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Kinetics of nicotinic acetylcholine ion channels in the presence of intravenous anaesthetics and induction agents

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1 Single channel currents activated by 250 nM acetylcholine were recorded from cell-attached patches of BC3H1 mouse tumour cells grown in culture. Channels were recorded in the absence and presence of alphaxalone, diazepam, etomidate, fentanyl, ketamine, meperidine, or propofol.

2 All of the anaesthetics tested shortened channel open time but did not alter single channel current amplitude. Drug concentrations calculated to reduce the time constant of open-time distributions by 50% were 99 μM alphaxalone, 66 μM diazepam, 57 μM etomidate, 26 μM fentanyl, 15 μM ketamine, 16 μM meperidine, or 81 μM propofol.

3 Ketamine, meperidine, and propofol reduced channel open time at concentrations comparable to plasma levels attained during therapeutic use of these agents, while alphaxalone, diazepam, etomidate, and fentanyl reduced channel open time only at levels higher than those encountered clinically.

4 The potency of these drugs in decreasing channel open time appears to be directly correlated with their octanol/buffer partition coefficients. In contrast to expectations, however, agents with higher partition coefficients were less potent in altering channel open time.

5 Ketamine and meperidine produced a prominent third component in closed-time distributions, which were otherwise well described by the sum of two exponential components. Alphaxalone, diazepam, and etomidate also produced a small third component, while no additional component was seen with propofol or fentanyl. These additional components probably arise from creation of an additional closed state of the channel.

6 We conclude that these agents are not altering channel properties merely by exerting non-specific effects via the lipid bilayer and that they are probably not all acting by similar mechanisms.

Keywords: Acetylcholine: receptor channels; analgesics: opioid: fentanyl, meperidine; anaesthetics: intravenous: etomidate, fentanyl, ketamine, meperidine, propofol; anaesthetics: steroid: alphaxalone; electrophysiology: flickering blockade; patch clamp; hypnotics: diazepam; etomidate; receptors: acetylcholine

Introduction

A wide variety of general anaesthetic compounds depress responses to acetylcholine (ACh). At the motor endplate, the decay rate of endplate currents is increased by potent volatile agents, long chain aliphatic alcohols, and barbiturates (Gage & Hamill, 1976; Torda & Gage, 1977; Gage *et al.*, 1978). Single channel recordings have confirmed that all of these agents decrease the open time of ACh-activated ion channels (Lechleiter & Gruener, 1984; Gate & McKinnon, 1985; Murrell *et al.*, 1991; Wachtel & Wegrzynowicz, 1991).

The mechanisms by which pharmacological agents decrease the open time of ACh-activated channels is not well understood. Some agents, such as barbiturates, are thought to enter the open channel to block it (Adams, 1976). Other drugs are thought to partition into the lipid phase of the membrane and alter fluidity in the vicinity of the channel (see reviews by Trudell, 1991; Ueda, 1991). For compounds with general anaesthetic properties, clinical potency in inducing general anaesthesia appears to be directly related to lipid solubility, usually measured as oil/water or octanol/buffer partition coefficient. This suggests that non-specific interactions with hydrophobic moieties may underlie some of the clinical effects of these drugs, and that anaesthetic solubility in the membrane may determine effectiveness.

At the single channel level, the potency of compounds with general anaesthetic properties may also be a function of lipid solubility. Alternatively, these agents may each be interacting with the channel in a unique fashion unrelated to lipid solubility.

The purpose of this study was to compare the effects of a variety of anaesthetic compounds and induction agents, with widely differing structures, in order to determine whether their potency in reducing the open time of ACh-activated channels is a non-specific function of lipid solubility, or whether their effects on ACh-activated channels exhibit structural specificity.

Methods

The methods used in these experiments have been described previously (Wachtel, 1988a; 1990). Patch clamp techniques were used to record single channel currents activated by ACh from cell-attached patches of BC3H1 mouse tumour cells grown in culture. BC3H1 is a muscle-like clonal cell line derived from a mouse cerebrovascular tumour. Cells express nicotinic receptors for ACh when allowed to differentiate in serum-free medium (Munson *et al.*, 1982; Olson *et al.*, 1983). ACh-activated channels in BC3H1 cells have been studied extensively by Sine & Steinbach (1984; 1986).

Cells were bathed in a Ringer solution containing (mM): NaCl 100, KCl 4, $CaCl_2$ 2, $MgCl_2$ 5 and HEPES 10, pH 7.4. Electrodes had resistances of 2–5 Mohm. They were filled with bathing solution containing 250 nM ACh plus anaesthetic agent. Experiments were performed at controlled room temperature (18–22°C).

Dilutions of diazepam (Valium, Roche), etomidate (Amidate, Abbott Laboratories), fentanyl citrate (Sublimaze,

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Janssen Pharmaceutica), ketamine (Ketaset, Aveco), and meperidine (pethidine; Demerol, Winthrop-Breon) were prepared daily. A 10 mM alphaxalone stock was prepared weekly by dissolving the powder (courtesy of Glaxo, Inc) in propylene glycol (1:10), and this stock was diluted daily to yield final concentrations of 20–500 μM in Ringer. The maximum concentration of propylene glycol in the final dilution was 1:200. A propofol (Aldrich) stock of approximately 500 μM was prepared by carefully adding 2 μl to 21 ml Ringer solution. The mixture was gently warmed and sonicated until the propofol was fully dispersed, then diluted daily. The exact concentration of this stock solution was determined by gas chromatography to be 493 μM.

Single channel opening events were recorded with the patch pipette hyperpolarized + 75 mV relative to cell resting potential, which was estimated to be − 59 mV based on a null potential of 0 mV for ACh-activated currents. Thus the potential across the membrane patch was − 134 mV. Signals were low pass filtered at 5 kHz, digitized at 20 μs per point and stored on an AST Premium 286 computer. Single channel events were analyzed to determine channel amplitude and open and closed durations with pCLAMP software package (Axon Instruments). Events containing more than one channel open at a time were not included in the analysis. Average channel amplitude was calculated from the mean amplitude of events > 500 μs in duration. Channel open-time and closed-time distributions were described as sums of exponential components. Parameters were estimated by the method of maximum likelihood (Wachtel, 1988b; 1991). Open-time distributions were corrected for missed openings less than 0.1 ms in duration, but were not corrected for undetected closed times. Similarly, closed-time distributions were corrected for missed closings less than 0.1 ms in duration, but were not corrected for undetected opening events.

Octanol/buffer partition coefficients of ketamine and alphaxalone were measured by gas chromatography because values could not be found in the literature. Aliquots of 10 mM ketamine (10 ml) or 50 mM alphaxalone (4 ml) in 0.1 mM phosphate buffer were mixed with equal volumes of octanol and shaken vigorously. The two phases were separated by centrifugation and the pH of the aqueous phase after centrifugation was 7.4. Samples of the aqueous phase (4 μl for ketamine, 10 μl for alphaxalone) were injected onto a gas chromatograph equipped with a 6-foot column packed with 3% OV-101 on 120/140 gas-chrom Q at 250°C and a flame ionization detector. Similar volumes of standard solutions of known concentrations were also injected. Retention times were 7.5 min for ketamine and 5.4 min for alphaxalone. The octanol/buffer partition coefficient was defined as the ratio of drug concentration remaining in the octanol phase, assumed to be 10 mM ketamine or 50 mM alphaxalone, to the concentration in the aqueous phase. Calculated partition coefficients were 60 for ketamine and 1900 for alphaxalone.

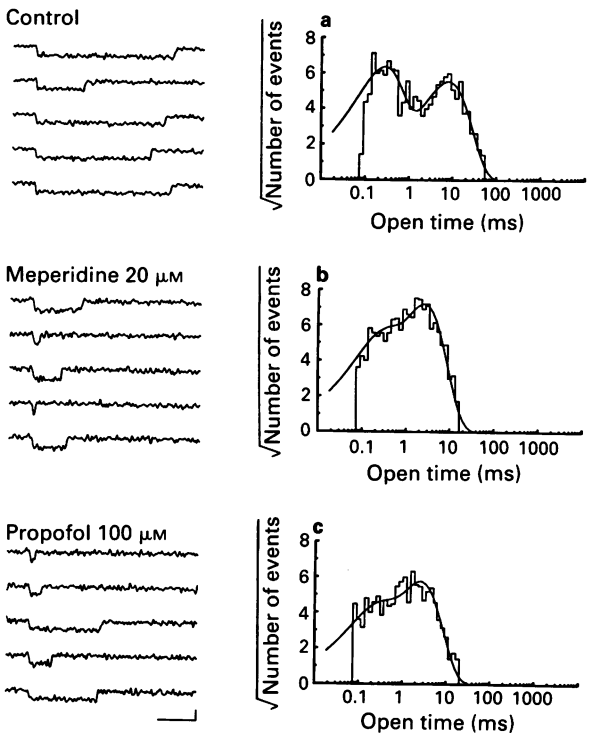

Figure 1 *Left panel*: representative single channel currents activated by 250 nM acetylcholine (ACh) from cell attached patches of BC3H1 cells. Opening events are shown as downward deflections. Channels were recorded in the absence of anaesthetic (control), or with meperidine (20 μM) or propofol (100 μM) in the recording electrode. In the presence of anaesthetic, opening events appeared much briefer in duration. Similar results were obtained with alphaxalone, diazepam, etomidate, fentanyl, and ketamine. Patch potential + 75 mV. Calibration marks 2 ms and 3 pA. *Right panel*: representative open-time histograms. Note logarithmic abscissa and square root ordinate scales. Each smooth curve is the square root of the probability density function corresponding to the sum of 2 exponential components, with time constants τ_f and τ_s ($\tau_f < \tau_s$) and fast component amplitude A_f. The probability density function for each component is exp {ln(t) − ln(τ) − exp [ln(t) − ln(τ)]} multiplied by the number of events in that component times the bin width (Sigworth & Sine, 1987). (a) ACh 250 nM. $\tau_f = 0.26$ ms, $\tau_s = 7.91$ ms, and $A_f = 0.55$. N = 770 events. (b) ACh 250 nM plus meperidine 20 μM. $\tau_f = 0.22$ ms, $\tau_s = 2.50$ ms, and $A_f = 0.27$. N = 852 events. (c) ACh 250 nM plus propofol 100 μM. $\tau_f = 0.21$ ms, $\tau_s = 2.43$ ms, and $A_f = 0.27$. N = 540 events. All of the anaesthetics tested shifted channel open-time distributions toward faster times.

Results

Figure 1 (left panel) shows examples of representative single channel currents activated by 250 nM ACh in the absence and presence of meperidine or propofol. With anaesthetic in the recording electrode, individual opening events appear briefer in duration. All of the agents tested produced qualitatively similar results and decreased the open duration of ACh-activated channels.

Amplitudes

None of the drugs tested altered mean single channel current amplitude, which was 2.68 ± 0.19 pA (mean ± s.d. from n = 49 patches) in the absence of anaesthetic. Channels were either non-conducting or fully-conducting, indicating that channel conductance was altered in an all or none fashion. This lack of effect on single channel amplitude suggests that the anaesthetics were not inducing a rapid flickering block of the open state that was not resolved by the recording system. An extremely rapid flickering block that was low-pass filtered would appear as an apparent reduction in single channel amplitude.

Open times

The distribution of open times from patches exposed to either 250 nM ACh or 250 nM ACh plus 20 μM meperidine or 100 μM propofol are shown in the right panel of Figure 1. Individual open times have been sorted into logarithmically spaced bins to form a histogram. For each of these patches the distribution of open times is well described by the sum of 2 exponential components, $A_f \exp(-t/\tau_f) + (1-A_f) \exp(-t/\tau_s)$, where t is open time and τ_f and τ_s are time constants ($\tau_f < \tau_s$). Similar distributions were obtained with the other agents. For these patches in which both components of open-time distributions could be clearly resolved, $\tau_f = 0.39 \pm 0.20$ (mean ± s.d. from $n = 26$ out of 39 patches) in the absence of anaesthetic and was not altered in any consistent manner by the agents tested.

For patches exposed to higher concentrations of anaesthetic, the faster component was not always apparent and a single time constant was adequate to describe the data. τ (or τ_s for 2-component distributions) was 6.92 ± 1.39 ms ($n = 39$) in the absence of anaesthetic and was decreased in a dose-dependent manner by all of the agents tested (Figure 2).

Based on these dose-response relationships, an EC_{50} was calculated for each anaesthetic (Table 1), where EC_{50} is the concentration that reduced τ_s, or τ, by 50%. EC_{50}s range from a low of 15 μM for ketamine to a high of 99 μM for alphaxalone, a difference of approximately 6 fold.

If the potency of a compound in reducing the open time of ACh-activated channels were solely a function of its concentration in the membrane, then drug potency should be directly correlated with lipid solubility. Figure 3 shows the relationship between EC_{50} and octanol/buffer partition coefficient, used as a measure of lipid solubility, for all of the agents tested. Indeed, the potency of these drugs in decreasing channel open time appears to be directly correlated with their partition coefficients. However, those agents with higher partition coefficients were actually less potent in altering channel open time. This is exactly the opposite of what would be predicted if the potency of a substance were determined merely by its tendency to partition into the membrane. Thus factors other than simple lipid solubility must underlie drug effects on these channels. In order to look for some evidence of a specific interaction between the anaesthetics and ACh-activated channels, as opposed to an interaction based on the colligative properties of drug molecules in the membrane, closed-time distributions were also examined.

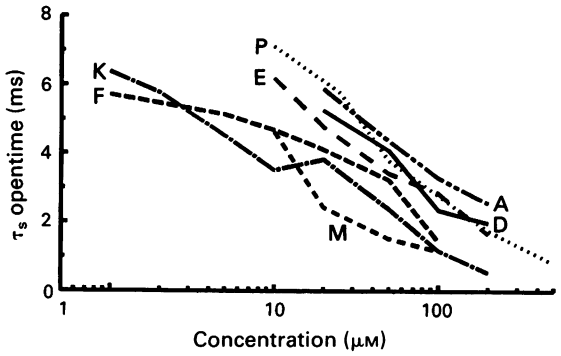

Figure 2 All of the agents tested decreased channel open time in a concentration-dependent manner. τ_s has been used as an indication of channel open time for those patches in which two exponential components were required to describe open-time distributions. Lines are labelled A = alphaxalone; D = diazepam, E = etomidate; F = fentanyl; K = ketamine; M = meperidine; P = propofol. Data points and error bars have been omitted for clarity. In the absence of anaesthetic, τ_s was 6.92 ± 1.39 ms ($n = 39$ patches). Drug concentrations that reduced τ_s by 50% are shown in Table 1.

Table 1 Comparison between EC_{50} values and peak blood levels usually attained after intravenous administration of therapeutic doses

	EC_{50} (μM)	*Peak plasma level (μM)*	*Intravenous dose*
Alphaxalone	99	12	0.4 mg kg^{-1}
Diazepam	66	70	20 mg
Etomidate	57	4.0	0.3 mg kg^{-1}
Fentanyl	26	0.06	50 μg kg^{-1}
Ketamine	15	8.4	2.5 mg kg^{-1}
Meperidine	16	3.6	50 mg
Propofol	81	34	2.5 mg kg^{-1}

Alphaxalone: Hillestad *et al.*, 1974; Sear & Prys-Roberts, 1979; diazepam: Reidenberg *et al.*, 1978; etomidate: Van Hamme *et al.*, 1978; Schuttler *et al.*, 1980; De Ruiter *et al.*, 1981; fentanyl: Hug, 1984; ketamine: Johnston *et al.*, 1974; Wieber *et al.*, 1975; meperidine: Mather *et al.*, 1974; propofol: Cockshott, 1985; Schuttler *et al.*, 1985; Kirkpatrick *et al.*, 1988; Shafer *et al.*, 1988.

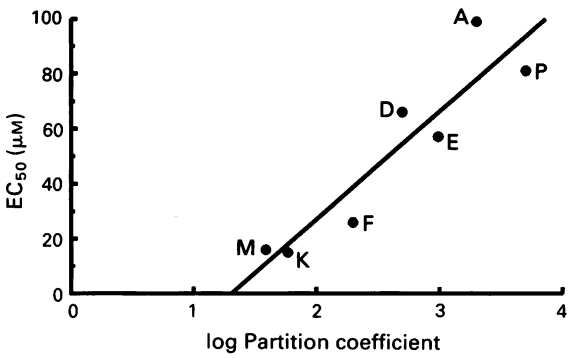

Figure 3 Relationship between the octanol/buffer partition coefficient of a drug and its EC_{50}, the concentration that reduces channel open time by 50% (listed in Table 1). Partition coefficients for alphaxalone (1900) and ketamine (60) were measured; values for diazepam (500), etomidate (1000), fentanyl (200), meperidine (40), and propofol (5000) are from the literature (Leo *et al.*, 1971; Heykants *et al.*, 1975; Hug, 1984; James & Glen, 1980). The EC_{50} for ketamine is from Wachtel (1988). Line is a least squares fit with correlation coefficient 0.92. In contrast to predictions, drugs with higher partition coefficients were less potent in decreasing channel open time.

Closed times

Figure 4 shows typical closed-time distributions for patches exposed to either 250 nM ACh or 250 nM ACh plus 20 μM meperidine or 100 μM propofol. Closed-time distributions for channels activated by ACh typically have 2 distinct components, a rapid closed time τ_f representing gaps within a burst, and a slower closed time τ_s representing gaps between bursts.

Channel closed-time distributions obtained in the presence of meperidine contained an additional intermediate component with time constant τ_i and relative amplitude A_i. At 20 μM meperidine, τ_i = 21.5 ± 9.1 ms and A_i = 0.39 ± 0.05 ($n = 5$). A similar new intermediate component was also observed with ketamine. Apparent differences in τ_s between patches do not necessarily reflect drug-induced changes in channel opening rate, but may simply be due to differences in the number of active channels in each patch.

No additional component in closed-time distributions was observed with propofol ($n = 34$) or fentanyl ($n = 29$). Alphaxalone, diazepam, and etomidate sometimes generated a small third component with amplitude less than 10%, although these findings have not yet been analyzed in detail.

Discussion

All of the agents tested decreased the open time of ion channels activated by ACh. Table 1 shows plasma levels normally attained during therapeutic use of these drugs. Ketamine, meperidine, and propofol altered channel properties at concentrations similar to those encountered clinically. Reductions in the open time of ACh-activated channels may be significant during use of ketamine, meperidine, and propofol, and may contribute to some of the clinical properties and side effects of these agents.

Alphaxalone, diazepam, etomidate, and fentanyl significantly reduced channel open time only at concentrations at least 8 times higher than clinical levels. Fentanyl, a potent synthetic opiate, altered channel properties at concentrations similar to those required for the other agents, but at

concentration 400 times greater than plasma levels. The lack of effect of alphaxalone, diazepam, and fentanyl at clinical concentrations is consistent with theories that these compounds exert their clinical effects by binding to specific receptor sites in the central nervous system. Alterations in the properties of ACh-activated ion channels are most probably unrelated to the clinical properties of these agents.

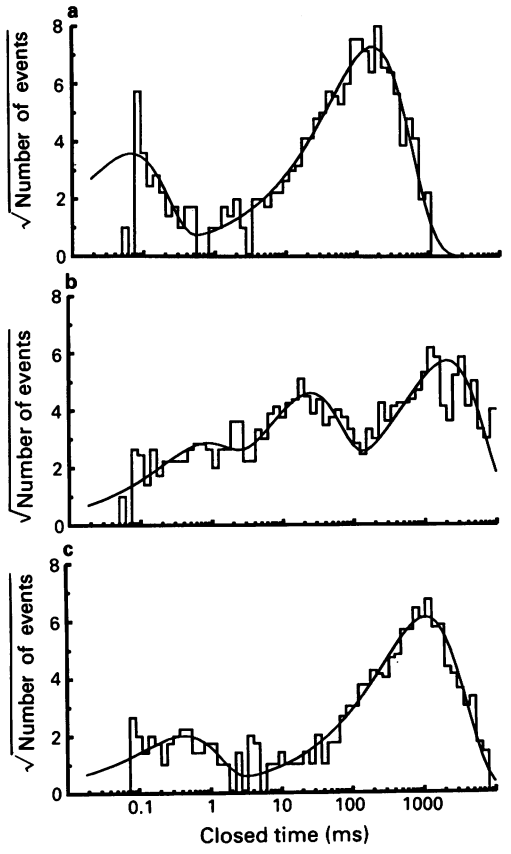

Figure 4 Representative closed-time histograms. Meperidine produces a prominent third component in the distributions, while propofol does not. (a) ACh 250 nM. The distribution of channel open times is described by a function that is the sum of two exponential components, with $\tau_f = 0.07$ ms, $\tau_s = 166$ ms, and $A_f = 0.19$. N = 763 events. (b) ACh 250 nM plus meperidine 20 μM. Three exponential components are required to describe the closed-time distribution, with $A_f = 0.11$, $\tau_f = 0.67$ ms, $A_i = 0.34$, $\tau_i = 23.6$ ms, and $\tau_s = 2032$ ms. N = 801 events. (c) ACh 250 nM plus propofol 100 μM. Two components are adequate, with $\tau_f = 0.43$ ms, $\tau_s = 1116$ ms, and $A_f = 0.10$. N = 545 events. Patch potential + 75 mV.

Lipid solubility, measured in terms of octanol/buffer partition coefficient, does not seem to be an important factor that determines the ability of a drug to decrease the open time of ACh-activated channels in these cells. This finding suggests that it is unlikely these drugs are acting merely by exerting non-specific effects via hydrophobic interactions.

The finding that ketamine and meperidine cause the appearance of a new, intermediate component in channel closed-time distributions suggests the appearance of a new closed state of the channel. Additional experiments are in progress to determine whether this new closed state may arise from block of the open channel by molecules of drug, and also to describe in more detail the changes in closed-time distributions produced by alphaxalone, diazepam, and etomidate.

Propofol and fentanyl, however, did not cause the appearance of an additional component in closed-time distributions, suggesting that a different mechanism may underlie their effects. Although it is possible that these agents were producing a third component in closed time distributions that was not resolved, this component would have to have represented less than 2–3% of events to remain undetected (Wachtel, 1991). The observed constancy of single channel amplitude indicates that these drugs were not inducing an extremely fast flickering type block of the open state that was unresolved due to bandwidth limitations. Upon filtering, a flickering block would appear as a single opening event with reduced amplitude. Such reduced amplitude events were not observed, even at higher drug concentrations that reduced channel open time by ten fold.

It is probably reasonable to conclude that the drugs tested here are not all acting by the same mechanism. In addition, some structural specificity is apparent, since lipid solubility alone is not an important factor in determining drug potency.

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