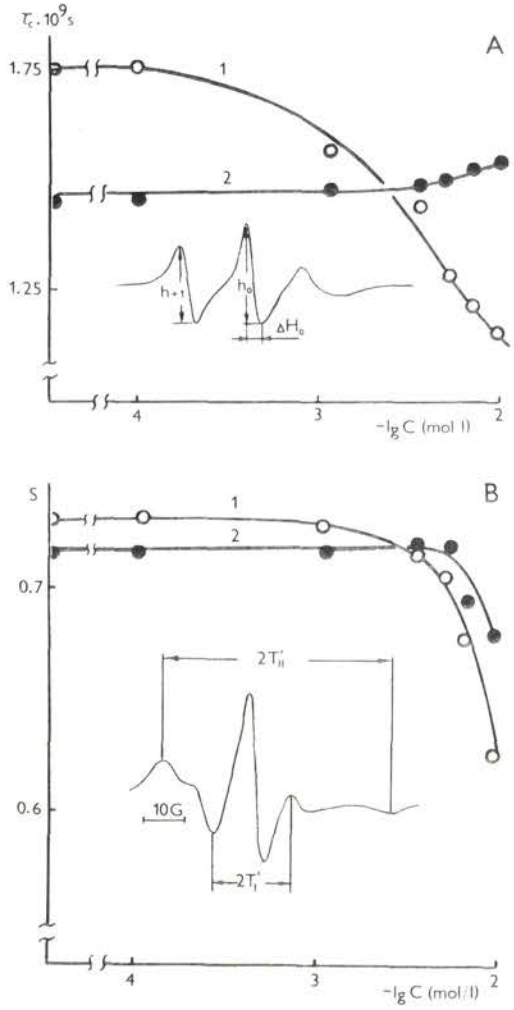

Fig. 2. Effect of tetracaine on the rotational correlation time τ_c of label NS 16(A) and on the order parameter S of label NS 5(B) in isolated synaptic membranes (1) and liposomes (2) prepared from membrane lipids. Also presented are the ESR spectra of the respective labels with the characteristics used for calculation of label mobility.

Lenaz et al. (1979) about the contribution of the protein-lipid interactions to the averaged microviscosity of the hydrophobic bilayer core and their disturbance by anesthetics. The order parameter of label NS5 located closer to the surface decreases upon tetracaine addition both in intact membranes and in liposomes.

This fact seems to indicate that protein-lipid interactions disturbed by tetracaine have much greater influence on mobility of hydrocarbon chains in membrane core than on motion of surface located parts of phospholipid molecules. Efficiencies of other drugs were tested with τ_c in synaptic membranes. In contrast to tetracaine, procaine and trimecaine at concentration range 0.1—10 mmol/l did not affect the value of rotational correlation time.

Going back to Fig. 1 and Table 1, it may be noted that the uptake systems for norepinephrine and choline as well as the sodium channels are blocked at tetracaine, procaine and trimecaine concentrations as low as 10^{-4} mol/l or less, at which there are no changes detectable in the surface or hydrophobic regions of the lipid bilayer. As far as the protein state is concerned, we have recently shown that in the presence of local anesthetics even in the millimolar concentration range the overall conformation of membrane proteins also remains unchanged, judging from the number of the surface thiol groups accessible to specific reagent 5-5′-dithiobisnitrobenzoic acid (Rakovich et al. 1981).

Thus, according to the model presented, the functional effects of tetraciane and possibly other charged tertiary amines are due to direct interaction with the sodium channels, transport systems or their microenvironment rather than to the events in the lipid phase of excitable membranes. Such a conclusion is quite consistent with the ideas based on the electrophysiological data, according to which local anesthetics suppress the action potential after binding to the sites located inside the sodium channels (Khodorov 1979) or they may be effective through changes in annular lipids (Lee 1977). An increase of fluidity of the lipid bilayer takes place at considerably larger anesthetic concentrations and is unlikely to be responsible for local anesthesia.

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