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Halothane-induced changes in acetylcholine receptor channel kinetics are attenuated by cholesterol

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The single-channel recording technique was used to investigate the role of membrane lipids in the action of general anesthetics on ion channels. We examined the effects of halothane on acetylcholine receptor channels in *Xenopus laevis* myocytes in which the plasma membrane cholesterol level had been changed by pretreatment with cholesterol-rich or cholesterol-free liposomes. We found that the alteration in acetylcholine receptor channel kinetics, elicited in the presence of clinically-relevant concentrations of halothane, is attenuated when membrane cholesterol is increased and enhanced when membrane cholesterol concentration is decreased. These findings support the idea that general anesthetics interact with synaptic receptor channels indirectly through the lipid domains in which these synaptic proteins are embedded.

Introduction

It has been postulated that general anesthetics produce the anesthetic state by blocking synaptic transmission through an alteration in the conformation of integral synaptic membrane proteins [1–6]. The primary site of action of general anesthetics may be either a hydrophobic pocket in such proteins or the lipophilic membrane domain in which these proteins are embedded. Thus, it is still unknown whether general anesthetics interact with functionally-relevant, synaptic proteins [7,8], or with the lipid matrix which surrounds them [9,10]. One approach for examining this controversy consists of monitoring the functional responses of single receptor channel proteins, to the action of general anesthetics, while perturbing the lipid membrane composition in which these proteins are embedded. If the interaction of general anesthetics is with membrane proteins alone, an alteration in membrane lipids which surround the proteins should not affect the functional response of these molecules to the anesthetic. Conversely, if general anesthetic action involves the lipid matrix, then changes in membrane lipids are predicted to affect specific changes in receptor channel function, which are modulated by anesthetics.

The advent of single-channel recording from synaptic receptor channels [11,12] has made the above mentioned approach feasible when combined with techniques which permit alterations in membrane lipid composition [13,14]. We have recently shown that the general anesthetic halothane shortens the mean open time (burst duration) of the acetylcholine receptor channels from embryonic *Xenopus* myocytes grown in culture [15]. This finding is consistent with previous work [16]

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showing, under similar conditions, an acceleration of the relaxation kinetics of endplate currents which constitute the ensemble behavior of a large population of acetylcholine receptors at the neuromuscular junction. Shortening of receptor channel open times could account for synaptic blockade if channel kinetics are accelerated to a point where current flow across the postsynaptic membrane is so limited as to result in failure to elicit an action potential in the postsynaptic cells in brain pathways critical for the maintenance of consciousness. We report here that the reduction in mean channel open time, by halothane, is altered depending on the concentration of cholesterol in the cell membranes. When the cholesterol concentration was elevated, by incubation of myocytes with cholesterol-rich liposomes, the reduction of channel open times by halothane was reduced, in a concentration-dependent fashion, in comparison to the general anesthetic's effect on untreated cells. Conversely, when the cholesterol concentration was lowered, the effect of halothane was accentuated in comparison to both untreated and cholesterol-enriched cells. Taken together, these results demonstrate that in the presence of a general anesthetic, synaptic receptor channel function depends on this lipid environment. These findings provide direct evidence in support of the role which membrane lipids play in the mechanism of action of general anesthetics.

Results and Discussion

Myocyte cholesterol levels were altered by incubating *Xenopus* muscle cells, grown in culture, with cholesterol-rich or cholesterol-free liposomes. Changes in cholesterol and experimental conditions are shown in Table I.

Cholesterol levels were significantly increased in myocytes treated with cholesterol-rich liposomes (Table I) when compared to untreated or cholesterol-free-treated cells. Of the total increase in cholesterol, at least 48% was due to cholesterol exchange between liposomes and muscle cells. It is highly likely that most of this cholesterol is contained within the sarcolemma since Lange and Ramos [21] have shown that a high proportion (80–95%) of total cell cholesterol is distributed within the plasma membranes of intact cells. Furthermore, addition of cholesterol to cells by incubation with cholesterol-containing liposomes results in an almost exclusive incorporation of the added sterol in the plasma membrane, with no

TABLE I

EFFECTS OF LIPOSOME TREATMENT ON CELL CHOLESTEROL

Cholesterol concentration is given in nmol/10^5 cells. Percent fusion and/or adhesion was determined by use of a non-exchangeable marker (10 μCi of [^{14}C]cholesterol oleate; Amersham Corporation, Amersham, U.K.) which remained with treated muscle cells after liposome incubation. Cholesterol analysis, by gas chromatography, showed that 0.8% of cholesterol-rich liposomes accounted for no more than 66 ± 4 nmol of cholesterol per 10^5 cells. Therefore, at least 48 ± 3% of the total cholesterol increase was due to cholesterol exchange. The increase in cholesterol subsequent to cholesterol-rich treatment was significant (P less than 0.005) when compared to untreated cells. C/P denotes the cholesterol to phospholipid ratio. Note that the increase in cell cholesterol corresponded to an increase in the C/P ratio of only 0.57. This was due to a concurrent increase in phospholipid levels. A similar, but smaller increase in phospholipids was also observed in cholesterol-free treated myocytes; numbers in parentheses refer to number of experiments. Values are means $\pm$ S.E. Methods: Cell cholesterol content was altered by use of the liposome incubation technique [14]. Liposomes were made from purified [17] phosphatidylcholine (80 mg; Sedary Research Laboratories, London, Ontario) with (40 mg; cholesterol-rich) or without (cholesterol-free) cholesterol (Sedary Laboratories). The lipid dispersions were sonicated, under ice and nitrogen, for 60 min (60 watts, Branson Sonicator, U.S.A.) in 10 ml of defined-medium [18] and then centrifuged (11 500 rpm, 40 min, 4°C; Sorvall centrifuge, SS-34 rotor) to remove titanium particles. The supernatant, containing unilamellar liposomes, was filter-sterilized (Millipore, 0.2 μm) and stored at 4°C until use [13]. Liposomes were diluted to a 10% suspension with defined medium, just prior to use, and added to *Xenopus* myocyte cultures for a period not exceeding 12 h. Cell cholesterol concentrations were determined by gas chromatography (glass column: 3% JXR on 100/120 mesh Gas-Chrom Q packing (Applied Science Laboratories, State College, PA); Shimadzu GC equipped with C-RIA integrator) after lipid extraction from cells by the method of Bligh and Dyer [19]. An internal stigmasterol standard was used to quantify the presence of cholesterol. Phospholipids were determined by the micro Bartlett assay [20].

Treatment	Cholesterol concn.	% fusion or adhesion	C/P ratio
None	6.9 ± 0.9 (9)	n.a.	0.16 ± 0.02 (4)
Cholesterol-free	6.0 ± 1.1 (6)	0.7 ± 0.1 (2)	0.10 ± 0.05 (2)
Cholesterol-rich	127 ± 38 (7)	0.8 ± 0.3 (3)	0.57 ± 0.20 (3)

detectable transfer to the cell interior over a 16-h period [21–23]. We have used a similar incubation of myocytes with cholesterol containing liposomes for a 12-h period. Therefore, our observed increase in myocyte cholesterol is most likely to be confined to the sarcolemma.

Incubation of myocytes with cholesterol-free liposomes slightly decreased cholesterol content but not by a significant amount (Table I). This may be due, in part, to the preexisting low levels of cholesterol in untreated cells. Poznansky and Czekanski [24] have shown that as the cholesterol/phospholipid (C/P) ratio decreases, cholesterol is more difficult to extract. Alternatively, this may be due to the relative inaccessibility of this pool of cholesterol to extraction. Liebel et al. [25] reported that liposome treatment did not lower cholesterol beyond 0.4 (C/P ratio) in a purified preparation of acetylcholine receptor reconstituted from *Torpedo*. They suggested that the cholesterol associated with the receptor protein is inaccessible to extraction by liposomes.

To determine whether changes in membrane cholesterol affected the interaction of the anesthetic with the acetylcholine receptor channel in these cells, we used the extracellular patch-clamp technique to measure single-channel amplitudes and burst durations (sequences of single-channel open times, interrupted by closed times shorter than 1 ms). The technique was identical to that previously described [15] and revealed, as before, two types of receptor channels on the basis of their conductance. Liposome treatment alone had no effect on single-channel conductance or burst duration for either channel type (Table II).

On exposure of liposome treated cells to halothane, channel current amplitudes were unaffected in agreement with our previous results on untreated cells [15]. Further, exposure to halothane reduced the mean burst duration of both high and low conductance channels in a concentration dependent manner. Results from a typical patch, derived from a cell exposed to cholesterol-free liposomes, are shown in Fig. 1. Most significantly, however, the halothane-induced reductions in liposome treated cells were quantitatively different in comparison to the reductions observed in patches from untreated cells. Specifically, cholesterol-free-treated patches showed enhanced reductions, at all concentrations of halothane, while cholesterol-rich-treated patches showed attenuated reductions during identical exposure to halothane. Fig. 2 summarizes these results for low-conductance (panel A) and high-conductance (panel B) channels and for pooled data in which both low- and high-conductance channels are combined (panel C). Thus, for example, a 4-fold increase in concentration of halothane (4%) is required to reduce acetylcholine receptor channel burst duration in cholesterol-rich-treated patches to a similar value observed (at 1% halothane concentration) in cholesterol-free-treated patches (Fig. 2, panel C).

These data show that liposome treatment of myocyte membranes modulated the effect of halothane on single receptor channel burst durations. Moreover, the extent of liposome fusion and/or adhesion to myocytes was the same re-

TABLE II

EFFECTS OF LIPOSOME TREATMENT ON ACETYLCHOLINE RECEPTOR CHANNEL PROPERTIES

Numbers in parentheses refer to number of patches. Values are means ± S.E. Conductance is given in pS and burst durations are in ms. Recording conditions are the same as those described in Fig. 1.

Treatment	Channel type	Conductance	Burst-duration
Untreated	low conductance	44 ± 7 (2)	2.2 ± 0.2 (4)
	high conductance	67 ± 7 (2)	0.8 ± 0.1 (4)
Cholesterol-free	low conductance	41 ± 7 (4)	2.6 ± 0.4 (14)
	high conductance	59 ± 9 (4)	0.9 ± 0.1 (14)
Cholesterol-rich	low conductance	44 ± 4 (8)	1.9 ± 0.3 (7)
	high conductance	63 ± 7 (8)	0.8 ± 0.1 (6)

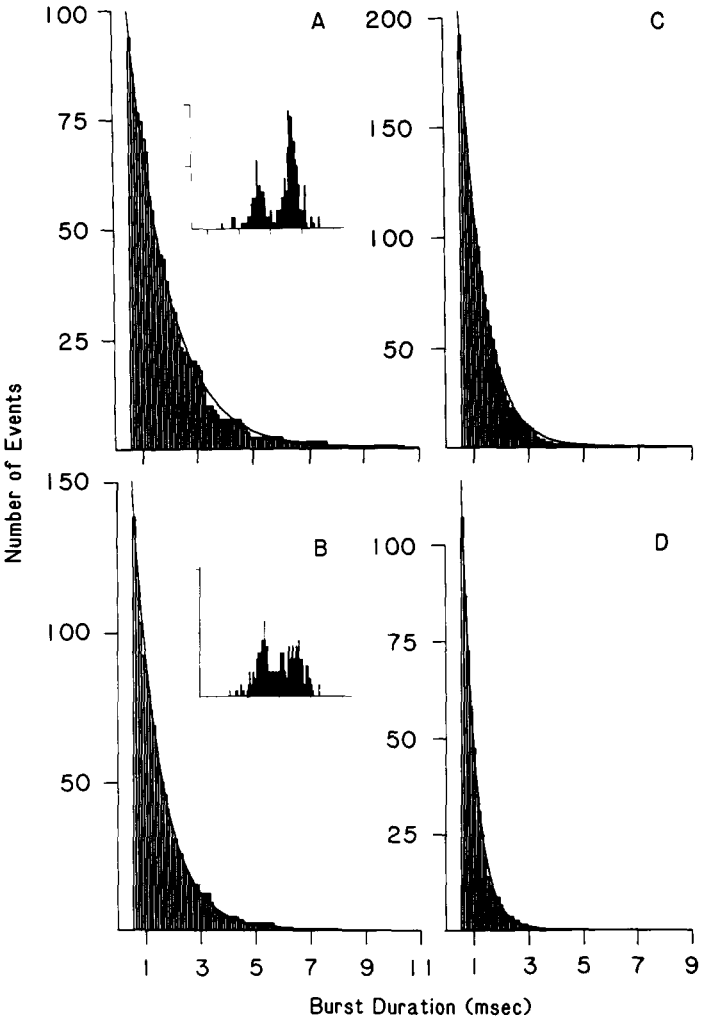

Fig. 1. Amplitude and cumulative burst-duration histograms from a cholesterol-free-treated cell-free patch. Amplitude histograms (inserts) reveal a bimodal distribution, which is maintained throughout the experiment. Mean amplitudes did not change significantly during the experiment (membrane potential was held at −90 mV, and the patch was continuously superfused with (in mM): K_2SO_4 70, KCl 4, Hepes-Na 8, (pH 7.4)). Ordinate and abscissa marks are 10, 20 events and 1, 3, 5, 7, 9 pA, respectively. Cumulative burst-duration histograms are plotted separately for the low-conductance channels (A) and for the high-conductance channels (C) under control conditions, and during exposure to 2% halothane (B and D, respectively). Burst durations were reduced from a mean value of 1.4 ms to 1.0 ms, for the low-conductance channels, and from 0.8 to 0.5 ms, for the high-conductance channels. Mean channel burst durations were estimated from the maximum likelihood method [26]. The bin width was 100 μs and each bin contained cumulative values of all events with burst durations greater than the value of a particular bin. Patch electrodes were filled with (in mM): NaCl 120, KCl 1.6, EGTA 1, Hepes-Na 8 (pH 7.4) and 0.2 μM acetylcholine.

gardless of the presence of cholesterol in the liposomes. This suggests that the modulation of burst-duration reduction by halothane was mediated by the level of cholesterol rather than the liposomal phospholipids.

One possible explanation for our findings is the decrease in anesthetic solubility produced by increased membrane cholesterol concentrations [27,28]. A decrease in anesthetic solubility is unlikely to fully account for our results since the increase in cholesterol concentration, that we measured, is expected to reduce the partitioning coefficient of halothane into the membrane by, at most, a factor of two [27]. Our data show that the halothane effect is reduced by a factor of four (cf. Fig. 2C). Similarly, it is unlikely that cholesterol produced a change in the affinity of acetylcholine for its receptor since similar cholesterol increases have been shown not to reduce the affinity of acetylcholine for the reconstituted receptor [29]. If such a reduction in affinity were to occur, in the system described here, we would expect channel kinetics to be affected in cholesterol-rich-treated cells even in the absence of the anesthetic. The conductance, as well as the burst duration values obtained under these conditions (Table I) were not different from those found in patches from untreated cells.

A more likely explanation for the interference, by cholesterol, in the action of halothane is a

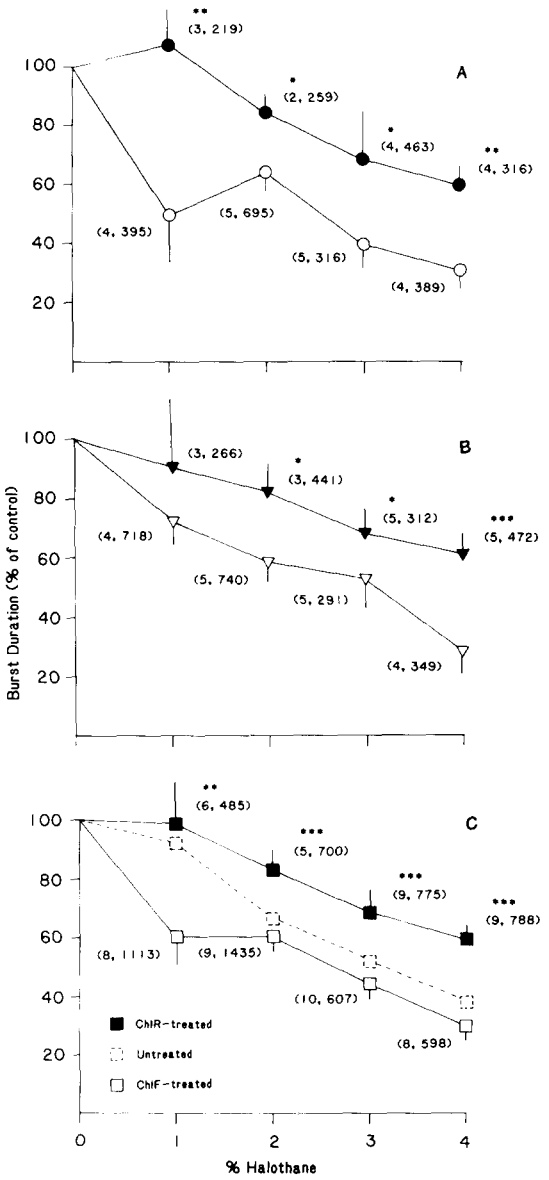

Fig. 2. Effects of halothane on burst-duration reductions of acetylcholine receptor channels from cells treated with cholesterol-free and cholesterol-rich liposomes. Mean burst durations are plotted separately for low-conductance (A) and high-conductance (B) channels from patches that were obtained from cholesterol-free (open symbols) and cholesterol-rich (closed symbols) treated cells. Combined data, for both channel types are plotted in C and compared to data from untreated (broken symbols) cells. Note that at all concentrations of halothane, reductions in burst durations are smaller in patches from cholesterol-rich-treated cells in comparison to patches from cholesterol-free-treated cells. Asterisks denote statistical significance (*, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.005$). Due to an inherent variability of mean burst duration from patch to patch [15], burst durations are expressed as % of control (no halothane) values and are means ± S.E. Numbers in parentheses refer to number of patches and events, respectively. Paired Student's *t*-tests were used to determine statistical significance.

perturbation in the membrane matrix in which the acetylcholine receptor is embedded. Cholesterol may interfere with the ability of the anesthetic to interact with critical membrane proteins (Miller, K.W., Third Int. Conference on Molecular and Cellular Mechanisms of Anaesthesia, Calgary, Alberta, Canada, 1984), or interfere with the anesthetic's action in modifying the lipid environment which surrounds the receptor protein [30]. A cholesterol stabilization of the active acetylcholine receptor conformational state was recently predicted from the preferential binding of androstane, a cholesterol analog, to the receptor protein [31]. Further, cholesterol has been shown to be an important lipid component in maintaining the acetylcholine receptor in the active form [32]. Our data therefore suggest that halothane may be acting at the lipid-receptor interface to destabilize the active conformation of the acetylcholine receptor channel.

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