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SINGLE CHANNEL ANALYSIS OF THE EFFECTS OF HALOTHANE
ON THE NICOTINIC ACETYLCHOLINE RECEPTOR CHANNEL

by

James Donald Lechleiter

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PHYSIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1984

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by James Donald Lechleiter entitled Single channel analysis of the effects of halothane on the nicotinic acetylcholine receptor channel and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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This dissertation is dedicated to the Stars!
I wish to extend my sincere appreciation and thanks to all who have supported me in the successful completion of my work. I would especially like to thank my advisor, Dr. Raphael Gruener, whose guidance, encouragement and perseverance were exceptional. I also thank my dissertation committee and in particular, I thank Dr. Michael Wells for his insights and remarkable assistance. I wish to acknowledge the generous help of Dr. Paul Brehm and Bob Reinking in setting up the patch clamp; Steve Moffett for writing the computer programs for data analysis, and Dr. Paul C. Johnson for his financial support and sincere interest in my work. In addition, I extend my appreciation to Judy Christianson, Susan Coffin and Wally Clark for their help.

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ADDENDUM CONCERNING STRUCTURE OF THIS DISSERTATION

This dissertation has been written in the format of a collection of manuscripts published (Chapters 2, 3 and 4) or to be published (Chapter 5) with a more extensive Introduction (Chapter 1) in accordance with guidelines adopted by the Department of Physiology (cf. Graduate Study in Physiology brochure, 1983; p. 22).

Because this structure is different from the conventional dissertation format, a certain amount of redundancy is present in the text, primarily in the sections detailing published manuscripts. For ease of reading this dissertation, the reader is alerted to redundancies on pp. 41-56.
ABSTRACT

Anesthesia, a state of being absent of sensation and consciousness, has been recognized since antiquity. Even today anesthesia is still best characterized by the lack of consciousness and sensation. Since anesthetic potency is correlated with lipid solubility, the site of action of general anesthetics has been thought to be hydrophobic in nature and to involve excitable membranes critical for interneuronal communications. Thus, general anesthetics may interact directly with functionally-relevant membrane proteins (via hydrophobic pockets) or indirectly, with the lipids surrounding these proteins.

To better understand the details of general anesthetic action, I examined how halothane interacts with a functional synaptic protein, the acetylcholine receptor channel embedded in the membranes of cultured Xenopus myocytes. Next, I examined how changing the lipid composition, of these membranes, affected this interaction. Using the extracellular patch-clamp technique, I found that halothane, at clinically-relevant concentrations, shortened the burst duration of single receptor channels without affecting their conductance. Moreover, the halothane-induced reduction of burst durations was significantly attenuated after pretreatment with cholesterol-rich liposomes which increased significantly the cholesterol content of these cells.

These findings provide the first direct support for the role of membrane lipids in the mechanism of GA action. In particular, I
demonstrated that increases in membrane cholesterol antagonize the
anesthetic action of halothane. Although direct action of cholesterol
on synaptic proteins cannot be ruled out, my data strongly suggest that
membrane lipids are involved at a critical, but as yet undefined, site
with which GAs interact. The exact manner by which increases in
membrane cholesterol antagonize GA action remains to be elucidated.
Anesthesia, a state of being absent of sensation and consciousness, has been recognized since antiquity (Goodman and Gilman, 1968). Since, present day methods of drug-induced anesthesia were unavailable and unknown in the ancient past, patients were often rendered temporarily unconscious by physical means. Although such methods, like striking a blow to the cranium, were effective, the resultant cerebral concussion was not always reversible.

Modern day use of anesthetics, to abolish pain associated with surgical procedures, began in the mid nineteenth century. At that time, the depth of anesthesia was arbitrarily divided into four stages: 1) analgesia (see appendix), 2) delirium, 3) surgical anesthesia, and 4) respiratory paralysis. Despite this descriptive detail, the mechanism of action of agents which produce the anesthetic state is still highly controversial. The anesthetic state is still best characterized by the absence of sensation and consciousness. Attempts to explain anesthesia in greater detail, and with a mechanistic approach, necessarily depend on an understanding of these processes which are still, however, not yet understood in their own right. This means that, of necessity, theories concerning anesthetic action are numerous, of diverse nature and still under intense study. A specific aspect of anesthetic action forms the focus of study of this
dissertation. It concerns the manner by which a general anesthetic (see appendix) interacts with a functional synaptic protein found in an excitable membrane and the effects that changing membrane lipid composition have on this interaction.

**Physiological Theories of Anesthetic Action**

The most basic functional unit of the central nervous system (CNS) is the neuron. Consequently, many properties of the CNS derive from the anatomical arrangement and the strength of synaptic connections among neurons. Since general anesthetics (GAs) disrupt normal functions attributed to the CNS (for example consciousness and the perception of pain), GAs can be thought to interfere with interneuronal communication (Sowton and Sherington, 1905). Indeed, Larrabee and Posternak (1952) first demonstrated that the site of GA action was at the synapse, the chemical transducing station between any two neurons. They found that chlorbutanol (see appendix for characteristics of anesthetics), chloroform, ether and sodium pentobarbitone blocked synaptic transmission at lower concentrations than those required to block axonal conduction. More recently, Richards and co-workers (1974; Richards, Russell and Smaje, 1975; Richards and White, 1975) have confirmed this finding. Working with the guinea-pig olfactory cortex and the dentate gyrus of the hippocampus (in vitro preparations which are monosynaptic systems), they demonstrated that both inhalation and intravenous GAs, at clinically relevant concentrations, left the presynaptic compound action potential unchanged while depressing the compound postsynaptic
potential. Although the synapse appears to be the primary site of GA action, several system components of the neuronal network may be labile sites of GA action. For example, axons branch extensively prior to forming synaptic connections, each successive branch being smaller in diameter. Since the susceptibility of nerves to anesthetic action is inversely proportional to axon diameter (Nathan and Sears, 1961), it is possible that impulse conduction, per se, may also be blocked in the terminal branches of axons (Seeman, 1972; Staiman and Seeman, 1974). Richards (1982) addressed this possibility by comparing the effects of the GA pentobarbitone and tetrodotoxin (a drug that blocks the sodium conductance mechanism but does not affect synaptic receptor channels [Kao, 1966]) on the guinea pig olfactory cortex. He showed that while both drugs depressed synaptic transmission, only pentobarbitone depressed compound postsynaptic potentials without affecting the latency or amplitude of presynaptic compound action potentials. Tetrodotoxin on the other hand, depressed synaptic potentials in parallel with a decrease in the amplitude and duration of presynaptic compound action potentials. Richards argued that GAs did not act by blocking impulse conduction in the terminal branches of axons, since pentobarbitone did not mimic the effects of tetrodotoxin.

Since the synapse has been identified as the primary site of action, GAs may be considered to alter synaptic transmission at various stages associated with trans-neuronal transmission.

General Anesthetic Effects on Transmitter Release

Synaptic transmission may be depressed by an anesthetic-induced
decrease in transmitter release at excitatory synapses or by an anesthetic-induced increase in transmitter release at inhibitory synapses. Unfortunately, the transmitters acting at specific central synapses still remain largely unknown. Thus, reports of general anesthetic effects on transmitter release must, as stated by Richards (1983), "rely on either neurochemical experiments with putative transmitter substances that are unsupported by the requisite physiological studies or physiological studies unsupported by the appropriate neurochemical experiments".

In the CNS, the amino acids, L-glutamate and L-aspartate are considered to be putative excitatory transmitters and gamma-aminobutyric acid (GABA) a putative inhibitory transmitter (McGeer, Eccles and McGeer, 1978). Collins (1979; 1980) reported that pentobarbitone decreased the release of $^{14}$C-labeled aspartate and glutamate while increasing the release of $^{14}$C-labeled GABA in slices of rat olfactory cortex. The ability of barbituates to inhibit the release of excitatory transmitters while enhancing the release of inhibitory transmitters has also been demonstrated in guinea pig cortical slices (Potashner et al. 1980), rat brain slices (Minchin, 1981) and in thalamic slices (Kendall and Minchin, 1982). In the peripheral nervous system, Matthews and Quilliam (1964) demonstrated that barbituates also decrease transmitter release in excitatory synapses. They found that electrically stimulated release of acetylcholine (ACh), measured by rat blood pressure (see appendix), was decreased in the presence of barbituates. In summary, the mechanism of GA action on excitatory and on inhibitory synapses is consistent with a decrease in synaptic
transmission produced by interference with transmitter release. Anesthetic inhibition of transmitter release has also been demonstrated indirectly by examining postsynaptic effects (these reports will be discussed in the subsection concerning postsynaptic conductance changes).

Conceivably, GAs could depress transmitter release by altering the concentration of transmitters in presynaptic vesicles or by altering the number of presynaptic vesicles which are released in response to a presynaptic depolarization. However, concentrations of \(^{14}\)C-labeled amino acids remain constant when cortical slices of guinea pig are exposed to GAs (Potashner et al. 1980). ACh concentrations (assayed by measuring the tension in frog muscle when exposed to brain extracts containing ACh) are actually increased in brain slices of rats, mice and rabbits when exposed to GAs (Crossland and Merrick, 1954). Further, synthesis and storage of transmitters in presynaptic terminals does not appear to be altered by GAs since post-tetanic potentiation (see appendix) and frequency potentiation are not affected by GAs (Somjen, 1963; Richards, 1972; 1973; Richards and White, 1975). Thus, it is likely that GAs depress transmitter release by altering the number of presynaptic vesicles that are released. More detailed investigations of this process are made difficult by the inaccessibility of CNS synapses.

Transmitter Binding to Postsynaptic Receptors

GAs may depress synaptic transmission by interfering with transmitter binding to postsynaptic receptors. For example, GAs may
alter transmitter binding such that the postsynaptic receptors are driven into nonfunctional conformational states. To examine this possibility, Young et al. (1978) investigated the effects of halothane, chloroform and diethyl ether on the binding of carbamoylcholine (see appendix) to the acetylcholine receptor (AChR) obtained from the electric organs of Torpedo californica. Young et al. found that these agents enhanced the binding of carbamoylcholine to the AChR such that the half-time for binding was decreased by a factor of ten. Young and co-workers suggested that this enhanced binding would result in the desensitization (see appendix) of postsynaptic receptors. Since the desensitized state of the postsynaptic receptor is nonfunctional, synaptic transmission would be depress. In support of this hypothesis, Sauter et al. (1980) have observed that inhalation GAs also enhance $^3$H-acetylcholine (ACh) binding to the AChR. Further, they found that high pressure application decreased transmitter binding, a finding which is consistent with the phenomenon of pressure reversal of anesthetic action (see discussion below; Classical Theories of Anesthetic Action). However, Sauter and co-workers also found a decrease in transmitter binding in the presence of clinical concentrations of non-inhalation GAs. These opposite findings demonstrate that different classes of GAs can produce opposite effects at the same functional site. Moreover, the same GA may produce different effects at different sites (see discussion below under postsynaptic conductance changes). In summary, GAs may affect changes in the binding of transmitters to postsynaptic receptors and thus depress synaptic transmission.
Postsynaptic Conductance Changes

GAs may also depress synaptic transmission by inhibiting postsynaptic conductance changes at excitatory synapses and/or by enhancing postsynaptic conductance changes of inhibitory synapses. In the CNS, studies have focused on the sensitivity of the postsynaptic membrane to putative neurotransmitters introduced by iontophoresis. Crawford and Curtis (1966), and later Galindo (1969), showed that barbituates reduced the sensitivity of neurones to the same concentration of glutamate in the cerebral cortex and the cuneate nucleus of cats. Halothane, however, was ineffective. This suggests that different anesthetics may have different mechanisms of action. Similarly, Richards and Smaje (1976) demonstrated that ether, methohexitone and pentobarbitone reduced the sensitivity of the olfactory cortex to glutamate while halothane had no effect. Richards and Smaje argued that since halothane does depress synaptic activity, halothane must work by reducing the amount of released transmitter. Zorychta, Esplin and Capek (1975) came to similar conclusions regarding the mechanism of action of halothane in the spinal cord.

The multiplicity of effects of GAs may, in part, be explained by different GA effects at various regions in the brain. For example, barbituates have been found to depress postsynaptic function in the cerebral cortex and the cuneate nucleus (Crawford and Curtis, 1966; Galindo, 1969). But, Weakly (1969) found that pentobarbitone and thiopentone did not affect postsynaptic function in the spinal cord. Rather, these GAs depressed synaptic transmission by decreasing the amount of transmitter released.
GAs have also been shown to depress the amplitude of excitatory postsynaptic potentials (EPSPs). Chalazonitis (1967) showed that clinical concentrations of halothane and ether decreased the amplitude of EPSPs in molluscan neurones. Similarly, the postsynaptic depolarization at the vertebrate neuromuscular junction is decreased by barbiturates (Adams, 1976), ether and halothane (Gage and Hamill, 1975), enflurane (Kennedy and Galindo, 1975) and by long-chain alcohols (Gage, McBurney and Van Helden, 1974). Each of these GAs have been shown to depress synaptic transmission by increasing the decay rate of postsynaptic currents (Gage, 1976). These data indicate that when excitatory transmitters interact with postsynaptic receptors, the postsynaptic macro-conductance is decreased. This may be due to a decrease in the conductance, per se, of the ionic channel associated with the postsynaptic receptor and/or to a decrease in the period of time the postsynaptic channels remain in the open conducting state (referred to as the single channel open-time). Indirect measurements, using noise analysis, indicate that both single channel conductance and open-time may be decreased by GAs (Landau et al. 1979). However, Adams (1976) has argued that an increased decay rate of postsynaptic currents may actually be due to GAs blocking the opened channel, thereby inhibiting current flow. In order to functionally resolve the mechanism of GA action at the postsynaptic membrane in more detail, a technique which can directly measure changes in postsynaptic conductance is required. Recently, such a technique has been developed. This technique, known as the single channel patch clamp,
allows direct measurements of single channel conductance and open time (Hamill et al. 1981).

First Study

To further advance our understanding of the mechanism of action of GAs, I chose to examine how GAs affect postsynaptic receptor function at the single channel level. To accomplish this, I used the patch clamp technique (Hamill et al. 1981) to directly observe the effects of halothane on the nicotinic AChR channel found in cultured *Xenopus laevis* muscle cells. This work is described fully in Chapters 2, 3 and 4. Although there is some overlap in the material that is presented here, each chapter has already been published and therefore, has been presented separately.

Although different molecular sites of GA action have been identified, the remarkable fact is that different GAs can act at the same site and produce similar effects on synaptic transmission. The similarities of anesthetic effects suggest that, at least some, GAs may share a common mechanism of action. In the second part of this introduction, I will discuss the similarities of GA action as related to their physiochemical interactions with cell membranes.

**Classical Theories of Anesthetic Action**

The Meyer–Overton Lipid Solubility Theory

General anesthesia can be produced by a diverse group of substances having no common chemical structure. This group includes inert gases, halogenated hydrocarbons, alcohols, barbiturates and
steroids (MacDonald and Wann, 1978). H. H. Meyer (1899) and E. Overton (1901) independently published identical theories of anesthetic action that, for the first time, linked the action of different GAs. They demonstrated that anesthetic potency was directly correlated with the lipid solubility of the anesthetic. Meyer (1937) summarized the Meyer-Overton lipid solubility theory as follows: "Narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipoids of the cell. This concentration depends on the nature of the animal or cell, but is independent of the narcotic." This hypothesis implied that a critical number of molecules must occupy the anesthetic site prior to anesthesia.

The Critical-Volume Theory

In 1954, Mullins suggested that the critical parameter responsible for narcosis was the volume occupied by an anesthetic. This modification of the Meyer-Overton theory is known as the critical-volume theory. Both of these theories correlate well with anesthetic potency. However, the best correlation for each theory occurs for anesthetic solubilities with different solvents. The critical-volume theory is best correlated with anesthetic potency when a more polar lipid solvent is used (Miller et al. 1972). Since both predictions of lipid solubility are in the range expected for membrane lipids (ibid), the two theories cannot be distinguished on this basis until the lipid solubility of the actual site of Ga action is known.

Johnson and Flagler (1950) first observed that hydrostatic pressure could antagonize an organism's response to anesthetics. They
demonstrated that tadpoles, anesthetized with 2-5% ethanol, resumed swimming when 200-300 atmospheres of pressure was applied. Similar pressure antagonism of anesthesia has been observed in newts and mice anesthetized with ether, nitrous oxide, halothane, pentobarbital and phenobarbital (Miller et al. 1973; Winter et al. 1976). The phenomenon of pressure antagonism of anesthetic action provides a test to distinguish between the Meyer-Overton and critical-volume theories since each theory predicts a different dependence of anesthetic potency on pressure. The critical-volume theory predicts that the volume expansion at the anesthetic site, due to the absorption of anesthetic molecules, may be reversed by increasing pressure thereby compressing the anesthetic-induced expansion. In contrast, the Meyer-Overton theory predicts pressure reversal because the anesthetic solubility decreases with increasing pressure (Krachevsky and Kasarnovsky, 1935), thereby reducing the number of anesthetic molecules at the site of action. These different theoretical predictions can be formulated such that each theory predicts a distinct linear dependence between anesthetic potency and pressure. Miller et al. (1973) showed that the critical-volume theory provided a much better linear correlation between anesthetic potency and pressure in comparison to the Meyer-Overton theory. Finally, the the lack of anesthetic effect of helium and neon provides an additional test to distinguish between these two theories. Since these two molecules are lipid soluble, the Meyer-Overton theory incorrectly predicts that they will be anesthetics. The critical-volume theory on the other hand provides an explanation for the non-anesthetic effects of some lipid soluble molecules. The
critical-volume theory predicts that when the volume expansion due to lipid soluble molecules is opposed by an equivalent compression at the site of GA action, due to the hydrostatic pressure of the lipid soluble molecules, no anesthetic effects will be observed. The non-anesthetic effects of helium and neon can be ascribed to their high coefficients of compressibility (Miller et al. 1973). In summary, it appears likely that the critical-volume theory is a more accurate description of the mechanism of GA action.

**Modern Theories of Anesthetic Action**

The correlation between anesthetic potency and lipid solubility implies that the site(s) of GA action is in the lipids of the cell membranes (Meyer, 1937). Currently, it is presumed that GAs act at hydrophobic sites within neuronal membranes and that this physical interaction depresses synaptic transmission (Seeman, 1972; Miller and Miller, 1974; Roth, 1979). However, cell membranes consist of proteins anchored in a 'fluid-like' matrix of lipids (Singer and Nicholson, 1972). It remains to be demonstrated whether GAs act directly on membrane proteins or indirectly, by interacting with membrane lipids (Franks and Lieb, 1982). Accordingly, theories may be divided into two classes, the protein theories and the lipid theories.

The Lipid Theories

**Expansion in Membrane Thickness.** The critical-volume theory suggests that membrane expansion is an integral component of the mechanism of anesthetic action. In fact, Kita et al. (1981) have shown that GAs increase the total volume of lipid bilayers by 0.2–0.6%.
Ashcroft et al. (1977) proposed that an increase in membrane thickness would distort lipid/protein interactions resulting in conformational changes which may interfere with protein function. They demonstrated that the addition of benzyl alcohol to black lipid membranes decreased membrane capacitance, a finding which is consistent with an increase in membrane thickness. However, recent work has shown that benzyl alcohol and octanol increase membrane capacitance when the black lipid membranes are carefully prepared free of lipid solvent (Ebihara et al. 1979; Elliott and Haydon, 1979; Reyes and Latorre, 1979). This suggests that membrane thickness may actually be decreased, although the increases in capacitance could be due to increases in dielectric constant (membrane capacitance is directly proportional to the dielectric constant and indirectly proportional to thickness; Reyes and Latorre, 1979). Direct measurements of membrane thickness, using X-ray diffraction and neutron scattering techniques (see appendix), show no changes in lipid bilayer thickness in the presence of nitrous oxide, cyclopropane or halothane (Franks and Lieb, 1978). But, X-ray diffraction measurements do show increases in membrane thickness when taken in the presence of n-alkane anesthetics (Padron et al. 1979; McIntosh et al. 1980). Haydon et al. (1977; 1980) found that n-alkanes also decreased capacitance in black lipid membranes and squid giant axons, consistent with an increase in membrane thickness. In summary, it appears that different GAs affect membrane thickness differently. Alcohols may decrease membrane thickness (depending on changes in the dielectric constant) while inhalation GAs appear to have no effect and n-alkanes appear to increase membrane thickness.
**Lateral Phase Separation Theory.** Membranes lipids can undergo reversible transitions from an ordered, gel-crystalline phase, to a disordered, liquid-crystalline phase. The physical state of membrane lipids depends on temperature and lipid composition (Lee, 1975; Sandermann, 1978). In particular, cholesterol and unsaturated fatty acids tend to depress the formation of the gel-phase lipids due to their inability to conform to an ordered molecular packing of membrane lipids (Shinitzky and Henkart, 1979; Cullis and DeKruijff, 1978).

Cooling on the other hand, tends to order the molecular packing of membrane lipids due to reduced molecular movements. The temperature at which a gel-to-liquid phase transition occurs is referred to as the transition temperature. Phospholipid mixtures, with different transition temperatures, can result in bilayers having both gel and liquid phases. The boundaries between gel and liquid phases are referred to as lateral phase separations. The bilayer can accommodate volume changes without disturbing the overall matrix of the lipid bilayer when lateral phase separations are present. This effect is accomplished by transforming some of the high-volume disordered lipids to lower-volume ordered lipids and is termed high lateral compressibility (Shimshick and McConnell, 1973). Since GAs lower the transition temperatures of lipids, GAs will alter or eliminate lateral phase separations (Hill, 1974; Jain et al. 1975; Lee, 1976). Trudell (1977) proposed that neuronal membranes contain lateral phase separations and suggested that GAs act by reducing the lateral compressibility of the membrane. The loss of lateral compressibility compromises the ability of the membrane to accommodate protein...
conformational changes which may be critical for interneuronal communication. This hypothesis, known as the lateral phase separation theory, suffers from the major criticism that, in mammalian cell membranes, gel-phase lipids have not been detected. It appears that the membrane lipid composition, primarily cholesterol and unsaturated fatty acids, prevents the existence of gel-phase lipids at physiological temperatures (Cullis and DeKruijff, 1978; Richards et al. 1980; Franks and Lieb, 1982).

**Lipid Annular Phase Transition Theory.** The immediate ring of lipids surrounding an integral membrane protein provides an interface between the protein and the bulk lipids and is termed the lipid annulus. Since membrane proteins have been shown to order their lipid annuli (Jost et al. 1973), it has been suggested that a change in the physical state of the lipid annulus may interfere with the function of the associated protein (Lee, 1976; Lenaz, 1975). Lenaz and co-workers (1979) compared the effects of butanol, halothane and ketamine on synaptosomal membranes to their effects on lipid vesicles obtained from lipids extracted from the same synaptosomal membranes. They found that the disordered effect of GAs, as measured by electron spin resonance (see appendix), was present only in the native synaptosomal membranes and not in the extracted lipid vesicles. They argued that the GAs disordered lipid annuli which would have been removed by the extraction procedure. They suggested that GAs act by disordering the lipid annuli of neuronal proteins (critical for synaptic transmission). This effect converts these proteins into nonfunctional or impaired conformational states. This theory of GA action is referred to as the lipid annular
phase transition theory. Lenaz and co-workers (1978) further investigated the effects of GAs on the enzymatic activity of erythrocyte acetylcholinesterase. This enzyme hydrolyzes ACh in its native state when it is membrane-bound and this activity is retained even after solubilization by detergents. In contrast, only membrane-bound acetylcholinesterase activity was inhibited by butanol, halothane and ketamine. Sensitivity to GAs was reestablished after the enzyme was reincorporated into a lipid environment. This finding lends further support to lipid annular phase transition theory and demonstrates the importance of the lipid environment of neuronal proteins critical for synaptic transmission.

Membrane Fluidity Hypothesis. As discussed above, membrane lipids exist in the fluid-phase. GAs have been postulated to act by increasing the 'fluidity' of the fluid-phase lipids. By partitioning into the lipid bilayer, GAs loosen the molecular packing of lipids thereby allowing greater molecular movement. The increase in 'fluidity' may produce conformational changes which inactivate neuronal proteins critical for synaptic transmission. Unlike the lipid annular phase transition theory, the membrane fluidity hypothesis does not require the presence of ordered lipid annuli. However, both theories attribute the mechanism of GA action to a disordering of membrane lipids which subsequently induces neuronal proteins to change into nonfunctional or impaired states. Metcalfe and co-workers (1968) first examined the effects of GAs on molecular movement within the membrane. Using nuclear magnetic resonance (NMR) techniques, they demonstrated that the lipid environment of benzyl alcohol became less restrictive to
movement (based on a narrowing line width of the resonance spectrum; see appendix) as the membrane concentration of alcohol was increased. Subsequent NMR work has demonstrated a fluidizing effect for omega-phenyl alcohols (Colley and Metcalfe, 1972), halothane and methoxyflurane (Shieh et al. 1975) and chloroform (Vanderkooi et al. 1977) in lipid bilayers. GA-induced increases in membrane fluidity have also been demonstrated by use of electron spin resonance (ESR) techniques. With this technique, fluidity increases are estimated from decreases in the order parameter (see appendix: electron spin resonance) calculated from the resonance spectra of spin labels. Trudell et al. (1973) have shown that clinical concentrations of halothane and methoxyflurane decreased the order parameter (fluidized) of spin labels within lipid bilayers. Similarly, increases in fluidity have been shown for chloroform, ether, ketamine, alcohols, alphaxalone, pentobaritone, chlorpromazine and cannabinol (Hubbell et al. 1970; Butler, Schneider and Smith, 1973; Trudell et al. 1975; Hsia and Boggs, 1975; Boggs, Yoong and Hsia, 1976; Miller and Pang, 1976; Chin and Goldstein, 1977; Richards et al. 1978; Pang and Miller, 1978). In addition, fluorescence depolarization measurements have been used to examine the effects of GAs on membrane fluidity. In this case, the degree of fluorescence polarization is dependent on the motion of the fluorescent probe (see appendix: fluorescent depolarization). Using this technique, Vanderrooi et al. (1977) showed that halothane, enflurane, ether, chloroform and ethanol increased fluidity in lipid bilayers, red blood cell membranes and in sarcoplasmic reticulum membranes. GA-induced increases in membrane fluidity were also shown
in mitochondrial membranes for butanol, halothane and ketamine (Lenaz et al. 1978b) and in synaptosomal membranes for halothane (Fellkofer and Sandhoff, 1980).

The membrane fluidity hypothesis predicts that molecules which do not produce anesthesia are inactive because of their inability to disorder (fluidize) membrane lipids. Consistent with this prediction, Lawrence and Gill (1975a) showed that the potent anesthetic steroid alphaxalone disordered membrane lipids to approximately the same extent as halothane. In contrast, the non-anesthetic steroid betaxalone produced only 6% of the membrane disordering produced by alphaxalone. These two steroids differ only in the configuration of a hydroxyl group.

The membrane fluidity hypothesis also predicts that GA action should be antagonized by any action that produces ordering of membrane lipids. Thus, application of hydrostatic pressure should antagonize the GA-induced increase in fluidity since compression opposes volume increases due to GA molecules. Consistent with this prediction, a direct antagonizing effect of pressure, and anesthetic-induced membrane disordering, has been demonstrated for gaseous, volatile, alcohol, steroid, amine and barbiturate general anesthetics (Johnson et al. 1973; Trudell et al. 1973b; Boggs et al. 1976; Chin et al. 1976).

In summary, the mechanism of GA action is consistent with the ability of GAs to disorder membrane lipids. However, a major criticism of the fluidity theory is that the magnitude of the fluidity effects is too small. Specifically, Pang et al. (1980) have calculated that increasing the temperature by 0.32°C will produce an equivalent
fluidity increase in lipid bilayers. Thus, it is argued that the membrane fluidity theory is unlikely to account for mechanism of GA action since such small changes in body temperature obviously do not produce anesthesia in animals. This argument presumes, however, that changes in body temperature are translated to changes in the fluidity of neuronal membranes. Even allowing for a direct effect, this argument presumes that increasing the temperature in lipid bilayers and in neuronal membranes will produce equivalent increases in membrane fluidity. In fact, Lenaz and co-workers (1979) have demonstrated that GA-induced increases in fluidity are quantitatively different in the two preparations. They found that butanol, halothane and ketamine fluidized synaptic membranes obtained from pig brain while these anesthetics produced insignificant fluidity increases in vesicles of lipids extracted from synaptic membranes. They argued that GAs disordered the lipid annuli surrounding proteins. When the proteins were removed from the synaptosomal membranes, no significant disordering effects could be observed in the presence of GAs due to the absence of lipid annuli. Similar differences in the ability of GAs to fluidize bilayers, with and without proteins, were reported using mitochondrial membranes (Lenaz et al. 1978). In addition, Firestone (1983) found that the incorporation of AChR proteins into lipid vesicles enhanced the the ability of GAs to increase membrane fluidity. Firestone calculated that the addition of proteins to lipid vesicles increased the temperature, which was required to equal a GA-induced increase in fluidity, by 1-2°C. Thus, an increase in temperature is unlikely to produce equivalent changes in lipid bilayers (without
proteins) and neuronal membranes.

The membrane fluidity theory also predicts that decreases in temperature should antagonize GA action. Spyropoulos (1957) reported that an ethanol-induced reduction in amplitude of the squid axon action potential (at 22°C) could be completely reversed by lowering the temperature to 4°C. Similarly, Richards et al. (1978) found the potency of butanol, in reducing the action potential amplitude, was also decreased by lowering the temperature (38°C-20°C). However, the anesthetic potency of benzyl alcohol was somewhat increased. Similarly, Halsey and Higgs (1976) did not find an antagonizing effect of temperature on the anesthetic effects of nitrous oxide or nitrogen. In fact, a decrease in body temperature, predicted to antagonize GA action in animals, is known to produce anesthesia in animals and to block neuronal conduction. These studies are further complicated by the dependence of chemical rate constants and anesthetic solubilities on temperature (Richards et al 1978). Thus, the current evidence does not convincingly support an antagonistic effect of temperature on anesthetic action.

The membrane fluidity theory can also be tested by altering the lipid composition of a test system. Lipid composition can affect the molecular packing of membrane lipids and consequently, the fluidity of membrane lipids. Since the volume of unsaturated bonds is greater than saturated bonds, increases in the concentration of unsaturated fatty acids will loosen the molecular packing of saturated fatty acids and therefore increase membrane fluidity. On the other hand, the rigid, planar ring structure of cholesterol acts to restrict the molecular
movement of lipids and therefore decreases fluidity (Shinitzky and Henkart, 1979; Cullis and DeKruijff, 1978). The effects of altering lipid composition on fluidization, produced by GAs, were examined on liposome permeability. De Gier et al. (1970) showed that liposomal permeability, induced by valinomycin (an antibiotic known to increase potassium permeability), increased with an increasing unsaturation of fatty acids and decreased when the cholesterol content was increased. In agreement with these findings, Pang et al. (1979) demonstrated that pentobarbital, halothane and butanol increased cation permeability in lipid vesicles and that each anesthetic also increased membrane fluidity. Further, the GA-induced increase in cation permeability was reversed by pressure (Johnson et al. 1973). As discussed above, these effects are consistent with the membrane fluidity theory.

The Protein Theories

The Protein Conformational Change Theory. The protein conformational change (PCC) theory proposes that the site of GA action is a hydrophobic region in neuronally relevant proteins (Eyring, Woodbury and D'Arrigo, 1973; Woodbury, D'Arrigo and Eyring, 1975). The interaction of GAs with the hydrophobic regions may cause a conformational change, in these proteins, resulting in their inactivation. Non-membrane proteins have been used to examine the direct action of GAs on proteins since non-membrane proteins are free of lipids. Schoenborn (1968) first investigated the effects of two anesthetics, xenon and cyclopropane, on the protein conformation of myoglobin. Using X-ray diffraction, he reported that xenon had little
effect on conformation while cyclopropane produced changes in the conformation of myoglobin. Thus, demonstrating that GAs could directly alter the conformation of a protein.

More recently, Barker et al. (1975) found that clinical concentrations of chloroform, halothane, ether and methoxyflurane caused small, but specific changes in the nuclear magnetic resonance (NMR) spectra of hemoglobin. These results suggested that GA/protein interactions were localized and specific. At higher anesthetic concentrations, the NMR spectrum exhibited more general changes suggesting interactions that were less specific. More detailed examinations of GA effects on hemoglobin were carried out by Brown, Halsey and Richards (1976; 1977). Using spin-echo Fourier-Transform NMR techniques (see appendix), smaller changes in the protein conformation of hemoglobin could be detected. They focused their measurements on the histidyl groups on the surface of hemoglobin since they were the only groups known to be derived from single protons (thus, simplifying the interpretation of NMR spectra). They found that GAs produced conformational changes in only some histidine residues. Since a general surface effect of GAs on hemoglobin would have been predicted to affect all histidine residues equally, they suggested that GAs interacted with specific hydrophobic pockets within hemoglobin. In support of GA interaction with hydrophobic pockets, they showed that GA–induced conformational changes could be correlated with the lipid solubility of the GAs.

Examination of the effects of GAs on bioluminescent systems has provided additional support for the PCC theory. Johnson et al. (1942)
first demonstrated that GAs inhibited bacterial luminescence and that this inhibition was reversed by pressure. More recently, methoxyflurane, chloroform and halothane have been shown to inhibit bacterial luminescence in vivo (Halsey and Smith, 1970; White and Dundas, 1972). Similarly, Ueda and Kamaya (1973) have demonstrated anesthetic inhibition of the firefly tail luminescence in vitro. The luminescence reaction is dependent on luciferase. Since this enzyme is water-soluble, it is assumed that it is lipid-free (McElroy, 1955). Consequently, it is argued that the GA-induced inhibition of luminescence is due to a direct interaction between GA and luciferase thus supporting the PCC theory. However, King and White (1976) have provided indirect evidence showing that luciferase may not, in fact, be lipid free. They found that GA-induced inhibition of bacterial luminescence showed an abrupt decrease (25%) when the temperature of the system was decreased 2°C below the growth temperature of the bacteria. They argued that this may be due to a phase change in membrane lipids although a temperature-induced protein conformation change could not be ruled out.

Recently, the PCC theory has been reexamined in light of the classical observations of GA action. As discussed previously, the critical volume hypothesis predicts that GA action will be antagonized by increases in hydrostatic pressure due to an opposing compression of GA-induced volume increases. Pressure reversal data is in excellent agreement with the predictions of the critical volume hypothesis (Miller et al. 1973). However, Franks and Lieb (1982) have reinterpreted the pressure reversal data in terms of a different
molecular model. They suggest that a single anesthetic molecule binds to a site on the protein and inactivates the protein. This model predicts that pressure will squeeze the GA molecule out of the protein site thereby antagonizing GA action. Using the same data as Miller et al. (ibid), Franks and Lieb showed that the PCC theory accounted for the pressure reversal data as convincingly as the critical volume hypothesis. Franks and Lieb (1984) have also reexamined the PCC theory in light of the correlation of anesthetic potency with lipid solubility. This classical observation by Meyer (1899) and Overton (1901) has been the major reason for the popular acceptance of lipid theories of anesthetic action. However, Franks and Lieb argue that this correlation is a necessary but not sufficient condition for the validity of any theory of anesthetic action. In support of this, they demonstrated that the GA-induced inhibition of firefly tail luminescence could, at the same time, also be correlated with the lipid solubility of GAs. Thus, the PCC theory is consistent with both the classical observations of pressure reversal and the correlation of anesthetic potency with lipid solubility.

Conclusions

The diversity of GA structure, action and sites of action makes a unifying theory of GA action unlikely. Rather, it appears that GA mechanisms may vary depending on the specific GA investigated and the specific site of GA action studied. In as much as the classical correlation of anesthetic potency with lipid solubility remains a rule to be observed in the investigation of any mechanism of GA action, I
chose to investigate the effects of lipid composition on the mechanism of GA action. Specifically, I altered the cholesterol content of Xenopus muscle cells grown in culture. I then determined whether the effects of halothane on the nicotinic AChR (described in Chapters 2, 3 and 4) were affected. This approach allowed me to examine the functional effects of a GA on a single molecular protein critical to synaptic transmission and to further probe the role of membrane lipid composition in the mechanism of GA action. This work is described in detail in Chapter 5.
CHAPTER 2

HALOTHANE SHORTENS ACETYLCHOLINE RECEPTOR CHANNEL KINETICS
WITHOUT AFFECTING CONDUCTANCE

Introduction

Although the exact mechanism(s) of action of general anesthetics (GAs) is still largely unknown, several hypotheses have been suggested to account for the ability of a diverse class of compounds to produce loss of sensation and consciousness. The lipid solubility theory was originally proposed because of the remarkable correlation between the oil/water partitioning coefficient and the potency of structurally-unrelated agents (Meyer, 1901; Overton, 1901; Meyer, 1937). More recently, several variants of this theory have been formulated (Mullins, 1954; Miller et al. 1973). Of these, the membrane fluidity theory explains anesthesia by the disordering of the lipid bilayer resulting in increased membrane fluidity (Metcalfe et al. 1968; Trudell et al. 1973; Gage and Hamill, 1975; Lenaz et al. 1979). This effect may change the behavior of membrane-bound proteins which are critical to the functioning of the neuron.

Despite the diversity of proposed mechanisms (for reviews, see refs. Roth, 1980; Franks and Lieb, 1982), it is now generally accepted that the synapse itself is the most likely site of action of GAs (Judge, 1983). This idea is derived from the finding that GAs block synaptic transmission at much lower concentrations than required for blockade of the action potential (Larrabee and Posternak, 1952). In as
much as central synapses are less accessible than peripheral ones, much of the evidence for the action of GAs on synaptic transmission has been derived from detailed studies of the neuromuscular junction. Thus, several GAs have been shown to reduce nerve-evoked post-synaptic depolarizations at various vertebrate endplates (Gissen et al. 1966; Waud and Waud, 1975; Gage and Hamill, 1976). A closer examination of the decrease in endplate potential amplitude, using the voltage clamp technique, has revealed this reduction to be due to an increase in the decay rate of miniature endplate currents (Gage and Hamill, 1976; Torda and Gage, 1977). Using the noise-analysis technique, Landau et al. (1979) surmised that endplate current reduction, at the vertebrate neuromuscular junction, likely reflects the shortening of the time which acetylcholine receptor (AChR) ion channels spend in the open configuration. These findings are consistent with the idea that general anesthetics act on synaptic processes and demonstrate unequivocally the usefulness of the neuromuscular junction as a prototypic synapse. However, these data do not yield detailed and direct information concerning the specific mechanism of GA action because the measurements are made on the entire ensemble of post-synaptic receptors. The recent development of the "patch-clamp", single channel technique (Neher et al. 1978; Hamill et al. 1981) provides direct measurements of single receptor channel activity.

I have used the patch-clamp, single channel technique to examine how a volatile anesthetic, halothane, affects the properties of single nicotinic AChR channels embedded in the membranes of embryonic *Xenopus* myocytes grown in cell culture. I found that
halothane, at clinically relevant concentrations, shortens the single channel burst durations (i.e. the time spent by the channel in the open, conducting configuration) without altering channel conductances.

**Materials and Methods**

Uninnervated muscle cells from *Xenopus laevis* embryos (stages 19-22) were grown in culture as described (Gruener and Kidokoro, 1982). Membrane patches were sealed with electrodes of 1-2 μm internal diameter with resistances of 3-15 Mohms. Electrodes were filled with a high sodium solution whose composition was 120 mM NaCl/1.6 mM KCl/1 mM CaCl₂/8 mM Hepes.HCl, pH 7.4, containing 0.1-0.4 μM acetylcholine (ACh). At this concentration of ACh, concurrent activation of multiple channels was rarely seen; when observed, these were omitted from the data base, facilitating data analysis. Channel events were recorded from cell-attached and cell-free patches. The patch-clamp circuit was built according to the design of Hamill et al. (1981). Single channel events were stored on FM magnetic tape (RACAL Store SD4; Fullerton, CA). Analogue records were digitized at 50-μsec intervals by a computer (Dynabase DB8/4; Milpitas, CA). A downward deflection in the current trace, lasting for a minimum of 0.6 msec (circuit time constant = 0.12 msec), and which occurred within an adjustable window (usually 2-15 pA) was considered to be a resolvable event. When an event was detected, the digitized record and its trailing baseline (usually 2.5 msec) were stored on a floppy disk. Amplitudes were calculated from the mean value of all digitized points at the peak of an event omitting the two initial and final points to cross the selected threshold. Each
event was then displayed and accepted or eliminated at the operator's discretion. The criterion for accepting an event opening was an abrupt transition from baseline to a constant current amplitude. The event was considered closed when the current level remained at baseline for at least 1 msec.

During experiments, performed at room temperature, cultures were continuously superfused (1-2 ml/min) with recording medium [high sodium solution: 120 mM NaCl/1.6 mM KCl/1mM/CaCl₂/8 mM HEPES.HCl, pH 7.4; for excised, cell-free patches, either 5 mM EGTA was added and CaCl₂ omitted. On occasion, a high potassium solution (70 mM K₂SO₄/24 mM KCl/8 mM HEPES.HCl was used]. Thymol-free halothane (Halocarbon Laboratories, Hackensack, NJ) was added to the perfusion medium by bubbling through a vaporizer (Forreger; Smithtown, NY) at concentrations of 1-4% [Percent of vaporized halothane mixed with air and bubbled through the recording medium reservoir; this concentration range corresponds to clinically relevant doses (Gage and Hamill, 1975) and to aqueous concentrations of 0.2-0.8 mM, in the recording chamber, as measured by ultraviolet spectroscopy (Blank and Thompson, 1980)]. After 4-7 minutes exposure to halothane, and following a similar control period, the anesthetic was washed away and channel events from the recovery period were usually recorded.

It has recently been shown (Sakmann et al. 1980; Colquhoun and Sakmann, 1981; Dionne and Leibowitz, 1982) that a given channel may open repeatedly when ACh is bound to its receptor. These repeated openings are called bursts since they are interrupted by short, sometimes incomplete, closures whose resolution depends, in part, on
the time-constant of the patch-clamp circuit. In order to distinguish between a burst, representing the activity of a single channel, and the opening of two different channels, Dionne and Leibowitz (1982) assembled opening-probability histograms. They showed that, at the neuromuscular junction of the garter snake, two successive openings appear as independent events (and hence likely to emanate from two separate channels) when the closed times exceed about 2 msec. As a conservative definition of the burst, I report my data as burst durations when a series of channel openings is interrupted by closed times shorter than 1 msec. A burst which is uninterrupted by closures is a special case of such events and is commonly referred to as the channel opentime. The Student t-test was used to determine statistical significance.

**Results**

When single channel events were assembled into histograms, on the basis of current amplitudes, a bimodal distribution was obtained (Figure 1; see also refs. Clark and Adams, 1981; Brehm et al. 1983). The resultant two populations of events are designated low-amplitude and high-amplitude channels. The low-amplitude channel has a relatively long opentime and a small amplitude, whereas the high-amplitude channel has a shorter opentime and a larger amplitude (see Figure 1 Insets). The bimodal amplitude distribution is maintained during exposure to halothane (Figure 1B) and during the recovery period following the washout of halothane (Figure 1C).

In another set of experiments, the mean channel amplitude from
Figure 1. Amplitude histograms of single channel events. Amplitude histograms, from a typical patch, reveal a bimodal distribution. When a separation value of 4.6 pA is chosen, by eye, two channel populations may be defined. These are called low-amplitude and high-amplitude channels. In this cell-free patch, the control mean amplitudes (±SD) of the low-amplitude and high-amplitude channels were $3.7±0.5$ pA (235 events) and $5.3±0.3$ pA (103 events), respectively (membrane potential was held at $-120$ mV; patch superfused with high sodium solution). These values remained constant at $3.6±0.6$ pA (134 events) and $5.5±0.6$ pA (103 events) during exposure of the patch to 3% halothane. During the recovery period, the respective amplitude values were $3.7±0.6$ pA and $5.8±0.6$ pA (104 and 76 events, respectively).

Insets: Representative recordings of single-channel events, before (A), during (B) and after (C) exposure to halothane. Because these traces are of short durations, they do not reflect the relative frequency of low- and high-amplitude channel events. Calibration bars are 6 pA and 5 msec.
four separate cell-free patches (membrane potentials were held at -120 mV and patches were superfused with high potassium solution; see Materials and Methods for composition) averaged 5.5±0.2 pA for the low-amplitude and 8.2±0.1 pA for high-amplitude channels (mean ± SEM; n = 4 patches) during the control period. When exposed to 4% halothane, the mean amplitudes remained unaltered at 5.4±0.3 pA and 8.2±0.3 pA, respectively.

The mean event amplitude, at a given membrane potential, and from a given patch, remained constant throughout the recording period (Figure 1) despite some variability, in the absolute amplitudes, from patch to patch. The total number of events, however, decreased as a function of time and exposure to halothane (Figure 1). When the current amplitude of each event (data as in Figure 1) is plotted against the corresponding single channel burst duration, the low-amplitude and high-amplitude channels clustered about their respective control amplitude values (Figure 2A). In the presence of halothane (Figure 2B), the distribution of burst durations was shifted to shorter values, and during the recovery period, the initial distribution of burst durations was almost completely reestablished (Figure 2C).

To quantitate the reduction in single channel burst durations, integrated histograms were assembled and fit with a single exponential (see legend of Figure 3). The mean burst duration was estimated from the inverse of the time constant of the fit (non-linear least squares method). A comparison of these histograms for the low-amplitude (Figure 3A and B) and high-amplitude channels (Figure 3C and D), before and after exposure to 3% halothane, shows a marked decrease in burst
Figure 2. Relationship between event amplitudes and their durations. Data (from the patch in Figure 1) are distributed into two distinct clusters on the basis of event amplitudes. During exposure to halothane (3%; panel B), the amplitude position of the clusters remains unaltered but the distribution of burst durations is shifted to shorter values. The initial distribution of burst durations is recovered (panel C) after washout of halothane. Events < 0.6 msec were excluded due to the limitation of the circuit's response time.
Figure 3. Integrated burst duration histograms. Burst durations (data as in Figure 1) are grouped on the basis of their amplitudes. The low-amplitude and high-amplitude channels are plotted separately during control conditions (panels A, C) and during exposure to halothane (panels B, D), respectively. The bin width of the histograms is 100 μsec, and each bin contains the cumulative values of all events with burst durations greater than the value of a particular bin. The mean channel burst duration was estimated from the inverse of the time constant of the fitted exponential N(t) = NTexp(-αt), using a nonlinear least-squares method, where N(t) is the number of events at time t, NT is the total number of events, and α is the time constant (Jackson et al. 1982; Brehm et al. 1984). An independent evaluation of burst durations, using the maximum likelihood method (Colquhoun and Sigworth, 1983), produced values that were numerically very close and statistically identical with those obtained from the method used here. Thus, the possible error introduced by this method appears to be very small.
durations from a mean value of 3.7 msec to 2.7 msec, and from 1.3 msec to 1.0 msec, respectively, for the two channel populations. Table 1 summarizes the concentration-dependent decrease in the mean burst duration for pooled data in which both low- and high-amplitude channel events were combined.

Table 1. Effects of halothane on mean burst duration

<table>
<thead>
<tr>
<th>% Halothane</th>
<th>Burst Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 ± 8* (3, 2, 1402)</td>
</tr>
<tr>
<td>2</td>
<td>67 ± 7** (1, 4, 1049)</td>
</tr>
<tr>
<td>3</td>
<td>52 ± 9** (1, 4, 647)</td>
</tr>
<tr>
<td>4</td>
<td>52 ± 14** (0, 4, 659)</td>
</tr>
</tbody>
</table>

Values are % of control and represent the mean ± SEM. Values in parentheses show numbers of cell-free patches, cell-attached patches, and events, respectively.
* Not significantly different from control.
** Significantly different from control (P < 0.005).

Even though these data (and those in Figure 4 below) were obtained from cell-attached and cell-free patches, the relative (% control) reduction, by halothane, of the burst durations was independent of patch type or its membrane potential. When the effects of halothane are plotted separately for the low-amplitude and high-amplitude channels (Figure 4), a differential effect is observed in which the reduction in the burst durations of low-amplitude channels (at 2% halothane) is significantly larger than that of the high-amplitude channels. Conversely, at the highest halothane concentration (4%), low-amplitude channels from two out of three patches showed a prominent slow component in the burst duration histogram. To obtain a
best least-squares fit to the low-amplitude channel data, at this concentration of halothane, a double exponential fit was utilized and the burst duration estimated from only the initial, fast time constant (dashed circle in Figure 4). The solid circle datum point, at 4% halothane, was derived from the single exponential fit and it contains the subpopulation of channel burst durations which were actually prolonged by the high concentration of halothane.

**Discussion**

The independence of anesthetic potency on size, shape and the chemical nature of GAs suggests that anesthetic action may depend on a non-specific interaction with excitable membranes. GAs, regardless of their molecular structure, produce disordering of lipid membranes (Pang et al. 1979; Mountcastle et al. 1978; but see Lieb et al. 1982), and this may lead to "fluidization" of the membrane in which receptor proteins are embedded (Gage and Hamill, 1975; Lenaz et al. 1979). Such a perturbation may be predicted to permit a faster relaxation of the altered conformation of the receptor protein induced by the agonist-receptor interaction. This would result in faster channel closures and therefore in a reduction of mean channel open time (Gage and Hamill, 1975). Consistent with this idea, Maleque et al. (1981) have reported that ketamine accelerated the decay rate of miniature endplate currents, at the frog neuromuscular junction, without affecting their conductances. Similarly, Haydon et al. (1983) have shown that halothane accelerates the current kinetics of the voltage-sensitive sodium channel in the squid giant axon, resulting in a reduction in inward current
Figure 4. Concentration-dependent effect of halothane on channel burst durations. Data from low-amplitude (circles) and high-amplitude (diamonds) channels are plotted separately as a function of halothane concentration. The reductions in burst durations (both low-amplitude and high-amplitude channels) are statistically different from their respective control values at 2% (p < 0.005), 3% (p < 0.05) and 4% (p < 0.005) halothane. At 2% halothane, the reduction in the burst durations of low-amplitude channels is significantly larger than that of the high-amplitude channels (p < 0.05). Burst durations are expressed as % of control (bars represent the SEM). Numbers, in parentheses, next to data symbols show the number of patches and the number of events used to calculate the respective values for each datum point. The asterisk (*) represents pooled low-amplitude channel data from 3 patches fit to a single exponential. In two of the three patches, a prominent slow component in the burst duration histograms was observed. These data could be better fit by a double exponential. When only the fast component is used, the data show a significant reduction, in burst duration, (broken symbol) as compared to control values.
flow. In contrast, Fernandez et al. (1982) reported that chloroform reduces the magnitude of sodium gating currents without affecting their kinetics. Recently, Nicoll and Madison (1982) reported that several GAs produced neuronal membrane hyperpolarization, albeit a quite high concentrations. In contrast, Lynch et al. (1982) showed that volatile anesthetics do not affect the resting potential of cardiac cells. Examination of inhibitory, GABA-ergic post-synaptic receptors revealed that when exposed to pentobarbital, mean channel opentimes were actually prolonged (Jackson et al. 1982). These diverse findings may reflect the action of different classes of anesthetics or the use of different preparations.

My data show that halothane shortens the burst durations of single ion channels, associated with the nicotinic AChR, in a concentration-dependent manner. These findings are consistent with previous reports on macro-endplate currents (Gage and Hamill, 1975; 1976), and noise analysis data (Landau et al. 1979). It is of interest to note here the findings of Gage and Hamill (1975) who reported that at high concentrations of halothane, miniature endplate currents had a rapid, early decay followed by a slower-than-normal decay. These data are consistent with my observations on the prolongation of low-amplitude channel burst durations under similar experimental conditions. In contrast to its effects on single channel burst duration, halothane does not appear to alter the single channel conductances at any of the tested concentrations. Thus, for a given patch, held at a constant membrane potential, event amplitudes are not altered by the presence of halothane. The shortening of the burst
durations of postsynaptic receptor channels, which I report here, would result in a decrease in the amplitude and duration of postsynaptic potentials. If this effect is sufficiently large, ion currents may fall below the threshold required for activation of the postsynaptic element and hence result in blockade of synaptic transmission.

During exposure to halothane, a decrease in the frequency of channel events was observed (compare Figure 1 A-C). This "drop-out" phenomenon was not observed for the high-amplitude channel events under prolonged (up to 15 minutes), control conditions. But under similar conditions, the low-amplitude channel event frequency decreased (within the first 5 minutes), and then leveled off. Since the current amplitudes of both low-amplitude and high-amplitude channels remain constant throughout the recording period, and since the mean low-amplitude and high-amplitude channel burst durations return to control values after washout of halothane, the drop-out phenomenon is unlikely to be due simply to membrane deterioration. One possible explanation for the drop-out phenomenon is that some receptors, located in the membrane patch, might migrate to the glass-membrane interface and remain trapped there. Ion Channel events from such receptors would therefore be undetectable. The more rapid disappearance of low-amplitude channel events, in my experiments, and the halothane-induced disappearance of high-amplitude channel events suggests an increase in the lateral mobility of the respective receptors. More likely, however, is the possibility that halothane, in addition to shortening channel burst durations, also changes the affinity of ACh for its
receptor (Young et al. 1978)). This effect would result in increased failure to activate channel events either because of a reduced probability of transmitter-receptor interaction or due to the involvement of receptor desensitization. Since I used low agonist concentrations (0.1-0.4 µM), and since I rarely saw simultaneous multiple channel openings, it is unlikely that the "drop-out" I observed was due to receptor desensitization.

While my results do not identify the site of action of halothane, as a general anesthetic, they provide useful information on the possible mode of action of the drug in attenuating or blocking synaptic transmission. The data I report here document directly that, in the presence of halothane, single AChR channels close more rapidly. In addition to the reduction in the burst duration, halothane may decrease or increase the number of brief closures (flickers) during a burst. In the former case, my findings of reduced burst durations demonstrate a net reduction in the open state configuration. In the latter case, the increased probability of linking additional openings into a given burst duration would tend to produce an increase rather than a decrease in the value of the burst. Thus, I would be underestimating the effect of halothane on reducing the burst durations. Clearly, further work is needed to better understand the mode of action of general anesthetics.
CHAPTER 3

EFFECTS OF HALOTHANE ON THE ACETYLCHOLINE RECEPTOR CHANNEL IN CULTURED XENOPUS MYOCYTES.

Introduction

General anesthetics, regardless of their molecular structure, cause disordering of membrane lipids and are believed to increase membrane fluidity (Pang et al. 1979; Lenaz et al. 1979). General anesthetics also reduce the amplitude and time-course of nerve-evoked endplate depolarizations (Gage and Hamill, 1976). The latter effect results from an increase in the decay rate of endplate currents, presumably due to the shortening of AChR channel opening times. We have used the patch clamp technique (Hamill et al. 1981) in order to determine what effects the volatile anesthetic halothane may have on the properties of single acetylcholine receptor (AChR) channels.

Materials and Methods

Uninnervated muscle cells from Xenopus laevis embryos (stages 19-22) were grown in culture as before (Kidokoro et al. 1980). Cultures were continuously superfused, at room temperature, with recording medium (in mM: 120 NaCl, 1.6 KCl, 1 CaCl₂, 8 Hepes, pH 7.4; for cell-free patches, EGTA 5 was added and CaCl₂ omitted) bubbled with air or air with vaporized (Forreger vaporizer) thymol-free halothane. Electrodes were pulled from glass pipettes (Drummond, 50 µl) to resistances of 3-10 M Ohms according to Hamill et al. (1981), and
filled with recording medium (diluted 10% with distilled water) containing 0.2 μM ACh.

Single channel currents were stored on FM tape (Racal Recorder, obtained from Racal Recorders Inc., Research Place, Rockville, Maryland). A 3 to 5 minute control record was obtained just prior to switching to halothane. The effects of halothane were monitored during the first five minutes of exposure to the anesthetic.

Our analysis is based on 3 cell-attached and 3 cell-free patches. Event amplitudes and durations were obtained from digitized records (100 μsec intervals). Opentime histograms were made using 100 μsec bins (opentime closures of less than 1 ms were ignored). The mean channel opentime was estimated as the time constant of the fitted exponential using a non-linear least squares method where \( N(t) \) is the number of events at time \( t \), \( N \) is the total number of events, and \( \alpha \) is the time constant. Events less than 0.6 ms were excluded due to the circuit's time constant (0.12 msec).

Results

Insets in figure 5 show typical oscilloscopic traces from a cell-attached patch just prior to (A) and during exposure to 2% halothane (B). The two event types seen in inset A, based on current amplitude, represent two populations of channels also observed by others (Clark and Adams, 1981; Hamill et al. 1981; Kidokoro et al. 1982). Amplitude histograms were assembled (not shown) and used to identify these populations. The smaller events are termed "m" channels, and the larger ones are called "j" channels. Exposure to halothane did
Figure 5. Control single channel current record (upper inset) from a cell-attached patch. The holding potential was -60 mV from resting potential. Two channel populations (see text) can be seen. The mean amplitude (± SD) of the small "m" channels is 6.8 ± 1.0 pA and of the larger "j" channels is 10.7 ± 1.3 pA (a separation amplitude of 8.2 pA was chosen by eye). The control "j" channel opentime histogram (A) has been fitted with an exponential curve whose time constant is 1.06 ms⁻¹ corresponding to a mean opentime of 1.0 ms. Similarly, the "m" channels (not shown) have an opentime of 2.2 ms. In 5B, a current record (lower inset) from the same patch after exposure to 2% halothane shows a predominance of short-duration events. The "j" channel opentime histogram has a time constant of 1.53 ms⁻¹ corresponding to a reduced mean opentime of 0.7 ms. The "m" channel mean opentime was reduced to 1.2 ms.
Figure 6. Effect of halothane on the mean open time of channel events from 6 patches. Intact and excised patches as well as "m" and "j" channels were combined for a given concentration of halothane. Paired Student t tests were done (four at each concentration) to determine significance. At higher halothane concentrations (2,3%), experimental points were significantly different ($p < 0.001$) from matched control values.
not affect the mean amplitude of either population nor those of patches exposed to higher concentrations of halothane.

The control mean "m" channel opentime, in this patch, was 2.2 ms and 1.0 ms for the "j" channels. On exposure to 2% halothane, channel opentimes were significantly reduced to 1.2 ms (p < 0.01) and 0.7 ms (p < 0.005), respectively. Inside-out cell-free patches (holding potential -120 mV) also had two populations of events whose opentimes were significantly reduced on exposure to halothane. Pooled data, including "m" and "j" channel opentime values from attached and cell-free patches, are plotted in figure 6 which shows a concentration-dependent reduction of channel opentimes by halothane.

Discussion

Anesthetic potency, which is best predicted by its lipid solubility, is essentially independent of the size, shape and chemical nature of general anesthetics (Kaufman, 1977). This suggests that the mechanism of anesthetic action is not directly related to a specific ligand to binding site interaction but may rather depend on a more general effect on membrane structure. General anesthetics are thought to exert their effects by disordering (or fluidizing) the lipids in which receptor proteins are embedded (Metcalfe et al. 1968; Gage and Hamill, 1975). Such fluidization is predicted to permit a faster channel closure resulting in a reduction of mean channel opentime (Gage and Hamill, 1975). Our direct measurements of receptor channel properties show that the channel amplitudes are unaffected but their
open times are significantly reduced, in a concentration-dependent manner, on exposure to halothane.
CHAPTER 4

EFFECTS OF A GENERAL ANESTHETIC ON THE ACETYLCHOLINE RECEPTOR CHANNEL PROPERTIES IN CULTURED XENOPUS MYOCYTES

Introduction

When acetylcholine (ACh) binds to its nicotinic receptor (AChR), the resultant permeability change is due to the opening of ion channels associated with the receptor. The period of time that such channels remain open is referred to as the opentime. Modulation of channel properties occurs during development (Michler and Sakmann, 1980) and in response to pharmacologic agents (Gage and Hamill, 1975) possibly as a result of changes in the microenvironment of the receptor. Thus, changes in membrane fluidity, for example, may be expected to affect channel kinetics.

General anesthetics, regardless of their molecular structure, increase membrane fluidity (Pang et al. 1979; Lenaz et al. 1979; Shieh et al. 1975) and reduce nerve-evoked postsynaptic depolarizations (Gage and Hamill, 1976). The latter effect results from an increase in the decay rate of endplate currents, presumably due to the shortening of AChR channel opentimes (Katz and Miledi, 1973). I investigated this possibility directly by measuring single channel currents using the extracellular patch clamp (Hamill et al. 1981). I propose that changes in receptor channel properties may result, under a variety of circumstances, from alterations in the membrane environment in which the receptor is embedded. Thus, in accord with Gage and
Hamill (1975) we suggest that general anesthetics affect receptor (synaptic) function by fluidizing the lipid microenvironment near the receptor. Such fluidization or disordering of membrane structure may permit a faster relaxation (closure) of receptor channels opened in response to the conformational change induced by the interaction of the receptor with the transmitter ligand. Similarly, during embryonic development, alterations in membrane lipids (Boland and Martonosi, 1974; Kutchai et al. 1978; Nagatomo et al. 1980) may account for the transition from slow to fast channels associated with the process of innervation (Sakmann and Brenner, 1978; Fischbach and Schuetze, 1980; Kullberg et al. 1981). The opposite effect, of increasing the AChR channel open time, as measured by the prolongation of the endplate current after denervation, was reported by Argentieri and McArdle (1981). These authors also reported a simultaneous change in membrane lipid composition consistent with a decrease in membrane fluidity.

Materials and Methods

Uninnervated muscle cells from Xenopus laevis embryos (stages 19-22) were grown in culture as previously described (Kidokoro et al. 1980). Experiments were carried out at room temperature. Cultures were continuously superfused with recording medium (in mM: 120 NaCl, 1.6 KCl, 1 CaCl₂, 8 Hepes, pH 7.4; for cell-free patches, 5 EGTA was added and CaCl₂ omitted) bubbled with air (control) or air with vaporized (Forreger vaporizer) halothane (2 and 3%). The flow rate was 1.1 ml/min and the chamber volume was 0.8 ml. Patch clamp electrodes were pulled from glass pipettes (Drummond, 50 μl) to resistances of 3-
10 M Ohms. Electrodes were lightly heat-polished, coated with sylgard, then re-polished just prior to the experiment. On several occasions, the sylgard coating was omitted. This resulted in a lower signal/noise ratio but otherwise did not affect the results. Electrodes were filled with recording medium (diluted 10% with distilled water) and 0.2 μM ACh. All solutions were filtered (0.2μm, Millipore). Single channel currents were recorded and stored on FM magnetic tape (Racal Recorder). A 3 to 5 minute control record was taken before switching to halothane. I began to observe the effects of halothane within the first two minutes of exposure; therefore, I report here data obtained at least 3 minutes after initiation of halothane exposure to insure equilibration (by a 4-fold replacement of the chamber volume). Washout began 3-5 minutes after recording in halothane. Recovery was noted within 10-20 minutes. With this protocol I collected at least 200 events per recording period.

My analysis of the effects of halothane on AChR channel properties is based primarily on cell-attached (n=2) and cell-free (n=2) patches. Each membrane patch was taken from a different cell and from a different one day old culture. All records were low-pass filtered (corner frequency=2.5 kHz ; Frequency Devices, model 901F) and digitized at 100 μsec intervals using a microcomputer (Dynabyte). The amplitudes, durations and inter-event intervals were computed from these digitized records. Channel events and closures composed of only one digitized point (= 100 μsec), were ignored because of the limit of resolution of our patch-clamp circuit (time constant = 0.12 msec).
Results

Figure 7 (top) shows a representative control record from a cell-attached patch [holding potential was -60 mV from resting potential (RP)]. Single channel events fell into 2 distinct "types" on the basis of current amplitudes in agreement with previous reports (Clark and Adams, 1981; Kidokoro et al. 1982). A histogram (figure 7; bottom) of the channel event amplitudes, from this patch, showed the characteristic bimodal distribution with means (± SEM) of 5.7±0.58 pA and 8.33±0.51 pA (a separation amplitude of 7 pA was chosen by eye). The corresponding mean opentimes were 2.19±0.34 msec and 1.46±0.14 msec. When this patch was exposed to 2% halothane (figure 8), the mean channel opentimes were significantly reduced to 1.72±0.42 msec and 1.10±0.10 msec, respectively.

Under the same experimental conditions, cell-free patches (inside-out; holding potential -120 mV) also showed two "types" of channel event amplitudes in control records. Exposure of these patches to halothane also resulted in a significant shift to shorter opentimes for all channel events; however, it was no longer possible to clearly distinguish two populations. After washout, channel opentimes returned to control values including the bimodal distribution. When the same current amplitude chosen to separate large from small control channels was used to separate channel types during halothane exposure, the opertime of both channel populations was reduced. These data are shown in Table 2. Reductions in channel opentimes are expressed as a percentage of the corresponding control mean opetime.
Figure 7. A control AChR single channel current record (top panel) from a cell-attached patch of a Xenopus myocyte in culture. The holding potential was ~60 mV from resting potential (RP). Two "types", large and small, of channels can be distinguished. Amplitude distribution of channel events from this patch (bin width is 0.1 pA; the number of events was 345). The means of each "type" of channel correspond to 5.7±0.58 pA and 8.33±0.51 pA, based on a separating current of 7 pA.
Figure 8. A current record (top) from a cell-attached patch (same as in figure 7) after exposure to 2% halothane. Note that all channel events now appear to be similar (compare with control in figure 7). Opentime histograms of control and halothane-treated patches. The mean channel opentime of the large (tall) current channels was reduced from $1.46 \pm 0.14$ msec to $1.1 \pm 0.1$ msec. Small (short) current opentimes were reduced from $2.19 \pm 0.34$ msec to $1.72 \pm 0.42$ msec.
Table 2. Effects of halothane on mean open times for cell-attached and cell-free patches.

<table>
<thead>
<tr>
<th>type of patch</th>
<th>channel type</th>
<th>2% halothane*</th>
<th>3% halothane*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell-attached</td>
<td>small</td>
<td>75.7% (2, 61)</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>large</td>
<td>82.5% (2, 119)</td>
<td>67.0% (1, 94)</td>
</tr>
<tr>
<td>cell-free</td>
<td>small</td>
<td>59.8% (1, 128)</td>
<td>65.0% (1, 117)</td>
</tr>
<tr>
<td></td>
<td>large</td>
<td>56.9% (1, 122)</td>
<td>38.5% (1, 83)</td>
</tr>
</tbody>
</table>

* values are expressed as % of control mean open times (# of patches, # of events)

In addition to the reduction in channel open time, halothane appears to affect the relative percentage of large-to-small current channels. Exposure of a cell-attached patch to halothane produced a shift from the control distribution (see figure 7) such that the number of small current channels was markedly decreased, while the number of large current channels was increased (figure 9). The percentage of large to small current channels increased from 56% (control) to 80% (in 2% halothane).

Discussion

In an attempt to test the hypothesis that the physical properties of the lipid environment influence the behavior of the AChR, we examined the effects of a general anesthetic, halothane, on the receptor channel open time. It is known that the anesthetic potency of an agent is best predicted by its lipid solubility in comparison to other general anesthetics and therefore, is essentially independent of its detailed size, shape and chemical nature (Franks and Lieb, 1982).
Figure 9. A histogram of current amplitudes (same as in figure 7) after exposure to 2% halothane. Using a separating amplitude of 7 pA, the population means correspond to 5.89±0.45 pA and 8.55±0.72 pA. The large current channels now represent 80% of the total, in comparison to 56% under control conditions.
This suggests that the mechanism of anesthetic action is not directly related to a specific ligand-protein interaction but rather to a more general effect on membrane structure. It is therefore highly probable that the anesthetic effect on receptor proteins is indirect in nature. It has been suggested that general anesthetics act by disordered or fluidizing (via volume perturbations) the membrane lipids in which the receptor protein is embedded (Metcalfe et al. 1968; Gage and Hamill, 1975; Lenaz et al. 1979). In agreement with this hypothesis, Gage and Hamill (1976) showed that many general anesthetics [which increase membrane fluidity (Pang et al. 1979; Lenaz et al. 1979; Shieh et al. 1975)] reduce the amplitudes of nerve-evoked postsynaptic depolarizations. They argued that an increase in membrane fluidity allows an open AChR channel to close (relax) more rapidly. Our results show that single AChR channels close more rapidly in the presence of halothane. These results are therefore in agreement with Gage and Hamill (1975) who obtained similar findings using endplate current measurements. Furthermore, by using the patch clamp technique, we were able to determine that the opentime of both channel types (large and small current) was reduced and that the relative percentage of large-to-small current channels was increased by halothane. The latter result is also consistent with the idea that an increase in membrane fluidity during development (Kutchai et al. 1976) may account for the transition from slow to fast channels, a process associated with synaptogenesis (Sakmann and Brenner, 1978; Fischbach and Schuetze, 1980; Kullberg et al. 1981).
Finally, a reduction in channel amplitudes (due to sealing of patches and/or the limitation on the response time of our patch clamp) may have affected the bimodal distribution of channel events. However, the maintenance of this distribution in halothane-exposed intact patches and during recovery suggests that the channel "types" may be differentially affected by halothane. That is, halothane causes a reduction in the number of small current channels while increasing the number of large current channels.
CHAPTER 5

HALOTHANE-INDUCED REDUCTIONS IN THE MEAN BURST DURATION OF ACETYLCHOLINE RECEPTOR CHANNELS ARE ATTENUATED BY INCREASES IN MEMBRANE CHOLESTEROL

Introduction

I have previously shown that, in the presence of halothane, the mean burst duration of single acetylcholine receptor (AChR) channels is reduced in a concentration-dependent manner (see Chapters 2, 3 and 4). In order to better understand the details of this action, I altered the lipid composition of Xenopus myocytes, by increasing and decreasing membrane cholesterol, and reexamined the effects of halothane on the single channel properties of AChRs. In light of the these experimental data, I examined the viability of current theories of GA action.

Materials and Methods

Single Channel Recording

Uninnervated muscle cells from Xenopus laevis embryos (stages 19–22) were grown in culture as previously described (Gruener and Kidokoro, 1982). Membrane patches were sealed with electrodes of 1–2 μm internal diameter with resistances of 3–10 Mohms. Electrodes were filled with a high sodium solution whose composition was (in mM): 120 NaCl, 1.6 KCl, 1 EGTA, 8 Hepes·HCl, pH 7.4. This solution contained 0.2–0.4 μM acetylcholine (ACh) for the activation of AChR channels. At this concentration, concurrent activation of multiple channels was
rarely seen; when observed, these were omitted from the data base, facilitating data analysis. Channel events were recorded from cell-free patches. The patch-clamp circuit was built according to the design of Hamill et al. (1981). Single channel events were stored on FM magnetic tape (RACAL Store SD4; Fullerton, CA). Analog records were digitized at 50-usec intervals by a computer (Dynabyte DB8/4; Milpitas, CA). A downward deflection in the current trace, lasting for a minimum of 0.6 msec (circuit time constant = 0.12 msec), and which occurred within an adjustable window (usually 2-15 pA) was considered to be a resolvable event. When an event was detected, the digitized record and its trailing baseline (usually 2.5 msec) were stored on a floppy disk. Amplitudes were calculated from the mean value of digitized points at the peak of an event omitting the two initial and two final points to cross the selected threshold. Each event was displayed and accepted or eliminated at the operator's discretion. The criterion for accepting an event opening was an abrupt transition from baseline to a constant current amplitude. The event was considered closed when the current level remained at baseline for at least 1 msec. The mean channel burst duration (see discussion below) was estimated using the maximum likelihood estimate for a single exponential (Colquhoun and Sigworth, 1983). With this method, the mean channel burst duration is equivalent to: (the total sum of resolvable burst durations [i.e. greater than 0.6 msec] divided by the total number of bursts) minus the minimum resolvable burst (0.6 msec).

It has recently been shown (Sakmann et al. 1980; Colquhoun and Sakmann, 1981; Dionne and Leibowitz, 1982) that a given channel may
open repeatedly when ACh is bound to its receptor. These repeated
openings are called bursts since they are interrupted by short,
sometimes incomplete, closures whose resolution depends, in part, on
the time-constant of the patch-clamp circuit. In order to distinguish
between a burst, representing the activity of a single channel, and the
opening of two different channels, Dionne and Leibowitz (1982)
assembled opening-probability histograms. They showed that, at the
neuromuscular junction of the garter snake, two successive openings
appear as independent events (and hence likely to emanate from two
separate channels) when the closed times exceed about 2 msec. As a
conservative definition of the burst, I report my data as burst
durations when a series of channel openings is interrupted by closed
times shorter than 1 msec. A burst which is uninterrupted by closures
is a special case of such events and is commonly referred to as the
channel open-time. The Student t-test was used to determine statistical
significance.

Experimental Protocol

During experiments, which were performed at room temperature,
cultures were continuously superfused (1-2 ml/min) with recording
medium (high potassium solution (in mM): 70 KSO\(_4\), 4 KCl, 2 4
HEPES.HCl, pH 7.4). Thymol-free halothane (Halocarbon Laboratories, Hackensack, NJ) was added to the perfusion medium by bubbling through a vaporizer
(Forreger; Smithtown, NY) at concentrations of 1-4% [Percent of
vaporized halothane mixed with air (flow rate: 0.1 liters/min) and
bubbled through the recording medium reservoir; this concentration
range corresponds to clinically relevant doses (Gage and Hamill, 1975) and to aqueous concentrations, in the experimental chamber, of 0.2-0.8 mM, as measured by ultraviolet spectroscopy (Blank and Thompson, 1980). After 4-7 minutes exposure to halothane, and following a similar control period, the anesthetic was washed away and channel events from the recovery period were usually recorded.

Liposome Treatment of Muscle Cells

Extraction of endogenous cholesterol or introduction of exogenous cholesterol into the sarcolemma was accomplished according to the procedures of Shattil and Cooper (1976). This method is based on the fusion and exchange of lipids between the cell plasma membrane and exogenous liposomes. Procedures for liposome preparation are described below. To lower the cholesterol levels, defined media (60% v/v Hepes buffered Dulbecco-modified Eagle's medium; 5 ug insulin/ml; 100 ug transferrin/ml; 20 nM progesterone; 100 uM putrescine) of muscle cell cultures were replaced with defined media containing cholesterol-free (ChIF) liposomes made from phosphatidylcholine (Sedary Research Laboratories, London, Ontario, CANADA; purification procedures are described below). Cell cultures were allowed to incubate with the liposomes for 12 hours. Cholesterol (Sedary Research Laboratories, London, Ontario, CANADA) levels were raised by incubating (same procedure as in ChIF liposome treatment) muscle cells for 12 hours with cholesterol-rich (ChIR) liposomes. Muscle cells were only treated with liposomes at 36 hours or 60 hours after plating. Patch-clamp experiments were performed after muscle cell cultures had been
incubated with liposomes for 12 hours.

Preparation of Cholesterol-Rich and Cholesterol-Free Liposomes

ChlR and ChlF lipid dispersions (80 mg phospholipid with or without 40 mg cholesterol) were lyophilized from chloroform under a stream of nitrogen. Lipids were then resuspended in 3 ml ethanol (95%), lyophilized a second time and placed under vacuum for 40 minutes to remove residual chloroform. Ten ml of defined medium (see composition above) was added to the dried lipids. ChlR and ChlF lipid dispersions were then sonicated at 60 watts (Branson Sonicator) for a total of 90 and 60 min, respectively. These sonication periods were interrupted every 5 min. with 1 min. cooling intervals (sonication was carried out in an ice bath under nitrogen). The sonicated lipid dispersions were centrifuged for 40 minutes at 4°C to remove titanium particles and multi-lamellar vesicles (Sorval Centrifuge RC2-B, SS-34 rotor, 11500 rpm). The supernates containing the uni-lamellar liposomes were sterilized by filtering (Millipore filters, 0.2 μm) and stored at 4°C (Shinitzky and Inbar, 1974). Prior to use, liposomes were diluted to 10% with defined medium.

Purification of Phosphatidylcholine

Phosphatidylcholine was placed onto a column of neutral alumina (Bio-Rad AG-7) which had been packed with chloroform. A column loading factor of 1 mg of phosphorous/g of alumina was used. The column was eluted with chloroform until no further organic material could be detected in the eluate. The column was then eluted with chloroform-methanol (85:15 v/v) and the eluate was collected in 5 ml fractions.
The phosphatidylcholine was located by spotting thin-layer plates and spraying the plates with phosphorous spray (Dittmer and Wells, 1969). Fractions containing phospholipid were then collected, the solvent evaporated and the phospholipids were dissolved in chloroform (20 mg phosphatidyl choline/1 ml chloroform). Phospholipid concentrations were checked by measuring the amount of phosphorous in the chloroform suspension by the macro Bartlett Phosphate assay (Dittmer and Wells, 1969). Briefly, phospholipid samples (approximately 1 umole, exact concentration to be determined) were evaporated to dryness under nitrogen. Perchloric acid (70%; 0.7 ml) was added and the samples were heat digested for 30 min. After cooling, ammonium molybdate (5%; 0.5 ml), ANSA reagent (1,2,4, aminoaphthal sulfonic acid; 0.4 ml) and water (to 10 ml) were added. The samples were vortexed and allowed to stand for 20 min. Light absorption of the samples were then read with a spectrophotometer (Beckman UV Visible, model 34; at 660 nm). Phosphorous was quantified by comparison with 1 umole phosphorous standards. Purity was checked by the presence of a single spot (detected with phosphorous spray; Dittmer and Wells, 1969) on thin-layer chromatography (chloroform-methanol-water, 95:35:4, v/v).

Determination of Whole Cell Cholesterol and Phospholipid Levels

Cholesterol was extracted from whole muscle cells by the method of Bligh and Dyer (1959). Methanol (2.5 ml) and chloroform (1.25 ml) were added to a 1 ml suspension of muscle cells (approximately 15000 cells as determined by a hemocytometer) and allowed to stand for 1 hr. Chloroform (1.25 ml) and saline (1 ml; 0.9% NaCl) were then added, the
suspension was vortexed and centrifuged (2300 rpm) to separate the polar and non-polar phases. The non-polar phase (bottom of tube), containing the cholesterol, was drawn off and evaporated to dryness under nitrogen. Stigmasterol (25.5 µg) was added for use as an internal standard. The lipids were resuspended in 50 µl of hexane prior to analysis by gas chromatography.

Cholesterol was determined by gas chromatography on a Shimadzu GC mini 1 gas chromatograph equipped with a Shimadzu C-RiA recording integrator. Glass columns (0.6 m, i.d. 2.6 mm) were filled with 3% JXR on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories). The chromatograph was run isothermally at 220°C. Cholesterol was quantified by the use of the internal stigmasterol standard.

Phospholipid concentrations were determined by the micro Bartlett Phosphate assay (Dittmer and Wells, 1969). Lipid samples were suspended in 0.4 ml of 70% perchloric acid and allowed to heat digest for 20 minutes. After cooling, ammonium molybdate (2.4 ml) and reducing agent (2.4 ml; 1,2,4, aminoaphthal sulfonic acid diluted 1:12 with water) were added and samples were boiled for 10 min. Samples were then cooled and read on a spectrophotometer (Beckman UV Visible, model 34) for light absorption at 630 nm. Phosphorous was quantitated by comparison with 0.05 µmole phosphorous standard.

Cholesterol changes can occur by exchange between muscle cells and exogenous liposomes or by fusion and/or sticking of liposomes to muscle cells. The percentage of liposome fusion and/or sticking was determined by adding 10 µCi of a non-exchangeable marker (14-C-cholesterol oleate; Amersham Corporation, Amersham, United Kingdom) to
the lipid dispersions prior to preparation of Ch1R or Ch1F liposomes (sonication procedures are described above). Counts of radioactivity (recorded by Beckman LS 7500), after muscle cells were incubated for 12 hours and washed with defined medium, were interpreted as counts due to the fusion and/or sticking of liposomes to muscle cells. The percentage of fusion and/or sticking was estimated by dividing the total number of counts in a 100 µl aliquot of cell suspension by the total number of counts in a 100 µl aliquot of liposome suspension that was used for cell incubation.

Cholesterol and phospholipid concentrations were reported per 10^5 muscle cells. The number of cells per aliquot of cell suspension was determined by using a hemocytometer.

**Results**

**Effects of Liposome Treatment on Cholesterol Content**

Muscle cell cultures were incubated with cholesterol-rich (Ch1R) or cholesterol-free (Ch1F) liposomes for 12 hours. Cells were then washed with defined medium, collected (triturated from the bottom of culture dishes and place in 10 ml glass tubes) and analyzed for cholesterol content. Ch1R-treated cells showed significant increases in cholesterol when compared to untreated and Ch1F-treated muscle cells (see table 3). Specifically, the cholesterol content averaged 127.0 ± 38 nmoles/10^5 cells (+ SEM; n=7) for Ch1R treatment whereas untreated and Ch1F-treated cells averaged 6.9 ± 0.9 (n=9) and 6.0 ± 1.1 (n=6) nmoles cholesterol/10^5 cells, respectively. In some of these experiments, the cholesterol:phospholipid (C/P) molar ratio for
Table 3. Effects of liposome treatment on cholesterol levels.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nmoles cholesterol*</th>
<th>% Fusion</th>
<th>C/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChlF</td>
<td>6.0 ± 1.1 (6)</td>
<td>0.7 ± 0.1 (2)</td>
<td>0.10 ± 0.05 (2)</td>
</tr>
<tr>
<td>untreated</td>
<td>6.9 ± 0.9 (9)</td>
<td>--</td>
<td>0.16 ± 0.02 (4)</td>
</tr>
<tr>
<td>ChlR</td>
<td>127.0 ± 38.0 (7)**</td>
<td>0.8 ± 0.3 (3)</td>
<td>0.57 ± 0.20 (3)</td>
</tr>
</tbody>
</table>

* cholesterol levels per 10^5 cells
** significantly different from untreated (p < 0.005)
Number in parentheses refers to the number of experiments

treated and untreated muscle cells was determined. I found that the
the C/P ratio in ChlR-treated cells was increased to 0.57 ± 0.2 (n=2).
The C/P ratios for untreated and ChlF-treated cells were 0.16 ± 0.02
(n=4) and 0.1 ± 0.05 (n=3), respectively.

To distinguish between cholesterol increases caused by fusion
versus exchange of cholesterol, with ChlR liposomes and muscle cells, I
determined the percentage of a non-exchangable liposome marker (14C-
cholesterol oleate) that remained with treated muscle cells after
liposome incubation. Since 14C-cholesterol oleate is non-exchangable,
the percentage of liposomes that remained with treated cells must be
attributed to liposome fusion and/or sticking (due to technical
limitations, fusion and sticking of liposomes to muscle cells could not
be distinguished). I found that 0.8 ± 0.3 % (n=3) of the ChlR
liposomes (see table 3) remained with muscle cells after extensive
washing. Cholesterol analysis, by gas chromatography, showed that this
percentage of ChlR liposomes contributed 66 ± 4 nmoles of cholesterol
per 10^5 cells. Alternatively, 52 ± 3 % of the total cholesterol
increase observed in ChlR-treated cells could be attributed to the
fusion and/or sticking of liposomes to muscle cells.
Effects of Liposome Treatment on Acetylcholine Receptor Channel Properties

Single-channel events, recorded from patches treated with ChlF or ChlR liposomes, were assembled into amplitude histograms (figure 10). In agreement with data from untreated patches (see figure 1), bimodal distributions were obtained on the basis of current amplitudes. The resultant populations are designated low-conductance (LC) and high-conductance (HC) channels. The LC channels have a relatively long open time whereas HC channels have shorter open times (figure 10 insets; compare with figure 1 insets).

The mean channel amplitudes (LC and HC channels) were not affected by liposome treatment (see table 4). For cell-free patches that were superfused with high potassium solution (see Material and Methods for composition) and held at membrane potentials of \(-90 \text{ mV}\), the mean LC channel amplitudes for ChlR-treated, ChlF-treated and untreated cells were \(4.1 \pm 0.1 \text{ pA (n=8 patches)}\), \(4.2 \pm 0.1 \text{ pA (n=15)}\) and \(4.2 \pm 0.1 \text{ pA (n=6)}\), respectively. Similarly, the HC channels had mean amplitudes of \(6.5 \pm 0.1 \text{ pA (n=8)}\), \(6.4 \pm 0.1 \text{ pA (n=15)}\) and \(6.6 \pm 0.2 \text{ pA (n=6)}\) for ChlR-, ChlF- and untreated cells, respectively. In addition, direct measurements of single channel conductance (see table 4) also showed no significant affects of liposome treatment on LC and HC channel conductance when compared to untreated cells.

Liposome treatment also had no significant effect on AChR channel burst durations (see table 4). As above, cell-free patches were held at \(-90 \text{ mV}\) and superfused with high potassium solution. The mean LC channel burst durations for ChlR-, ChlF- and untreated cells were
Figure 10. Amplitude histogram of single-channel events from a patch treated with cholesterol-free liposomes. Amplitude histograms reveal a bimodal distribution (compare figure 1), which is maintained throughout the experiment. The mean amplitude of LC and HC channels, during each experimental period, is not significantly different. In this cell-free patch, the control mean amplitudes (± SD) of the LC and HC channels were 4.3 ± 0.6 pA (103 events) and 6.5 ± 0.6 pA (196 events), respectively (membrane potential was held at -90 mV; patch superfused with high potassium solution). These values remained constant at 4.2 ± 0.7 pA (146 events) and 6.3 ± 0.5 pA (107 events) during exposure to 2% halothane. During the recovery period, the respective amplitude values were 4.2 ± 0.7 pA and 6.4 ± 0.4 pA (76 and 108 events, respectively). Insets. Representative recordings of single-channel events before (A), during (B), and after (C) exposure to halothane. Calibration bars are 6 pA and 2 msec.
Table 4. Effects of cholesterol on acetylcholine receptor channel properties

<table>
<thead>
<tr>
<th>treatment</th>
<th>channel</th>
<th>amplitude (pA)</th>
<th>conductance (pS)</th>
<th>burst duration (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChlF</td>
<td>LC</td>
<td>4.2 ± 0.1 (15)</td>
<td>41 ± 7 (4)</td>
<td>2.6 ± 0.4 (14)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>6.4 ± 0.1 (15)</td>
<td>59 ± 9 (4)</td>
<td>0.9 ± 0.1 (15)</td>
</tr>
<tr>
<td>untreated</td>
<td>LC</td>
<td>4.2 ± 0.1 (6)</td>
<td>44 ± 7 (2)</td>
<td>2.2 ± 0.2 (4)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>6.6 ± 0.2 (6)</td>
<td>67 ± 7 (2)</td>
<td>0.8 ± 0.1 (4)</td>
</tr>
<tr>
<td>ChlR</td>
<td>LC</td>
<td>4.1 ± 0.1 (8)</td>
<td>44 ± 4 (8)</td>
<td>1.9 ± 0.3 (7)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>6.5 ± 0.1 (8)</td>
<td>63 ± 7 (8)</td>
<td>0.8 ± 0.1 (6)</td>
</tr>
</tbody>
</table>

Number in parentheses refers to the number of patches. Values are reported mean ± SEM.

1.9 ± 0.3 msec (n=7), 2.6 ± 0.4 msec (n=14) and 2.2 ± 0.2 msec (n=4), respectively. The mean HC channel burst durations were 0.8 ± 0.1 msec (n=6), 0.8 ± 0.1 msec (n=15) and 0.9 ± 0.1 msec (n=4), respectively.

Effects of Cholesterol on Halothane Action

The bimodal distribution and mean channel amplitudes (LC and HC channels), seen in untreated cells (see figure 1), are maintained during exposure to halothane (figure 10 B) and the recovery period following washout of halothane (figure 10 C). During exposure to 2% halothane, the LC and HC channels had mean amplitudes of 98% (% of control) and 97% and during recovery, 98% and 98%, respectively. A current amplitude value of 5.4 pA was chosen by eye to separate the two channel populations. In another set of experiments (patches were exposed to 4% halothane), the LC channel amplitudes remained unchanged for patches treated with ChIR- or ChlF-treated cells. However, the HC channels showed significant (p < 0.05) reductions in mean amplitudes to
97 ± 1% in ChlR-treated cells and to 95 ± 2% in ChlF-treated cells (4 patches).

The total number of events decreased as a function of time and exposure to halothane for both ChlR- and ChlF-treated (figure 11) patches. The same drop-out phenomenon is seen for channels in untreated cells (figure 11; see also Brehm et al. 1984). However, the extent of the drop-out phenomenon is not statistical different when comparing cells treated and untreated with liposomes or when comparing cells under control conditions versus exposure to halothane.

When the current amplitude of each event (data as in figure 10) is plotted against the corresponding single-channel burst duration, the LC and HC channels cluster about their respective control mean amplitude values (figure 12 A). During exposure to halothane (figure 12 B), the distribution of burst durations (both LC and HC channels) for liposome treated cells was shifted to shorter values in accord with data from untreated cells (cf. figure 2 B). The initial distribution of burst durations is fully recovered after washout of halothane (figure 12 C).

Halothane-induced reductions in AChR burst durations were quantified by the maximum likelihood method for a single exponential (Colquhoun and Sigworth, 1983). The mean burst duration was determined by the inverse of the time constant of the exponential estimate. Cumulative histograms of burst durations were then assembled and fit with the exponential estimated by the maximum likelihood method. A comparison of these histograms during control (figure 13 A and C) and after exposure to 2% halothane (figure 13 B and D), for a ChlF-treated
Figure 11. Channel event frequency for LC and HC channels. Channel event frequencies are plotted separately during control conditions (A and B) and during exposure to 3% halothane (C and D). During control conditions, the rate of LC channel (circles) frequency begins to decrease after about 3 minutes for myocytes treated with ChlR liposomes whereas both LC channels from both ChlF-treated and untreated myocytes show a decrease in the rate of channel frequency (drop-out) after the first minute. HC channels (diamonds) show about a 30% drop in the channel frequency rate for liposome treated cells compared to untreated cells. During halothane exposure, the LC and HC channel frequency show an initial increase within the first minute of halothane followed by a decrease in the frequency. Data are expressed as a percent of the number of events (± SEM) occurring within the first minute of control (first bin). During halothane exposure, the first bin refers to the number of events occurring in the last minute under control conditions. Numbers refer to the number of patches.
Figure 12. Relationship between event amplitudes and their durations for a patch treated with cholesterol-free liposomes. (A) Data (from the patch in figure 1) are distributed into two distinct clusters on the basis of current amplitudes. During exposure to 2% halothane (B), the amplitude position of the clusters remains unaltered but the distribution is shifted to shorter values. During recovery (C), the initial distribution reappears.
Figure 13. Cumulative burst duration histograms from a patch treated with cholesterol-free liposomes. Burst durations (same patch as in figure 1) are grouped on the basis of their amplitudes. The LC and HC channels are plotted separately during control conditions (A and C) and during exposure to 2% halothane (B and D). The bin width of the histogram is 100 μsec, and each bin contains the cumulative values of all events with burst durations greater than the value of a particular bin. The mean channel burst duration was estimated from the maximum likelihood method (see Materials and Methods). Burst durations are reduced from a mean value of 1.4 msec to 1 msec for the LC channels and from 0.8 msec to 0.5 msec for HC channels.
patch, shows a marked reduction in the mean burst durations. Burst duration are reduced from a mean value of 1.4 msec to 1 msec for LC channels and from 0.8 msec to 0.5 msec for the HC channels. Figure 14 A summarizes the reductions in the mean burst durations for pooled data in which both LC and HC channel events were combined. In accord with data from untreated cells, patches from liposome treated cells show a concentration-dependent reduction in AChR burst durations. However, the the halothane-induced reductions are significantly different when comparing ChlR-, ChlF- and untreated muscle cells. During exposure to 1 % halothane, ChlR-treated patches showed no significant reduction in the mean burst duration of AChRs (99 ± 12 % of control; n=6 patches) whereas patches from ChlF-treated cells showed a significant reduction in AChR channel burst durations to 61 ± 9 % (n=8). In the presence of 4 % halothane, patches from ChlR- and ChlF-treated cells both showed significant reductions in mean burst durations. The mean burst duration in ChlR-treated patches was reduced to 60 ± 5 % (n=9) while the mean burst duration in ChlF-treated cells was reduced to 31 ± 4 % (n=8). It is of particular interest to note that four times as much halothane (4%) was required to reduce AChR channel burst durations in ChlR-treated patches (61 ± 4 %) to a similar value (60 ± 9 %) that was observed at 1% halothane for ChlF-treated patches. The effects of halothane on AChR burst durations are plotted separately for LC and HC channels in figure 14 B and C. In accord with the pooled data, the halothane-induced reduction in burst duration is generally antagonized for ChlR-treated cells and augmented for ChlF-treated cells when compared to untreated cells. Note in particular that for LC channels,
Figure 14. Comparison of burst durations from patches treated with cholesterol-free (ChIF) and cholesterol-rich (ChIR) liposomes in the presence of halothane. (A) Data from LC and HC channels were pooled and plotted as a function of halothane concentration. A concentration-dependent reduction in mean burst durations is observed for ChIR-, ChIF-treated and untreated patches. Note however, that the halothane-induced reductions in burst durations are significantly different when comparing patches treated with ChIR and ChIF liposomes. In addition, the reduction in burst durations at 1% halothane, for ChIF-treated patches is significantly greater than untreated cells (p < 0.025). Similarly, the burst duration reductions from ChIR-treated patches, at 2 and 4% halothane, are significantly different from untreated patches (p < 0.05). In panels B and C, LC channel (circles) and HC channel (diamonds) data are plotted separately as a function of halothane concentration. Halothane exposure results in a concentration-dependent reduction of burst durations for both LC and HC channels in all myocytes. Note the halothane-induced reduction in burst duration is smaller in ChIR than in ChIF treated myocytes. Asterisks mark statistical significance (*, p < 0.05; **, p < 0.025; ***, p < 0.005). Burst durations are expressed as a % of control (± SEM). Numbers in parentheses refer to the number of patches and number of events, respectively.
Figure 14. Comparison of burst durations from patches treated with cholesterol-free (ChlF) and cholesterol-rich (ChlR) liposomes in the presence of halothane.
not even four times as much halothane can produce similar reductions in burst duration for ChlR-treated patches when compared the burst duration reduction observed at 1% halothane for ChlF-treated patches. Similarly, three times as much halothane is required to produce similar reductions in HC channel burst durations in ChlR-treated cells when compared to burst duration reductions in ChlF-treated cells at 1% halothane.

Discussion

The classical observation by Meyer (1899) and Overton (1901), that anesthetic potency is correlated with lipid solubility, led to the suggestion that general anesthetics (GAs) act at hydrophobic sites within excitable membranes and that this physical interaction blocks membrane excitability (Seeman, 1972; Miller and Miller, 1975; Roth, 1979).

The objectives of my dissertation were to: 1) examine the manner by which a GA interacts with a functional synaptic protein in an excitable membrane, and 2) examine the effects of changing the membrane lipid composition on this interaction. To accomplish these objectives, I examined how the volatile GA halothane affects the single channel behavior of the nicotinic acetylcholine receptor (AChR) channels in Xenopus muscle cells grown in culture. I then altered the lipid composition, in these cells, by increasing and decreasing membrane cholesterol content through cell incubation with cholesterol-rich and cholesterol-free liposomes and reexamined the effects of halothane on these treated cells.
Data derived from these experiments are used to evaluate the current theories of mechanisms of actions of GAs.

Effects of Halothane on the Acetylcholine Receptor Channel

A GA-induced decrease in the amplitude of postsynaptic potentials (see Chapter 1, Postsynaptic Conductance Changes) can result from a decrease in ion current flow across the membrane. Current flow depends on both the conductance state and open time of an ion channel. Gage and Hamill (1975; 1976; 1978) suggested that GAs decrease postsynaptic depolarizations by producing a faster relaxation, from the open to the closed conformational state, of the postsynaptic ion channels. They demonstrated that halothane, ether and octanol increased the rate of decay of postsynaptic currents at the neuromuscular synapse. The increased decay rate of postsynaptic currents is consistent with a decrease in the mean open time of the AChR channel (Katz and Miledi, 1973). However, decreases in AChR channel conductance could also depress postsynaptic depolarizations. In fact, Landau et al. (1979) found that methoxyflurane decreased both the mean conductance and open time of the AChR channel using noise analysis measurements (see appendix). In contrast, Maleque et al. (1981), using the same technique, reported that ketamine decreased the mean open time, but did not affect the conductance of the AChR channel.

In addition to changes in the properties of postsynaptic channels, GAs may also depress postsynaptic depolarizations by blocking channels in the open state (sequential blocking model; Adams, 1976). In this case, the open channel becomes plugged by GA molecules and
prevented from conducting. Thus, the total time that a channel is open remains unaltered, but the total time a channel may conduct is actually shortened. The reduction in channel conducting time predicts an increase in the decay rate of postsynaptic currents and a decreased mean opentime as measured by noise analysis (Maleque et al. 1981).

In order to distinguish between the various possibilities, I used the patch clamp technique to examine the effects of halothane on the single channel properties of the AChR channel. With this technique, both the opentime and conductance state of single receptor channels can be monitored directly. Since a given channel may open repeatedly when acetylcholine is bound to its receptor (Dionne and Liebowitz, 1982), I report my data as burst durations, which represent the activity of a single channel. Note that a single burst, caused by short closures of a single channel, is not distinguishable from a single burst caused by blockade of the open channel. In the latter case, however, the mean burst duration is predicted to increase when the concentration of channel blockers (and thus the number of apparent closures due to blocking) is increased.

**Halothane Effects on Acetylcholine Receptor Channel Burst Duration and Conductance.** My data show that halothane shortens the burst durations of both low- (LC) and high-conductance (HC) AChR channels (see figure 4). These data are consistent with results obtained from noise analysis of postsynaptic currents as discussed above. Further, they support the hypothesis that GAs act by producing a more rapid transition of the channels from the open to the closed state. In contrast, my data do not support the sequential blocking
model of GA action, at least with respect to halothane. In particular, the blocking model predicts that as the concentration of GAs is increased, the open channel will be plugged more frequently (i.e. more apparent single channel closures) during a burst and the mean burst duration will be increased. To the contrary, I found that the mean burst duration of LC and HC channels decreased as the halothane concentration was increased (see figure 4).

In contrast to its effects on single channel burst durations, halothane did not change the single channel conductance of either LC or HC channels (see figure 1). Thus, for a given patch, at the same membrane potential, the single channel event amplitudes remained the same regardless of the halothane concentrations tested. However, under conditions of altered lipid composition of the cell membranes, high concentrations of halothane (4 %) appeared to slightly reduce the single channel conductance of HC channels (see discussion below; Effects of Liposome Treatment on Halothane Action).

In summary, the findings of the shortening of postsynaptic burst durations provide a direct molecular explanation of the previously reported decrease in the amplitude and duration of postsynaptic potentials (Gage and Hamill, 1975; 1976). More generally, it can be argued that if the reductions of postsynaptic burst durations, reported here, were sufficiently large and were to occur in critical synapses in the CNS, then postsynaptic depolarizations would below the threshold required for the activation of postsynaptic elements and synaptic transmission would be blocked. This blockade could inactivate specific neuronal circuits involved in pain sensation
and the maintenance of consciousness. For example, the brain stem reticular system has been suggested to play a major role in the control of consciousness (Angel, 1980). The brain stem appears to be a way station, for both ascending (sensory) and descending (motor) pathways, between the cerebral cortex and the spinal cord. The reticular neuronal network, referred to as the sensorimotor modulation system (Angel, 1980), may modulate the level of consciousness. In particular, ascending reticular nerve fibers appear to govern electrocortical arousal (e.g. awakening from sleep). Electrical stimulation of the reticular system desynchronizes electrocortical activity. This desynchronization, and possibly the level of consciousness, has been shown to be suppressed by barbiturates (Angel, 1980).

**Acetylcholine Receptor Channel Event Frequency.** Analysis of single channel records also allows measurements of frequency of event occurrences. Under control conditions, the event frequency of the HC channels remained constant for periods up to 15 minutes after patch sealing (see figure 11). Under the same conditions, in contrast, the LC channel event frequency decreased to about 30% within 5 minutes and then remained constant. Using the same preparation and technique, Brehm et al. (1984) found a decrease in the channel event frequency for both channel types, although the LC channels dropped out at a faster rate. This drop-out phenomenon could be a general response of postsynaptic receptor channels to agonist application (e.g. receptor desensitization). Alternatively, the drop-out phenomenon may be in response to experimental manipulations and could result from membrane deterioration or movement of AChRs to the glass-membrane interface and
their entrapment there (ion channel events can only be detected within the patch). Each of these possibilities is discussed below.

Desensitisation of AChRs receptors, which is a process in which receptors undergo a reversible conformational change from the active to an inactive state, could be a contributing factor to the drop-out phenomenon. High agonist concentrations (10–100 uM) have produced desensitization of AChRs in frog (Sakmann et al. 1980; Ogden and Colquhuon, 1983). Since I used very low agonist concentrations (0.1–0.4 uM) and since I rarely saw simultaneous multiple channel openings, it is unlikely that receptor desensitization was a contributing factor to the drop-out phenomenon which I observed.

To make single channel measurements, a very tight seal (referred to as the giga-seal) is established between the cell membrane and the recording glass electrode tip. The formation of the giga-seal often requires applying suction, to the patch, which pulls the cell membrane into contact with the electrode tip. The resultant membrane distortion may damage the integrity of the patch, resulting in some deterioration. If membrane distortion results in deterioration, it is expected that this be a continuous process. However, I found that the mean current amplitudes of LC and HC channels remained constant throughout the recording period (see figure 1). In addition, the mean burst durations of LC and HC channels returned to control values after washout of halothane. It is therefore unlikely that membrane deterioration was a contributing factor to the drop-out phenomenon.

The drop-out phenomenon may have been caused by a lateral movement of AChRs to the glass-membrane interface where channel events
cannot be detected. AChRs have been shown to migrate within cell membranes (Axelrod et al. 1978). Assuming an AChR diffusion constant of $10^{-10}$ cm$^2$/sec (Axelrod et al. 1978) and particle diffusion due to brownian motion, an AChR could diffuse approximately 1 micron in 25 sec or 5 microns in 10 minutes. Since the patch electrode tip is 1-2 microns in diameter, the time-course for AChR drop-out parallels the time-course expected for movement to the glass-membrane interface. Therefore, diffusion of AChRs to the glass-membrane interface could be a contributing factor in the drop-out phenomenon.

Halothane Effects on Acetylcholine Receptor Channel Event Frequency. General anesthetics may act by decreasing the number of channel openings, thereby decreasing postsynaptic depolarizations. In fact, after exposure to halothane, I observed a pronounced decrease in the frequency of channel events (figure 11; cf. figures 1A, 1B and 1C). Although the effects of halothane alone cannot account for the drop-out phenomenon (see above), it may have contributed to it by fluidizing the membrane. Fluidization would increase AChR mobility and therefore accelerate the drop-out rate. This is consistent with my findings (see figure 11). It is also conceivable that desensitization has contributed partially to the drop-out phenomenon during exposure to halothane. Inhalation GAs have been shown to enhance the binding of carbamoylcholine (an AChR agonist) and ACh to AChRs in Torpedo californica (Young et al. 1978; Sauter et al. 1980). Such enhanced binding may have produced some desensitization by increasing the apparent agonist concentration.
Effects of Increased Membrane Cholesterol

Since anesthetic potency is correlated with lipid solubility, the site of GA action is most likely to be hydrophobic in nature. Consequently, GAs may interact directly with functionally relevant membrane proteins, through hydrophobic pockets, or indirectly through GA interaction with the lipid environment of these proteins (Franks and Lieb, 1982; 1984). If GA action is produced through interactions with lipids, then alterations in this membrane component are expected to affect the action of GAs.

In order to further understand the role of membrane lipids in the mechanism of action of GAs, I altered the lipid composition of Xenopus muscle cells by increasing or decreasing the cholesterol content of these cells. I accomplished this by incubating these cells with cholesterol-rich (ChIR) and cholesterol-free (ChIF) liposomes and examining the effects of halothane on the single channel properties of the AChR channel. With data derived from these experiments, I examined the viability of current lipid and protein theories of GA action.

**Effects of Liposome Treatment on Membrane Cholesterol.** Muscle cells treated with ChIF liposomes showed only a slight decrease in cholesterol (0.10; cholesterol:phospholipid molar ratio [C/P]) whereas cells treated with ChIR liposomes showed a significant increase in whole cell cholesterol (0.57; C/P ratio) when compared to untreated cells (0.16; C/P ratio; see table 3). The absence of a significant cholesterol decrease in ChIF treated cells may be due, in part, to the initial low levels of cholesterol in untreated cells. Liebel et al. (1983) have reported that liposome treatment could not
lower cholesterol levels beyond 0.40 (C/P ratio) in AChRs reconstituted from Torpedo. They argued that this pool of cholesterol may be inaccessible to liposome extraction. Since the cholesterol level in untreated cells was only 0.16 (see table 3), it is also possible that this constituted an inaccessible pool of cholesterol for liposome extraction.

Since increases in cholesterol levels may have been due to fusion and/or exchange of cholesterol, I determined the maximum amount of cholesterol that could be attributed to fusion and/or adhesion (the fusion of liposomes with membranes could not be distinguished from the adhesion of liposomes) of liposomes to muscle cells. I found that only 52% of the increase in cholesterol could be attributed to fusion and/or adhesion (see Results subsection, Effects of Liposome Treatment on Cholesterol Content in Chapter 3). Alternatively, approximately half (48%) of the cholesterol increase must be attributed to exchange between ChlR-liposomes and muscle cells.

Increases in whole cell cholesterol may be due, in part, to increases in intracellular levels of cholesterol. However, Poznansky and Lange (1978) have found that the exchange of cholesterol between red blood cell ghosts and ChlR liposomes is limited to the outer portion of the plasma membrane. Further, the flip-flop of cholesterol from the outer half to the inner half of the membrane occurred very slowly (half time in excess of 6 days). Therefore, it is likely that the increase in whole cell cholesterol, which I measured, is confined to the outer half of the sarcolemma.
**Effects of Cholesterol on Acetylcholine Receptor Channel Event Frequency.** Under control conditions, the rate of LC channel drop-out, in patches from cells treated with ChlF liposomes, is the same as that observed in untreated patches (see figure 11). In contrast, the extent of drop-out of LC channels is attenuated by previous treatment of myocytes with ChlR liposomes. This attenuation of drop-out is also seen during exposure to halothane. A similar analysis of HC channels shows that about a 30% drop-out (within 2-3 min) occurs under all conditions including exposure of patches to halothane. Despite these apparent trends, statistically significant differences were not found. Because of the large intrinsic variability in this parameter, no conclusions can be drawn, at present, concerning the underlying mechanism (s) responsible for this phenomenon. Suffice it to say that for reasons given above, I have eliminated the possibility that the drop-out phenomenon is due to membrane deterioration and consider it unlikely that receptor desensitization can fully account for it. Therefore, it is still possible that migration of receptors to the periphery of the patch and membrane distortion, by formation of the patch, remain as the most likely contributing causes to this behavior.

Previously, I reported (see Chapter 2) that, in agreement with Brehm et al. (1984), the LC channel drop-out rate in untreated cells, is faster than the HC channel drop-out rate. This difference disappeared on exposure to halothane (see figure 11) and I now show that other perturbations of the cell membrane (increased or decreased cholesterol) are also associated with a loss of distinction between the LC and HC channel drop-out behavior. It can be concluded that any
alterations in membrane structure interfere, perhaps, with the environment surrounding the two channel types. Such disturbances in structure may tend to reduce the apparent difference in channel type behavior with regard to the drop-out phenomenon.

**Effects of Cholesterol on Acetylcholine Receptor Channel Properties.** Increases in membrane cholesterol may affect AChR channel properties during control periods. For example, the maintenance of ion permeability in reconstituted AChRs has been shown to require the presence of cholesterol (Dalziel et al. 1980; Criado et al. 1982). Dalziel et al. (1980) found that 25-50 mole% cholesterol is required to produce maximum agonist-induced ion flux in AChR-enriched membranes reconstituted from Torpedo. It is therefore possible that the increase in cholesterol, observed in my data (from 0.16 in untreated cells, to 0.57 in cholesterol-enriched cells; see table 3), may have affected the single channel properties of AChRs in muscle cells. Specifically, changes in cholesterol may affect the affinity of ACh for the AChR, the opentime of AChR channels or their conductance. Dalziel et al. (1980) found that when the cholesterol levels were increased, in AChR preparations reconstituted in lipid bilayers, no changes in the affinity of ACh for the AChR occurred. Therefore, it is unlikely that increases in membrane cholesterol produced a change in the affinity of ACh for its receptor in the muscle cell membranes investigated here. Similarly, increases in membrane cholesterol produced no significant effects on the mean control burst durations and amplitudes of AChR channels (see table 4).
Effects of Liposome Treatment on Halothane Action. In contrast to the inability of halothane to affect single channel conductance in untreated cells, halothane slightly decreased the conductance of HC channels in both ChlR and ChlF treated muscle cells. In the presence of 4% halothane, the conductance of HC channels was reduced by about 5% in ChlR treated cells and by about 3% in ChlF treated cells. Halothane exposure did not affect the conductance of LC channels at any of the tested concentrations. Since a decrease in HC channel conductance is observed for both types of liposome treatment, the presence of cholesterol cannot account for this effect. However, it is possible that the HC channel is more susceptible to the fusion and/or adhesion of liposomes.

In general, my data show that incubation of Xenopus myocytes with ChlR liposomes antagonizes the halothane-induced reduction in burst duration in both LC and HC channels (see figure 14). On the other hand, treatment with ChlF liposomes appears to augment the halothane-induced reduction of burst durations. In addition to the direct interference of cholesterol with the action of halothane (see discussion below; Viability of Current Lipid and Protein Theories of Anesthetic Action), other factors may have affected the efficacy of halothane. For example, fusion and/or adhesion of liposomes to muscle cells may have interfered with the mechanism of halothane action. However, the extent of liposome fusion and/or adhesion to myocytes may be assumed to be the same regardless of the presence of cholesterol in these liposomes (see table 3). Consequently, the effects of liposome fusion and/or adhesion, per-se, should be similar. Since the effects
of liposome treatment were not found to be the same, however, the presence of cholesterol appears to be responsible for the difference in halothane action in ChlR- and ChlF-treated muscle cells.

Another possibility is that the cholesterol-induced antagonism of halothane action may have been due to a decrease in the solubility of halothane in the altered membranes. Cholesterol has been shown to decrease the partitioning coefficient of halothane in liposome preparations (Simon et al. 1979; Smith et al. 1981). A decrease in lipid solubility would result in the presence of fewer anesthetic molecules at the site of action, and consequently, in a reduced efficacy of halothane effect. The cholesterol levels, which I found in ChlR- and ChlF-treated muscles cells, were 36 and 9 mole%, respectively (see table 3). Using these data, I estimated that an equivalent change in cholesterol levels in lipid bilayers, reported by Simon et al. (1979), could decrease the halothane partition coefficient by a factor of less than 2, at most. However, the data reported here show that four times as much halothane is needed to shorten the AChR burst duration in ChlR-treated cells when compared to the burst duration reduction at 1% halothane in ChlF-treated cells. Therefore, a decrease in the partitioning coefficient of halothane would account for less than 50%, at the worst, of the observed effects of cholesterol on halothane action. Further, fusion and/or adhesion of liposomes to myocytes cannot account for the effects of cholesterol on halothane action. Therefore, antagonism of halothane action by increases in membrane cholesterol and augmentation of halothane action by its decrease may be concluded to result from direct interference in the
mechanism of action of halothane. Consequently, these data may be used to test the viability of various protein and lipid theories of anesthetic action (see discussion below; Viability of Lipid and Protein Theories of Anesthetic Action).

Viability of Current Lipid and Protein Theories of Anesthetic Action

Expansion in Membrane Thickness Theory. GAs have been shown to increase membrane thickness and this effect has been postulated to interfere with the normal function of proteins critical to the processes of membrane excitability (Ashcroft et al. 1977). Haydon et al. (1977) have shown that n-alkanes increase lipid bilayer thickness to a much larger extent when the cholesterol levels are increased from 0 to 29 mole%. Further, they demonstrated that the ability of n-alkanes to block impulse propagation was directly correlated with their ability to increase membrane thickness in cholesterol-enriched lipid bilayers. Therefore, this theory predicts that increasing the cholesterol content in membranes will augment the ability of GAs to increase membrane thickness. The data I present here are in direct contradiction to this prediction. Specifically, I found that increases in membrane cholesterol antagonize the action of halothane on the AChR channel burst duration.

Moreover, the ability of GAs to increase membrane thickness does not appear to be a general phenomenon. For example, Franks and Lieb (1981) have shown that halothane does not increase membrane thickness in cholesterol-free lipid bilayers or in cholesterol-rich red blood cell membranes. The results reported here emphasize the fact
that an increase in membrane thickness cannot alone account for GA action.

**Lateral Phase Separation Theory.** GAs have been proposed to act by partitioning into membrane lipids thereby reducing their lateral compressibility. This effect could compromise the ability of intrinsic membrane proteins to undergo conformational changes (Trudell, 1977). Since membrane cholesterol depresses gel-phase lipids, an increase in cholesterol content would increase lateral compressibility and consequently, may antagonize the action of halothane on the burst duration of AChR channel. However, increases in lateral compressibility should also affect the burst durations of AChR channels, per-se. Since I found no effect of increased membrane cholesterol on AChR burst durations during control periods, it is unlikely that changes in lateral compressibility contribute significantly to the postulated mechanism of GA action.

**Lipid Annulus Phase Transition Theory.** GAs have also been proposed to act by disordering the lipid annuli of proteins thereby interfering with normal protein function (Lee, 1976; Lenaz et al. 1977). Spin-labeled studies on AChR membrane fragments from Torpedo support the existence of an immobilized lipid component (the lipid annulus; see Chapter 1, Lateral Phase Transition Theory) surrounding the AChR (Bienvenue et al. 1977; March and Barrantes, 1978; Marsh et al. 1981). In addition, Andreasen and McNamee (1980) have shown that increases in fatty acid unsaturation inhibit ion permeability of reconstituted AChRs. They argued that increased levels of unsaturated fatty acids may disorder the immobilized lipid annulus of the AChR
thereby interfering with its function. One possible explanation for the halothane-induced reduction in AChR channel burst duration is that halothane may disorder the AChR lipid annulus. The lipid annulus phase separation theory predicts that GAs will be inactive when they are unable to disorder the lipid annulus. My data show that increased membrane cholesterol antagonizes the action of halothane on the AChR burst duration. Since cholesterol has been shown to order membrane lipids (Shinitzky and Henkart, 1978; Cullis and DeKruijff, 1978), cholesterol is expected to interfere with the ability of halothane to disorder the AChR lipid annulus thereby antagonizing the halothane-induced reduction in AChR channel burst duration. My data are therefore consistent with the lipid annulus phase transition theory.

In addition to cholesterol antagonism during halothane exposure, increases in membrane cholesterol may be expected to order the AChR lipid annulus during control periods. Consequently, it is expected that the burst duration of AChR channels would be increased when the lipid annuli are more ordered. Despite this expectation, I found that control burst durations in cholesterol-enriched membranes were not different from those in untreated cells (see table 4). It is of interest to note here that Andreasen and McNamee (1980) found that increases in saturated fatty acids (which also order membrane lipids; Shinitsky and Henkart, 1978) had little effect on ion permeability of reconstituted AChRs. Therefore, the inability of cholesterol to alter control AChR burst durations is consistent with the findings of Andreasen and McNamee (ibid).
Membrane Fluidity Theory. GAs have also been postulated to act by fluidizing the fluid-phase lipids thereby impairing or inactivating neuronal proteins which are critical for synaptic transmission (Metcalfe et al. 1968; Trudell et al. 1973). Gage and Hamill (1975) suggested that fluidization of the membrane, in which the AChR channel is embedded, may permit faster relaxation of the open channel to the closed state. Since cholesterol has been shown to order membrane lipids, increases in membrane cholesterol are predicted to antagonize the action of halothane on AChR burst duration. Consistent with this prediction, my data show that increases in membrane cholesterol antagonize the halothane-induced reduction in AChR burst duration. However, this theory also predicts that cholesterol, in and of itself, should affect AChR burst durations. As discussed above, increases in membrane cholesterol order membrane lipids. Consequently, I would expect that a decrease in membrane fluidity would produce a prolongation of the burst duration of AChR channels. Since I observed no changes in AChR burst duration in the presence of increased cholesterol levels, it is unlikely that membrane fluidity changes can account for changes in the burst durations of AChR channels during exposure to halothane. Thus, my data do not support the membrane fluidity theory.

The Protein Conformational Change Theory. GAs have been proposed to interact directly with membrane proteins which are critical for synaptic transmission (Eyring et al. 1973; Woodbury et al. 1975; Franks and Lieb, 1982). In order for this mechanism of GA action to be viable for AChR channels, this theory must account for the cholesterol-
induced antagonism of halothane action on the AChR channel burst duration. Increases in membrane cholesterol have been shown to affect the partition coefficients of GAs (Simon et al. 1979; see discussion above). However, the effect on partition coefficients appears to be too small to account for the halothane effects observed in this studied (see discussion above). Miller (1984) proposed that cholesterol may displace halothane molecules from its site of action on membrane proteins. This hypothesis predicts that increases in membrane cholesterol will antagonize the action of GAs by removing GAs from the site of action. My data do not rule out this possibility; however, a mechanism by which increased cholesterol levels can prevent halothane binding to proteins must first be more clearly defined.

In summary, the data presented here are most consistent with the lipid annulus phase transition theory but also demonstrate that other theories of GA action cannot be ruled out. It may be impossible, in fact, to construct a unitary theory of anesthetic action. This may be all-the-more true given the large diversity in the chemical structures of general anesthetics and the still to be clearly defined targets of these agents in the central nervous system.
APPENDIX A

GLOSSARY OF TERMS AND TECHNIQUES

Analgesia. A state of being in which noxious stimuli are perceived but not interpreted as pain. This state is usually accompanied by sedation without loss of consciousness.

Receptor Desensitization. A process in which receptors may undergo a reversible conformational change from the active to an inactive state. The process is observed as a loss of receptor sensitivity to a ligand.

Electron Spin Resonance. An electron possesses spin angular momentum and consequently, a spin magnetic moment. The spin magnetic moment can take two directions with respect to an applied magnetic field. The two spin orientations are separated by a discrete energy level. An unpaired electron can oscillate between these two orientations when the energy of an applied magnetic field matches the energy difference between the two spin magnetic moments. This condition is referred to as electron spin resonance. The energy spectra at which resonance occurs provides information on the motion of the spin label (the molecule that carries the unpaired electron) and on the environmental polarity of the spin label. These spectra are quantified in terms of an order parameter. An order parameter near one is interpreted as a rigid environment in which the probe (spin-label) motion is very restricted. An order
parameter of zero suggests that the probe movement is isotropic and indicates a very fluid environment.

Fluorescence Spectroscopy. The use of fluorescent probes to investigate the physical state of bimembranes. Fluorescence polarization and intensity of the membrane probe is measured with a spectrophotofluorimeter. The fluorescent probe is excited with polarized light. The polarization of the fluorescent emission gives information on the mobility of the probe within its membrane environment. In a more fluid environment, the excited fluorescent probe is able to rotate to a greater degree than in a more rigid environment. This randomizes light emission and reduces the fluorescent polarization. Conversely, the fluorescent probe rotates less in a rigid environment before emission therefore maintaining, to a greater extent, fluorescent polarization.

General Anesthesia. A state of being in which consciousness and the sensation of pain are abolished while pulmonary ventilation and cardiac function are maintained. Mechanisms of action of general anesthetics are unknown, but many theories have been proposed.

Iontophoresis. A technique which allows very accurate delivery of drugs by utilizing the ionic charge of the drugs. The drug is injected through a micropipet by passing pulses of current.

Local Anesthesia. A state of being in which the perception of pain is locally abolished by axonal conduction block (primarily by blocking inward current flow of the action potential) without
affecting the individual's state of consciousness.

**Noise Analysis.** Application of agonist to an excitable membrane (e.g., acetylcholine applied to the postsynaptic membrane of muscle) results in membrane current fluctuations (noise). The current fluctuations are presumed to reflect random variation in the number of open ion channels. If one assumes the underlying unitary events (single channel openings) are square pulses (i.e., either open or closed) of variable duration and that the channels open independently, then the amplitude of the unitary event can be derived from the variance of current fluctuations about the mean of the total agonist-induced conductance. The mean open time of the unitary events can be derived from analysis of the frequency (by means of Fourier analysis) of the current fluctuations.

**Nuclear Magnetic Resonance.** Analogous to electrons, nuclei may also possess two spin magnetic moments in an applied magnetic field. Similarly, when the energy of an applied magnetic field matches the energy difference between the two spin orientations, the nucleus will resonate between the two spin magnetic moments. However, the nuclear magnetic moments are much weaker than electron magnetic moments due to the larger mass of nuclei. Consequently, the energies (radio frequencies) at which nuclear magnetic resonance (NMR) occur are much lower than electron magnetic resonance. NMR spectra give information about the motion and chemical environment of the nuclear probe. A narrow line width indicates an unrestrictive environment for
Neutron Diffraction. In this technique, neutrons are passed through a crystalline sample. Since neutrons have wavelike properties, they produce a diffraction pattern after passing through the crystal. Neutron diffraction provides information on the nuclear dimensions within molecular structure.

Rat Blood Pressure. A technique in which the concentration of acetylcholine is determined by injecting samples (containing acetylcholine) into the rat and monitoring rat blood pressure. The amount of acetylcholine is quantified by running a standard curve of rat blood pressures against known concentrations of acetylcholine.

Spin-echo Fourier-Transform Nuclear Magnetic Resonance. With this technique, nuclear magnetic spectra can be analyzed in terms of signal frequency, chemical shift, line width and line frequency. The latter parameters are controled by the decay times $T_1$ and $T_2$ which indicate the lengths of time taken for an individual nucleus to dissapate its spin energy to another similar nucleus ($T_2$) and to the molecular lattice ($T_1$). The spin-echo method is particularly sensitive to these relaxation parameters (derived from line width and frequency) such that small changes in protein conformation can be detected that would not otherwise have been observed with changes in the chemical shift.

X-ray Diffraction Crystallography. In this technique, a beam of X-rays is passed through a crystal. Based on the diffraction pattern
of the scattered X-rays, molecular distances can be measured in the original crystal. X-rays are scattered more by electron-rich heavy atoms. Consequently, the technique provides more information on the atomic dimensions of molecular structure.
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