*Proc. Natl. Acad. Sci. USA*
Vol. 81, pp. 2929–2933, May 1984
Neurobiology

# Halothane shortens acetylcholine receptor channel kinetics without affecting conductance

(general anesthetics/patch clamp/myocytes/cell culture)

JAMES LECHLEITER AND RAPHAEL GRUENER*

Department of Physiology, College of Medicine, University of Arizona, Tucson, AZ 85724

*Communicated by S. Hagiwara, January 26, 1984*

ABSTRACT **The extracellular patch-clamp technique was used to examine how halothane, a general anesthetic, affects the properties of single nicotinic acetylcholine receptor channels of embryonic *Xenopus* skeletal muscle cells grown in culture. Under control conditions, single-channel events showed a bimodal distribution on the basis of current amplitudes. This distribution was maintained during exposure to halothane and its washout. In addition, the mean current value of the low- and high-amplitude channels was unaffected by the presence of the anesthetic at clinically relevant concentrations. In contrast, halothane shortened the burst durations of both channel types in a concentration-dependent manner. This shortening of burst durations may be an expression of the more rapid relaxation of the channel protein to the nonconducting state, possibly due to the disordering effect of the anesthetic on membrane lipids in which the receptor protein is embedded. This functional change, in the behavior of the synaptic receptor, provides further direct information on the mode of action of general anesthetics.**

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 (1–3). More recently, several variants of this theory have been formulated (4, 5). Of these, the membrane fluidity theory explains anesthesia by the disordering of the lipid bilayer resulting in increased membrane fluidity (6–9). This effect may change the behavior of membrane-bound proteins that are critical to the functioning of the neuron.

Despite the diversity of proposed mechanisms (for reviews, see refs. 10 and 11), it is now generally accepted that the synapse itself is the most likely site of action of GAs (12). This idea is derived from the finding that GAs block synaptic transmission at much lower concentrations than required for blockade of the action potential (13). Inasmuch 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 postsynaptic depolarizations at various vertebrate end plates (14–16). A closer examination of this decrease in end plate potential amplitude, using the voltage-clamp technique, has revealed the reduction to be due to an increase in the decay rate of miniature end plate currents (16, 17). Using the noise-analysis technique, Landau *et al.* (18) surmised that end plate current reduction, at the vertebrate neuromuscular junction, likely reflects the shortening of the time that acetylcholine receptor (AcChoR) ion channels spend in the open configuration. These findings are consistent with the idea that GAs 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 postsynaptic receptors. The recent development of the patch-clamp, single-channel technique (19, 20) provides direct measurements of single receptor channel activity.

We have used the patch-clamp, single-channel technique to examine how a volatile anesthetic, halothane, affects the properties of single nicotinic AcChoR channels embedded in the membranes of embryonic *Xenopus* myocytes grown in cell culture. We 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 (21). Membrane patches were sealed with electrodes of 1–2 $\mu$m in internal diameter with resistances of 3–15 M$\Omega$. Electrodes were filled with a high sodium solution whose composition was 120 mM NaCl/1.6 mM KCl/1 mM $CaCl_2$/8 mM Hepes·HCl, pH 7.4, containing 0.1–0.4 $\mu$M acetylcholine (AcCho). At this concentration of AcCho, 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.* (20). Single-channel events were stored on FM magnetic tape (RACAL Store SD4, Fullerton, CA). Analogue records were digitized at 50-$\mu$sec 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 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 acceptance was an abrupt transition from baseline to a constant current amplitude. The event was consid-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GA, general anesthetic; AcCho, acetylcholine; AcChoR, AcCho receptor.

*To whom reprint requests should be addressed.

ered 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/1 mM $CaCl_2$/8 mM Hepes·HCl, pH 7.4; for excised, cell-free patches, 5 mM EGTA was added and $CaCl_2$ was omitted. On occasion, a high potassium solution (70 mM $K_2SO_4$/4 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 (8) and to aqueous concentrations of 0.2–0.8 mM, in the recording chamber, as measured by ultraviolet spectroscopy (22)]. After 4–7 min of 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 (23–25) that a given channel may open repeatedly when AcCho is bound to its receptor. These 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. To distinguish between a burst, representing the activity of a single channel, and the opening of two different channels, Dionne and Leibowitz (25) 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, we

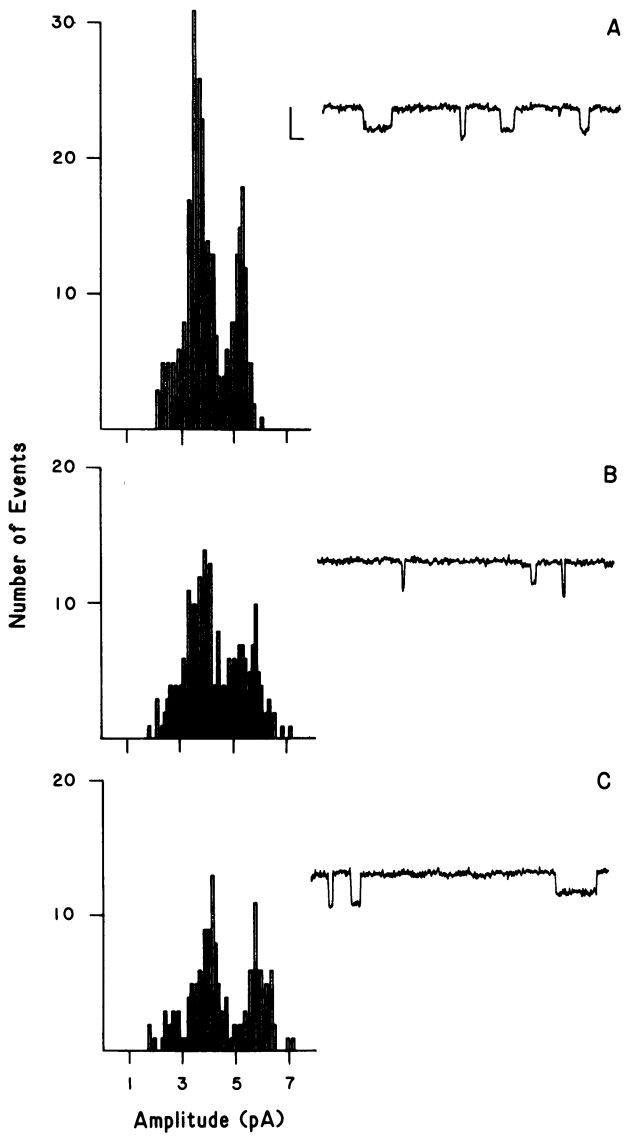


FIG. 1. Amplitude histograms of single-channel events. Amplitude histograms, from a typical patch, reveal a bimodal distribution. When a separation value of 4.6 pA is chosen, by eye, two channel populations may be defined. These are called low-amplitude and high-amplitude channels. In this cell-free 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.

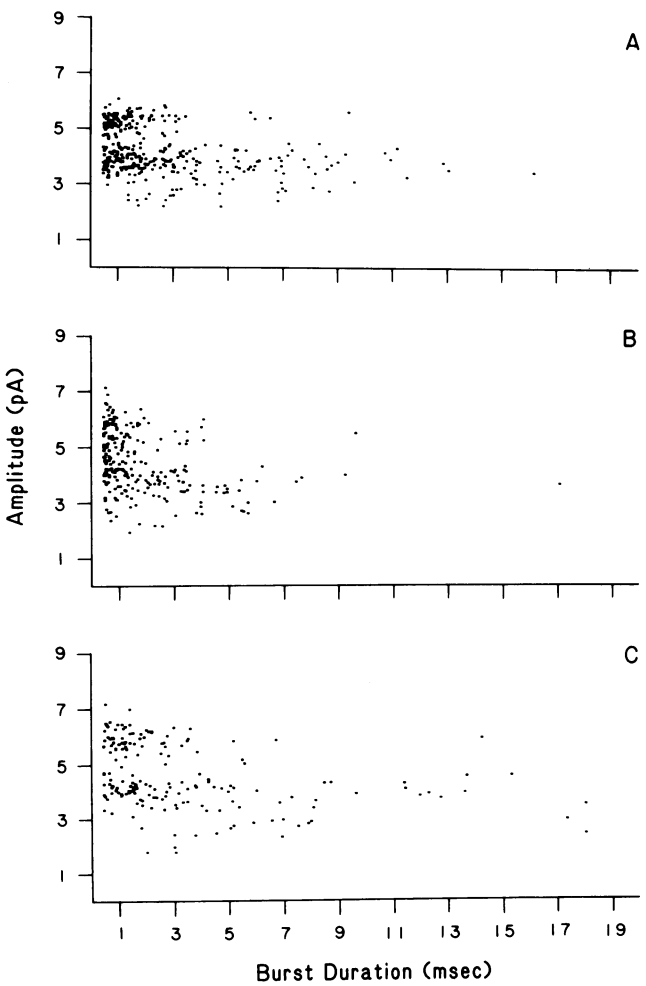


FIG. 2. Relationship between event amplitudes and their durations. (*A*) Data (from the patch in Fig. 1) are distributed into two distinct clusters on the basis of event amplitudes. During exposure to halothane (3%; *B*), the amplitude position of the clusters remains unaltered but the distribution of burst durations is shifted to shorter values. The initial distribution of burst durations is recovered (*C*) after washout of halothane. Events <0.6 msec were excluded due to the limitation of the circuit's response time.

report our data as burst durations when a series of channel openings is interrupted by closed times shorter than 1 msec. A burst that 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.

## RESULTS

When single-channel events were assembled into histograms, on the basis of current amplitudes, a bimodal distribution was obtained (Fig. 1; see also refs. 26 and 27). The resultant two populations of events are designated low-amplitude and high-amplitude channels. The low-amplitude channel has a relatively long open time and a small amplitude, whereas the high-amplitude channel has a shorter open time and a larger amplitude (see Fig. 1 *Insets*). The bimodel amplitude distribution and the mean amplitude values are maintained during exposure to halothane (Fig. 1*B*) and the recovery period following the washout of halothane (Fig. 1*C*).

In another set of experiments, the mean channel amplitude from 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 ± 0.1 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 ± 0.2 pA and 8.2 ± 0.2 pA, respectively.

The mean event amplitude, at a given membrane potential, and from a given patch, remained constant throughout the recording period (Fig. 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 (Fig. 1). When the current amplitude of each event (data as in Fig. 1) is plotted against the corresponding single-channel burst duration, the low-amplitude and high-amplitude channels clustered about their respective control amplitude values (Fig. 2*A*). In the presence of halothane (Fig. 2*B*) 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 (Fig. 2*C*).

To quantitate the reduction in single-channel burst durations, integrated histograms were assembled and fit with a single exponential (see legend of Fig. 3). The mean burst duration was estimated from the inverse of the time constant of the fit (nonlinear least squares method). A comparison of these histograms for the low-amplitude (Fig. 3 *A* and *B*) and high-amplitude channels (Fig. 3 *C* and *D*), before and after exposure to 3% halothane, shows a marked decrease in burst 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.

Even though these data (and those in Fig. 4 below) were obtained from cell-attached and cell-free patches, the rela-

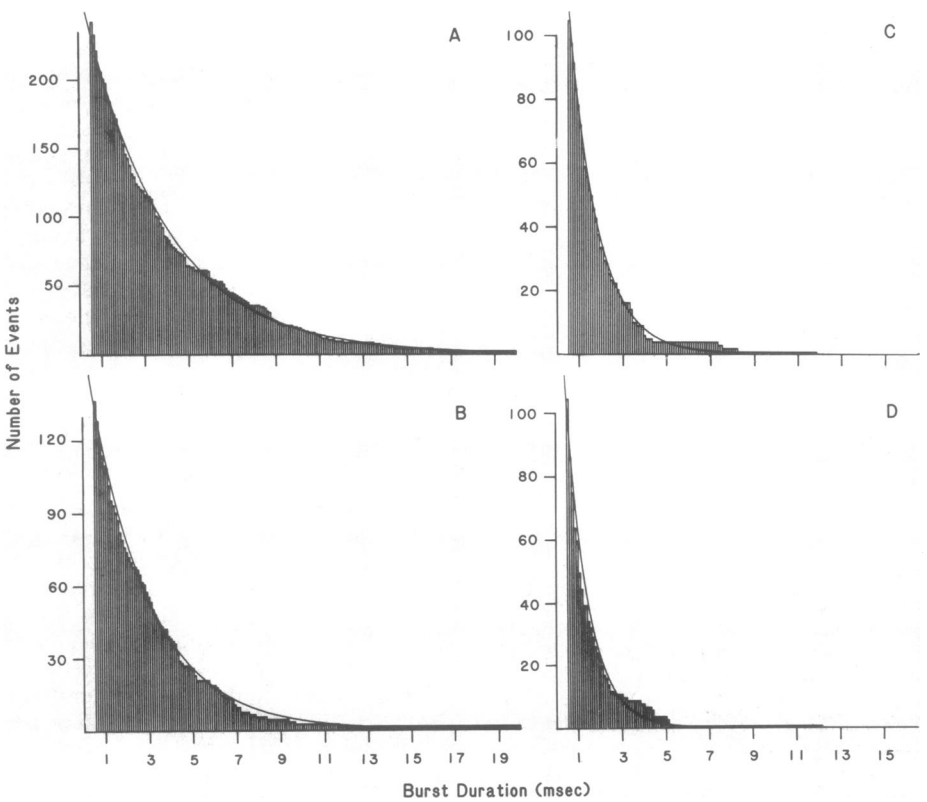


FIG. 3. Integrated burst duration histograms. Burst durations (same patch as in Fig. 1) are grouped on the basis of their amplitudes. The low-amplitude and high-amplitude channels are plotted separately during control conditions (*A* and *C*) and during exposure to halothane (*B* and *D*), respectively. 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 $N(t) = N_T \exp(-\alpha t)$, using a nonlinear least squares method, where $N(t)$ is the number of events at time $t$, $N_T$ is the total number of events, and $\alpha$ is the time constant (28, 29). An independent evaluation of burst durations, using the maximum likelihood method (30), 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.

Table 1. Effects of halothane on mean burst duration

| % halothane | Burst duration |
|---|---|
| 1 | 95 ± 8* (3, 2, 1402) |
| 2 | 67 ± 7† (1, 4, 1049) |
| 3 | 52 ± 9† (1, 4, 647) |
| 4 | 52 ± 14† (0, 4, 659) |

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 ($P < 0.005$).

tive (% 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 (Fig. 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 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 was estimated from only the initial, fast time constant (dashed circle in Fig. 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 that 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

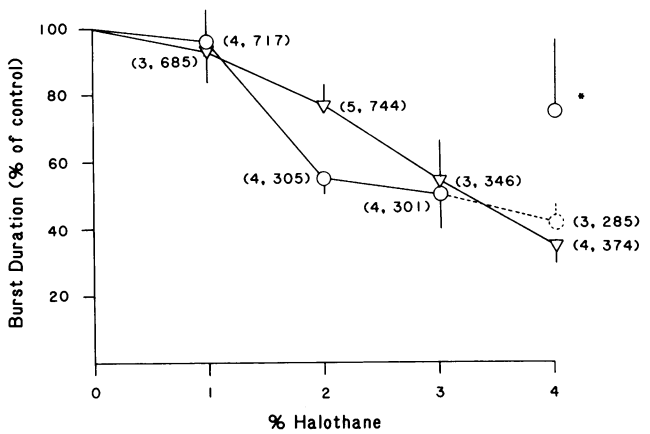


FIG. 4. Concentration-dependent effect of halothane on channel burst durations. Data from low-amplitude (○) and high-amplitude (▽) channels are plotted separately as a function of halothane concentration. The reductions in burst durations (both low-amplitude and high-amplitude channels) are statistically 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 three 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.

may depend on a nonspecific interaction with excitable membranes. GAs, regardless of their molecular structure, cause disordering of lipid membranes (refs. 31 and 32; but see ref. 33), and this may lead to "fluidization" of the membrane in which receptor proteins are embedded (8, 9). 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 (8). Consistent with this idea, Maleque *et al.* (34) have reported that ketamine accelerated the decay rate of miniature end plate currents, at the frog neuromuscular junction, without affecting their conductances. Similarly, Haydon and Urban (35) 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 flow. In contrast, Fernandez *et al.* (36) reported that chloroform reduces the magnitude of sodium gating currents without affecting their kinetics. Recently, Nicoll and Madison (37) reported that several GAs produced neuronal membrane hyperpolarization, albeit at quite high concentrations. In contrast, Lynch *et al.* (38) showed that volatile anesthetics do not affect the resting potential of cardiac cells. Examination of inhibitory, "GABA-ergic" postsynaptic receptors revealed that when exposed to pentobarbital, mean channel open times were actually prolonged (28). These diverse findings may reflect the action of the different classes of anesthetics or the use of different preparations.

Our data show that halothane shortens the burst durations of single ion channels, associated with the nicotinic AcChoR, in a concentration-dependent manner. These findings are consistent with previous reports on macroend plate currents (8, 16) and noise-analysis data (18). It is of interest to note here that Gage and Hamill (8) reported that at high halothane concentrations, miniature end plate currents had a rapid, early decay followed by a slower-than-normal decay. These data are consistent with our 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 we report here, would result in a decrease in the amplitude and duration of postsynaptic potentials, and if this effect were sufficiently large, ion currents may fall below the threshold required for activation of the postsynaptic element and result in blockade of synaptic transmission.

During exposure to halothane, a decrease in the frequency of channel events was observed (compare Fig. 1 *A–C*). This "drop-out" phenomenon was not observed for the high-amplitude channel events under prolonged (up to 15 min), control conditions. But under similar conditions, the low-amplitude channel event frequency decreased (within the first 5 min) 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 our experiments, and the halothane-induced disappearance of high-amplitude channel events may reflect an increase in the lat-

eral 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 AcCho for its receptor (39). 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 we have used low agonist concentrations (0.1–0.4 $\mu$M), and since we rarely saw simultaneous multiple channel openings, it is unlikely that the drop-out we observed was due to receptor desensitization.

Although our findings do not identify the site of action of halothane as a GA, they provide useful information on the possible mode of action of the drug in attenuating or blocking synaptic transmission. The data we report here document directly that, in the presence of halothane, single AcChoR 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, our findings of reduced burst durations demonstrate a net reduction in the open state configuration. In the latter case, the increased probability of linking additional open times into a given burst duration would tend to produce an increase rather than a decrease in the value of the burst. Thus, we 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 GAs.

We are grateful to Steve Moffett for writing the computer programs and to Drs. T. Blanck, P. Brehm, R. Brinton, and D. Kreulen for their constructive suggestions. Thymol-free halothane was a gift from Mr. Lou Ferstanding (Halocarbon Laboratories, Hackensack, NJ). This study was supported by a Basic Research Science grant to R.G. from the Arizona Health Sciences Center and by National Institutes of Health Training Grant HL07249 to the Department of Physiology.

1. Meyer, H. H. (1901) *Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol.* **46,** 338–346.
2. Overton, E. (1901) *Studien uber die Narkose* (Fischer, Jena, G.D.R.).
3. Meyer, K. H. (1937) *Trans. Faraday Soc.* **33,** 1062–1068.
4. Mullins, L. J. (1954) *Chem. Rev.* **54,** 289–323.
5. Miller, K. W., Paton, W. D. M., Smith, R. A. & Smith, E. B. (1973) *Mol. Pharmacol.* **9,** 131–143.
6. Metcalfe, J. C., Seeman, P. & Burgen, A. S. V. (1968) *Mol. Pharmacol.* **4,** 87–89.
7. Trudell, J. R., Hubbell, W. J. & Cohen, E. N. (1973) *Biochim. Biophys. Acta* **291,** 321–327.
8. Gage, P. W. & Hamill, O. (1975) *Neurosci. Lett.* **1,** 61–65.
9. Lenaz, G., Curatola, G., Mazzanti, L., Bertoli, E. & Pastuszko, A. (1979) *J. Neurochem.* **32,** 1689–1695.
10. Roth, S. H. (1980) *Can. Anaesth. Soc. J.* **27,** 433–439.
11. Franks, N. P. & Lieb, W. R. (1982) *Nature (London)* **300,** 487–493.
12. Judge, S. E. (1983) *Br. J. Anaesth.* **55,** 191–200.
13. Larrabee, M. G. & Posternak, J. M. (1952) *J. Neurophysiol.* **15,** 91–114.
14. Gissen, A., Karis, J. H. & Nastuk, W. L. (1966) *J. Am. Med. Assoc.* **197,** 770–774.
15. Waud, B. E. & Waud, D. R. (1975) *Anesthesiology* **42,** 275–280.
16. Gage, P. W. & Hamill, O. P. (1976) *Br. J. Pharmacol.* **57,** 263–272.
17. Torda, T. A. & Gage, P. W. (1977) *Br. J. Anaesth.* **49,** 771–776.
18. Landau, E. M., Richter, J. & Cohen, S. (1979) *Mol. Pharmacol.* **16,** 1075–1083.
19. Neher, E., Sakmann, B. & Steinbach, J. H. (1978) *Pflügers Arch.* **375,** 219–228.
20. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100.
21. Gruener, R. & Kidokoro, Y. (1982) *Dev. Biol.* **91,** 86–92.
22. Blank, T. J. J. & Thompson, M. (1980) *Anesth. Analg.* **59,** 481–483.
23. Sakmann, B., Patlak, J. & Neher, E. (1980) *Nature (London)* **286,** 71–73.
24. Colquhoun, D. & Sakmann, B. (1981) *Nature (London)* **294,** 464–466.
25. Dionne, V. E. & Leibowitz, M. (1982) *Biophys. J.* **39,** 253–261.
26. Clark, R. B. & Adams, P. R. (1981) *Soc. Neurosci. Abstr.* **7,** 838a.
27. Brehm, P., Moody-Corbett, F. & Kidokoro, Y. (1983) *Soc. Neurosci. Abstr.* **9,** 1180a.
28. Jackson, M. B., Lecar, H., Mathers, D. A. & Barker, J. L. (1982) *J. Neurosci.* **2,** 889–894.
29. Brehm, P., Moody-Corbett, F. & Kullberg, R. (1984) *J. Physiol. (London)*, in press.
30. Colquhoun, D. & Sigworth, F. J. (1983) in *Single-Channel Recording*, eds. Sakmann, B. & Neher, E. (Plenum, New York), pp. 233–257.
31. Pang, K., Chang, T. & Miller, K. W. (1979) *Mol. Pharmacol.* **15,** 729–738.
32. Mountcastle, D. B., Biltonen, R. L. & Halsey, M. J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4906–4910.
33. Lieb, W. R., Kovalycsik, M. & Mendelsohn, R. (1982) *Biochim. Biophys. Acta* **688,** 388–398.
34. Maleque, M. A., Warnick, J. E. & Albuquerque, E. X. (1981) *J. Pharmacol. Exp. Ther.* **219,** 638–645.
35. Haydon, D. A. & Urban, B. W. (1983) *J. Physiol. (London)* **341,** 429–439.
36. Fernandez, J. M., Bezanilla, F. & Taylor, R. E. (1982) *Nature (London)* **297,** 150–152.
37. Nicoll, R. A. & Madison, D. V. (1982) *Science* **217,** 1055–1056.
38. Lynch, C., III, Vogel, S., Pratila, M. G. & Sperelakis, N. (1982) *J. Pharmacol. Exp. Ther.* **222,** 405–409.
39. Young, A. P., Brown, F. F., Halsey, M. J. & Sigman, D. S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4563–4567.