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Fast and slow interactions of n-alkanols with human 5-HT_3A receptors: Implications for anesthetic mechanisms

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ABSTRACT

This is part of a continuing patch-clamp study exploring molecular actions of anesthetics and systematically varied related substances on 5-HT_3A receptors as prototypes of ligand-gated ion channels. Specifically, n-alkanols, related to but simpler in structure than propofol, were studied to explore the complex actions of this leading intravenous anesthetic.

Outside-out patches excised from HEK 293 cells heterologously expressing human 5-HT_3A receptors were superfused with even-numbered n-alkanols (ethanol through n-tetradecanol) of different concentrations. Fast solution exchange for varying durations allowed separation of drug actions by their kinetics.

Compared with propofol the electrophysiological responses to n-alkanols were not much simpler. n-Alkanols produced fast and slow inhibition or potentiation of current amplitudes, and acceleration of current rise and decay time constants, depending on exposure time, concentration, and chain-length of the drug. Inhibition dominated, characterized by fast and slow processes with time constants separated by two orders of magnitude which were similar for different n-alkanols and for propofol. Absolute interaction energies for ethanol to n-dodecanol (relative to xenon) ranged from −10.8 to −37.3 kJ mol^{-1}.

No two n-alkanols act completely alike. Potency increases with chain length (until cutoff) mainly because of methylene groups interacting with protein sites rather than because of their tendency to escape from the aqueous phase. Similar wash-in time constants for n-alkanols and propofol suggest similar mechanisms, dominated by the kinetics of conformational state changes rather than by binding reactions.

1. Introduction

Why is ethanol in contrast to propofol such a poor general anesthetic, although both drugs share several physicochemical characteristics? Though known to mankind for millennia ethanol has never made it into routine clinical practice, while propofol has become the most widely used clinical intravenous anesthetic since its introduction less than fifty years ago.

This and future papers are to explore the differences between the intravenous anesthetic propofol and inhalation anesthetics while trying to understand at the same time which molecular characteristics of alcohols – which in several ways have physicochemical properties intermediate between these drug classes – may prevent them from being good general anesthetics. Although not differing much in their lipophilicity, intravenous anesthetics appear to have generally greater hypnotic potency than inhaled anesthetics. They act also more potently on ion channels, of which the ligand-gated ion channels are generally more susceptible than voltage-gated ion channels [1].

There is agreement in the anesthetic literature that there is disagreement as to which ion channels are relevant to general anesthesia, depending on which prominent author you read. Any but superficial consideration of what defines general anesthesia will result in reluctance to state exclusively which ion channels are relevant to general anesthesia. Particularly also the failure of the drug industry to profit from the molecular genetic approach to synthesizing better and specific drugs has led to a new appreciation of the importance of so-called "dirty drugs", acting at many receptors. General anesthetics are dirty drugs par excellence, which have been shown already to act on a number of different receptors.

This is part of a systematic study exploring molecular actions of anesthetics and anesthetic-like substances on membrane proteins [1], here on 5-HT_3A receptors as prototypes of ligand-gated ion channels. In contrast to other ligand-gated ion channels, the homopentameric 5-HT_3A receptor forms functional ion channels, which simplifies subsequent kinetic analyses. It also has a slow onset kinetic thus allowing the resolution of fast wash-in kinetics of anesthetic agents which would be difficult to detect for faster activating ligand-gated ion channels. Kinetic models emerging from systematic studies on 5-HT_3A

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receptors can subsequently be extended and tested on other ligand-gated ion channels.

In a series of studies, both published [2–5] and under way, anesthetic target sites are characterized and mapped by a systematic variation of anesthetic-related drugs serving as probes, in contrast to the complementary approach of site-directed mutagenesis.

These and similar studies of voltage-gated sodium and potassium channels [6,7] as well as other proteins [8–10] suggest that several but weak interactions with many proteins are characteristic of general anesthetic action, involving both polar and nonpolar, both specific and nonspecific interactions [1]. The drug with the weaker polar group appears to be the better anesthetic: thus propofol (its phenolic hydroxyl group shielded by two isopropyl neighbors on either side) rather than phenol, and diethyl ether rather than ethanol (the ether oxygen being less polar than a hydroxyl group) have been in clinical use [11]. A hypothesis based on the homeostasis of lipophilic substances has been proposed as a mechanism of anesthesia that would allow many anesthetic actions to occur simultaneously, yet not result in physiological chaos [11].

Regarding propofol we found that it was considerably less potent than other intravenous anesthetics in its actions, yet the actions of propofol and structurally closely related phenol derivatives were complex, affecting both the amplitudes and the kinetics of 5-HT induced currents [2–4]. The combination of patch-clamp of excised outside-out patches with a fast solution exchange system allowed the kinetic separation of different anesthetic actions on 5-HT_3A receptors as well as the examination of their current kinetics, leading to the identification of at least two separate actions with time constants in the tens of milliseconds range and in the seconds range, respectively. Whereas overall actions on 5-HT_3A receptors were inhibitory, an enhancing component of anesthetic action could be detected in some phenol derivatives. This component is normally masked by an overall suppression in equilibrium conditions. Underlying mechanisms appeared to involve the phenolic hydroxyl group, hydrophobic interactions, and steric restrictions [4].

In this paper, n-alkanols are investigated because as simple acyclic alcohols they are related to propofol but are less complex in structure. Thus they share with propofol and similar phenol derivatives a common structure consisting of a hydrophobic moiety and a polar hydroxyl group (–OH). They differ from propofol and are simpler in that they do not possess branched hydrocarbons and, not being aromatic, lack delocalized electrons. The balance of hydrophobic and polar interactions can be shifted in the direction of hydrophobic properties becoming increasingly more dominant when the chain length of the n-alkanol is increased, without the structure of the n-alkanol changing. By contrast, in the series of alkylated phenols, the structure does change as the hydroxyl groups of 2-isopropylphenol and of propofol are surrounded by one or two isopropyl groups, respectively; this may compromise the accessibility of the hydroxyl group compared with n-alkanols [4].

Steric interactions and/or molecular size have been suggested as potential causes preventing propofol from having the current-enhancing effects on 5-HT_3A receptor channels described above [4]. n-Alkanols may be suitable probes to distinguish between these possibilities as they have been reported to also cause potentiation of 5-HT induced currents, with a molecular cutoff occurring around n-hexanol [12,13].

Using our previous experimental approach the aims of this study were (i) to identify separate components of action of n-alkanols and to test whether the greater structural simplicity of n-alkanols is reflected in less complex current responses, (ii) to quantitate the contributions of polar and non-polar groups to the interaction energy and to test whether physicochemical functional groups can be correlated with electrophysiological action, (iii) to compare the actions of n-alkanols with those of propofol and phenol derivatives, and (iv) to provide the basis for future comparisons with ongoing studies where the balance between hydrophobic and polar contributions is shifted, alternatively, by changing the polar groups, resulting in structurally even simpler inhalation anesthetics such as dialkyl-ethers and n-alkanes.

2. Materials and methods

2.1. Cell culture

Transfected human embryonic kidney 293 cells stably expressing human 5-HT_3A receptors [14] were grown as mono-layers on culture plates (NUNC, Wiesbaden, Germany) in DMEM Nutrient Mix F12 medium containing 10% heat-inactivated fetal calf serum, penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), geneticine (0.75 μg ml^{-1}) and glutamine (292 μg ml^{-1}). The cells were cultured at 37 °C in a humidified atmosphere (5% CO_2) and afterwards transferred to 35 mm Petri dishes (NUNC). The cells were ready for electrophysiological experiments 7-11 days after transfer.

2.2. Drugs and solutions

Ethanol, n-butanol and n-hexanol were obtained from Merck (Darmstadt, Germany), n-octanol from Fluka Chemie AG (Buchs, Switzerland), n-decanol, n-dodecanol and n-tetradecanol from Sigma-Aldrich (Munich, Germany). 5-HT (serotonin creatinine sulfate complex) was obtained from Sigma (Munich, Germany). 30 μM (unless specified otherwise) 5-HT solutions were prepared daily from 25 mM aqueous stocks (stored at −20 °C). Drug solutions for ethanol, n-butanol, n-hexanol and n-octanol were prepared daily by dissolving the pure substance in extracellular solution. When n-decanol was prepared in this manner, it had to be stirred for at least 48 h to ensure that it had completely dissolved. n-Decanol was also prepared from ethanolic stock (10 mM) by dilution, resulting in a maximal ethanol concentration of 4.3 mM. Both ways of preparing n-decanol solutions resulted in $IC_{50}^{steady\text{-}state}$ values for current inhibition that were not significantly different. n-Dodecanol and n-tetradecanol solutions were only prepared from ethanolic stock solutions (10 mM), resulting in a maximal ethanol concentration of 1.7 mM.

2.3. Electrophysiology

Currents through the 5-HT_3A receptor were measured in excised outside-out patches with the patch-clamp technique. Before starting patch-clamp recordings, the culture medium was replaced by 'extracellular' solution of the following composition (mM): NaCl 150; KC1 5.6; $CaCl_2$ 1.8; $MgCl_2$ 1.0; HEPES 10; D-glucose 20; and pH 7.4. The extracellular solution used for superfusion contained no D-glucose. Patch pipettes with resistances of 2–6 MΩ were manufactured from borosilicate glass capillaries (Kwik-FilTM, World Precision Instruments, U.S.A.) using a pipette puller (List L/M-3P-A) and filled with intracellular solution composed of (mM): KC1 140, EGTA 10, $MgCl_2$ 5, HEPES 10, and pH 7.4. Experiments were performed at room temperature (18–22 °C). For current measurements we used a patch-clamp amplifier (EPC-7, List Electronic, Darmstadt, Germany) in combination with an external low pass filter set at 1 kHz (either Frequency Devices, MA, U.S.A. or LPBF-48DG, NPI Electronic, Tamm, Germany). Data were digitally recorded at a sampling rate of 2 kHz with a Digidata 1200 (Axon Instruments, Foster City, CA, U.S.A.) interface and protocols of the current responses were recorded with Clampex 6 software (Axon). 500 ms before 5-HT exposure, the membrane potential was hyperpolarized from 0 to −100 mV.

2.4. Drug application modes

The drug application system was a multi-tube superfusion system (RSC 200, Biologic, France) with a flow rate of 1–2 ml/min. It contained a rotating head equipped with five or nine separate glass capillaries as perfusion pipets, allowing the application of five (5-tube configuration) or nine (9-tube configuration) different solutions. With the RSC 200 superfusion system, maximally two (never three or more) solutions

flow simultaneously at any one time (only one solution flows onto the patch and in its immediate vicinity).

Its stepping motor was set to complete a rotation to a new position within 32 ms. Considering that it takes currents induced by 30 μM 5-HT about 20 ms to reach their peak, the fastest wash-in data point that can be reported by the system is 20 ms when 5-HT and the drug are applied simultaneously, and 52 ms (i.e. 32 ms + 20 ms) when the drug and 5-HT are applied successively. In order to minimize loss of lipophilic drugs, all parts of the superfusion system were made of inert materials like Teflon® and glass, except for a short piece of tubing and valve provided with the superfusion system (RSC 200, Biologic, France) and the initial drip chamber (Intrafix SafeSet, B. Braun Melsungen AG, Germany) serving as reservoir [3].

The reproducible positioning of the patch in relation to the perfusion pipet is critical. It is verified during an experiment by checking many parameters of the kinetic responses of the elicited currents (delay of response, number of rise time constants and decay time constants, inhomogeneities in the current responses). When an outside-out patch was excised from a smooth, angularly shaped cell, extracellular solution was applied for a period of 60 s before starting the measurements. Between each application the patch was 'washed out' with extracellular solution. Because of the typical slow recovery of 5-HT_3A receptors from desensitization following 5-HT exposure, a minimum of one minute was allowed to elapse before the next current was elicited. If the duration of the application of the n-alkanol exceeded one minute, the same amount of time was allowed for wash-out before another current was recorded.

Each experimental run with the 5-tube configuration consisted of a total of three control currents (30 μM 5-HT only) and three currents under drug application (30 μM 5-HT and drug), alternating between control and drug. To compensate for rundown effects, the three currents under control conditions and the three currents during drug exposure were averaged, respectively, before further analysis ($\tau_{rundown}$ = 550 ± 230 s, n = 6 patches under control conditions only, with no drug exposure, each patch lasting at least three time constants). In the case of n-tetradecanol (4 min wash-in), it was not always possible to have three successive runs each of control and drug application (requiring a patch lifetime of at least 24 min); therefore, to compensate for rundown effects the controls bracketing a drug application were averaged before further analysis.

The 9-tube configuration allowed the application of three different drug concentrations to the same patch. An experimental run always followed the sequence: control, drug (concentration 1), control, drug (concentration 2), control, drug (concentration 3), control, drug (concentration 1), … and was repeated as long as the patch lasted, always starting with the lowest drug concentration. To compensate for rundown effects, the controls bracketing a drug application were averaged before further analysis.

When two current traces resulting from a drug being applied in two different modes were compared in the same figure (Fig. 1), they were corrected for rundown by multiplying the second (drug) trace by the ratio of the respective control currents. This correction was typically of the order of 5–10%.

The 9-tube configuration was also used to measure the effect of n-alkanol on currents elicited by 1, 2 or 30 μM 5-HT, respectively. In this case, an experimental run in the open-channel application followed the sequence control (30 μM 5-HT), control (1 μM 5-HT), n-alkanol + 1 μM 5-HT, control (2 μM 5-HT), n-alkanol + 2 μM 5-HT, n-alkanol + 30 μM 5-HT, control (30 μM 5-HT), … , and was repeated as long as the patch lasted.

Three different protocols of drug application were used:

- *Steady-state application*: continuous exposure to the drug 60 s before and during the application of 5-HT.
- *Wash-in application*: similar to steady-state application except that the 60 s wash-in period is replaced by a wash-in period varying between 52 ms and 270 s.
- *Open-channel application*: no drug application prior to the 5-HT pulse, drug application only simultaneously with 5-HT.

2.5. Data analysis and statistics

Analysis was performed with pClamp 8 (Axon, Foster City, CA) and GraphPad Prism 5 software (GraphPad, CA, USA), which was also used for the creation of graphics.

Whenever possible, the time courses of 5-HT induced currents were fitted simultaneously for onset and decay with a biexponential function (pClamp 8, Axon), yielding the time constants for current onset (τ_{rise}) and current decay (τ_{decay}). The decay of currents from this subtype of 5-HT_{3A} receptor can generally be described by a single time constant [14]. Rarely did currents have to be fitted separately for the onset or decay phases, using a single exponential function respectively.

We choose to use the terms τ_{rise} (instead of $\tau_{activation}$) for current onset and τ_{decay} (instead of $\tau_{desensitization}$) for current decay because in the presence of n-alkanols we can no longer assume that the rising and decaying phases of the 5-HT evoked current simply reflect activation and desensitization processes modified directly by the drug. Instead, other blocking processes, independent of activation and desensitization mechanisms, may contribute to modified time constants of the rising and decaying phases, respectively.

The concentration-response curves were fitted by the Hill-Equation $i = [1 / (1 + c^n / IC_{50}^n)]$, i is the remaining peak current as fraction of the maximal (control) current, c is the drug concentration, n is the Hill coefficient, and IC_{50} is the drug concentration causing half-maximal effect.

Wash-in curves were fitted with a biexponential decay function of time (t):

$$I(t) = I_\infty + F_{fast} * (1 - I_\infty) * exp\left(-t/\tau_{fast}\right) + \left(1 - F_{fast}\right) * (1 - I_\infty) * exp(-t/\tau_{slow}), \quad (1)$$

where $I(t)$ is normalized such that $I(t = 0) = 1$, $I_\infty = I(t \to \infty)$, and F_{fast} is the fraction that the fast component contributes to the total effect. Wash-in curves were fitted in two different ways. i) Unrestricted fit with free parameters: independently varying the parameters I_∞, F_{fast}, τ_{fast} and τ_{slow} for each n-alkanol, and ii) restricted fit with shared parameters: varying τ_{fast} and τ_{slow} but keeping them the same for each n-alkanol, and freely varying the parameters I_∞ and F_{fast} for each n-alkanol.

The free energy of transfer of n-alkanols between the input phase (gas or aqueous) and the target site(s), $\Delta\mu^{\theta,CH_2}$, can be obtained as the slope of a linear regression when the mole-fraction of the n-alkanol is plotted against the number of methylene groups it contains according to the formula [15]:

$$\log x_{in,50\%}^{ROH} = \frac{n\Delta\mu^{\theta,CH_2}}{RT} + const. \quad (2)$$

where

$$const. = \log a_{site,50\%}^{ROH} + \frac{\left(\Delta\mu^{\theta,OH} + \Delta\mu^{\theta,CH_3}\right)}{RT} \quad (3)$$

$x_{in,50\%}$ is the mole fraction of the n-alkanol *ROH* in the input phase at which it causes 50% suppression of the peak current; log is the natural logarithm; n is the number of methylene groups in the n-alkanol; $\Delta\mu^\theta$ is the standard free energy (or standard chemical potential) of transferring the respective functional group from the input phase to the site of anesthetic action; and $a_{site,50\%}$ is the corresponding chemical activity at the site of anesthetic action. The assumption is made that the interaction takes place when the n-alkanol is in equilibrium with its target site(s) and that the activities at the site of anesthetic action are equal for all n-alkanols when they cause 50% suppression [15].

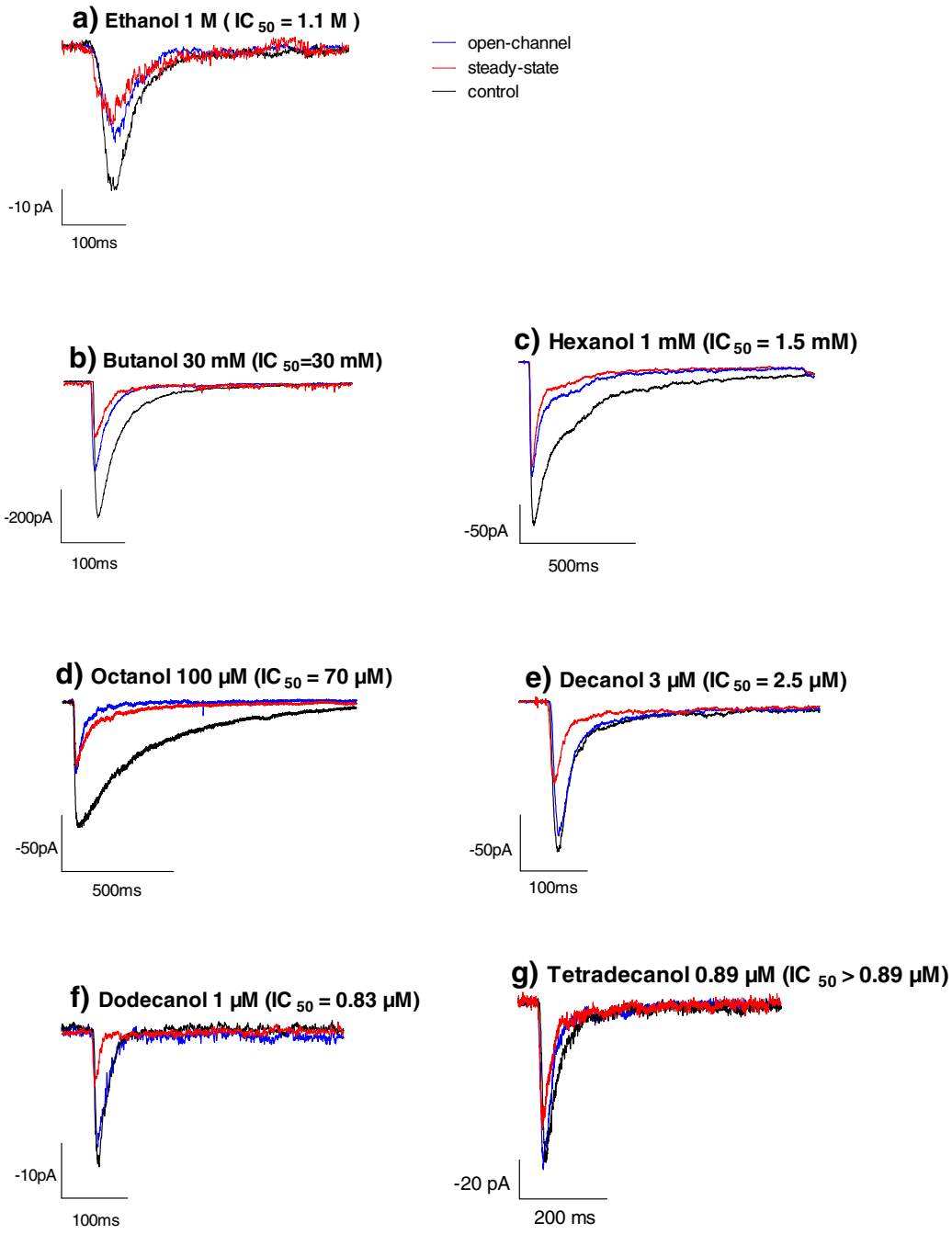

Fig. 1. Current responses to open-channel and steady-state applications of n-alkanols differ and depend on chain-length. Representative original traces of 5-HT (30 µM)-induced currents under control conditions and with drug given in either open-channel or steady-state application modes. For any given n-alkanol, open-channel as well as steady-state applications were recorded at the same n-alkanol concentration (close to the respective $IC_{50}^{steady\text{-}state}$) and from the same patch as the control current (see Materials and methods). Note that n-tetradecanol at the highest achievable aqueous concentration caused less than 50% current reduction in the steady-state application.

The difference in free energies of transfer of two drugs between the gaseous phase and the 5-HT_3A receptor target site, $\Delta\mu^{\theta,drug1} - \Delta\mu^{\theta,drug2}$, at the concentration where the drugs have 50% effect is:

$$\Delta\mu^{\theta,drug1} - \Delta\mu^{\theta,drug2} = RT \cdot \left(\log x_{gas,\ 50\%}^{drug1} - \log x_{gas,\ 50\%}^{drug2}\right) = RT \cdot \log\left(P_{50}^{drug1} / P_{50}^{drug2}\right) \quad (4)$$

where $x_{gas,\ 50\ \%}^{drug}$ is the mole fraction of the drug at 50% effect and P_{50}^{drug} is the corresponding partial pressure of the drug according to the ideal gas law, and it is assumed that at 50% effect the activities of both substances are the same at the receptor target site [15].

Results are reported as means ± standard deviations (SD). Differences between single data points were tested for significance with Student's t tests (Microsoft Excel and GraphPad Prism5). Differences were considered significant when p values for the respective test were less than 0.05. Linear regression fits were performed with GraphPad Prism5, r^2 being the square of the linear correlation-coefficient. IC_{50} values and their 95% confidence intervals for concentration-response curves were obtained from curve fits with GraphPad Prism5. The fit parameters of the wash-in curves, their 95% confidence intervals and R^2 values (quantifies goodness of fit) were also obtained with GraphPad Prism5.

3. Results

n-Alkanols by themselves (in the absence of 5-HT) did not induce any significant current responses. For each of the five n-alkanols from n-butanol to n-dodecanol any current response was below 3.5% of the amplitude of the subsequently 5-HT-induced current in the same patch (4–6 patches and between 12–14 measurements for each n-alkanol) and thus below the level at which currents could be resolved reliably.

When 5-HT_3A receptor channels in excised outside-out patches were exposed to n-alkanols, the currents elicited by 5-HT (30 μM) were reduced when compared to the respective control currents (Fig. 1). 30 μM 5-HT represents a concentration at which the response of the 5-HT_3A receptor channel is close to maximal; it serves as reference both with regard to channel functionality as well as for comparing the actions of many other anesthetics and anesthetic-like substances. Current traces are shown for n-alkanol concentrations close to their respective IC_{50} values for steady-state application ($IC_{50}^{\text{steady-state}}$, see below). These traces do not always show 50% effect because of experimental variation and the steepness of the concentration-response curve in this region. As the chain length of the n-alkanol increased, inhibition in the open-channel application became increasingly less such that n-alkanols longer than n-decanol had no significant effect on the peak current any longer.

The traces in Fig. 1 show clearly that 5-HT induced currents are inhibited more after 60 s of n-alkanol exposure (steady-state application) than after approximately 20 ms (open-channel application). In order to determine how long it takes for the inhibition by n-alkanols to be complete, wash-in experiments were conducted in which 5-HT_3A receptors were exposed to n-alkanols for durations between 20 ms (open channel application) and 60 s (steady-state application). However, in order to compare different n-alkanols at equivalent potencies, their $IC_{50}^{\text{steady-state}}$ concentrations had to be determined first. These were established by bracketing the $IC_{50}^{\text{steady-state}}$ values with concentrations below and above (Fig. 2). Importantly, in most experiments these three concentrations were applied to the same patch, considerably reducing the number of experiments necessary to achieve statistical significance.

Concentration-response curves for open channel applications are shifted to higher concentrations compared with those for steady-state applications (Fig. 2). No attempt was made to bracket $IC_{50}^{\text{open-channel}}$ values for all n-alkanols in the open-channel application since limitations of aqueous solubility prevented their measurements for the higher n-alkanols.

IC_{50} values for the two different applications were obtained by curve fitting to the Hill equation (Fig. 2, Table 1). The potencies of n-alkanols increase with chain length, both for open-channel (until n-decanol) and for steady-state applications (until n-dodecanol).

These concentration-response curves do not show a monotonic progression from short-chain to long-chain n-alkanols: they overlap for ethanol, are separated for n-butanol by a factor greater three, overlap again for n-hexanol, and only then begin to progressively separate for longer-chain n-alkanols (Table 1). This suggests several, superimposing interactions (see Discussion).

The potency for inhibition increases with the chain length of the n-alkanol, each additional methylene group increasing potency by a factor of 5.1 (from ethanol to n-decanol, Table 1). The interaction energy responsible for this increase can be estimated from the free energy, $\Delta\mu^{\theta,CH_2}$, resulting when a methylene group is transferred between the aqueous phase and the target site(s). $\Delta\mu^{\theta,CH_2}$ can be obtained as the slope of a linear regression when the mole-fraction of the n-alkanol is plotted against the number of its methylene groups (see Eqs. (2) and (3) in Materials and methods). When the free energy is calculated using the experimental $IC_{50}^{\text{steady-state}}$ values, it contains both terms for the n-alkanol interacting with water (aqueous solution from which it is applied) and with the sites of the anesthetic target (see Eq. (3) in Materials and methods). By considering n-alkanols in the gas phase instead of in aqueous solution, it becomes possible to obtain a free energy term that reflects only the interaction with the anesthetic target. This involves the assumption that n-alkanols do not interact with each other in the gaseous phase (which would be true at low concentrations). The partial gas pressure, P_{50}, in equilibrium with the aqueous IC_{50} can be calculated from the IC_{50} by using the water/gas partition coefficients of Table 2. The reciprocal of the P_{50} thus reflects absolute rather than relative (to the aqueous phase) potency.

When the mole-fraction of the n-alkanol in the gas phase is plotted against the number of methylene groups it contains, the resulting slope yields a free energy change per methylene group of -3.27 kJ mol^{-1} (Fig. 3), in contrast to -3.97 kJ mol^{-1} when the mole-fraction of the n-alkanol is plotted in the aqueous phase instead. Whereas the former free energy value should reflect exclusively the interaction with the anesthetic site of action, the latter free energy value also contains contributions from n-alkanols interacting with water.

Apart from n-alkanols suppressing the peak amplitudes of 5-HT induced currents, they also affected their kinetics, which could be resolved in our excised patch configuration. The time constants of current onset (τ_{rise}) and current decay (τ_{decay}) for 5-HT (30 μM) induced currents were in line with those expected for outside-out patches [19]. Relative values of the time constants at n-alkanol concentrations close to the IC_{50} (open-channel and steady-state application) are shown in Fig. 4. Both current onset as well as current decay is generally accelerated by n-alkanols.

Loss of activity and cutoff may simply reflect a drug not reaching its site of action. There are several reports that long-chain n-alkanols may take time to reach their full effect. Uptake of n-alkanol by lipid bilayers sometimes required 15 min to attain complete equilibrium [20]. Experiments with long-chain n-alkanols at various concentrations of lobster nerve membrane vesicles showed that the inhibitory effect appeared to depend somewhat on the membrane lipid concentrations of the preparation [21]. Size-dependence of wash-in time constants had been reported for the inhibition of sodium currents and of potassium currents in squid giant axons where the axolemma membrane is surrounded by several layers of Schwann cells [22].

If anesthetic protein sites are in chemical equilibrium with other lipophilic phases, e.g. within lipid bilayers or other proteins with lipophilic or hydrophobic domains, then filling these would delay reaching an equilibrium concentration. These problems should be minimized by using an excised outside-out membrane preparation in conjunction with a fast solution exchange system delivering a continuous large flow of undepleted n-alkanol solution in close vicinity to the membrane patch.

Concentrations of n-alkanols at or close to their $IC_{50}^{\text{steady-state}}$ values were washed in for different durations, varying between 52 ms and up to 270 s. The resulting curves averaged over several patches for each n-alkanol showed biphasic effects which could be well fitted by biexponential decay (see Fig. 5). There was a fast and a slow effect of peak current inhibition, except for n-hexanol, where a phase of fast inhibition was followed by a phase of potentiation that led to a partial restoration of the peak current.

Except for n-dodecanol, the fast effect was more or less complete before 52 ms (the fastest wash-in time that could be recorded, see Materials and methods), implying that the fast time constant cannot be much slower but probably is faster than 52 ms. This was confirmed by running unrestricted curvefits (see Materials and methods) which produced fast time constants faster than 52 ms for the n-alkanols up to n-decanol (see free parameters, Table 3). Consistent with the limited time resolution, in most of these fits their confidence intervals could not be determined (Table 3).

Missing confidence intervals could also be a reflection of the fitting procedure containing more free parameters than can be determined by the data. Free parameters can be reduced by noticing that there is no clear trend for either the fast or the slow time constants from the

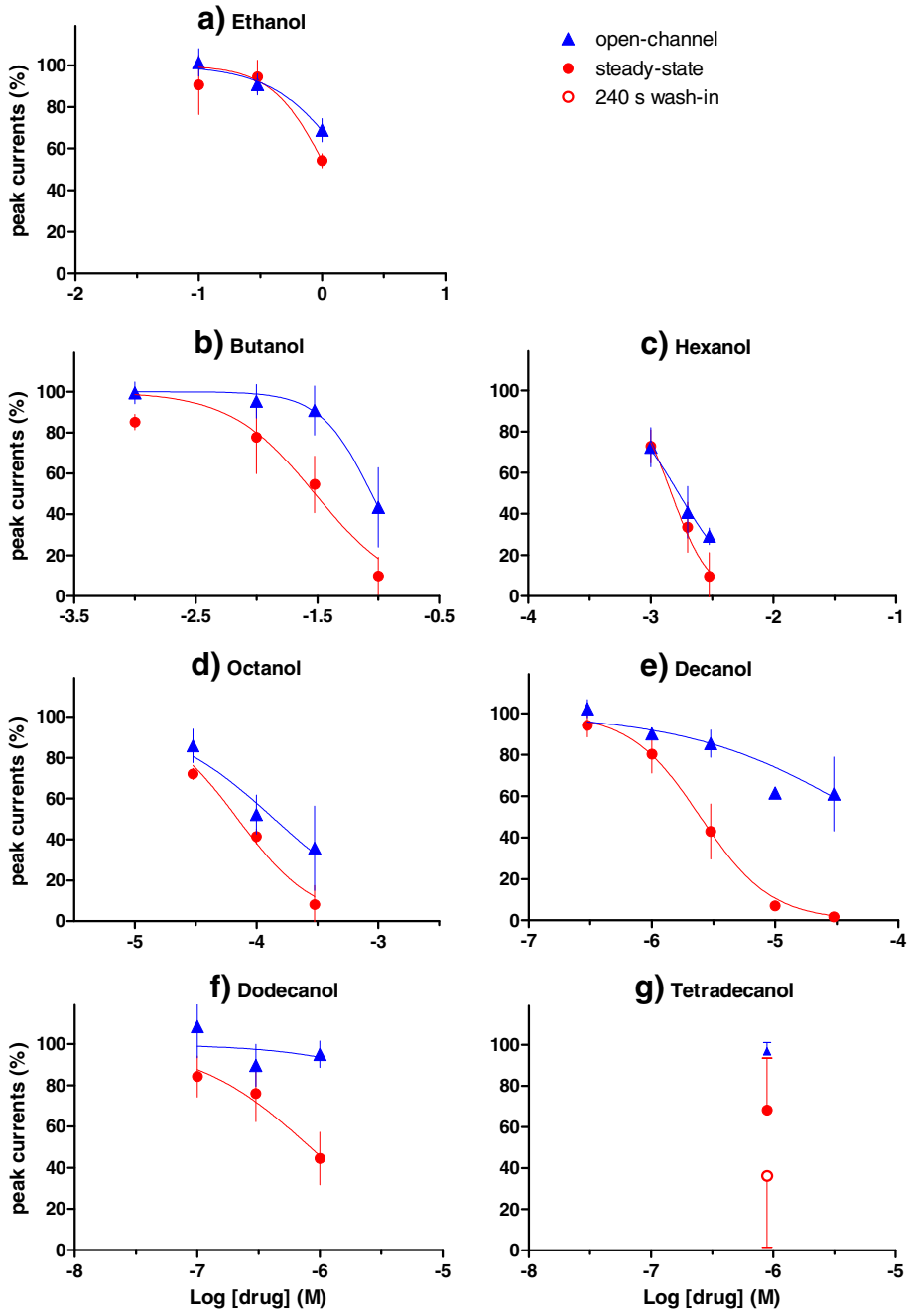

Fig. 2. Concentration-response curves for long-chain n-alkanols become insensitive for short applications compared with long applications. Inhibition by n-alkanols of the peak amplitudes of 5-HT (30 μM)-induced currents in open-channel and steady-state applications, normalized to the respective control currents in the absence of drug. The curves have been fitted to Hill equations (fit parameters in Table 1). At the highest concentration achievable for n-tetradecanol, suppression in the standard (1 min) steady-state application was less than 50% but not yet complete (filled circle, n = 18 patches, significantly different from control). Therefore, the wash-in time was increased to 4 min (open circle, n = 18 patches, significantly different from control and from the 1 min data point) when the suppression appeared complete (see text); note the large standard deviation.

Table 1
IC_{50} values and Hill coefficients n_H for brief and long applications of n-alkanols.

n-Alkanol	Molecular formula	$IC_{50}^{steady\text{-}state}$ (M)	n_H	$IC_{50}^{open\text{-}channel}$ (M)	n_H
Ethanol	C_2H_5OH	1.1 ± 0.25	-2.0 ± 1.1	1.7 ± 0.64	-1.4 ± 0.6
Butanol	C_4H_9OH	$3.0 * 10^{-2} \pm 7.8 * 10^{-3}$	-1.2 ± 0.4	$8.8 * 10^{-2} \pm 1.8 * 10^{-2}$	-2.1 ± 0.8
Hexanol	$C_6H_{13}OH$	$1.5 * 10^{-3} \pm 2.0 * 10^{-4}$	-2.8 ± 0.8	$1.7 * 10^{-3} \pm 2.3 * 10^{-4}$	-1.7 ± 0.6
Octanol	$C_8H_{17}OH$	$7.0 * 10^{-5} \pm 1.3 * 10^{-5}$	-1.4 ± 0.3	$1.4 * 10^{-4} \pm 6.2 * 10^{-5}$	-0.9 ± 0.5
Decanol	$C_{10}H_{21}OH$	$2.5 * 10^{-6} \pm 3.6 * 10^{-7}$	-1.5 ± 0.4	$5.6 * 10^{-5} \pm 4.8 * 10^{-5}$ $(3 * 10^{-5})$	-0.6 ± 0.2
Dodecanol	$C_{12}H_{25}OH$	$8.3 * 10^{-7} \pm 3.4 * 10^{-7}$	-0.9 ± 0.4	$(1 * 10^{-6})$	/

Aqueous IC_{50} values and Hill coefficients, n_H, were obtained from fitting the concentration-response curves in Fig. 2 to Hill equations (±95% confidence intervals). Values in parentheses represent, respectively, the highest experimentally employed concentrations (but at which the effects were still less than 50% in the open channel application).

Table 2
Absolute potencies P_{50} and corresponding free energy changes for interactions of n-alkanols with 5-HT_{3A} receptors.

n-Alkanol or phenol	P_{50} (atm)	$\Delta\mu^{\theta,drug} - \Delta\mu^{\theta,xenon}$ (kJ mol^{-1})	Water/gas partition coefficient	Aqueous solubility (M)
Ethanol	$5.8 * 10^{-3}$	−10.8	4680	$2.2 * 10^{1}$
Butanol	$2.5 * 10^{-4}$	−17.3	2880	$8.5 * 10^{-1}$
Hexanol	$2.2 * 10^{-5}$	−23.8	1700	$5.8 * 10^{-2}$
Phenol	$5.5 * 10^{-7}$	−33.1	70,800	$8.8 * 10^{-1}$
Octanol	$1.7 * 10^{-6}$	−30.4	1000	$4.2 * 10^{-3}$
Decanol	$1.3 * 10^{-7}$	−36.9	468	$2.3 * 10^{-4}$
Dodecanol	$1.0 * 10^{-7}$	−37.3	204	$2.2 * 10^{-5}$
Propofol	$3.8 * 10^{-8}$	−39.7	11,500	$7.0 * 10^{-4}$

P_{50}, were calculated from the IC_{50} [Table 1, phenol and propofol from a previous publication [4]] by using the water/gas partition coefficients in column 4 for n-alkanols and phenol [16], and for propofol calculated from Henry's Law Constant [17]. The water/gas partition coefficient for n-dodecanol was extrapolated from n-decanol, using the ratio of n-nonanol and n-decanol [16]. Free energy changes for a substance are expressed relative to the inert gas xenon and were calculated from P_{50} values (for n-alkanols obtained from the correlation in Fig. 3; for xenon from [18]), see Eq. ((4) in Materials and methods. A tenfold potency change corresponds to a free energy change of 5.7 kJ mol^{-1}. Aqueous solubilities were taken from [17].

preceding (unrestricted) curvefits to strongly depend on the chain length of the n-alkanol (Table 3; see Discussion). In the extreme case, all fast time constants and, with a different value, all slow time constants could be identical for the different n-alkanols.

In order to test whether the present data are compatible with such a hypothesis, the wash-in data were refitted (Fig. 5f) by restricting free parameters, with all n-alkanols sharing the same fast time constant and the same slow time constant, respectively. Now there were only two unrestricted parameters for each n-alkanol left (see Materials and methods), yet despite these limitations the resulting (restricted) curvefits (dotted lines in Fig. 5a–e, and Table 3) did not deviate much from the unrestricted fits (solid lines in Fig. 5a–e, and Table 3) with only slightly deteriorated values for R^2. However, the confidence intervals of the two time constants have become sufficiently narrow to indicate that they are significantly different by a factor of about seventy. Furthermore, except for n-dodecanol, all the fast time constants of the unrestricted fits (Table 3) lie within or close to the confidence interval of this time constant for the restricted fit (Table 3). Again, the fast time constant (24 ms) of the restricted fit did not exceed the resolution limit of 52 ms for the wash-in, and even its upper limit of the confidence interval remained close to 52 ms. Except for n-hexanol, the slow time constants of the unrestricted fit (Table 3) are also close to the confidence interval for that time constant of the restricted fit (Table 3).

The slow time constant of the restricted fit, estimated to be 1.7 s, confirms the previous analysis that the time constants of fast and slow inhibition are separated by almost two orders of magnitude on the time axis, consistent with the hypothesis that there are two distinct processes of wash-in.

Returning to the issue of n-hexanol data points demonstrating significant potentiation (see Fig. 5b), the two different ways of curve fitting each reproduced this potentiation (see below). Furthermore,

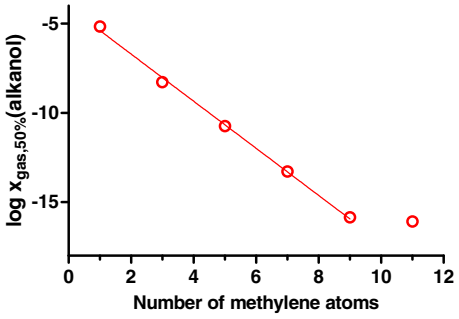

Fig. 3. Free energy change per methylene group of n-alkanols. The logarithms of the mole fractions $x_{gas,50\%}$ of n-alkanols required to suppress the peak of the 5-HT_3A receptor current by 50% are plotted versus the number of methylene groups in the molecule (not the number of carbon atoms). The linear regression shown yields a free energy change per methylene group of −3.27 kJ mol^{-1}, where $r^2 = 0.998$; the n-dodecanol point has not been included in the regression calculation.

when individual patches exposed to n-hexanol were considered, they each showed this potentiating component beyond 1 s wash-in duration as well (data not shown). Normally, however, individual patches did not all last long enough to contain all the time points of a wash-in curve for any of the n-alkanols shown in Fig. 5. Nevertheless, four out of eight patches contributing to the wash-in curve for n-hexanol (Fig. 5b) had a sufficient number of data points so they could be fitted individually, each patch exhibiting a potentiation with a similar time course as that shown in Fig. 5b. Slow inhibition by n-hexanol could not be detected and if present would be masked by a dominating potentiation with a similar time course (Fig. 5b).

Saturating concentrations of n-tetradecanol produced significant inhibition after wash-in for one minute (Figs. 1 and 2). However, inhibition became significantly stronger when the wash-in time was increased from one minute (32 ± 25% , n = 18 patches) to four minutes (64 ± 35% , n = 18), suggesting wash-in time constants of the order of one minute or more. The determination of such long time constants proved experimentally more difficult, at least partly because of a large variability such as is indicated by the large standard variations above. However, rundown was not accelerated in the presence of n-tetradecanol (7.9 ± 3.0 min, n = 10 patches compared with 9.1 ± 3.8 min, n = 6 patches in the absence of n-tetradecanol; each patch lasting longer than two times the rundown time constant), suggesting that the variability did not originate from an increase of irreversible effects (such as rundown) on the current amplitude.

While our wash-in data showed potentiation caused by n-hexanol, only indirect evidence of a similar potentiation was detectable for wash-in of n-butanol (see Discussion). However, potentiation has been described for n-alkanols below n-hexanol in whole cell experiments [12,13], where amplitudes of currents elicited by low 5-HT concentrations were increased by n-alkanols. Therefore, we examined the effects of n-butanol in outside-out patches on currents elicited by low 5-HT concentrations.

The two series of currents shown in Fig. 6a and b correspond to the same patch but recorded at different lifetimes of the patch. This apparently had an impact on how potentiation of the current amplitude was observed. While the amplitudes of currents induced by our standard concentration of 30 μM 5-HT were still reduced in the presence of 20 mM n-butanol, consistent with our other data (Figs. 1 and 2), currents induced by 1 μM or 2 μM 5-HT could be potentiated (Fig. 6). In this example, typical of other patches as well, currents recorded from the patch did not show potentiation of the maximal peak initially (Fig. 6a), but did so 16 min later (Fig. 6b). It should be noted that all traces in this figure were recorded from the same patch, and that they do not show an arbitrary variability but a systematic progression in their behavior, with the data at 1 μM 5-HT supporting those recorded at 2 μM 5-HT.

This observation may be related to the fact that the kinetics (τ_{rise} and τ_{decay}) of 5-HT induced control currents had accelerated in this patch, common for 5-HT induced currents recorded from a patch as it

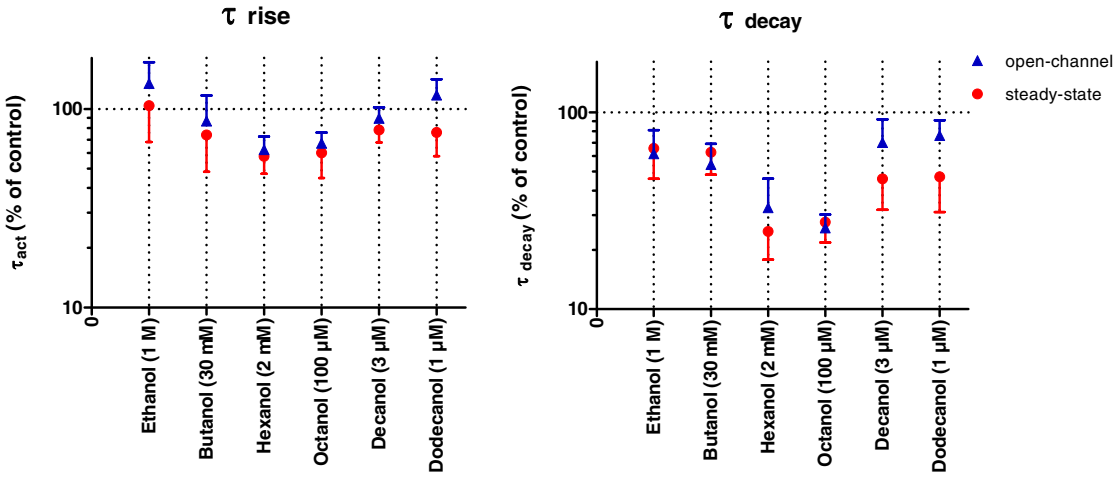

Fig. 4. Current kinetics are accelerated by n-alkanols for both applications. n-Alkanol concentrations were close to their respective $IC_{50}^{steady\text{-}state}$ value. For each patch, the time constant of current onset (τ_{rise}) or current decay (τ_{decay}) during drug application was normalized by the respective time constant for the control current (control: $\tau_{rise} = 9.4 \pm 8.2$ ms, 111 measurements in 51 patches; $\tau_{decay} = 114.7 \pm 102.5$ ms, 110 measurements in 51 patches). All data are significantly different from 100% (paired Students t test), except for τ_{rise} for ethanol (both applications) and τ_{rise} (open-channel application) for n-butanol and for n-dodecanol.

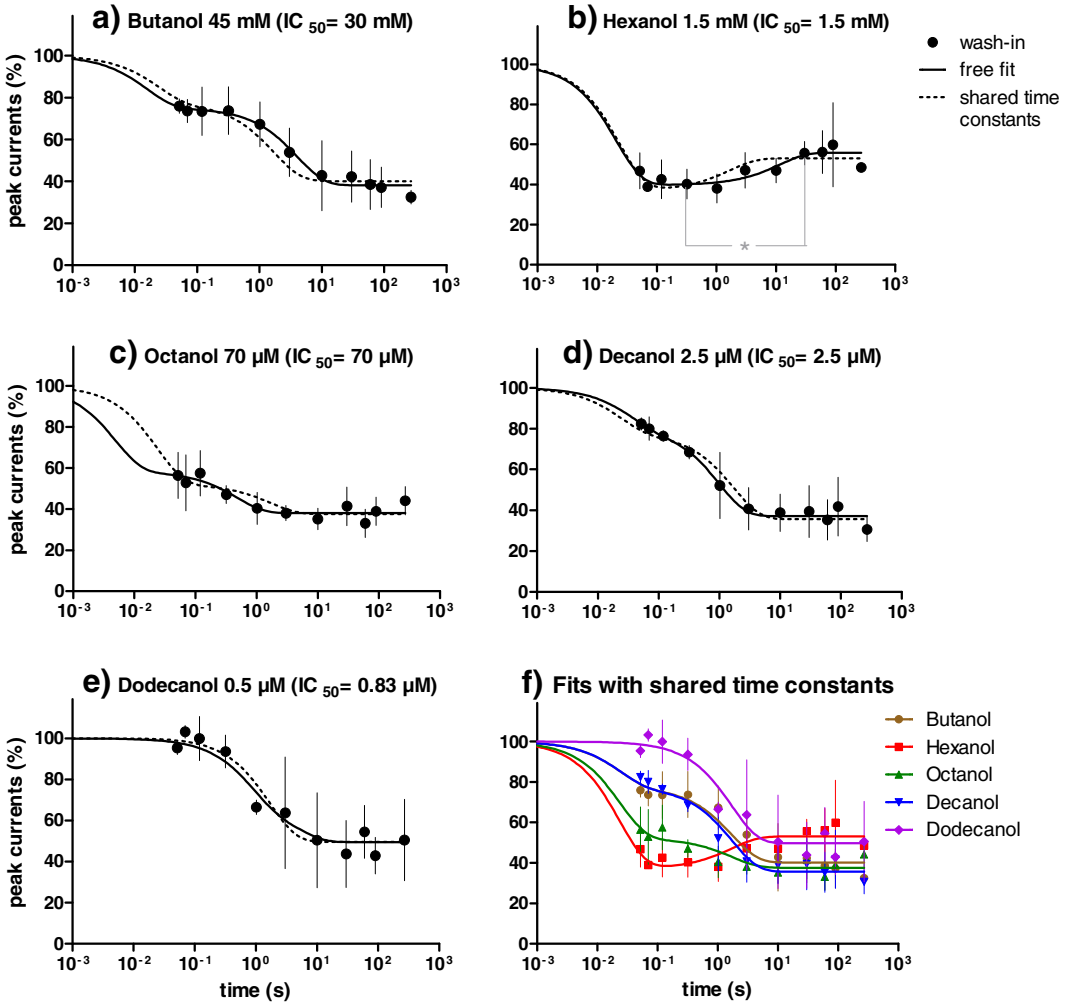

Fig. 5. Biphasic kinetics of n-alkanol actions. The peak current (normalized to control before wash-in) remaining after wash-in of the n-alkanol (at a concentration close to the $IC_{50}^{steady\text{-}state}$) is plotted versus wash-in duration. Note that for n-hexanol (Fig. 5b), an initial inhibition of peak amplitude is followed by a second process of potentiation, leading to a partial recovery (see text); the asterisk denotes that the difference between the two indicated data points (before begin and after completion of potentiation) is significant (unpaired t test); the first point was chosen because the fast process was complete and the second process had not begun yet; the second point was chosen after the second process was complete and a plateau had been reached again. Each curve represents between 5–10 patches. Parameters of the fitted curves (solid lines: each n-alkanol fitted by their own set of parameters; dotted lines: all n-alkanols share the same two time constants; see Materials and methods) are given in Table 3. Experimental data and the fits (shared time constants) for all n-alkanols (from Fig. 5a–e) are superimposed in the last subfigure (Fig. 5f).

Table 3
Kinetic parameters for the biphasic wash-in of n-alkanol actions.

n-Alkanol parameters: Confidence interval: Confidence interval:	τ_{fast} (ms) Free/shared Free parameters Shared parameters	F_{fast} (%) Free/shared Free parameters Shared parameters	τ_{slow} (s) Free/shared Free parameters Shared parameters	Plateau (%) Free/shared Free parameters Shared parameters	R^2 Free/shared
Butanol	16/24 (–) (14–71)	40/38 (29–52) (27–48)	4.1/1.7 (2.2–24.5) (1.2–2.7)	38/40 (34–43) (36–44)	0.74/0.71
Hexanol	21/24 (11–260) (14–71)	137/134 (115–158) (111–157)	11.6/1.7 (–) (1.2–2.7)	56/53 (50–62) (48–58)	0.38/0.31
Octanol	5/24 (–) (14–71)	67/77 (47–88) (62–92)	0.48/1.7 (–) (1.2–2.7)	38/38 (34–42) (32–43)	0.51/0.47
Decanol	34/24 (–) (14–71)	31/35 (6–56) (23–46)	0.99/1.7 (0.56–4.69) (1.2–2.7)	37/36 (32–42) (30–41)	0.83/0.81
Dodecanol	830/24 (–) (14–71)	64/0 (0–100) (0–10)	4.8/1.7 (–) (1.2–2.7)	49/50 (43–56) (45–54)	0.72/0.71

Fit parameters for the two types of fits, both of which fit the biphasic data shown in Fig. 5. Data are displayed as indicated in the header row. Fast and slow time constants were determined either as free parameters (independent for each n-alkanol) or as shared parameters (each n-alkanol has the same fast and the same slow time constant; see Materials and methods). Their 95% confidence intervals are given in the two rows below; (–) indicates that no confidence interval could be determined. Additional free parameters were the fraction of the fast effect (F_{fast}, percentage of the total effect) from which the fraction of the slow effect can be calculated ($F_{slow} = 100\% - F_{fast}$), and the value for the plateau current left after the wash-in was complete. The fraction of the slow effect for n-hexanol is negative since it represents a potentiation. Values for R^2 are also shown (see Materials and methods). Note that the confidence intervals for the shared time constants have become very narrow compared with those for the free time constants, supporting the conclusion that the fast and slow time constants of wash-in differ by a factor of about seventy for all n-alkanols. The other confidence intervals (for F_{fast} or plateau) have mostly changed comparatively little between the two different types of *fit*.

ages. This may suggest the hypothesis that the kinetics of control currents can influence the observed pharmacological response. The test of this hypothesis requires grouping currents according to the kinetics of their control currents and then averaging the observed pharmacological response of these groups. Although we regularly had patches that were potentiated and others that were not, these experiments were not pursued in this paper, because of the large number of patches needed to reach statistical significance because of the small currents involved. However, similar observations were made with n-hexanol and n-octanol (but not with ethanol), strengthening the hypothesis.

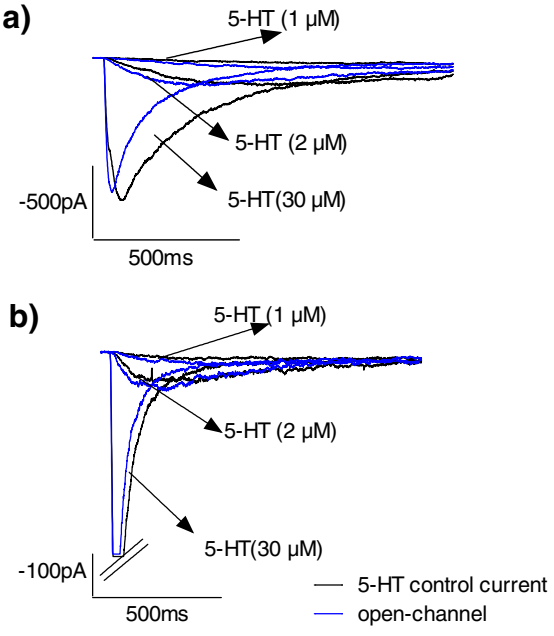

Fig. 6. Example of potentiation at low [5-HT]. Currents may or may not show potentiation, in this case by 20 mM n-butanol (open-channel application), depending on [5-HT] and on current kinetics. In Fig. 6a n-butanol (blue trace) potentiates at 1 μM 5-HT but potentiation is not as clear cut at 2 μM 5-HT. Although the current rises sooner and the peak (2 μM 5-HT) is reached earlier (blue trace, its maximal amplitude is about the same as for the control current (black)). The traces of Fig. 6b have been recorded 16 min later. The respective control currents have become faster than in Fig. 6a (current kinetics tend to accelerate during the lifetime of a patch) and now both currents appear potentiated at both low 5-HT concentrations (1 and 2 μM). The current at 30 μM 5-HT remains still suppressed by n-butanol though this is not shown in this particular figure (compare also Fig. 1b) because, in order to increase resolution, currents in Fig. 6b were recorded at a gain where the peak of the 30 μM 5-HT-induced current saturated (indicated by the double slash).

4. Discussion

4.1. Alkanols still show many interactions

Reviewing the experimental results reveals that the actions of the n-alkanols are not much simpler than those of propofol and the phenol derivatives [4]. n-Alkanols affect different current parameters simultaneously, such as amplitude (Figs. 1 and 2, Table 1) as well as onset and decay time constants and their fractional effects (Figs. 1 and 4) in such a way that none is identical to any other in all their actions. Being able to separate drug effects that would normally appear superimposed (Fig. 5), it became possible to distinguish fast and slow inhibitory as well as potentiating effects.

In the nicotinic acetylcholine receptor, which is closely related to the 5-HT_3 receptor, at least three different mechanisms of action of n-alkanols have been suggested, comprising the channel lumen, an allosteric site on the protein, and a site involving either the membrane lipid or the lipid/protein interface [23]. However, their relative contributions have not been quantitated. Attempts to quantitatively pry apart different mechanisms for 5-HT_3 receptors have also been hampered because of incomplete or even contradictory experimental data. An impractical large number of experiments would be required in order to prove with statistical significance that three or even more (ever smaller) superimposing effects are independent. This is suggested from the experience of other experiments involving the separation of only two effects, where evidence had been presented that fast and slow inhibitory actions on 5-HT_3 receptor appear to be independent and additive for propofol and other phenol derivatives [4] as well as for ifenprodil [24]. Lack of experimental data has to be considered also in the context of the mathematical model that has been suggested for 5-HT_3A receptors. In its reduced form it still has eight free parameters, more than can be determined experimentally [25].

In addition, there are several examples of contradictory data. While amplitude potentiation for n-hexanol was observed in N1E 115 whole cells [13] it was not detected in *Xenopus* oocytes [26]. Furthermore, the amplitude potentiation for short-chain n-alkanols observed in *Xenopus* oocytes preparation [26] is much larger than that reported in a whole cell preparation [13], and it is even smaller in the excised outside-out patch study presented here. Inconsistent results from different experimental approaches may be partly a methodological problem related to time resolution: the IC_{50} of the inhibitory action of morphine on 5-HT_3A receptors differed by a factor of more than three when measured within the same laboratory in either the whole cell mode [27] or in the excised outside-out patch mode [28]. Similarly, competitive action of metoclopramide at 5-HT_3A receptors was detectable in (slow) radioligand binding studies but not in (fast) excised outside-out patches [19]. In this context, differences in the kinetics of control currents as illustrated by exemplary n-butanol experiments (Fig. 6) may determine how potentiation in current amplitude at low 5-HT concentrations is detected, suggesting the hypothesis that the kinetics of control currents can influence the observed pharmacological response.

Particularly when processes have similar kinetics it becomes even more difficult to separate them, such as a superposition of fast inhibition and a postulated fast potentiation for n-butanol as suggested in the following. It has been observed above that n-butanol does not quite fit the trend in the progression from short-chain to long-chain n-alkanols (Figs. 1, 2 and 5). Here the fast inhibitory component is less pronounced than for ethanol, n-hexanol or n-octanol, perhaps because it may be offset by an additional component of potentiation, similar to the one detected for n-hexanol, but faster. Therefore, the fast inhibitory component may appear less pronounced for n-butanol (Fig. 5a) than for n-hexanol (Fig. 5b) because it simply reflects a superposition of independent processes of potentiation and fast inhibition such that inhibition dominates. The future comparison of different but related homologues may help to support or discard this hypothesis.

Trying to characterize individual molecular actions underlying the observed effects becomes all the more difficult the more other confounding effects and actions there are. Therefore, the strategy has been adopted instead to first compare both the overall and the distinct effects of drugs possessing different chemical functional groups, but using the same methodology throughout. Having identified chemical groups that are associated with distinct functional effects might then help in the future to study different functional effects in isolation.

4.2. Absolute potencies and free energies

Despite their many actions the overall effect of n-alkanols on the peak of the 5-HT-induced current is inhibition (Fig. 2). The potency for inhibition increases with the chain length of the n-alkanol (Tables 1 and 4). Thus each methylene group added to an n-alkanol causes it to interact more strongly with its target(s). The chain length dependence of the $IC_{50}^{\text{steady-state}}$ values (Table 1) yields a free energy change of -3.97 kJ mol^{-1} per methylene group from the aqueous phase to the site of anesthetic action, whereas the free energy change per methylene group from the gas phase to the site of anesthetic action is -3.27 kJ mol^{-1} (Fig. 3). These values are typical for free energies of transferring methylene groups of n-alkanols into lipid bilayers or hydrocarbon/aqueous interfaces [15]. There are two noteworthy results: first, the main contribution to the free energy of transfer stems from the interaction of the methylene with anesthetic sites (-3.27 kJ mol^{-1}) and not from leaving the aqueous environment (-0.70 kJ mol^{-1}). Thus van der Waals forces appear to be much more important in this interaction than hydrophobic forces. This was already indicated by the fact that the water–gas partition coefficients for n-alkanols were much less chain length dependent than the potencies at the 5-HT_3A receptor (Table 2): whereas the partition coefficient decreases by merely a factor of ten between ethanol and n-decanol, the corresponding $IC_{50}^{\text{steady-state}}$ value decreases by more than five orders of magnitude. Second, the methylene groups interact more strongly (by -0.93 kJ mol^{-1}) with the ligand-gated 5-HT_3A receptor than with a voltage-gated sodium channel [15]. If this result is confirmed for other ion channels as well, then the 2.1-fold potency difference observed between voltage-gated and ligand-gated ion channels for volatile anesthetics [1] may in part (factor of 1.4) result from methylene groups interacting more strongly with ligand-gated ion channels. By considering and comparing other homologous series, estimates of free energies for other chemical functional groups may be obtained.

4.3. Cutoff

Using an excised outside-out membrane preparation in conjunction with a fast solution exchange system resulted in two wash-in time constants that varied little (less than a factor of ten, for the unrestricted fit, and less than a factor of three, for the restricted fit, as suggested by the confidence intervals in Table 3) between different n-alkanols until and including n-decanol (Table 3 and related text), suggesting that the values of these time constants are not dependent on filling a lipophilic site in equilibrium with the anesthetic site of action. If the time constants reflected simple binding then they would be expected to be roughly inversely proportional to the concentration at which they were recorded [29], thus being of the order of 30 when two neighboring n-alkanols are compared or by a factor of 90,000 between n-butanol and n-dodecanol. Thus wash-in time constants that vary little between different n-alkanols suggest that they are determined by transitions between conformational states (such as resting as well as fast and slow desensitized states) rather than by binding reactions.

However, loss of inhibitory potency was pronounced beyond n-dodecanol, although some inhibitory activity was still observed for n-tetradecanol by us (Figs. 1 and 2, but note the much higher concentration by comparison) and others [13]. The overall trend in the progression from short-chain to long-chain n-alkanols was the fading contribution of the fast inhibitory component (Table 3), so that for n-dodecanol no more fast inhibition was detectable. The fading of one inhibitory component while another remained had been observed before for potassium channels in the same membrane where no such fading had been observed for simultaneously present sodium channels, thus ruling out the n-alkanol not adequately reaching its site of action [22].

Do these findings imply that two different binding pockets or interaction sites of limited molecular dimensions are responsible for fast and slow inhibitory processes? To complicate matters further, the significant acceleration of the decay time constant (Fig. 4) suggests that some fast action of n-dodecanol still remains. Furthermore, the concept of binding pockets of limited dimensions becomes even more problematic as molecular size cannot be the exclusive reason for long-chain n-alkanols losing their inhibitory potency [30]: Anandamide (N-arachidonoylethanolamine), a linear molecule consisting of 22 carbon atoms and terminated, as the n-alkanols are, by a hydroxyl group is still capable of inhibiting 5-HT_3A receptors [31], although it is almost twice the size of n-dodecanol. Possessing additional polar (amide) groups and four double bonds make it much more water soluble. Similarly, propofol in contrast to n-dodecanol (both containing 12 carbons) still retains fast inhibitory action on current amplitude. In this context it may be relevant that propofol also is more water soluble than n-dodecanol (Table 2). Furthermore, the n-alkanols cut off at n-tetradecanol, the alkenols at octadecenol, the n-alkanes at n-decane, and the perfluoroalkanes at butane [30], correlating well with their respective aqueous solubilities. The same is found when the cutoff is compared between n-alkanols and cycloalkanemethanols [32]. In addition, the large variability observed in the data in the presence of n-tetradecanol (see Results) must also be considered. It is well known that long-chain n-alkanols are difficult to work with because they adhere to the apparatus and significant quantities may partition into membranes [33]. Despite the use of the fast superfusion

system, therefore, it is possible that some not yet fully understood artifact(s) based on macroscopic properties of n-alkanols, such as aqueous solubility or adsorption to interfaces, may be responsible for observed cutoffs in inhibition rather than their molecular interaction with protein sites of limited dimensions.

This should be less of a problem for cutoff in potentiation. However, while there is agreement that cutoff for potentiation occurs at lower chain-length of n-alkanols, there is disagreement as to where this should be. Whole cell recordings from 5-HT_3 receptors in N1E-115 report n-hexanol potentiating current amplitude [13], but potentiation by n-hexanol or higher n-alkanols was neither detected in 5-HT_3A receptors [12] nor in 5-HT_3(A + B) receptors expressed in *Xenopus* oocytes [26]. We have detected evidence of potentiation for n-hexanol (Fig. 5b) and, at low 5-HT concentration, also for n-butanol (Fig. 6) in some patches. Furthermore, analogous to some of the other examples we have discussed before, potentiation may still be present even for larger homologues but be masked because of a simultaneous superposition by a larger inhibitory process. Thus potentiation observed in excised outside-out patches has always been small at best, despite the much better kinetic resolution than that obtainable in whole cell measurements. This together with the already discussed differences in kinetic resolution or even in functional endpoints defining potentiation contributes to the uncertainty in determining cutoff points for potentiation.

Obviously the conclusions on cutoff points are not clear cut. There may be in principle at least three cutoff points if we add the cutoff in fast inhibition to those for inhibition and potentiation previously proposed for 5-HT_3 receptors [13]. The implications for molecular interaction sites, however, remain very unclear. Not only may macroscopic effects cause distortions but it has also been argued before that considering cutoff in function only without complementary binding measurements was an untenable approach for mapping binding site sterics [34].

4.4. Comparison between n-alkanols and some phenol derivatives

n-Alkanols (Table 1) and the phenol derivatives [4] have in common that the overall effect is current inhibition and that during wash-in at least two components with distinct time constants separated by approximately two orders of magnitude (Table 3) can be distinguished and are similar to those for propofol (35 ms and 4.8 s, [4]). However, while for the n-alkanols both processes are inhibitory except for n-hexanol (where the slower process is potentiating), only propofol showed two inhibitory processes, while phenol and 2-isopropylphenol caused fast amplitude potentiation, followed by a slower inhibitory component.

For both propofol and n-alkanols, the acceleration of the decay (τ_{decay}) time constant of the 5-HT-induced current washes in very fast and is present in the open-channel application already. n-Alkanols and propofol [4] also have in common that the concentration-response curves for slow inhibition of the current amplitude are more potent than the corresponding curves for the fast inhibition (Table 1).

However, there was no evidence of strong potentiation as observed for 2-isopropylphenol and phenol [4] that remains detectable in the steady-state application. These phenol derivatives caused pronounced amplitude potentiation even at 30 μM 5-HT and substantially slowed the time constant of current decay also in the steady-state application. In contrast, none of the n-alkanols slowed the decay time constant or potentiated the amplitude of currents induced by high concentrations of 5-HT (30 μM). Instead, decay time constants were generally accelerated (Fig. 4). Fig. 6 provided an example of current kinetics apparently determining whether or not amplitude potentiation is observed. This raises the question whether differences in current kinetics (whole cell patches feature currents that are of the order of 50 times slower than those of excised patches, and currents recorded from *Xenopus* oocytes are even slower) may be the reason why at most amplitude potentiation seen in our experiments is weak while in whole cell recordings larger potentiation has been observed [12,13,26].

Another example of weak potentiation is the slow potentiation that follows a fast inhibition by n-hexanol (Fig. 5b). These two processes superimpose so that the total effect under equilibrium conditions is inhibition. This type of slow potentiation was not detected for other n-alkanols. It may have been present but masked by a stronger slow inhibition. Separation becomes difficult because the relative contribution of all components to the overall effect depends on drug concentration and exposure time as well as on the chain-length of the n-alkanol (Figs. 4 and 5, Table 3).

The reason for the absence of strong potentiation in n-alkanols may be that the phenolic hydroxyl group is able to form stronger hydrogen bonds than the n-alkanolic hydroxyl group, consistent with previous observations that potentiation of 5-HT currents was much stronger for trichloroethanol than for ethanol [35], the former also being capable of forming stronger hydrogen bonds than the latter.

Both phenol and n-hexanol each possess six carbon atoms and a hydroxyl group but differ in the aromatic ring structure of the phenol. Similarly, propofol and n-dodecanol also correspond to each other, possessing twelve carbon atoms and a hydroxyl group each. However, whereas the potency (referred to the gas phase) increases with chain length between n-hexanol and n-dodecanol by a factor of 220, it increases by only a factor of 42 between phenol and propofol. Electronegativity of and steric hindrance by the two isopropyl groups on either side of the phenolic hydroxyl group may be responsible. This is consistent with earlier reports of a reduced potency of propofol [4]. If this reduced reactivity (relative to other phenol derivatives) between propofol and 5-HT_3 receptors is also characteristic of its interactions with anesthetic sites considered important for anesthesia (e.g. $GABA_A$ receptors), it could imply that in clinical use propofol has fewer side effects than other phenol derivatives. This may have been one of the reasons why it has been found superior to more than fifty other phenol derivatives during in vivo testing [36].

5. Conclusions and outlook

In conclusion, when the structurally simpler n-alkanols are compared with propofol and related phenol derivatives much of the complexity of modulation of function by these drugs is retained. Each of these alcohols and phenols possess partly similar but overall distinct spectra of effects, none of them is identical to any other of these drugs in all its actions. The aqueous potency increase per methylene group of the n-alkanol is due mainly to interactions of the methylene group with its anesthetic target and to a lesser extent due to its partitioning out of the aqueous phase because of its hydrophobicity. Absolute interaction energies (relative to xenon) increased from -10.8 kJ mol^{-1} for ethanol to -37.3 kJ mol^{-1} for n-dodecanol.

Potentiation by the n-alkanols is weaker than that by phenol, consistent with the less reactive hydroxyl group of n-alkanols suspected to play a role in this interaction. Different cutoffs in function have been detected but their molecular interpretation remains uncertain. Propofol is less potent relative to other related phenol derivatives when compared with the series of n-alkanols. Wash-in time constants that vary little between different n-alkanols suggest that they are determined by transitions between conformational states rather than by binding reactions. Further studies with other series of homologues possessing weaker or no polar groups are needed in order to confirm these conclusions, to identify the origin of the two time constants, to characterize the role of the hydroxyl group in inhibition and potentiation, and to further explore the nature of cutoff in potency.

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