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The effects of temperature on the interactions between volatile general anaesthetics and a neuronal nicotinic acetylcholine receptor

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1 Completely isolated identified neurones from the right parietal ganglion of the pond snail *Lymnaea stagnalis* were investigated under two-electrode voltage clamp. Neuronal nicotinic acetylcholine receptor (AChR) currents were studied at low acetylcholine concentrations ($\leq$200 nM).

2 Inhibition of the ACh-induced currents by three volatile general anaesthetics (halothane, isoflurane and methoxyflurane) and the specific inhibitor (+)-tubocurarine was studied as a function of temperature (over the range 4-25°C).

3 The inhibition by the volatile anaesthetics increased (inhibition constants decreased) with decreasing temperature while the inhibition by (+)-tubocurarine did not change significantly near room temperature, but decreased at lower temperatures. The (+)-tubocurarine inhibition appeared to be competitive in nature and showed no significant voltage-dependence.

4 The van't Hoff plots (logarithms of the dissociation constants against reciprocal absolute temperature) were linear for the anaesthetics, but markedly non-linear for (+)-tubocurarine. From these plots, values for the changes in the standard Gibbs free energy $\Delta G^{\circ}_{water \rightarrow AChR}$, enthalpy $\Delta H^{\circ}_{water \rightarrow AChR}$, entropy $\Delta S^{\circ}_{water \rightarrow AChR}$ and heat capacity $\Delta C^{\circ}_{p\ water \rightarrow AChR}$ were determined. Tubocurarine was found to bind very much tighter to the receptor than the volatile anaesthetics due, entirely, to a favourable increase in entropy on binding.

5 A comparison between the temperature-dependence of the anaesthetic inhibition of the ACh receptor and that of general anaesthetic potencies in animals indicates that the temperature-dependence of animal potencies might be simply accounted for in terms of changes in anaesthetic/receptor binding.

Keywords: General anaesthesia; inhalational anaesthetics; tubocurarine; ion channel; thermodynamics; enthalpy of binding; entropy of binding; heat capacity; identified molluscan neurones

Introduction

It has been known for nearly a century that the potencies of general anaesthetics change with body temperature. This was first observed with tadpoles (Meyer, 1901), but subsequently shown for a variety of other animals including goldfish (Cherkin & Catchpool, 1964), crustaceans (McKenzie *et al.*, 1992) and mammals (Eger *et al.*, 1965; Regan & Eger, 1967; Munson, 1970; Steffey & Eger, 1974; Vitez *et al.*, 1974; Eger & Johnson, 1987; Antognini, 1993). Surprisingly, for a given anaesthetic, the temperature-dependence of animal potency appears to be rather similar across species, suggesting that there may be some common underlying explanation. An understanding of what this might be may shed some light on the molecular mechanisms underlying the actions of general anaesthetics (Franks & Lieb, 1982; Simon, 1993). It may also have some relevance to clinical practice, since the administration of general anaesthetics during surgery under hypothermic conditions is now routine.

There appears to have been a general reluctance in the field to pursue an explanation of the effects of temperature on animal potencies, in contrast to the great efforts that have been made to understand the effects of pressure (e.g. the pressure-reversal of anaesthesia), and only a handful of studies have been published. One possible reason for this is the feeling that the effects of temperature on an organism, particularly a homeotherm such as a mammal, are likely to be so complex that a simple explanation is unlikely. Nonetheless, one thing is certain: general anaesthetics must act by binding to, or partitioning into, certain target sites in the central nervous system. Studying the way in which temperature affects the interactions between anaesthetics and the putative targets in the central nervous system may provide information on the molecular nature of these targets, and on the extent to which the temperature effects in animals can be accounted for in simple molecular terms.

We have previously shown that the temperature-dependence of binding of volatile general anaesthetics to the anaesthetic-sensitive enzyme firefly luciferase is very different from the temperature-dependence of their partitioning into lipids (Dickinson *et al.*, 1993). (For example, the transfer of halothane from aqueous solution to the firefly enzyme is exothermic, while partitioning of halothane into lipid bilayers is endothermic). In this paper we extend these studies, and present results on the temperature-dependence of the binding of three volatile general anaesthetics to a transmembrane protein - a neuronal nicotinic acetylcholine receptor (AChR). We contrast the effects of temperature on anaesthetic binding with the effects of temperature on the binding of a specific inhibitor of nicotinic AChRs, (+)-tubocurarine. We have chosen to use an ion channel receptor, found in certain identified neurones of the pond snail *Lymnaea stagnalis*, which is inhibited by a wide range of general anaesthetics (McKenzie *et al.*, 1995) with a sensitivity broadly similar to that found in animals. Furthermore, using this system, accurate inhibition measurements can be made over a period of several hours with a single neurone.

Methods

Electrophysiology

Electrophysiological measurements were made using methods similar to those described previously (Franks & Lieb, 1991a;

McKenzie *et al.*, 1995). *Lymnaea stagnalis* snails (1.3 to 4.5 g) were obtained from Blades Biological (Cowden, Kent). All of the measurements were made on a group of three or four neurones which had a yellow-speckled appearance and lay adjacent to one another and to the right of the neurosecretory region in the ventral portion of the right parietal ganglion. The cells were completely isolated from the right parietal ganglion, which had been dissected away intact from the CNS. Identified neurones were isolated by impaling them with two microelectrodes and then slowly moving the ganglion away until the cell and its axons pulled free. This left the cell suspended in a bath (volume ~ 50 μl) which was perfused with solution at a rate of 1–2 ml min^{-1}. Under these conditions, solution exchange around the cell was rapid, and equilibration was generally complete in a few seconds or less. A two-electrode voltage clamp (Axoclamp 2A, Axon Instruments, Foster City, California, U.S.A.) was used, with the current record filtered (10 Hz, −3 dB) by an 8-pole Bessel filter. The current and voltage records were digitised and stored on a computer or video tape. Electrodes were fabricated with 1 mm filamented glass capillaries (Clark Electromedical Instruments, Reading, Berkshire) and were filled with 2.5 M potassium chloride. Electrode resistances were usually in the range 5–30 MΩ.

The temperature of the solution in the bath was controlled in the range 4–25°C by means of a Peltier cooler below the bath and could be typically set with a precision of about 0.2°C. The solutions flowing into the bath were cooled by passing them through polyethylene tubing inside a copper heat exchanger situated between the Peltier device and the recording bath. The temperature was measured with a thermocouple, located close to the cell in the recording bath and connected to a digital thermometer (model 1408K, Digitron Instrumentation, Hertford).

With electrodes filled with potassium chloride, the ACh-induced Cl^- current approached zero as the membrane potential approached zero (McKenzie *et al.*, 1995), the reversal potential having shifted from the normal chloride reversal potential of −70 mV due to increased levels of chloride within the cell. This allowed large ACh-induced Cl^- curents to be measured at a standard holding potential of −80 mV, where contributions from anaesthetic-activated potassium currents (Franks & Lieb, 1988; Franks & Lieb, 1991b) were minimized.

In order to minimize desensitization of the ACh-activated current, low concentrations (≤200 nM) of ACh were used. The anaesthetics methoxyflurane and isoflurane applied on their own did not appreciably affect the resting current. Consequently, these agents were co-applied with ACh without any pre-exposure. Nonetheless, both these agents were applied alone at each temperature to check that there was no significant effect. Halothane, on the other hand, often activated a small inward current when applied alone in the absence of ACh. Halothane was therefore pre-applied before application of ACh, to establish a new baseline. In most cells (6 out of 8), (+)-tubocurarine had no effect when applied alone and was co-applied with the ACh. In two cells, however, (+)-tubocurarine induced a small inward current; for these cells, (+)-tubocurarine was pre-applied to establish a new baseline. The inhibition was calculated with ACh-induced control currents before and after the administration of anaesthetic or (+)-tubocurarine.

The inhibition by anaesthetics was square-dependent (Franks & Lieb, 1991a; McKenzie *et al.*, 1995) and was fit by the equation:

$$\sqrt{\frac{I_o}{I}} = 1 + \frac{A}{K_i} \quad (1)$$

where I_o/I is the ratio of control to inhibited current at an anaesthetic concentration A, and K_i is the inhibition constant (the apparent anaesthetic/receptor dissociation constant). This is consistent with two anaesthetic molecules being able to bind to the receptor (each with an inhibition constant K_i), but only one molecule being necessary for inhibition (Franks & Lieb, 1984; Franks & Lieb, 1991a; McKenzie *et al.*, 1995).

The inhibition by (+)-tubocurarine was linear rather than square-dependent and was fit by the equation:

$$\frac{I_o}{I} = 1 + \frac{c}{K_i} \quad (2)$$

where I_o/I is the ratio of control to inhibited current at a (+)-tubocurarine concentration c, and K_i is the (+)-tubocurarine/receptor inhibition constant. This is consistent with only one molecule of (+)-tubocurarine binding to the receptor.

At each temperature, K_i values (means ± s.e.means) were determined as described previously (McKenzie *et al.*, 1995) using Eqn. 1 for anaesthetics or Eqn. 2 for (+)-tubocurarine. The temperature was then changed and the process repeated. Measurements of K_i were typically made at 4 different temperatures. Usually the experiment was started at room temperature (~20°C), the cell then cooled to the lowest temperature (~5°C), rewarmed to ~15°C, and then cooled again to ~10°C. Even after 3 to 4 h, if the cell was then restored to room temperature, there was no significant difference in the observed inhibition.

HEPES ($pK_a = 7.55$) was chosen as the buffer because of its low temperature coefficient; buffer pH increased by only 0.3 units on cooling from 25°C to 5°C. Nonetheless, in order to check that changing pH over this range had no effect on inhibition, the effect of isoflurane was studied at room temperature at both pH 7.4 and pH 7.7; no significant change in K_i was observed.

Calculation of thermodynamic parameters

From the temperature-dependence of the inhibition constant K_i for inhibition of the ACh current by anaesthetic or (+)-tubocurarine, values of the changes in the standard Gibbs free energy $\Delta G^o_{water \rightarrow AChR}$, enthalpy $\Delta H^o_{water \rightarrow AChR}$, entropy $\Delta S^o_{water \rightarrow AChR}$ and heat capacity $\Delta C^o_{p_{water \rightarrow AChR}}$ were determined for the transfer of 1 mol of anaesthetic or (+)-tubocurarine from the aqueous phase to the receptor. The standard state was 1 molar anaesthetic or (+)-tubocurarine. In experiments with anaesthetics, the van't Hoff plots (of $\ln K_i$ against 1/T, where T is the absolute temperature) were linear. The data were fitted (unweighted least-squares) to the equation

$$\ln K_i = A + \frac{B}{T} \quad (3)$$

For each cell, the thermodynamic parameters $\Delta G^o_{water \rightarrow AChR}$, $\Delta H^o_{water \rightarrow AChR}$ and $\Delta S^o_{water \rightarrow AChR}$ were calculated by use of

$$\Delta G^o_{water \rightarrow AChR} = R(AT + B) \quad (4)$$

$$\Delta H^o_{water \rightarrow AChR} = RB \quad (5)$$

$$\Delta S^o_{water \rightarrow AChR} = -RA \quad (6)$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}) and A and B are the fitted constants. Mean values and associated errors (s.e.means) of $\Delta G^o_{water \rightarrow AChR}$, $\Delta H^o_{water \rightarrow AChR}$ and $\Delta S^o_{water \rightarrow AChR}$ were obtained by combining data from several cells.

The van't Hoff plots for (+)-tubocurarine inhibition showed clear non-linearity. These data were fitted (unweighted least-squares) to the equation

$$\ln K_i = A + \frac{B}{T} + \frac{C}{T^2} \quad (7)$$

where A, B and C are the fitted constants.

For each cell the thermodynamic parameters were calculated by use of

$$\Delta G^{\circ}_{water\rightarrow AChR} = R\left(AT + B + \frac{C}{T}\right) \quad (8)$$

$$\Delta H^{\circ}_{water\rightarrow AChR} = R\left(B + \frac{2C}{T}\right) \quad (9)$$

$$\Delta S^{\circ}_{water\rightarrow AChR} = R\left(-A + \frac{C}{T^2}\right) \quad (10)$$

$$\Delta C^{\circ}_{pwater\rightarrow AChR} = -\frac{2RC}{T^2} \quad (11)$$

Mean values and associated errors (s.e.means) of $\Delta G^{\circ}_{water\rightarrow AChR}$, $\Delta H^{\circ}_{water\rightarrow AChR}$, $\Delta S^{\circ}_{water\rightarrow AChR}$ and $\Delta C^{\circ}_{pwater\rightarrow AChR}$ were obtained by combining data from several cells.

Solutions

The composition of normal saline was: (mM) NaCl 50, KCl 2.5, $CaCl_2$ 4, $MgCl_2$ 4, HEPES 10 and glucose 5; titrated to pH 7.4 at 25°C with NaOH. Anaesthetics and (+)-tubocurarine (chloride salt) were dissolved in normal saline, with and without ACh. The volatile anaesthetics were made up as fractions of saturated solutions in normal saline. The concentrations of the saturated solutions were taken as: isoflurane 15.3 mM (Franks & Lieb, 1991a) halothane 18.0 mM (Seto *et al.*, 1992) and methoxyflurane 9.1 mM (Seto *et al.*, 1992). The sources of the anaesthetics were as follows; halothane (May and Baker, Dagenham, Essex), isoflurane and methoxyflurane (Abbott Laboratories Ltd., Queenborough, Kent). All chemicals other than anaesthetics were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset).

Results

Temperature-dependence of the uninhibited ACh-induced current

The control ACh-induced Cl^- current almost invariably (24 out of 27 cells) increased with decreasing temperature. The extent of the increase was, however, quite variable. This was perhaps due to the counteracting effects of changing intracellular Cl^- concentration at the start of the experiments (which tended to increase the current with time) and general 'run-down' (which tended to reduce the current with time) over the very long time-course of the average experiment (2–4 h). Figure 1 shows the result of pooling all of the data. The slope of the line corresponds to a change of about 3% per degree.

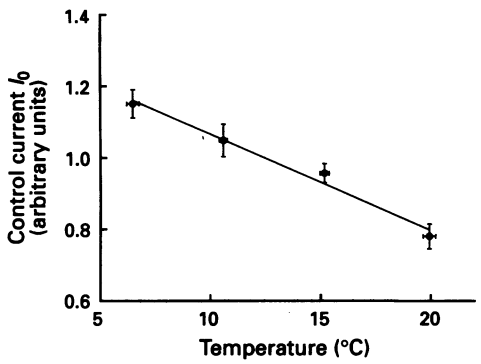

Figure 1 Change in control acetylcholine-induced (150 nM) current with temperature. The data are from 27 cells and have been normalised by setting the control currents to be the same at an arbitrary temperature (12°C) in the middle of the range. Because the exact experimental temperatures varied from cell to cell, results from different cells have been combined by averaging data obtained at similar temperatures. The error bars are s.e.means and the line is an unweighted least-squares straight-line fit to the data. The slope of the line corresponds to a change of about 3% per degree.

Temperature-dependence of the anaesthetic-inhibition of the ACh-induced current

All three anaesthetics inhibited the ACh-induced current more potently as the temperature was lowered. Representative

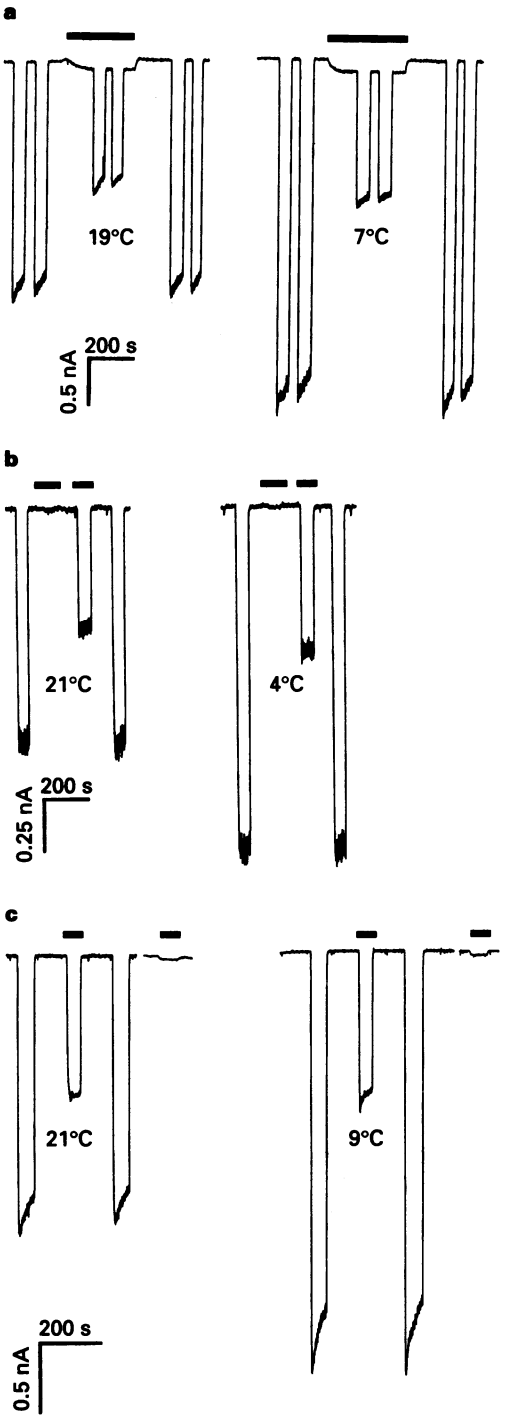

Figure 2 Inhibition of the acetylcholine-induced (150 nM) current by volatile general anaesthetics at different temperatures. Representative examples from three different neurones showing the inhibition by (a) halothane at 0.7 mM, (b) isoflurane at 0.16 mM and (c) methoxyflurane at 0.23 mM. The solid bars indicate the application of the anaesthetic. Only in the case of halothane did the anaesthetic induce an appreciable current on its own. In experiments with halothane, the anaesthetic was pre-applied so that an accurate baseline could be established. The membrane potential was −80 mV.

switches into ACh, with and without anaesthetic, are shown at two different temperatures for halothane (Figure 2a), isoflurane (Figure 2b) and methoxyflurane (Figure 2c). Only in the case of halothane did the anaesthetic on its own cause an appreciable change in the resting current (see Figure 2).

Inhibition constants K_i were determined for the three volatile anaesthetics over a range of temperature between 4–25°C using five to seven cells for each anaesthetic. The van't Hoff plots ($\ln K_i$ against reciprocal absolute temperature) were linear; Figure 3 shows representative van't Hoff plots from individual cells for the three anaesthetics. Table 1 gives mean values for the thermodynamic parameters $\Delta G^o_{water \rightarrow AChR}$, $\Delta H^o_{water \rightarrow AChR}$ and $\Delta S^o_{water \rightarrow AChR}$, together with their associated errors (s.e.means).

Inhibition of the ACh-induced current by (+)-tubocurarine

The ACh-induced current was sensitive to (+)-tubocurarine in the micromolar range. Figure 4a shows the dose-response relationship for the inhibition of the control current at a low (200 nM) concentrations of ACh. The data were fitted to a Hill equation of the form: % inhibition $= 100c^h/(c^h + K_i^h)$, where c is the concentration of (+)-tubocurarine, h is the Hill coefficient and K_i is the (+)-tubocurarine/receptor inhibition constant. The fit gave (means ± s.e.means) $h = 0.98 \pm 0.04$ and $K_i = 0.99 \pm 0.03$ μM. At a high ACh concentration (5 μM), 1 μM (+)-tubocurarine gave no significant inhibition ($n = 3$ cells) of the peak ACh-induced current (see lower inset to Figure 4a). At low concentrations of ACh the inhibition (calculated from the quasi-steady-state *I*-V curves, see Figures 4b–c) by (+)-tubocurarine showed no significant voltage-dependence ($n = 5$ cells).

Temperature-dependence of the (+)-tubocurarine inhibition of the ACh-induced current

The inhibition by (+)-tubocurarine, in contrast to that by the volatile general anaesthetics, did not change significantly near room temperature, and decreased at lower temperatures, giving markedly non-linear van't Hoff plots. A representative example is shown in Figure 4d. Thermodynamic parameters were calculated for each of eight cells, and mean values of $\Delta G^o_{water \rightarrow AChR}$, $\Delta H^o_{water \rightarrow AChR}$, $\Delta S^o_{water \rightarrow AChR}$ and $\Delta C^o_{pwater \rightarrow AChR}$ and their associated errors (s.e.means) are given in Table 1.

Discussion

Effects of temperature on control current

The control ACh-induced currents generally increased with decreasing temperature (about 3% per degree). Although (as mentioned in the Methods section) some uncontrolled time-dependent changes may have influenced the exact magnitude of this temperature-dependence, we are in little doubt of the qualitative trend. Since it has been well-established (Sine & Steinbach, 1984; Quartararo & Barry, 1988; Dilger *et al.*, 1991) that the unitary conductance of muscle-type ACh channels decreases with decreasing temperature (with a $Q_{10} \approx 1.4$), the opposite trend we observe is probably due to a change in the channel kinetics and/or an increase in the binding affinity of acetylcholine for the receptor. Our present data do not allow us to distinguish between these possibilities. However, the change in the control current itself is of little consequence for the analysis described here, because anaesthetic and (+)-tubocurarine inhibitions were always calculated using control currents observed at the same temperature.

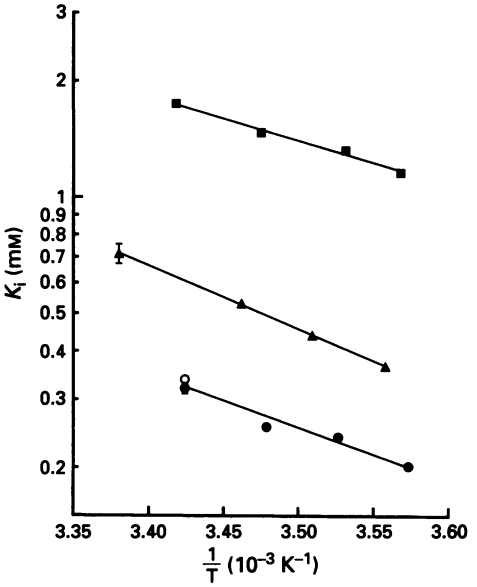

Figure 3 Temperature-dependence of binding of volatile anaesthetics isoflurane (●), halothane (■) and methoxyflurone (▲) to the acetylcholine receptor (AChR). Representative van't Hoff plots of K_i (in mM on a logarithmic scale) against reciprocal absolute temperature. The lines are unweighted least-squares straight-line fits. Each line corresponds to data from an individual cell. (The data shown here were from three different cells). Each data point is the mean of between four and six K_i determinations, and the error bars are s.e.means. The two K_i values for isoflurane at room temperature ($1/T \approx 3.41 \times 10^{-3} K^{-1}$) were determined at the start (●) and end (○) of the experiment. Where error bars are not shown, they were smaller than the size of the symbols. For each anaesthetic, data from between five and seven cells were combined to give the mean values and errors for the thermodynamic parameters $\Delta G^o_{water \rightarrow AChR}$ $\Delta H^o_{water \rightarrow AChR}$ and $\Delta S^o_{water \rightarrow AChR}$ which are listed in Table 1.

Temperature-dependence of anaesthetic inhibition

All three of the volatile anaesthetics, halothane, isoflurane and methoxyflurane, became more potent (i.e. K_i became smaller) as the temperature was lowered. When plotted on van't Hoff

Table 1 Changes in standard Gibbs free energy, enthalpy, entropy and heat capacity for transfer of anaesthetics and (+)-tubocurarine from aqueous buffer to the acetylcholine receptor at 20°C

Inhibitor	n_{cells}	$\Delta G^o_{water \rightarrow AChR}$ (kJ mol^{-1})	$\Delta H^o_{water \rightarrow AChR}$ (kJ mol^{-1})	$\Delta S^o_{water \rightarrow AChR}$ (J mol^{-1} K^{-1})	$\Delta C^o_{pwater \rightarrow AChR}$ (kJ mol^{-1} K^{-1})
Halothane	5	−15.6 ± 0.1	−20 ± 2	−16 ± 8	a
Isoflurane	7	−19.2 ± 0.1	−24 ± 3	−17 ± 9	a
Methoxyflurane	5	−18.0 ± 0.1	−33 ± 3	−50 ± 9	a
Tubocurarine (at 20°C)	8	−33.1 ± 0.1	−2 ± 6	108 ± 21	−2.2 ± 0.5
Tubocurarine (at 10°C)	8	−31.6 ± 0.2	21 ± 4	187 ± 15	−2.4 ± 0.5

The standard state is 1 M anaesthetic or (+)-tubocurarine. The errors are standard errors based on n_{cells}. For each cell there were, typically, 20 measurements of inhibition constant K_i over the temperature range 4–25°C. [a]Not significantly different from zero.

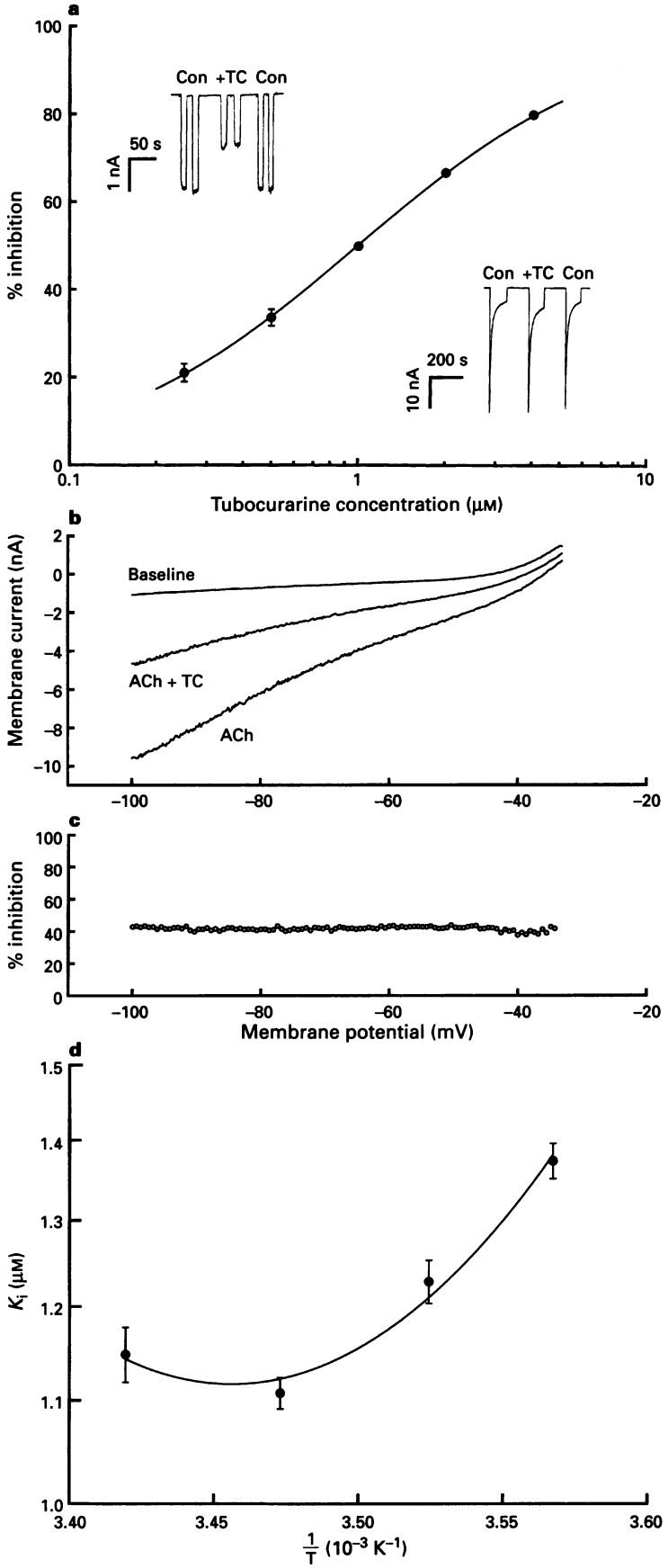

Figure 4 Inhibition of the acetylcholine-induced current by (+)-tubocurarine. (a) The dose-response relationship with 200 nM ACh. The data are from three different cells and the errors are s.e.means. The line is an unweighted least-squares fit to a Hill equation of the form: % inhibition = $100c^h/(c^h + K_i^h)$, where c is the concentration of (+)-tubocurarine, h is the Hill coefficient and K_i is the (+)-tubocurarine inhibition constant. The fit gives $h = 0.98 \pm 0.04$ and $K_i = 0.99 \pm 0.03$ μM. The inset at the top left illustrates a typical segment of data showing the inhibition by 1 μM (+)-tubocurarine at 200 nM ACh. The inset at the bottom right illustrates the lack of effect of 1 μM (+)-tubocurarine at 5 μM ACh. In the insets, Con and +TC refer to the ACh control and (+)-tubocurarine-inhibited responses, respectively. (b) The inhibition of the ACh-induced (200 nM) current by (+)-tubocurarine showed no significant

plots, the logarithm of the inhibition constants K_i decreased linearly with reciprocal absolute temperature (see Figure 3). The inhibition by halothane and isoflurane showed a similar temperature-dependence (about 4% per degree), while the inhibition by methoxyflurane was slightly more sensitive to temperature (changing by about 6% per degree). The linearity of the van't Hoff plots shows that the thermodynamic parameters $\Delta H^o_{water \rightarrow AChR}$ and $\Delta S^o_{water \rightarrow AChR}$ remain constant with temperature, and thus there is no significant change in heat capacity on binding (i.e. $\Delta C^o_p = (\partial H/\partial T)P \approx 0$). It should be pointed out, however, that the error in our determination of $\Delta C^o_{pwater \rightarrow AChR}$ is ~ 0.5 kJ mol^{-1} K^{-1} and thus changes in heat capacity of this magnitude or less would not have been detected. For example, the decreases in heat capacity one might have anticipated (Tanford, 1980; Abraham & Matteoli, 1988) for the transfer of small apolar molecules from water to a hydrophobic site are of about this size (e.g. for ethane being transferred from water to *n*-hexadecane, ΔC^o_p is approximately -0.25 kJ mol^{-1} K^{-1} (Abraham & Matteoli, 1988)) and would not have been measurable in our experiments.

As can be seen from the thermodynamic parameters in Table 1, the temperature-dependence of the anaesthetic K_i values correspond to enthalpy changes on binding of between -20 to -33 kJ mol^{-1}. This is most simply interpreted as the anaesthetics making somewhat more favourable interactions at their binding sites on the receptor than in water. What little *in vitro* work that has been published on the effects of temperature on the interactions of general anaesthetics with putative anaesthetic targets has mostly used non-volatile agents, and the ΔH^o values observed (or which can be calculated) appear to depend both on the system and the anaesthetic (Harper *et al.*, 1983; Bradley & Richards, 1984; Lohse *et al.*, 1984; McLarnon & Quastel, 1984; Prince & Simmonds, 1992; Kosk-Kosicka & Roszczynska, 1993). There are, however, some studies on model systems where direct comparisons can be made, in which the enthalpies of binding of volatile anaesthetics to lipid bilayers (Smith *et al.*, 1981) and the firefly luciferase enzyme (Dickinson *et al.*, 1993) have been determined. These values are listed in Table 2, together with the results of the present study. It is clear from the comparison that the enthalpy changes on binding of anaesthetics to the ACh receptor are similar to those observed for binding to the soluble enzyme firefly luciferase, but very different (in both sign and magnitude) to the changes found for partitioning into lipid bilayers. This means that, as the temperature is reduced, the inhibition of the ACh receptor increases even though the concentration of volatile anaesthetics in lipid bilayers actually decreases. This is consistent with the view that these anaesthetics are acting on the ACh receptor by directly binding to the protein molecule itself, rather than acting by dissolving into the lipid bilayer portion of the membrane (Franks & Lieb, 1991a; Dickinson *et al.*, 1994).

Tubocurarine inhibition and its temperature-dependence

Tubocurarine is a well-characterized antagonist of nicotinic AChRs, and it can act both as a competitive inhibitor and as a channel-blocker, depending upon the source of the receptor (Marty *et al.*, 1976; Manalis, 1977; Colquhoun *et al.*, 1979; Lipscombe & Rang, 1988; Akaike *et al.*, 1989; Bertrand *et al.*, 1990). Our observed ACh-induced currents were sensitive to (+)-tubocurarine in the micromolar range but exhibited no significant voltage-dependence (see Figure 4), suggesting that (+)-tubocurarine was not acting as a channel-blocker. Although we did not investigate the mode of inhibition in any detail, the lack of effect of (+)-tubocurarine at a high concentration of ACh, compared to its potent effect at low concentrations of ACh (see insets to Figure 4a), indicated it was acting as a competitive-inhibitor, as has been found with some other neuronal nicotinic AChRs (Lipscombe & Rang, 1988; Akaike *et al.*, 1989; Bertrand *et al.*, 1990). Despite the fact that there appear to be two equivalent ACh binding sites (McKenzie *et al.*, 1995), we found the inhibition by (+)-tubocurarine increased linearly with concentration and the Hill plot (Figure 4a) had unit Hill coefficient, suggesting only a single (+)-tubocurarine molecule was involved in the inhibition.

The temperature-dependence of the inhibition by (+)-tubocurarine was strikingly different to that observed with the volatile general anaesthetics. Not only were the van't Hoff plots non-linear (see Figure 4d), but, over most of the temperature range, the potency of (+)-tubocurarine decreased with decreasing temperature. A decrease in potency with decreasing temperature has been observed with (+)-tubocurarine acting on AChRs from the rat neuromuscular junction (Dar-

Table 2 Enthalpies of transfer of general anaesthetics from the aqueous phase to animals, the acetylcholine receptor, firefly luciferase and lipids

	$\Delta H^o_{water \rightarrow AChR}$ (kJ mol^{-1})			
Anaesthetic	*Animal*	*AChR*[c]	*Luciferase*[d]	*Lipid*[e]
Halothane	-11 ± 3[a]	-20 ± 2	-18 ± 2	$+11 \pm 1$
Isoflurane	-19 ± 9[b]	-24 ± 3	–	+16
Methoxyflurane	-22 ± 8[a]	-33 ± 3	-24 ± 1	$+1 \pm 2$

[a]Dog: calculated (Dickinson *et al.*, 1993) using the data of Eger *et al.* (1965), Regan & Eger (1967) and Steffey & Eger (1974).
[b]Rat: calculated using the data of Vitez *et al.* (1974).
[c]This study.
[d]Dickinson *et al.* (1993).
[e]Calculated (Dickinson *et al.*, 1993) using the data of Smith *et al.* (1981) for bilayers of phosphatidylcholine/phosphatidic acid/cholesterol (no error is given for isoflurane because data at only two temperatures were published).

voltage-dependence ($n = 5$ cells). Quasi-steady-state *I*-V curves were generated by slowly (3 mV s^{-1}) ramping the membrane potential from -100 mV to -35 mV. In this example, the (+)-tubocurarine concentration was 1.42 μM. (c) The % inhibition of the ACh-induced current by (+)-tubocurarine, calculated using the data in (b), was essentially constant with membrane potential. (d) Temperature-dependence of binding of (+)-tubocurarine to the AChR at 150 nM ACh. Representative van't Hoff plot of K_i (in μM on a logarithmic scale) against reciprocal absolute temperature for an individual cell. The line is an unweighted least-squares second order polynomial fit. Each data point is the mean of between four and five K_i determinations, and the error bars are s.e.means. Data from eight cells were combined to give the mean values and standard errors for the thermodynamic parameters $\Delta G^o_{water \rightarrow AChR}$, $\Delta H^o_{water \rightarrow AChR}$, $\Delta S^o_{water \rightarrow AChR}$ and $\Delta C^o_{pwater \rightarrow AChR}$ which are listed in Table 1.

veniza *et al.*, 1979) and on rat diaphragm (Horrow & Bartkowski, 1983). The curvature in the van't Hoff plots means that the enthalpy of binding did not remain constant with temperature; the mean value of $\Delta H^{o}_{water \rightarrow AChR}$ changed from roughly zero at room temperature to about $+20$ kJ mol^{-1} at 10°C (see Table 1). This change in enthalpy with temperature corresponds to a change in heat capacity on binding of about -2.3 kJ mol^{-1} K^{-1}. Although, more often than not, van't Hoff plots of ligands binding to membrane receptors are linear within experimental error (see e.g. Weiland *et al.*, 1979; Quast *et al.*, 1982; Ruiz-Gómez *et al.*, 1989; Maksay, 1994), curvilinear van't Hoff plots are not uncommon, and decreases in heat capacity on binding of between about 1–6 kJ mol^{-1} K^{-1} have been reported, for example, for ligands binding to muscarinic ACh receptors (Gies *et al.*, 1986), β-adrenoceptors (Morin *et al.*, 1984) and glucocorticoid receptors (Eliard & Rousseau, 1984). For the transfer of small apolar molecules to hydrophobic solvents, a decrease in the heat capacity is the rule rather than the exception (Tanford, 1980; Abraham & Matteoli, 1988), and such changes (typically ~0.5 kJ mol^{-1} K^{-1}) are often interpreted in terms of the 'hydrophobic effect' and a change in water structure around the molecule upon transfer. To what extent this effect contributes to the decrease in the heat capacity we have observed when (+)-tubocurarine binds to the ACh receptor is difficult to say; there may well be a conformational change when (+)-tubocurarine binds to the receptor (we only observe a single molecule binding to a receptor which has two ACh binding sites), so it is possible that some of the change in heat capacity is associated with a change in protein conformation.

Comparison of anaesthetic and (+)-tubocurarine binding

As we have argued elsewhere (Dickinson *et al.*, 1993), the binding of an anaesthetic to a target site can never, in isolation, be meaningfully-described as being either 'enthalpically' or 'entropically' driven. This is because the relative magnitudes of ΔH^{o} and ΔS^{o} are dependent upon the arbitrary choice of standard state. For example, given the data in Table 1 one might be tempted to say that the binding of isoflurane to the AChR was largely enthalpically driven ($\Delta H^{o}_{water \rightarrow AChR} = -24$ kJ mol^{-1}), with a small entropy change opposing the transfer ($-T\Delta S^{o}_{water \rightarrow AChR} = +5$ kJ mol^{-1}). However, this would be to forget that the choice of 1 molar as the standard state, while widely used, is completely arbitrary. Any reference concentration could have been chosen with equal validity. If a different standard state is chosen, then the calculated values of ΔG^{o} and ΔS^{o} change (by the same amount) while the values of ΔH^{o} and ΔC^{o}_{p} remain constant (since they are independent of standard state). Using one particular standard state a binding reaction may appear to be 'entropically-driven', while using another it may appear to be 'entropically-driven'. The fallacious claims that particular binding interactions are either 'entropically-driven' or 'entropically-driven' are widespread in the literature, and often lead to unwarranted molecular interpretations in terms of, for example, changes in water structure or protein conformational changes.

What can be justified, however, is a comparison of one binding interaction with another. For example, an interpretation of the changes $\Delta\Delta G^{o}_{water \rightarrow AChR}$ and $\Delta\Delta S^{o}_{water \rightarrow AChR}$ can be made because these changes are not dependent on the choice of standard state. (For example, the binding of a group of γ-aminobutyric acid type A agonists might be said to be relatively more entropically-driven than a group of antagonists (Maksay, 1994), but neither group can be said to be entropically-driven in an absolute sense). Thus while we cannot make molecular sense of the relative values of $\Delta H^{o}_{water \rightarrow AChR}$ and $\Delta S^{o}_{water \rightarrow AChR}$ for any one anaesthetic, we can contrast the binding of (+)-tubocurarine with the volatile agents, even though these agents probably bind to quite different sites. From Table 1 it is evident that the thermodynamics of the interactions of anaesthetics and (+)-tubocurarine with the AChR are very different. For the anaesthetics the transfer is enthalpically favourable, while for (+)-tubocurarine the reverse is the case. Moreover, the entropy changes for the binding of (+)-tubocurarine are considerably more positive than those for the binding of the anaesthetics. Thus (+)-tubocurarine binds very much tighter to the receptor than the anaesthetics due, entirely, to a favourable change in entropy. This may be due to changes in the water of hydration of the site and/or inhibitor on binding, or due to conformational changes in the receptor.

Comparison with animal potency data

One of the main reasons for undertaking this work was to see if the temperature-dependence of general anaesthetic potencies in animals could be simply accounted for in terms of the molecular interactions of these agents with putative target sites such as nerve ion channels. This amounts to a comparison of the enthalpy changes that we have observed for the anaesthetics binding to the AChR and those derived from animal potency changes (from van't Hoff plots calculated using gas phase data, together with gas/water ΔH^{o} values). As can be seen from Table 2 there is surprisingly close agreement between the enthalpy values associated with receptor binding and those derived from animal potency measurements, although the anaesthetics show a somewhat larger temperature-dependence for inhibiting the AChR than that found with animals. This comparison suggests that, all other things being equal, the simple mechanism of enhanced receptor binding as the temperature is lowered is sufficient to account for the changes in anaesthetic potencies that are observed in animals. Of course it is far from certain that all other things are equal as the temperature changes, and the effects of temperature *per se* on synaptic transmission are bound to complicate this simple picture. Nonetheless, it will be interesting to see if the changes in anaesthetic binding with temperature that we have observed with both the AChR and the luciferase enzyme are found with other proteins and ion channels.

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