

Isoflurane Causes "Flickering" of the Acetylcholine Receptor Channel: Observations Using the Patch Clamp

Roger S. Brett, M.D., Ph.D.,* James P. Dilger, Ph.D.,* Kathrynne F. Yland, B.A.†

The authors investigated the effects of a volatile anesthetic (isoflurane) on the functional properties of a well characterized membrane ion channel (the acetylcholine receptor channel), using the patch clamp electrophysiological method together with local microperfusion of isoflurane. They found that isoflurane causes single acetylcholine receptor channels to "flicker" rapidly between open and closed states, reminiscent of the kinetic pattern induced by local anesthetics. The mean amplitude of currents flowing through open channels was unchanged. Although these observations initially suggested that isoflurane blocks open ion channels, closer analysis of the "isoflurane pattern" revealed features inconsistent with classical open channel block: the duration of bursts of channel openings is shortened, and that of the brief closures within bursts lengthens as the concentration of isoflurane is increased. The authors suggest that isoflurane exerts its characteristic effects on the kinetic properties of acetylcholine receptor channel by an allosteric mechanism. In addition, and apart from their mechanistic significance, these effects may underlie the known potentiation by isoflurane of curariform neuromuscular blockade. (Key words: Acetylcholine: receptor channels. Anesthetics, volatile: isoflurane. Mechanisms of anesthesia. Receptors: acetylcholine.)

This article is accompanied by an editorial. Please see: Strichartz GR: Through the eye of the channel. ANESTHESIOLOGY 69:155-156, 1988.

THE MECHANISM‡ of general anesthesia is unknown. Current theories favored lipid¹⁻³ or protein⁴⁻⁶ sites of anesthetic action, and, at both of these sites, a variety of physical processes have been proposed. Regardless of the exact nature of the site and action of anesthetic

* Assistant Professor, Department of Anesthesiology and Department of Physiology and Biophysics.

† Technician, Department of Anesthesiology.

Received from the School of Medicine, State University of New York at Stony Brook, Stony Brook, New York. Accepted for publication March 1, 1988. Dr. Brett is a recipient of the B.B. Sankey Anesthesia Advancement Award (I.A.R.S.) and a Starter Grant from the American Society of Anesthesiologists, Committee on Research. Dr. Dilger is supported by grant NS21581 from the National Institutes of Health. Preliminary account presented at the Annual Meeting of the International Anesthesia Research Society, March 14-18, 1987, Lake Buena Vista, Florida.

Address reprint requests to Dr. Brett: Department of Anesthesiology, School of Medicine, SUNY at Stony Brook, Stony Brook, New York 11794-8480.

‡ For convenience, we use the term "mechanism" in the singular, recognizing that, in fact, a multiplicity of mechanisms are likely to contribute to the anesthetic state.⁷

drugs, it is clear that the anesthetic state must necessarily be associated with a perturbation of normal signaling processes within the central nervous system; processes which, in turn, are mediated by the concerted activity of ion channels. Unfortunately, an important obstacle to further progress in understanding the phenomenon of anesthesia arises from our limited knowledge of these central neuronal channels, which now appear to exist in a much greater variety of functional types than previously suspected.⁸ By contrast, the descriptions of several more familiar ion channels of the peripheral nervous system, including the acetylcholine receptor channel and the voltage-dependent sodium channel, have recently been dramatically improved.⁹⁻¹⁵ At present, it seems reasonable to proceed with the investigation of anesthetic mechanisms by undertaking detailed examinations of the effects of specific anesthetics on ion channels whose structural and functional properties are already well described.

We are pursuing this approach using the recently introduced "patch-clamp" technique¹⁶⁻¹⁸ to record the minute electrical currents that flow through single acetylcholine receptor channels. Here, we report that isoflurane alters the kinetic properties of these channels, giving rise to clear-cut "bursts" of many brief openings; whereas the channels normally open and close in a much simpler pattern, consisting of one or two long openings followed by a long closure (the meaning of the terms "brief" and "long" in the present context will be quantitatively defined below). In the presence of isoflurane, the average duration of these bursts of channel activity is shorter than under control conditions (despite a larger number of openings per burst), and the brief closures within a burst are longer. The unitary conductance of the channel is not altered. We have previously reported a portion of these findings in abstract form;¹⁹ we now present a fuller account, and briefly discuss their mechanistic significance.

We have chosen to study the acetylcholine receptor channel for two reasons. First, we did so because it is a channel whose chemical structure and biophysical properties are known in exquisite (although still incomplete) detail.^{9,11-15} This means that the effects of anesthetics on its activity can be interpreted in the context of an already mature body of information. Second, it is the prototype of the class of neurotransmitter-gated channels, which are generally believed to be more sensitive

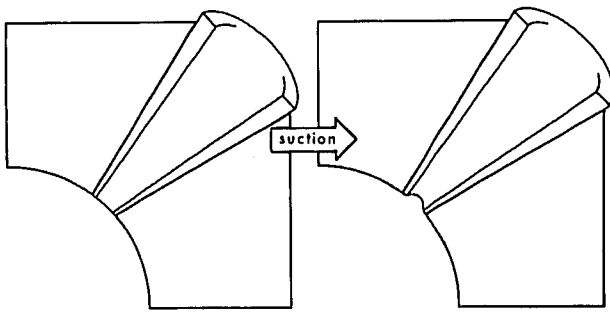


FIG. 1. A schematic illustration of the formation of a high resistance seal in a patch clamp experiment. In this lateral cross-sectional view, the surface of the cell and the tip of a patch micropipette are shown. At left, the pipette has been positioned (using a micromanipulator, not shown) so that it just touches the surface of the cell membrane. After application of gentle suction to the interior of the pipette (right), a small patch of membrane is drawn into the pipette.

to perturbation by general anesthetics than the voltage-gated channels.^{20,21}

The electrophysiological method used here (the "patch clamp") requires introductory comment, as it is likely to be unfamiliar to much of the present readership. For many decades (since, in fact, the dawn of the "receptor" concept²²), physiologists have sought to understand how the binding of a neurotransmitter (such as acetylcholine) to its receptor is transduced into an electrical signal. Among the most fundamental questions were the following: What is the magnitude of the unitary signal that arises from each channel opening event? How long does it last? How many such events make up the "macroscopic" signal, that is, the endplate current? Finally (and perhaps, from the clinical viewpoint, most importantly), how do drugs affect this transduction process?

Approximate answers to these questions were first given in the early 1970s, when several groups carried out spectral analyses of the extra electrical "noise" seen when acetylcholine was present at the surface of endplate membranes.²³⁻²⁶ They estimated that individual acetylcholine receptor channels had an electrical conductance in the range of tens of picosiemens (one picosiemens = 10^{-12} Ohm⁻¹), and that, on average, the acetylcholine-bound channels opened for a duration of several milliseconds. These measurements, elegant as they were in concept and execution, were necessarily indirect, and relied on acceptance of certain reasonable (but unverifiable) assumptions. A direct method of observing ion channel activity was needed.

Such a method—called the "patch clamp" (because the voltage across a tiny patch of membrane is "clamped" to values chosen by the investigator)—was provided in rough form by Neher and Sakmann in 1976,¹⁶ and has more recently (1981) been dramatically

improved by the same workers and their colleagues.¹⁷ In brief, the technique involves lowering the heat-polished tip of a glass micropipette gently onto the surface of a cell membrane, and applying light suction to the micropipette interior, as illustrated schematically in figure 1. With luck, the membrane patch covered by the micropipette tip will be pulled slightly up into the aperture, and, for reasons not yet fully understood, a tight seal will often be formed between the membrane bilayer and the glass micropipette tip. Under optimal circumstances, the electrical resistance of such a seal may reach 100 gigaohms (one gigaohm = 10^9 Ohms) or more. It is this very high resistance, in parallel with the greatly reduced capacitance of a membrane patch having an area of only a few square micrometers, that allows resolution of the microscopic ionic currents (their magnitudes range from tenths to tens of picoamperes, depending on channel type) that intermittently flow through any channels that happen to have been trapped in the membrane patch. Many important technical variations on this basic method have now been described, but these are beyond the scope of the present study. The essential point, for our purposes, is that the behavior of the "trapped" acetylcholine receptor channels can be examined either under control conditions (that is, in the presence of acetylcholine only) or in the presence of an anesthetic.

In general, there are two rather different experimental approaches to the problem of comparing the normal behavior of membrane channels with their behavior in the presence of a drug. One approach is to collect data from a control group of membrane patches, and compare them with data from another group of patches exposed to the drug. The other is to collect control data from a patch, then expose the same patch to the drug and collect more data. Both approaches have drawbacks, which become particularly vexing in the case of volatile agents. In earlier experiments,¹⁹ we chose the first approach, hoping to achieve precise control of the isoflurane concentration by including the anesthetic in our recording micropipettes. Although we succeeded in observing the effects we now recognize as characteristic of isoflurane's action,¹⁹ the method proved cumbersome because of patch-to-patch variability. Moreover, because of diffusion, we remained uncertain that the actual drug concentration within the micropipette tip was well controlled during the course of a typical experiment. We, therefore, abandoned the requirement for precise control of concentration and, using individual membrane patches, developed a more reliable method of observing the onset of, and recovery from, isoflurane's characteristic effect. The method of local microperfusion, described below, achieves this.

Materials and Methods

Clonal BC3H-1 cells were cultured using previously described methods.²⁷ These cells, originally derived from a murine intracranial tumor,²⁸ were chosen for study because the nicotinic acetylcholine receptors they express are similar to those found in skeletal muscle cells and have been extensively characterized from pharmacological,²⁷ biochemical,²⁹ and metabolic³⁰ standpoints. The results shown here were obtained during study of cells that had been maintained for 2-4 days in medium containing 0.5% fetal calf serum to inhibit cell division and promote differentiation. For electrophysiological recording, culture medium was replaced with an extracellular solution at room temperature (21.5° C.) containing 150 mM NaCl, 5.6 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, and 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer and the culture dish mounted on the stage of an inverted microscope (fig. 2) equipped with a Hoffman differential interference optical system and supported on an air table for isolation from environmental vibration. Patch (recording) micropipettes were fabricated from borosilicate glass using a two-stage vertical pull, heat-polished under microscopic visualization, coated near the tip with an acrylic paint, and filled with a solution containing 140 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, and either 250 or 500 nM acetylcholine chloride. Both extracellular and micropipette solutions were adjusted with NaOH or KOH to a pH of 7.3. Micropipette resistances ranged between 2 and 5 megaohms.

Pipettes for local microperfusion of cells with isoflurane solutions were fabricated using the method described above, omitting the heat-polishing step. These pipettes were filled with an extracellular solution having the same composition as the bath solution, but also containing 0.45, 2, or 5 mM isoflurane (equivalent to vapor concentrations of 0.9, 3.9, and 9.7 volumes percent, respectively). These solutions were prepared by dilution of saturated (15 mM)⁶⁶ isoflurane solutions using glass syringes.

The recording and perfusion micropipettes were then mounted on separate micromanipulators and moved into a position several micrometers above the cell membrane, while applying gentle suction to the perfusion micropipette to avoid leakage of isoflurane prior to collection of control data. The recording (patch) micropipette was then lowered onto the membrane surface and suction applied to the pipette interior to establish a high-resistance (30-60 gigaohms) seal. If this step was successful, the position of the perfusion micropipette was again adjusted, while maintaining

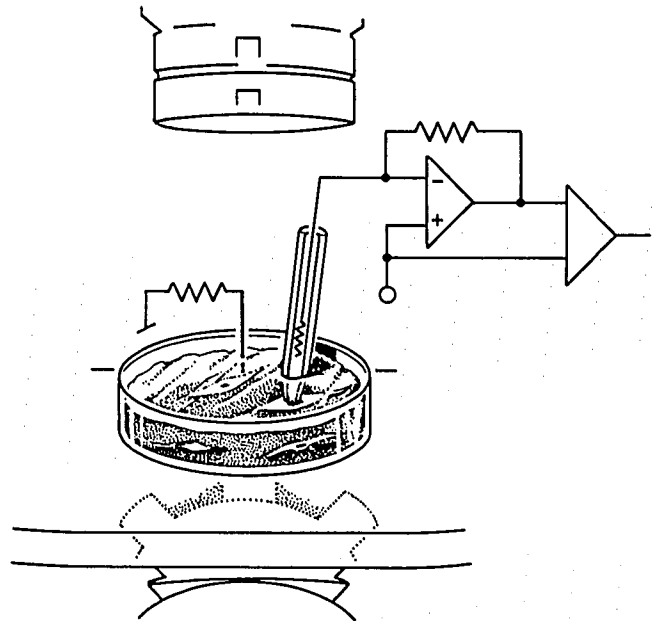


FIG. 2. A diagram of the major components of the patch clamp experiment. Cells, grown in a plastic tissue culture dish, are viewed through an inverted microscope. The patch micropipette (mounted on a micromanipulator, not shown) has been sealed onto one of the cells as illustrated in figure 1. A silver chloride wire is used to provide electrical continuity between the saline solution inside the pipette and a current-voltage converter/amplifier.

gentle suction, to point directly toward the area surrounding the isolated membrane patch, as shown in figure 3. The patch micropipette voltage was set to 70 mV relative to extracellular ground (thus hyperpolarizing the patch from the resting cell membrane potential) and

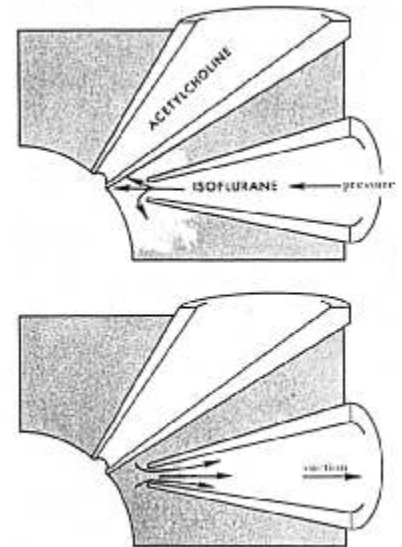


FIG. 3. The local microperfusion technique of applying and removing isoflurane. A patch of membrane containing acetylcholine receptor channels has been isolated by the upper pipette, which contains acetylcholine to activate the channels. The lower pipette contains a solution of isoflurane in saline.

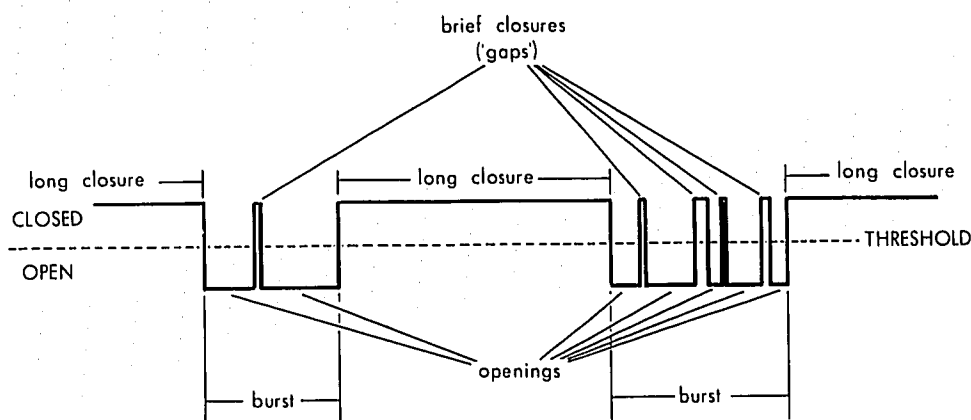


FIG. 4. Schematic representation of acetylcholine receptor channel currents, illustrating the kinetic nomenclature adopted here. On the left, a burst of two openings separated by one brief closure is shown. On the right, a burst of five openings separated by four brief closures is shown. The two bursts are separated by a long closure. Although not illustrated here, a burst may consist of a single opening.

several hundred unitary acetylcholine receptor channel currents were recorded. The membrane area surrounding the patch was then immediately perfused with isoflurane solution by applying pressure to the microperfusion pipette interior (fig. 3), and channel currents were again recorded. Finally, to remove isoflurane from the bath solution in the vicinity of the membrane patch, suction was again applied to the microperfusion pipette until recovery from the drug effect became visually apparent (usually 5–30 s), and a final recording of channel currents obtained. In some experiments, this cycle was repeated several times.

Membrane currents were amplified using a List EPC-7 current-voltage converter headstage and amplifier (List Electronics, Darmstadt, FRG), digitized on line using a Concept 2000 analog interface (Concept 2000, Burlington, Vermont) and stored by a minicomputer (model PDP-11/73, Digital Equipment Corporation, Maynard, MA) on a large-volume Winchester disc drive. The current signal was filtered at 3000 Hz and sampled at 50 μ s per point. Subsequent analysis consisted of measurement of the amplitudes and durations of single channel currents, compilation of these data into histograms, and fitting of the histograms using standard Gaussian and exponential functions. The measurement step was facilitated by a computer program that searched for and displayed on a high-resolution graphics monitor all transitions across a threshold (here, one-half the mean current amplitude; cf. fig. 4) determined by the operator. The displayed event, if accepted by the operator, was then measured and stored as a list of three numbers (position, amplitude, duration) for further analysis. For each experimental condition, the mean single channel current amplitude was obtained from the midpoint of a Gaussian function fitted to the distribution of amplitudes. The significance of differences between amplitude distributions was tested using one-way analysis of variance. The mean event durations were obtained from the time constants of single (open

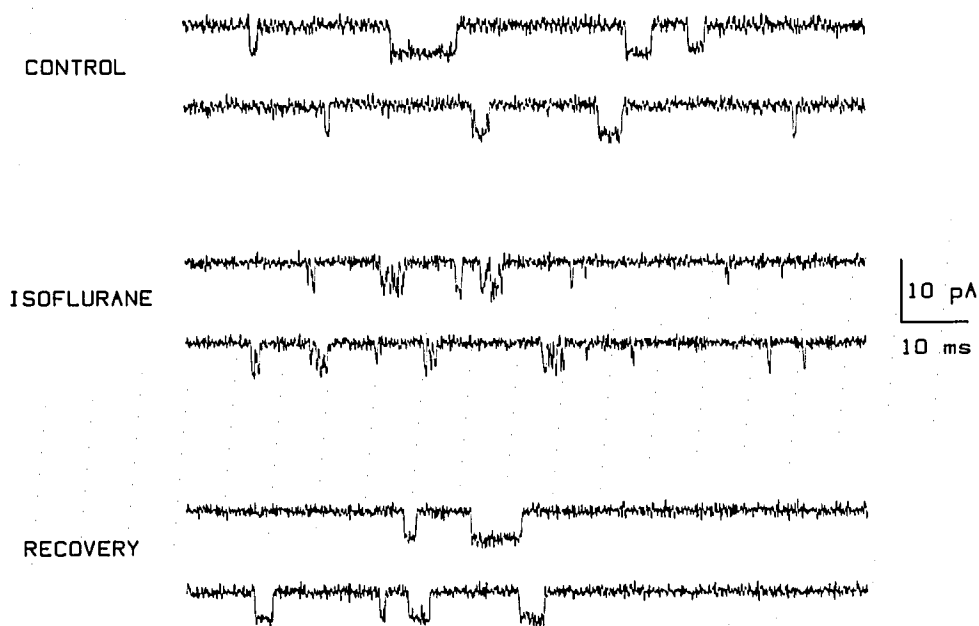
durations and burst durations) or double (closed durations) exponential functions fitted to the distributions of event durations using the method of maximum likelihood.³¹

Several historical ambiguities exist in the nomenclature used by various investigators to identify ion channel events for statistical analysis. The most important of these concerns the distinction between the "open duration" (or "open time") and the "burst duration" (or "burst time"). Confusion arose because channel events which appeared to be single openings using early recording methods subsequently proved, at higher temporal resolution, to have a complex structure of "brief closures" or "gaps,"³² as shown schematically in figure 4. Some investigators have, therefore, abandoned the term "open duration" (in recognition of the fact that some of the brief closures may always elude detection) and adopted the term "apparent open duration" ("apparent open time") for the time between a downward threshold crossing (channel opening) and the next upward threshold crossing (channel closure) at a given bandwidth. For brevity, we will retain the simpler term "open duration," bearing in mind the implications of a finite bandwidth. The term "burst duration," on the other hand, remains unambiguous, as long as a criterion time for discriminating between the brief gaps within bursts and the longer closures between bursts is stated. We define the "burst duration" as the time between a channel opening following a closed time longer than t_{crit} and the next channel closure to a closed time longer than t_{crit} , where t_{crit} is a criterion time obtained from the fitted distribution of all closed durations.³²

Results

The kinetic properties of acetylcholine receptor channels within membrane patches were rapidly and reversibly altered by local microperfusion of the surrounding membrane area with isoflurane solutions. Fig-

FIG. 5. Examples of unitary acetylcholine receptor channel currents before, during, and after local microperfusion with 5 mM isoflurane, illustrating the characteristic features (see text) of isoflurane's action. Channel openings are represented by downward deflections of the current trace. When isoflurane is removed (bottom panel), the channels are indistinguishable from those observed under control conditions.



ure 5 illustrates the characteristic features of this effect: openings occur more frequently, are briefer, and are more obviously grouped into "bursts." Using 2 or 5 mM isoflurane, we found that this pattern was always qualitatively evident within several tens of seconds after starting the perfusion, and dissipated at a similar rate after stopping it; we did not, however, make systematic measurements of on- and offset rates. Recovery of the native kinetic properties of the channels after removal of isoflurane was usually complete (fig. 5, lower panel; table 1). Although the basic "isoflurane pattern" observed during local microperfusion with the two higher concentrations was easily recognizable on line, subsequent analysis revealed additional subtle features, which could also be discerned in records obtained during perfusion with a much lower concentration (0.45 mM) of isoflurane.

An example of the analysis of 1185 channel events recorded from a single membrane patch before and during local microperfusion with 5 mM isoflurane is given in figure 6. As expected, the distribution of closed durations (figs. 6a, b) could not be adequately fitted to a single exponential function, but was satisfactorily approximated by the sum of two exponential components. The faster component, corresponding to the "flickering" closures within a burst, had a time constant in the range of 50–250 microseconds. Figure 6a shows that isoflurane increased both the average duration and frequency of these brief closures. The slower component (fig. 6b) had a time constant in the range of tens of milliseconds, reflecting the intervals between bursts, and is presumably determined by the acetylcholine concentration and the (unknown) number of receptor channels in the patch. These intervals became shorter in

TABLE 1. Summary of the Effects of Isoflurane on the Properties of Acetylcholine Receptor Channels

	Control	Isoflurane			Recovery
		0.45	2	5	
Perfused isoflurane concentration (mM)	0	0.45	2	5	0*
Mean duration of brief closures (ms)	0.04	0.08	0.09	0.24	0.05
Mean open duration (ms)	2.9	1.6	1.6	0.34	3.0
Mean burst duration (ms)	3.9	2.6	2.3	1.0	3.6
Brief closures (within bursts) per ms (%)	100	191	1130	1560	135
Mean duration of long closures (%)	100	77	48	37	134
Opening frequency (%)	100	143	268	386	86
Single channel current (%)	100	96	90	88	92
Number of experiments	5	2	1	2	4

The indicated concentration of isoflurane was present in the perfusion micropipette. The asterisk following "0" in the Recovery column indicates a nominal zero (see methods). The designation "(%)" indicates values expressed as percent of control. Each experiment was

performed on a separate patch and each patch was obtained from a cell in a new culture dish. The single channel properties were obtained from records containing at least 200 channel events.

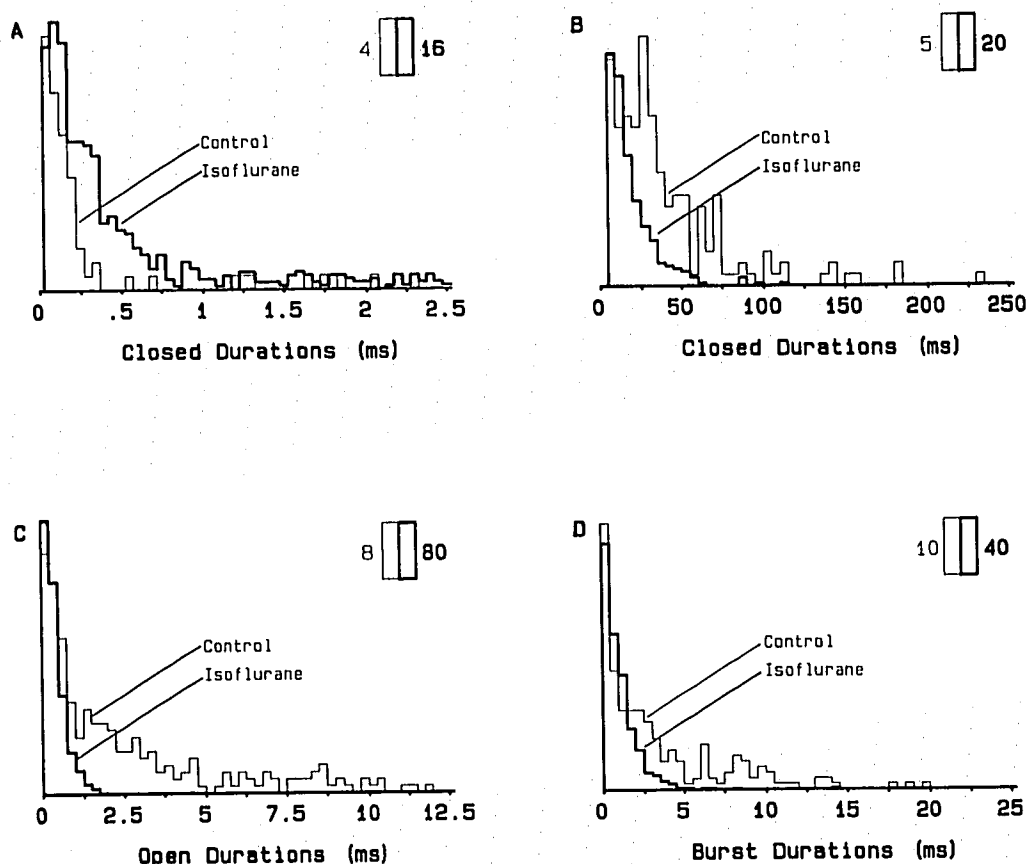


FIG. 6. Event duration histograms generated from a local microperfusion experiment using 5 mM isoflurane. Control conditions (before application of isoflurane) are illustrated with thin lines; the heavy lines show the distribution of events during perfusion with isoflurane. The ordinate represents the number of events having durations within the range indicated by the abscissa. Calibration bars for the ordinate are shown in the upper right hand corner of each panel (left number for control, right for isoflurane). A. The fast component of the distribution of closed durations (bin width = 50 μ s). B. The slow component of the distribution of closed durations (bin width = 5 ms; first bin omitted). C. The distribution of open durations (bin width = 250 μ s). D. The distribution of burst durations (bin width = 500 μ s).

the presence of isoflurane (cf. table 1), indicating that channels opened more frequently.

In the example shown, the average open duration (fig. 6c) was shortened about nine-fold in the presence of isoflurane. The average burst duration (fig. 6d) was also shortened, but to a smaller extent. The net result of these kinetic shifts is to produce a characteristic "flick-

ering" appearance. A quantitative measure of this effect is obtained by dividing the number of brief closures per burst by the mean burst duration (see table 1, brief closures (within bursts) per ms).

The electrical conductance of the acetylcholine receptor channel, unlike its kinetic properties, appears unaffected by isoflurane. Figure 7 shows the distribution of single channel current amplitudes before and during local microperfusion with 5 mM isoflurane. The small difference between the two distributions is statistically insignificant. Although we cannot exclude the possibility of a real but quantitatively minor effect of isoflurane on channel conductance, our data are most simply interpreted to mean that the anesthetic has no effect on this parameter.

Table 1 summarizes the effects of isoflurane solutions on the distribution of closed, open, and burst durations, as well as the single-channel current amplitudes and the opening frequency of acetylcholine receptor channel events recorded from five exemplary membrane patches. The mean event durations for each patch were obtained using the analytical procedures illustrated in figure 6. Despite the fact that precise control of isoflurane concentration is not expected using the local microperfusion technique, an approximate concentration dependence is apparent in each of the measured kinetic

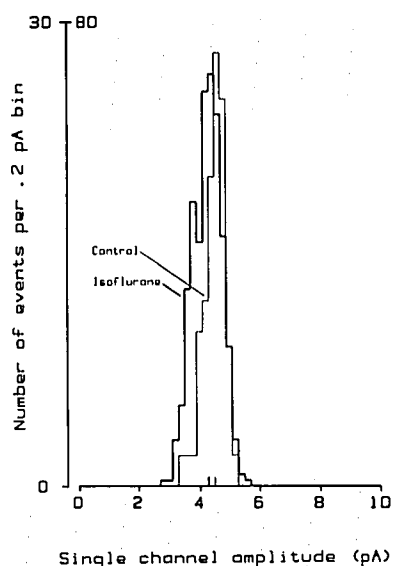


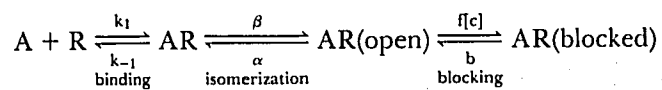
FIG. 7. Current amplitude histograms generated from an experiment using 5 mM isoflurane. Histograms representing both control (thin line) and isoflurane (heavy line) conditions are shown. The observed single channel amplitudes were accumulated in 0.2 pA bins. The mean (\pm standard deviation) single channel current was 4.5 ± 0.4 pA under control conditions and 4.3 ± 0.5 pA in the presence of isoflurane. The difference is statistically insignificant.

parameters. The right-hand column shows that each parameter returned toward its control value after withdrawal of isoflurane.

Discussion

Although more than a century has gone by since the discovery that certain gases and volatile liquids can reversibly abolish consciousness, we still know very little about their fundamental action on the nervous system. The "Meyer-Overton Rule," which calls attention to the close relationship between the potency of a general anesthetic and its lipid solubility,^{33,34} only underscores our ignorance: although it suggests strongly that the "anesthetic site" is hydrophobic in character, such a site could be present either in the bulk lipid of membrane bilayers¹⁻³ or, as more recently proposed,⁴⁻⁶ in hydrophobic domains within proteins. Let us examine our data from the point of view of one who wishes to discriminate between these opposing interpretations of the Meyer-Overton Rule. We will conclude that rigorous discrimination is not yet possible, but that the data tend to exclude at least two popular, but opposing, hypotheses. These are the open channel blocking hypothesis and the membrane fluidity hypothesis.

Perhaps the most striking feature of isoflurane's effect on the kinetic processes of the acetylcholine receptor channel is the "flickering" or "bursting" phenomenon seen in the presence of the drug. Qualitatively, this pattern resembles the effect of local anesthetics on the same channel,³⁵⁻³⁷ and has been widely recognized as evidence supporting the open channel blocking model proposed by Adams to explain the actions of barbiturate³⁸ and local³⁹ anesthetics on acetylcholine receptor channels. § Over the past decade, this model has provided a robust conceptual framework for many investigations of drug actions on acetylcholine receptor channels. In brief, the model appends an additional closed (blocked) state to the classical schema⁴⁵ for activation of the channel, as follows:



where A represents an agonist molecule, R a receptor, and [c] the concentration of the putative blocking agent. The rate constants k_1 , k_{-1} , β , α , f , and b define the proposed binding, isomerization, and blocking pro-

cesses shown above. ** According to the model, drug molecules interact with the channel only in its open, ion-conducting state, presumably (although not necessarily) by physically entering and plugging the conducting pathway. In contrast to an earlier proposal,⁵⁰ it is envisioned here that the blocking event results in complete, rather than partial, occlusion of the channel. If the model correctly describes the action of isoflurane, several qualitative †† predictions should be met. First, the open duration of the channel should be shortened in the presence of the drug. This is expected because the open channel can now close *via* either of two pathways: it can close either in the "normal" way, governed by the rate constant α (see model); or it can close by becoming blocked, according to the concentration-dependent rate constant f . Second, if the blocking events are brief compared to the normal open duration of the channel—but not too brief to be resolved, given the frequency limitations imposed by signal amplification and filtration—then channel openings should take on a "flickery" appearance due to brief interruptions of current flow by the putative blocking molecule. Thus, they should appear on the oscilloscope as "bursts" of many short openings, rather than single long openings that may occasionally be interrupted by a brief closure or two. Third, the single channel current amplitude should not be altered by the drug. As we have shown (figs. 5-7), each of these predictions is fulfilled.

Two additional predictions of the model, however, are not fulfilled. First, the duration of the bursts should be prolonged by increasing the concentration of isoflurane. This prediction arises from solution of the first-order rate equations generated by the model, and has been repeatedly derived by others.^{35,36,51} Although perhaps less intuitively obvious than the shortening of individual channel openings, the necessary lengthening of the burst duration may be appreciated by inspection of the model. Since the blocked channel cannot close directly, but must first become unblocked, passing back

** This relatively early model does not reflect the full complexity of acetylcholine receptor channel kinetic processes as revealed by recent patch-clamp studies. For example, experiments at low temperature have shown that as many as three open states of the acetylcholine receptor channel in BC3H-1 cells can be distinguished;⁴⁶ indeed, a higher resolution analysis of our own data suggests that, even at room temperature, more than one open state may be present. The existence of several "desensitized" (closed) states⁴⁷ and the fact that efficient channel opening requires the sequential binding of two acetylcholine molecules^{48,49} are similarly ignored by the model. These considerations do not, however, diminish its value as an initial test.

†† In fact, the model generates these same predictions (as well as others describing the expected voltage-dependence of the channel blocking process) in a quantitative form, but detailed tests of the fit to these predictions are beyond the scope of this report and will be presented elsewhere.

§ During roughly the same period, similar models were being introduced by Strichartz,⁴⁰ Courtney,⁴¹ and Hille^{42,43} to explain the actions of local anesthetics on axonal sodium channels. All of these arose, to a greater or lesser extent, by analogy to Armstrong's analysis⁴⁴ of potassium channel blockade by quaternary ammonium ions.

through the open state, blocking events will tend to postpone the closing isomerization by which the burst is terminated. Second, the duration of the blocking events themselves (the brief closures) should be independent of the drug concentration. Contrary to prediction, however, our data indicate that the bursts become shorter and the brief closures become longer as the concentration of isoflurane is increased (fig. 6; table 1). Hence, we must either abandon the effort to describe isoflurane's effect using an open channel blocking model, or superimpose additional mechanisms on the basic action described by the model. For example, it is possible that blocked channels may close directly (at a relatively slow rate) without passing back through the open state,³⁹ or may enter long blocked states as well as brief ones.

Correct or incorrect in the present instance, the open channel blocking model is an example, perhaps the example *par excellence*, of anesthetic mechanisms that envision a specific interaction between an individual anesthetic molecule and a known molecular target (here, an ion channel). That our data fail in several respects to support this model is, in a sense, something of a disappointment, if only because a strong confirmation of the model would allow a rather large class of hypotheses—those that postulate a “unitary” physical chemical action on lipid bilayers—to be excluded from further consideration. Let us now briefly examine one of these, the membrane fluidity $\ddagger\ddagger$ hypothesis, to see whether it offers any obvious advantages over theories based on specific interactions.

The concept that increases in membrane fluidity might account for the acceleration of post-synaptic currents observed in the presence of anesthetics was introduced by Gage and Hamill in 1975⁵² (and reviewed by the same investigators in 1981²¹), and has recently been invoked to explain an acceleration by halothane of acetylcholine receptor channel kinetics in frog muscle membranes.^{53,54} That anesthetics do, in fact, fluidize or “disorder” lipid bilayers^{55–59} is not in dispute. Rather, it is the stronger claim that increased fluidity *per se* causes the observed changes in the function of acetylcholine receptor channels (and, hence, by analogy, that of the ion channels underlying conscious or behavioral states) that we will examine. The magnitude of these fluidizing effects, variously defined, has typically been rather small at clinically effective anesthetic concentrations.⁶ This in itself, of course, does not seriously weaken the hypothesis, since one is free to speculate that small in-

creases in fluidity may be sensitively coupled to physiologically important changes in ion channel function.¹ It is reasonable to insist, however, that any functional changes attributed to pharmacologically increased membrane fluidity be at least similar, if not identical, to those observed when fluidity is increased by physical means, as by raising temperature. We have investigated the kinetic properties of acetylcholine receptor channels in BC3H-1 membranes at temperatures ranging from 20–35°C; $\S\S$ although we observe, as expected,⁶⁰ a several-fold acceleration of channel closing rates as the temperature is increased, the “flickering” activity characteristic of isoflurane is not reproduced. Thus, it seems unlikely that the kinetic pattern observed in the presence of the anesthetic is due to a change in membrane fluidity.

If, as we suspect, neither ion channel blockade nor altered membrane fluidity can satisfactorily account for the effects of isoflurane on acetylcholine receptor channel function, what alternative mechanisms should be explored? Perhaps isoflurane becomes bound to sites on the receptor channel other than the ion conducting pathway, and exerts its effects on the kinetic behavior of the channel by an allosteric mechanism. Studies of the inhibition of the soluble enzyme luciferase⁶ by volatile anesthetics have strengthened the concept that functionally important anesthetic binding sites can exist within hydrophobic domains of a pure protein, providing an alternative to lipid-based interpretations of the Meyer-Overton rule. Unfortunately, the function of an ion channel, unlike that of an enzyme, cannot be examined in the absence of its lipid environment, so the results of analogous studies of the acetylcholine receptor channel must be less incisive. However, the available biochemical data^{61,62} suggest that, in addition to the acetylcholine and α -bungarotoxin binding sites located in relatively hydrophilic domains of the α -subunit, there exist at least two additional classes of binding sites in hydrophobic domains of the channel protein. These sites recognize a variety of agents known as “noncompetitive inhibitors” of the acetylcholine response, including chlorpromazine, phencyclidine, and bupivacaine. The locations of these sites within the three-dimensional structure of the receptor channel protein are not well mapped, but there is evidence that a unique high-affinity drug-binding site exists within the mouth of the ion channel itself, whereas a number of lower-affinity sites are found within the membrane-spanning portion of the channel, perhaps at the protein/lipid interface. The present observation that ion channels within a membrane patch are promptly affected by changes in the concentration of isoflurane bathing the

$\ddagger\ddagger$ We disclaim responsibility for the unfortunate ambiguities and controversy associated with the use of the term “fluidity” in reference to biological membranes. Although this term has often been used without precise definition in the literature of anesthetic mechanisms, it remains conceptually important as a central example of the “unitary” hypotheses.

$\S\S$ Dilger JP, Brett RS, Poppers DM, unpublished observations.

larger membrane area outside the patch indicates that the drug can reach (and leave) its site/s of action by lateral diffusion within the membrane bilayer. Thus, it is likely that the sites at which isoflurane exerts its action exist at or near the lipid/protein interface, although a site within the channel cannot be excluded. It is intriguing to note that, in the relatively few cases in which the effects of uncharged noncompetitive inhibitors have been examined at the single channel level, "flickering"^{37,63,64} and shortening of the open duration,^{37,63-65} but not lengthening of the burst duration, have been reported.

Finally, although we did not set out to obtain physiological data *per se*, it is reasonable to ask whether our results shed any light on clinically observable phenomena. The local microperfusion method described here leaves considerable uncertainty in the estimate of the membrane concentration to which isoflurane rises during perfusion, but it is clear that typical effects on the acetylcholine receptor channel are detectable when the aqueous drug concentration is no higher than 450 μM , comparable to that associated with the anesthetic state.^{¶¶} Thus, it seems likely that the potentiation of d-tubocurarine-induced neuromuscular blockade⁶⁷ by isoflurane is mediated, at least in part, by a reduction of the average open duration of the acetylcholine receptor channels that remain available for activation (that is, those unoccupied by d-tubocurarine molecules). Hence, the endplate current will suffer both a reduction in amplitude (due to d-tubocurarine) and an accelerated decay (due to isoflurane). Both factors will contribute to a reduction of the net charge transfer across the endplate membrane, resulting in impairment of neuromuscular transmission.

¶¶ Our conversions of aqueous to gas-phase concentrations are based on the temperature-dependent isoflurane water/gas partition coefficients quoted by Firestone *et al.*⁶⁶

The authors wish to thank Ms. Claire Mettwie for excellent technical assistance in the preparation and maintenance of BC3H-1 cells, and Mr. David Poppers for helpful participation in some of these experiments.

References

1. Miller KW, Roth SH: Inside the "black box," Molecular and Cellular Mechanisms of Anesthetics. Edited by Roth SH, Miller KM. New York, Plenum Press, 1986, pp 261-266
2. Haydon DA, Elliott JR, Hendry BM, Urban BW: The action of nonionic anesthetic substances on voltage-gated ion conductances in squid giant axons, Molecular and Cellular Mechanisms of Anesthetics. Edited by Roth SH, Miller KM. New York, Plenum Press, 1986, pp 267-277
3. Elliott JR, Haydon DA: Mapping of general anesthetic target sites. *Nature* 319:77-78, 1986
4. Franks NP, Lieb WR: Mapping of general anaesthetic sites (reply to Elliott and Haydon). *Nature* 319:78, 1986
5. Franks NP, Lieb WR: Do direct protein/anesthetic interactions underlie the mechanism of general anesthesia? *Molecular and Cellular Mechanisms of Anesthetics*. Edited by Roth SH, Miller KW. New York, Plenum Press, 1986, pp 319-329
6. Franks NP, Lieb WR: What is the molecular nature of general anaesthetic target sites? *Trends in Pharmacological Sciences* 8:169-174, 1987
7. Urban BW: Modifications of excitable membranes by volatile and gaseous anesthetics, *Effects of Anesthesia*. Edited by Covino BG, Fozzard HA, Rehder K, Strichartz G. Bethesda, American Physiological Society, 1985, pp 13-28
8. Hille B: *Ionic Channels of Excitable Membranes*. Sunderland, Sinauer, 1984
9. Mishina M, Kurosaki T, Tobimatsu T, Morimoto Y, Noda M, Yamamoto T, Terao M, Lindstrom J, Takahashi T, Kuno M, Numa S: Expression of functional acetylcholine receptor from cloned cDNAs. *Nature* 307:604-608, 1984
10. Noda M, Shimizu S, Tanabe T, Takai T, Kayano T, Ikeda T, Takahashi H, Nakayama H, Kanaoka Y, Minamino N, Kangawa K, Matsuo H, Raftery MA, Hirose T, Inayama S, Hayashida H, Miyata T, Numa S: Primary structure of Electrophorus electricus sodium channel deduced from cDNA sequence. *Nature* 312:121-127, 1984
11. Sakmann B, Methfessel C, Mishina M, Takahashi T, Takai T, Kurasaki M, Fukuda K, Numa S: Role of acetylcholine receptor subunits in gating of the channel. *Nature* 318:538-543, 1985
12. Mishina M, Tobimatsu T, Imoto K, Tanaka K-I, Fujita Y, Fukuda K, Kurasaki M, Takahashi H, Morimoto Y, Hirose T, Inayama S, Takahashi T, Kuno M, Numa S: Location of functional regions of acetylcholine receptor α -subunit by site-directed mutagenesis. *Nature* 313:364-369, 1985
13. Kistler J, Stroud RM, Klymkowsky MW, Lalancette RA, Fairclough RH: Structure and function of an acetylcholine receptor. *Biophys J* 37:371-383, 1982
14. Finer-Moore J, Stroud RM: Amphipathic analysis and possible formation of the ion channel in an acetylcholine receptor. *Proc Natl Acad Sci USA* 81:155-159, 1984
15. Young EF, Ralston E, Blake J, Ramachandran J, Hall ZW, Stroud RM: Topological mapping of acetylcholine receptor: Evidence for a model with five transmembrane segments and a cytoplasmic COOH-terminal peptide. *Proc Natl Acad Sci USA* 82:626-630, 1985
16. Neher E, Sakmann B: Single channel currents recorded from membrane of denervated frog muscle fibres. *Nature* 260:799-802, 1976
17. Hamill OP, Marty E, Neher A, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391:85-100, 1981
18. Sakmann B, Neher E: *Single-channel Recording*. New York, Plenum Press, 1983
19. Flanigan KE, Brett RS: Effects of isoflurane on single acetylcholine receptor channel kinetics (abstract). *Anesth Analg* 66:S58, 1987
20. Larrabee MG, Posternak JM: Selective action of anaesthetics on synapses and axons in mammalian sympathetic ganglia. *J Neurophysiol* 15:91-114, 1952
21. Gage PW, Hamill OP: Effects of anesthetics on ion channels in synapses. *Neurophysiology IV, International Review of Physiology* 25:31-45 1981
22. Langley JN: On nerve endings and on special excitable substances in cells. *Proc Roy Soc Lond [Biol]* 78:170, 1906
23. Katz B, Miledi R: Membrane noise produced by acetylcholine. *Nature* 226:962-963, 1970

24. Katz B, Miledi R: Further observations on acetylcholine noise. *Nature* 232:124-126, 1971
25. Katz B, Miledi R: The characteristics of 'end-plate noise' produced by different depolarizing drugs. *J Physiol (Lond)* 230:707-717, 1973
26. Anderson CR, Stevens CF: Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J Physiol (Lond)* 235:655-691, 1973
27. Sine SM, Taylor P: Functional consequences of agonist-mediated state transitions in the cholinergic receptor. *J Biol Chem* 254:3315-3325, 1979
28. Schubert D, Harris AJ, Devine CE, Heinemann SF: Characterization of a unique muscle cell line. *J Cell Biol* 61:398-413, 1974
29. Boulter J, Patrick J: Purification of an acetylcholine receptor from a nonfusing muscle cell line. *Biochemistry* 16:4900-4908, 1977
30. Patrick J, McMillan J, Wolfson H, O'Brien JC: Acetylcholine receptor metabolism in a nonfusing cell line. *J Biol Chem* 252:2143-2153, 1977
31. Colquhoun D, Sigworth FJ: Fitting and statistical analysis of single-channel records, *Single-Channel Recording*. Edited by Sakman B, Neher E. New York, Plenum Press, 1983, pp 191-263
32. Colquhoun D, Sakmann B: Fast events in single-channel currents activated by acetylcholine and its analogues at the frog end-plate. *J Physiol (Lond)* 369:501-557, 1985
33. Meyer HH: Zur Theorie der Alkoholnarkose. I. Welche Eigenschaft der Anästhetika bedingt ihre narkotische Wirkung? *Arch Exp Path Pharmacol* 42:109, 1899
34. Overton E: Studien Über die Narkose, zugleich ein Beitrag zur allgemeinen Pharmakologie. Jena, G. Fischer, 1901
35. Neher E, Steinbach JH: Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J Physiol (Lond)* 277:153-176, 1978
36. Neher E: The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anaesthetics. *J Physiol (Lond)* 339:663-678, 1983
37. Ogden DC, Siegelbaum SA, Colquhoun D: Block of acetylcholine-activated ion channels by an uncharged local anaesthetic. *Nature* 289:596-598, 1981
38. Adams PR: Drug blockade of open end-plate channels. *J Physiol (Lond)* 260:531-552, 1976
39. Adams PR: Voltage jump analysis of procaine action at frog end-plate. *J Physiol (Lond)* 268:291-318, 1977
40. Strichartz GR: The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. *J Gen Physiol* 62:37-57, 1973
41. Courtney KR: Mechanism of frequency-dependent inhibition of sodium currents in frog myelinated nerve by the lidocaine derivative GEA 968. *J Pharmacol Exp Ther* 195:225-236, 1975
42. Hille B: The pH-dependent rate of action of local anesthetics on the node of Ranvier. *J Gen Physiol* 69:475-496, 1977
43. Hille B: Local anesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J Gen Physiol* 69:497-515, 1977
44. Armstrong CM: Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J Gen Physiol* 58:413-437, 1971
45. del Castillo J, Katz B: Interaction at end-plate receptors between different choline derivatives. *Proc Roy Soc Lond [Biol]* 146:369-381, 1957
46. Sine SM, Steinbach JH: Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by low concentrations of agonist. *J Physiol (Lond)* 373:129-162, 1986
47. Sakmann B, Patlak J, Neher E: Single acetylcholine-activated channels show burst-kinetics in the presence of desensitizing concentrations of agonist. *Nature* 286:71-73, 1980
48. Katz B, Thesleff S: A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J Physiol (Lond)* 138:63-80, 1957
49. Adams PR: An analysis of the dose-response curve at voltage-clamped frog endplates. *Pflügers Arch* 360:145-153, 1975
50. Steinbach AB: A kinetic model for the action of Xylocaine on receptors for acetylcholine. *J Gen Physiol* 52:162-180, 1968
51. Colquhoun D, Hawkes AG: The principles of the stochastic interpretation of ion-channel mechanisms, *Single-channel Recording*. Edited by Sakman B, Neher E. New York, Plenum Press, 1983, pp 135-175
52. Gage PW, Hamill OP: General anaesthetics: Synaptic depression consistent with increased membrane fluidity. *Neurosci Lett* 1:61-65, 1975
53. Lechleiter J, Moffett S, Gruener R: Effects of halothane on the acetylcholine receptor channel in cultured *Xenopus* myocytes. *Biophys J* 45:15-16, 1984
54. Lechleiter J, Gruener R: Halothane shortens acetylcholine receptor channel kinetics without affecting conductance. *Proc Natl Acad Sci USA* 81:2929-2933, 1984
55. Trudell JR, Hubbell WL, Cohen EN: The effect of two inhalation anesthetics on the order of spin-labelled phospholipid vesicles. *Biochim Biophys Acta* 291:321-327, 1973
56. Boggs JM, Yoong T, Hsia JC: Site and mechanism of anesthetic action I. Effect of anesthetics and pressure on fluidity of spin-labelled lipid vesicles. *Mol Pharmacol* 12:127-135, 1976
57. Harris RA, Groh GI: Membrane disordering effects of anesthetics are enhanced by gangliosides. *ANESTHESIOLOGY* 62:115-119, 1985
58. Miller KW, Braswell LM, Firestone LL, Dodson BA, Forman SA: General anesthetics act both specifically and nonspecifically on acetylcholine receptors, *Molecular and Cellular Mechanisms of Anesthetics*. Edited by Roth SH, Miller KM. New York, Plenum Press, 1986, pp 261-266
59. Ueda I, Hirakawa M, Arakawa K, Kamaya H: Do anesthetics fluidize membranes? *ANESTHESIOLOGY* 64:67-71, 1986
60. Magleby KL, Stevens CF: A quantitative description of end-plate currents. *J Physiol (Lond)* 223:173-197, 1972
61. Changeux J-P, Revah F: The acetylcholine receptor molecule: Allosteric sites and the ion channel. *Trends in Neurosciences* 10:245-250, 1987
62. Karlin A, Dipaola M, Kao PN, Lobel P: Functional sites and transient states of the nicotinic acetylcholine receptor. *Society of General Physiologists Series*, Vol. 41. New York, Wiley-Interscience, 1987, pp 43-65
63. Varanda WA, Aracava Y, Sherby SM, VanMeter WG, Eldefrawi ME, Albuquerque EX: The acetylcholine receptor of the neuromuscular junction recognizes mecamylamine as a non-competitive antagonist. *Mol Pharmacol* 28:128-137, 1985
64. Gage PW, McKinnon D: Effects of pentobarbitone on acetylcholine-activated channels in mammalian muscle. *Br J Pharmacol* 85:229-235, 1985
65. Changeux J-P, Pinset C, Ribera AB: Effects of chlorpromazine and phencyclidine on mouse C2 acetylcholine receptor kinetics. *J Physiol (Lond)* 378:497-513, 1986
66. Firestone LL, Miller JC, Miller KW: Tables of Physical and Pharmacological Properties of Anesthetics, *Molecular and Cellular Mechanisms of Anesthetics*. Edited by Roth SH, Miller KM. New York, Plenum Press, 1986, pp 455-470
67. Miller RD, Eger EI II, Way WL, Stevens WC, Dolan WM: Comparative neuromuscular effects of Forane and halothane alone and in combination with d-tubocurarine in man. *ANESTHESIOLOGY* 35:38-42, 1971