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Pressure Effect on the Membrane Action of a Nerve-Blocking Spin Label

(anesthetics/anesthesia/conformation change/receptor/synaptosomes)

J. C. HSIA AND J. M. BOGGS

Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8

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ABSTRACT **A reversible nerve-blocking spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) was used to study the nature of anesthetic-binding sites in nerve membranes as a function of pressure. The nerve-blocking effect of TEMPO is enhanced under pressure. At atmospheric pressure, TEMPO blocks nerve conduction by solubilizing in the apolar region of the nerve membrane. However, the nerve-conduction-block by TEMPO at 150 atm of helium was related to the binding of TEMPO to a pressure-induced high-affinity polar site in the nerve membrane. The new TEMPO-binding site could not be detected in lipid model membranes and, thus, the involvement of membrane protein in the new site was inferred. Pressure may induce a nerve membrane conformation change in the presence of TEMPO.**

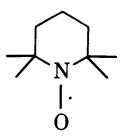
The observation that under different pressure, a single anesthetic, i.e., TEMPO, was capable of blocking nerve conduction by binding to two different sites within the nerve membrane, supports the view that there are multiple anesthetic receptor sites, which differ in chemical composition and location within the nerve membrane. These sites, when occupied by different classes of anesthetics, produce the general phenomenon of nerve-conduction block. The enhancement of nerve-conduction block by pressure may be due to the increased concentration of TEMPO in the new site in the nerve membrane under pressure.

One of the major unsolved problems in the molecular pharmacology of anesthesia is the mechanism by which nerve conduction is blocked by anesthetics. In order to understand the mechanism of anesthesia, it is useful to give a general definition of an anesthetic as "a drug which reversibly blocks the action potential of the nerve." Thus it is possible to compare the mechanism of action of a wide variety of nerve-blocking drugs such as tranquilizers, anticonvulsants, antihistamines, steroids, antiarrythmics, narcotics, vasodilators, and sedatives (1).

The general correlation of anesthetic potency to the nonaqueous/aqueous partition coefficient supports the classical idea that the membrane anesthetic interaction is hydrophobic (1). Some general phenomena of these anesthetics have been observed, such as membrane expansion, fluidization, and disordering. Most of the published evidence tends to support the view that the lipid region of nerve membrane is the primary site of anesthetic action, and the phenomenon of anesthesia may be associated with an increase in the volume or fluidity of the lipid region of nerve membrane. However, attempts to correlate the nerve-blocking activity of these anesthetics with their membrane concentrations and volume occupation reveal a variation of 43-fold and 8.5-fold, respectively (1). These discrepancies could be accounted for if different classes of anesthetics act on different sites within the nerve membrane. In this communication we show that binding of a single anesthetic to two different sites within the nerve membrane can produce similar blocking effects.

The use of helium or hydrostatic pressure has been one of the most unique experimental approaches for testing certain molecular theories of anesthesia. Reversal of the anesthetic state by pressure has been demonstrated on whole animals and isolated nerve (2, 3; Roth, S. H., Smith, R. A. & Paton, W. D. M., manuscript in preparation). Recently pressure was found to enhance the effect of halothane on a marine amphipod (4). Therefore, any theory of anesthesia must account for the mechanism by which the anesthetic state is reversed or enhanced by pressure. A study of this phenomenon on spin-labeled phospholipid vesicles as a model for the hydrophobic region of nerve membranes showed that anesthetics increase the fluidity of the hydrocarbon region while pressure decreases it (5). Our experimental approach is to study the pressure effect by directly monitoring the behavior of anesthetic spin-label molecules in nerve membranes. Recently Trudell *et al.* (6) have reported a study using this approach on phospholipid vesicles and showed that pressure does not cause a significant change in the distribution of the anesthetic between the lipid bilayer and the aqueous phase. However, phospholipid vesicles may not be an adequate model for the nerve membrane and, indeed, we demonstrate in this study that there is a dramatic difference in behavior of the spin-label anesthetic in nerve and synaptosomal membranes as compared to lipid bilayers.

A water-soluble, lipophilic spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) reversibly blocked the action potential of isolated rat phrenic and frog sciatic nerves



TEMPO

with a median effective dose (ED_{50}) of 6.4 mM and 13mM, respectively. The nerve-blocking effect of TEMPO on frog sciatic nerve was significantly enhanced under 100 atm of helium (Roth, S. H., Staiman, A., Boggs, J. M. & Hsia, J. C., manuscript in preparation). Since TEMPO retains its activity

Abbreviations: ESR, electron spin resonance; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; ED_{50}, median effective dose. In this paper, 50 mol % means one mol of cholesterol per one mol of phosphatidylcholine.

under pressure, we now have a unique opportunity to examine the pressure effect on the behavior of TEMPO in nerve membranes. The specific advantage of using a spin-label anesthetic is that the paramagnetic resonance of a nitroxide spin label depends on its motion and on the polarity of the solvent (7). Under ideal conditions the resonance peaks due to the distribution of the spin label in two or more environments can be resolved (8, 9). Thus, the use of water-soluble, lipophilic spin labels permits the simultaneous determination of the distribution and motional characteristics of the spin label in a biological membrane and the polarity of the membrane-binding sites.

By monitoring the distribution and environment of TEMPO in nerve and model membranes as a function of pressure, we show: (*a*) at atmospheric pressure, blocking of the nerve action potential is related to the solubilization of TEMPO in the apolar region of the nerve membrane; (*b*) pressure has no significant effect on the TEMPO concentration in the apolar region of nerve and lipid model membranes; (*c*) however, in hydrated nerve and synaptosomes, pressure reversibly induces a redistribution of TEMPO from the aqueous phase to a high-affinity polar site in the membrane, and if free TEMPO in the aqueous phase is limited, TEMPO migrates from the apolar site to the new site.

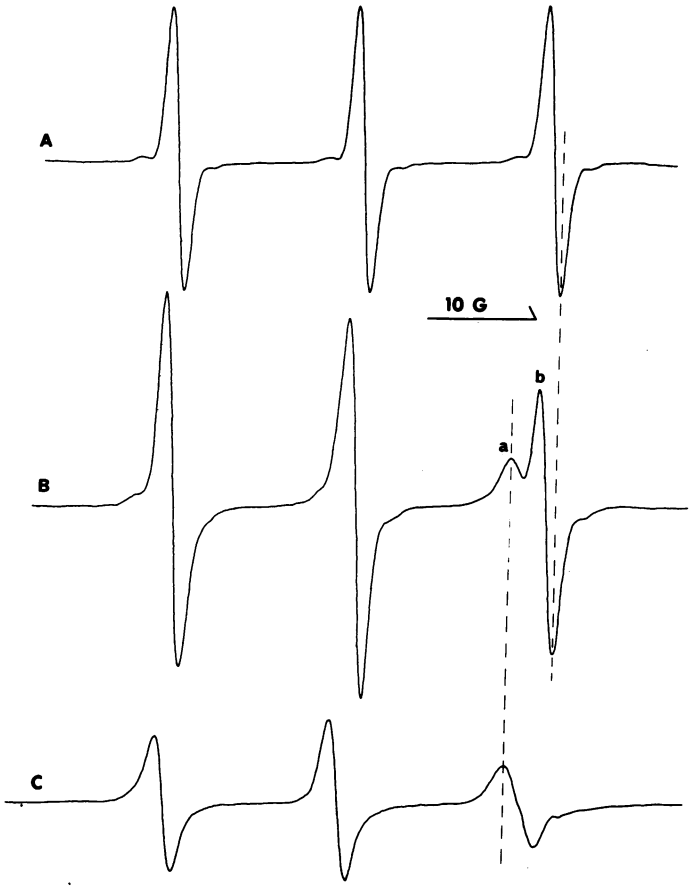


FIG. 1. ESR spectra of TEMPO in Ringer's solution. (*A*) Free TEMPO; (*B*) in the presence of egg phosphatidylcholine vesicles (hyperfine line *a* is part of the spectrum due to TEMPO in the apolar region of the lipid bilayer and *b* is part of the spectrum due to TEMPO in the aqueous phase); (*C*) ESR spectrum of TEMPO in the apolar region of egg phosphatidylcholine obtained by subtracting spectrum *A* from spectrum *B* with the use of a Fabri-Tek 1072 instrument computer. *Vertical broken line* marks identical magnetic field position.

MATERIALS AND METHODS

2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO), melting point 36.5°, was prepared as described (10). Egg phosphatidylcholine was purchased from Pierce Chemical Co. Cholesterol was recrystallized in methanol, melting point 148.5°. All chemicals used are reagent grade. A thick-walled cylindrical quartz cell (outer diameter 8 mm, inner diameter 0.8 mm) and high-pressure system were constructed by Drs. S. A. Boggs and J. MacTaggart, Dept. of Physics, University of Toronto. A helium tank (99.9%) containing 2200 lb./inch^2 was from Matheson of Canada, Ltd.

Lipid dispersions containing various amounts of cholesterol were prepared by hand dispersion of 5–10 mg of lipid in 0.1 ml of 6.4 mM TEMPO in Ringer's solution. Rat-brain synaptosomal membranes were a gift from M. Chou-Wong and P. Seeman. Rat phrenic nerve was freshly dissected from a decapitated rat and immediately transferred to Ringer's solution. Frog sciatic nerve was freshly dissected and desheathed. Spin labeling was achieved by equilibrating the membranes and phrenic nerve preparation in 6.4 mM TEMPO in Ringer's solution, while 13 mM TEMPO was used for frog sciatic nerve. A stainless steel wire and hypodermic needle were used to transfer the nerve and membrane suspensions, respectively, to the high pressure cell. TEMPO was reduced by the phrenic nerve membrane under aerobic conditions, but when the oxygen was displaced by nitrogen or helium it was reoxidized. When the signal intensity had stabilized, the spectra were recorded. The pressure effect on

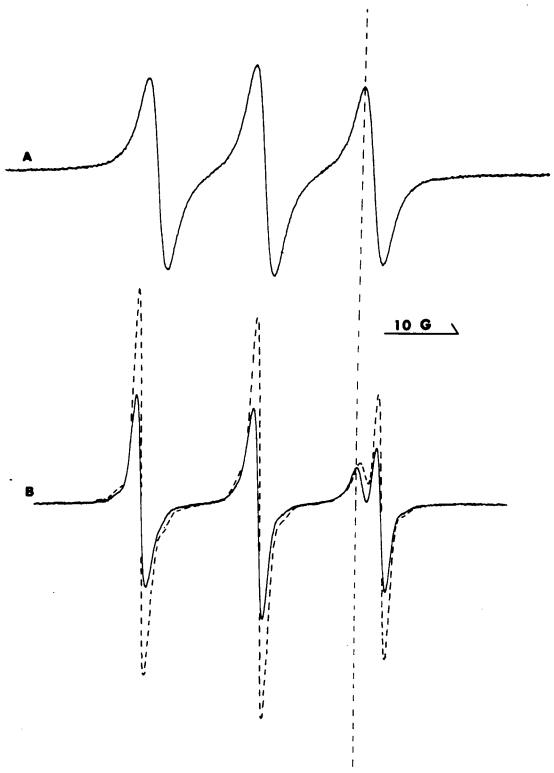


FIG. 2. ESR spectra of TEMPO (*A*) in rat phrenic nerve, sealed in a tissue cell (essentially only intracellular water is present); (*B*) in rat phrenic nerve (——) and in rat-brain synaptosomes (– – –) in Ringer's solution. (The volume of the Ringer's solution was adjusted relative to that of the nerve membrane so that only limited free TEMPO is present in the aqueous phase.) *Vertical broken line* marks identical magnetic field position.

TABLE 1. *ESR parameters of TEMPO in various systems*

	a_0 (G)	Δg_0* (G)
Ringer's solution	17.2	0
Octanol	16.1	0.2
Hexane	15.1	3.9
Egg phosphatidylcholine	15.9	1.0
Egg phosphatidylcholine + 33 mol/% cholesterol	15.5	0
Egg phosphatidylcholine + 50 mol/% cholesterol	15.4	0
Phrenic nerve	15.3	0

* Δ g_0 is the downfield shift of the center lines of TEMPO in various solvents relative to that in Ringer's solution.

the electron spin resonance (ESR) spectra was recorded at least three times to ensure reproducibility and reversibility of the results. All ESR spectra were recorded on a Varian X-band E-6 ESR spectrometer. Constant temperature of the sample was maintained by passing a stream of air through the ESR cavity at ambient temperature. Spectral subtraction in Fig. 1 was done with the use of a Fabri-Tek model 1072 instrument computer.

RESULTS

Resonance Spectra of TEMPO in Lipid Model Membrane. Fig. 1*A* shows the resonance spectrum of TEMPO in Ringer's solution. The resonance spectrum of TEMPO in egg phosphatidylcholine vesicles shown in Fig. 1*B* is similar to that observed by Hubbell and McConnell (8). Hyperfine lines *a* and *b* are each part of the separate three-line spectra due to TEMPO in the apolar region of the phospholipid vesicles and the surrounding aqueous phase, respectively. The resonance spectrum of TEMPO in egg phosphatidylcholine (Fig. 1*C*) was resolved by subtracting an equivalent concentration of free TEMPO in the aqueous phase (Fig. 1*A*) from Fig. 1*B* with the use of a Fabri-Tek model 1072 instrument computer. The apolar environment in egg phosphatidylcholine shifts the g_0 value down-field by 1.0 G and decreases the isotropic hyperfine splitting a_0 from 17.2 to 15.9 G.

Similar resonance spectra of TEMPO bound to lipid phase could be obtained by equilibrating an orientated lipid multibilayer film with TEMPO, draining and partially drying with a stream of N_2. Due to rapid isotropic motion of TEMPO, its resonance spectrum was orientation independent. The isotropic splitting a_0 and shift in g_0 for egg phosphatidylcholine with and without cholesterol are compared to these values in octanol and hexane in Table 1. TEMPO in phosphatidylcholine–cholesterol experiences a more apolar environment similar to that in hexane, although this is not reflected by a downfield shift in g_0.

Resonance Spectra of TEMPO in Nerve and Synaptosomal Membranes. The resonance spectrum of TEMPO in rat phrenic nerve equilibrated with 6.4 mM TEMPO in anaerobic Ringer's solution with excess aqueous phase removed is shown in Fig. 2*A*. A nearly pure nerve-membrane bound spectrum is obtained. The apolar environment of the TEMPO-binding site decreases a_0 from 17.2 G in the Ringer's solution to 15.3 G but does not shift g_0, thus resembling a hydrocarbon region of phospholipid with high cholesterol content (Table 1).

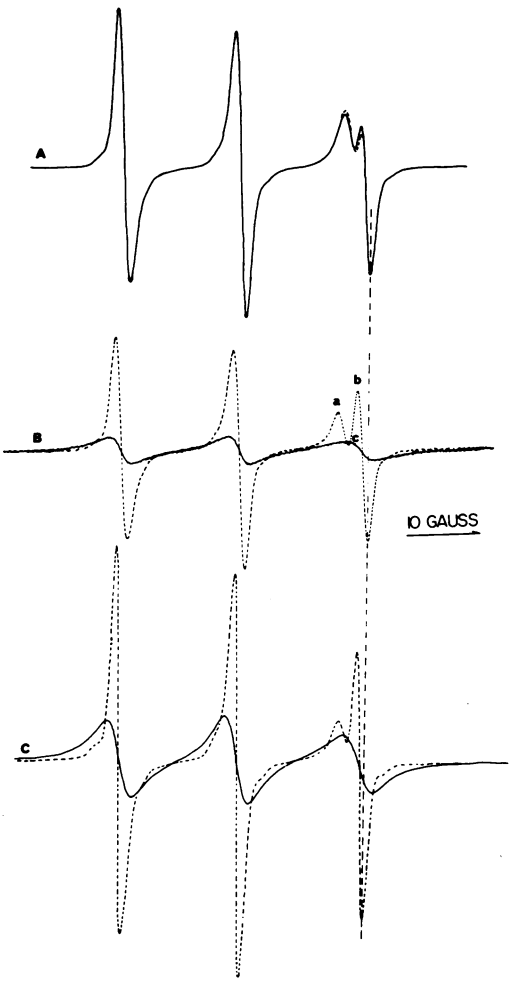


FIG. 3. ESR spectra of TEMPO (*A*) in vesicles of egg phosphatidylcholine + 33 mol % cholesterol at 1 atmosphere (- - -) and under 150 atm of helium (——); (*B*) in rat phrenic nerve in Ringer's solution at 1 atm (- - -) and under 150 atm of helium (——) (under pressure hyperfine lines *a* and *b* are converted to line *c*); (*C*) in rat-brain synaptosomes in Ringer's solution at 1 atm (- - -) and under 150 atm of helium (——). Volume of Ringer used in (*B*) and (*C*) is identical to that shown in Fig. 2*B*. A similar pressure effect thus exists in intact nerves and isolated nerve membranes. *Vertical broken line* marks identical magnetic field position.

Fig. 2*B* shows the superposition of the resonance spectra of TEMPO in hydrated phrenic nerve and synaptosomal membranes, showing that peak *a* (as defined in Fig. 1*B*) for synaptosomes is located upfield from that in nerves. The difference in the position of peak *a* indicates a larger isotropic splitting a_0 in synaptosomes than that in phrenic nerve. Nevertheless the a_0 values of TEMPO in synaptosomes and phrenic nerve indicate TEMPO binds to an apolar site within the nerve membrane.

Pressure Effect on the Resonance Spectrum of TEMPO in Model and Nerve Membranes. The resonance spectra of TEMPO in aqueous dispersions of egg phosphatidylcholine with 50 mol % cholesterol under 1 atm and 150 atm of helium are shown in Fig. 3*A*. The height of peak *b* (as defined in Fig. 3*B*) under pressure increases slightly with a corresponding decrease in the height of peak *a*, indicating

that pressure squeezes some TEMPO out of the membrane into the aqueous phase. A similar effect has also been reported recently by Trudell *et al.* (5). This slight decrease in solubility is also seen in pure egg phosphatidylcholine vesicles and is probably due to compression of the apolar region of the bilayer under pressure, as monitored by an increase in the order parameter of spin-labeled lipids (4).

When nerve membrane was studied in the presence of excess free TEMPO in the surrounding aqueous phase, 150 atm of helium had no detectable effect on peak *a* (spectrum not shown). This observation is consistent with the results shown in Fig. 3.1., i.e., pressure has an insignificant effect on the concentration of TEMPO in the apolar region of the nerve membrane. Contrary to the effect on phospholipid vesicles (Fig. 3.1), pressure caused a significant decrease in the height of peak *b*. It will be clear from the discussion below that the decrease in height of peak *b* is due to the absorption of free TEMPO from the aqueous phase into the nerve membrane.

When free TEMPO in the aqueous phase was limited or the volume of Ringer's solution is small, where both peaks *a* and *b* are resolved as shown in Fig. 3*B*, a dramatic pressure effect is observed. Under 150 atm of helium both peaks *a* and *b* disappear and a new peak *c* is observed. This pressure effect on the resonance spectrum is completely reversible. Furthermore, this pressure effect can be demonstrated reversibly in isolated synaptosomal membranes (Fig. 3*C*). The isotropic hyperfine splitting a_0 of the spectrum increases under pressure to 17.0 G in phrenic nerve and 16.9 G in sciatic nerve and in synaptosomes, as compared to 15.3 G in the apolar site of the nerve membrane (Table 1). The increase in a_0 indicates the new TEMPO-binding site in the nerve membrane is polar. In addition, the new polar site in the nerve membrane appears to have greater affinity for TEMPO than the apolar site. This is evident in the migration of TEMPO from the aqueous phase and the membrane apolar site to the pressure-induced polar site, resulting in a disappearance of both peaks *a* and *b* (Fig. 3*B* and *C*). The decrease in the resonance peak heights (peaks *c* in Fig. 3*B* and *C*) under pressure can be attributed to the increased magnetic dipolar interactions as a result of localization of TEMPO in the new polar site.

In the presence of excess free TEMPO, we have shown that pressure does not affect the TEMPO concentration in the apolar site; therefore the location and chemical composition of the pressure-induced polar site must be different from that of the apolar site within the nerve membrane. Binding of TEMPO to the pressure-induced polar site results in an enhancement of the blocking of nerve conduction in the presence or absence of free TEMPO in the aqueous phase (Boggs, J. M. & Hsia, J. C., in preparation). Therefore, the polar site must be an active receptor site for certain anesthetics.

DISCUSSION

The most significant result from the present work is that under different pressure of helium, TEMPO blocks nerve conduction by binding to two distinctively different sites in the nerve membranes. This experimental evidence lends support to the concept that there are multiple anesthetic receptor sites that differ in their chemical composition and/or location within the nerve membrane. These sites, when combined with certain anesthetics, produce the general phenomenon of nerve-conduction-block.

The pressure-induced migration of TEMPO to a new polar site may be due to a conformation change in the nerve membrane induced by pressure, probably involving membrane protein(s), since this effect could not be detected in phospholipid bilayer model membranes. The significant enhancement of nerve-blocking effect under pressure (Roth, S. H., Staiman, A., Boggs, J. M. & Hsia, J. C., in preparation) may be due to the increased concentration of TEMPO in the new site or a greater efficacy of TEMPO at the new site in blocking the Na^+ conductance channel. Other drugs, such as gaseous anesthetics, whose actions are reversed under pressure may be squeezed out of the membrane, or binding to both the apolar and polar site may have antagonistic effects. The pressure-induced conformation change may not occur in the absence of certain drugs such as TEMPO since 136 atm of helium by itself has no effect on nerve conduction (Roth, S. H., Smith, R. A. & Paton, W. G. M., in preparation).

Trudell *et al.* (5) have recently supported the previous proposal of Metcalfe *et al.* (11), Johnson and Bangham (12), and Johnson and Miller (13) that anesthesia is due primarily to the increased fluidity produced by anesthetics in the membrane lipid and that pressure reversal is due to the recompression of the hydrocarbon region of the lipid, thus decreasing the fluidity. However, pressure reversal of anesthetic action on lipid model systems has not actually been demonstrated. Trudell *et al.* (5) have shown that pressure increases the order parameter of lipid bilayers to the same extent in the presence or absence of anesthetics. Similarly, Johnson *et al.* (14) have shown that pressure has nearly identical effects on the cation permeability in liposomes in the presence and absence of anesthetics. It is reasonable to conclude that phospholipid bilayer vesicles have not been proven an adequate model for studying the mechanism of anesthesia and the pressure effect on nerve membranes

NOTE ADDED IN PROOF

We have found that TEMPO is not completely reoxidized in the phrenic nerve and it is reduced slowly by sciatic nerve and synaptosomal membranes. This reduction stops when pressure is applied. Thus the effective TEMPO concentration in the membrane in these experiments is much lower than indicated. If reduction is prevented similar results are obtained. This will be reported in more detail in a subsequent publication. This does not change the conclusions reached in this paper.

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