Erythrocyte Membrane Expansion Due to the Volatile Anesthetics, the 1-Alkanols, and Benzyl Alcohol

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The extent to which semiturgid red cells could enter the tip of a glass micropipette of approximately 1-μm internal diameter was used to measure the area expansion of freshly drawn erythrocytes. The pipette was mounted in a gas-tight glass observation chamber so that one single cell at a time could be observed while it was being perfused, either with buffer alone or with buffer containing an anesthetic at physiologic concentration. The effect of the drugs was studied at room temperature at doses of 1, 2, and 4 times ED₉₀ for tadpoles. The drugs studied were: halothane, methoxyflurane, diethyl ether, fluroxene, isoflurane, hexanol, heptanol, octanol, decanol, dodecanol, tridecanol, tetradecanol, hexadecanol, and benzyl alcohol. The measured area expansion ranged between 0.13% and 0.62%. The measured expansion closely approximated the expansion expected by incorporation of the molecules. Tetradecanol and hexadecanol are not anesthetic to tadpoles, but they did expand the membrane. Therefore, expansion may not be related to anesthesia. (Key words: Alcohol: membrane effects. Anesthetics volatile: halothane; diethyl ether; methoxyflurane; isoflurane; fluroxene. Theories of anesthesia: membrane effects.)

The expansion of the red blood cell membrane due to absorption of anesthetic substances has been a useful model for the study of a number of theories concerning the mechanism of action of anesthetics. However, information from direct measurement of the expansion of the living cell has been very difficult to obtain; consequently, membrane surface area expansion has been measured by indirect techniques. From such studies it was noted that the measured volume expansion of the membrane, for both general and local anesthetics, was much greater than that calculated from the volume occupied by the anesthetic molecules. This observation has been seminal to a number of theories for the mechanism of action of anesthetics. It is therefore important to determine the expansion caused by the various anesthetics, as accurately as possible, in order to see if the use of more refined techniques will modify the existing data and thus favor some theories for the mechanism of action of anesthetics while making others less likely.

In this paper, we report measurements made by a technique which accurately measures small changes in the membrane surface area of a single cell as it is being exposed to the anesthetic and is undergoing direct visual observation.

Materials and Methods

A micropipette was mounted in a gas-tight perfusion chamber constructed as described previously, except that Teflon® and stainless steel tubing were used, instead of polyethylene, where it was exposed to the anesthetic.

Red blood cells were suspended in hypotonic buffer at an osmolarity just sufficient to allow a small amount of membrane slackness (150 mOsm). The excess membrane allowed the cell to be caught and held on the tip of the micropipette, and also allowed any changes in total cell surface area to be determined by noticing any change in length of the tongue of the cell membrane that protruded into the pipette. The rest of the cell assumes a spherical shape outside the pipette; the diameter of this portion was also measured and included in the calculation of area change. Changes in tongue length were observed and recorded photographically and by videotaping.

The fractional increase in surface area is given by the
equation:

$$\frac{\delta A}{A} = D_p \cdot \delta L (1 - D_p/D_s)/(D_p^2 + D_p \cdot L - D_p^2/4)$$

where \(D_p\) is the pipette diameter, \(D_s\) is the diameter of the spherical portion of the cell outside the pipette, \(L\) is the initial length of the tongue, and \(\delta L\) is the observed change in length. This equation is derived from the geometry of the captive cell on the assumption that the volume remains constant and that terms containing second and third order changes in the diameter, \(D_s\), can be neglected.

The ratio \(\delta A/A\) is dimensionless and, therefore, the scale of the measurements does not matter so long as they are self-consistent. However, the glass pipette acts like a cylindrical lens which magnifies the dimension \(D_p\) but not \(L\) or \(D_s\). Since the measurements were made from photographic enlargements, it was necessary to know the actual inside diameter of the pipette and the photographic magnification in order to correct for re-
fraction. The error due to photographic enlargement was less than 1%. One pipette was used in all experiments, and it had an internal diameter of 0.92 ± 0.01 μm and an external diameter of 2.26 ± 0.02 μm measured by scanning electron microscopy. The hydrostatic pressure required to hold the cell and to sphere the portion outside the pipette was never greater than 3 cmH₂O. Therefore, both transfer of water across the membrane and stretching of the membrane could be considered negligible.


The unit for measurement of the dose response was ED₅₀ for tadpoles. This value was obtained for heptanol, ether, fluorozone, and methoxyflurane in our laboratory using tadpoles (Rana pipien berlandieri, stage 40–47, Nesco, Wisconsin). A group of tadpoles was exposed to the anesthetic, and the dose which caused loss of righting reflex in half of them as described by Pringle and Miller[1] was recorded. Published values for ED₅₀ were used for the other substances.[3,10] Tridecanol is only a partial anesthetic, so ED₅₀ was the best that could be achieved to represent unit dose. Tetradecanol and hexadecanol are nonanesthetic to tadpoles, so the dose was selected by comparison with tetradeanol and hexadecanol which are anesthetics.

The gaseous anesthetic agents were used in the liquid form and dissolved in phosphate-buffered saline to give a concentration of ED₅₀ × 4. Gas-tight glass syringes with Teflon® seals were used for serial dilution to ED₅₀ × 2, and ED₅₀ × 1. After dilution, the solution remained capped in the syringe until used for perfusion of the cell. ED₅₀ refers to tadpoles at room temperature in each case.

Photomicrographs were made of the untreated cell in buffer, and then of the same cell while buffer containing the anesthetic was being perfused. Attempts were made to measure changes in diameter of the spherical portion of the cell, because this information would provide a means of distinguishing between area and volume changes, but the changes were so small that they were below the limits of resolution of the light microscope.

In order to increase the reliability of the results, a dose-response curve relating membrane area expansion to anesthetic concentration was plotted for each agent. The three concentrations of anesthetic in buffer, obtained by serial dilution as described above, were used in sequence. The lowest concentration was used first, and this value was always well within the range used in clinical anesthesia. The highest concentration was used last. This sequence was adopted to avoid any possibility of absorption and consequent carry over in the Teflon® tubing used to connect the chamber to the delivery syringe. As a control for our experiment, the cell was perfused with plain buffer before and after perfusion with the anesthetic agent to see if it returned to its initial value. With the volatile anesthetics, the concentration in the buffer emerging from the chamber was compared with the concentration entering the chamber using gas chromatography. The chamber was completely cleared of all solutions containing anesthetic after each experiment and all tubing was replaced.

Results

The measured values of area expansion at ED₅₀ are shown in Table 1, together with the regression slope and correlation coefficient of the mean expansion at each concentration of anesthetic used. The number of cells at each concentration is also listed. The area expansion at ED₅₀ for those agents considered to be anesthetics ranged between 0.13% for methoxyflurane and 0.62% for fluorozone.

Figure 1 shows the dose-response curves for isoflurane which is typical for the various agents examined. If the same cell was exposed repeatedly to the same concentration of anesthetic, no variation in the measured expansion could be detected. For different cells there was some variation, possibly due to age or cholesterol content. For example, in a typical experiment with isoflurane at a concentration of 1 × ED₅₀, the measured values were: 0.31%, 0.39%, 0.22%, 0.34%, and 0.31%. This set of readings gave a mean of 0.31% and a standard deviation of ± 0.06. For this concentration of isoflurane, the expansion was completely reversible when the chamber was purged with buffer to remove the anesthetic. For higher concentrations of the anesthetics, only partial reversibility was observed. At ED₅₀ × 4, the expansion was about 75% reversible.

Thus, at clinically used doses, the expansion is directly proportional to the dose. The anesthetic potency for the 1-alkanol series shows an almost exponential increase as the length of the carbon chain increases. This can be accounted for by the corresponding increase in partition coefficient between the aqueous buffer and the lipid bilayer. The decreasing solubility in the buffer is compensated by the increasing solubility in the lipid so
Table 1. Area Expansion as Measured and Calculated

<table>
<thead>
<tr>
<th>Agent</th>
<th>Unit Dose (μM)</th>
<th>Number of Cells</th>
<th>Measured Expansion %</th>
<th>Regression Slope</th>
<th>Correlation Coefficient</th>
<th>Partition Coefficient Lipid/Water</th>
<th>Isotropic</th>
<th>Anisotropic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halothane</td>
<td>230</td>
<td>7</td>
<td>0.22</td>
<td>0.20</td>
<td>0.98</td>
<td>35.0*</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>210</td>
<td>5</td>
<td>0.13</td>
<td>0.14</td>
<td>0.95</td>
<td>77.6†</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>Ether</td>
<td>9,100</td>
<td>7</td>
<td>0.27</td>
<td>0.25</td>
<td>0.95</td>
<td>96.2‡</td>
<td>0.15</td>
<td>0.23</td>
</tr>
<tr>
<td>Fluoroxene</td>
<td>2,900</td>
<td>7</td>
<td>0.02</td>
<td>0.02</td>
<td>1.00</td>
<td>78.6‡</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>290</td>
<td>5</td>
<td>0.31</td>
<td>0.32</td>
<td>1.00</td>
<td>61.0†</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>Hexanol</td>
<td>700</td>
<td>5</td>
<td>0.23</td>
<td>0.24</td>
<td>0.99</td>
<td>13.0*</td>
<td>0.07</td>
<td>0.11</td>
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<tr>
<td>Heptanol</td>
<td>190</td>
<td>5</td>
<td>0.26</td>
<td>0.27</td>
<td>0.99</td>
<td>35.0†</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>Octanol</td>
<td>60</td>
<td>6</td>
<td>0.35</td>
<td>0.35</td>
<td>1.00</td>
<td>150.0**</td>
<td>0.30</td>
<td>0.45</td>
</tr>
<tr>
<td>Decanol</td>
<td>13</td>
<td>8</td>
<td>0.31</td>
<td>0.29</td>
<td>0.97</td>
<td>1975.0†</td>
<td>0.32</td>
<td>0.48</td>
</tr>
<tr>
<td>Dodecanol</td>
<td>5.4</td>
<td>4</td>
<td>0.42</td>
<td>0.42</td>
<td>1.00</td>
<td>5355.0†</td>
<td>0.44</td>
<td>0.66</td>
</tr>
<tr>
<td>Tridecanol</td>
<td>37.0</td>
<td>4</td>
<td>0.87</td>
<td>0.87</td>
<td>1.00</td>
<td>387.0**</td>
<td>0.25</td>
<td>0.38</td>
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<tr>
<td>Tetradecanol</td>
<td>35.0</td>
<td>7</td>
<td>0.37</td>
<td>0.37</td>
<td>1.00</td>
<td>1222.0</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>Hexadecanol</td>
<td>40.0</td>
<td>5</td>
<td>0.57</td>
<td>0.57</td>
<td>1.00</td>
<td>1975.0†</td>
<td>0.32</td>
<td>0.48</td>
</tr>
<tr>
<td>Benzy alcohol</td>
<td>2,000.0</td>
<td>4</td>
<td>0.24</td>
<td>0.23</td>
<td>0.99</td>
<td>2.3*</td>
<td>0.03</td>
<td>0.05</td>
</tr>
</tbody>
</table>
| * Red blood cell membrane. Seeman.6
| † Egg phosphatidylcholine 64%/egg phosphatic acid 4%/cholesterol 35%. Smith et al.16
| ‡ Egg phosphatidylcholine or dioleylophytidylcholine or dilauryleptidylcholine. Simon et al.17 Cited by Kita et al.12
| § Olive oil. Miller and Smith.5
| ¶ Egg phosphatidylcholine 96%/phosphatic acid 4%. Smith et al.16
| †† Egg phosphatidylcholine. Jain et al.18
| ‡‡ Egg phosphatidylcholine cholesterol. Colley and Metcalfe.15
| §§ Red blood cell cholesterol. Seeman et al.20
| †† Red blood cell membrane. Colley et al.21

that the concentration of anesthetic in the bilayer for a given area increase remains about constant.

Because of insolubility in water of dodecanol, tridecanol, tetradecanol, and hexadecanol, these were first dissolved in a small amount of ether. The ether solution was then used to disperse the alcohol in the buffer. Control experiments with this same amount of ether but no alcohol were done. The effect of the ether was found to be negligible at the concentrations used. It is of interest to note that the alcohols, tetradecanol and hexadecanol, which are not considered to be anesthetics, did enter the membrane and caused expansion comparable to that found with lower order alcohols at dose levels which do produce anesthesia.

Discussion

The erythrocyte membrane has long been the preferred biologic model for determination of expansion associated with anesthetic agents.1,2,4,6,7 The methods used by earlier workers to measure membrane expansion were indirect methods which relied upon the fact that an increase in surface area of a red blood cell permits that cell to achieve a larger volume prior to hypotonic lysis. This larger cell volume was measured either by the Coulter principle or deduced from the osmotic change required to lyse the cells. Using the former method, the value found for octanol at a concentration of 7.4 × 10⁻⁴ M was 6% area increase. This dosage was twelve times the dose we used for ED₅₀. At this concentration, our method gives a 3.6% area increase. Seeman11 obtained an expansion of 1.0% for benzyl alcohol at a concentration of 1.6 × 10⁻² M. This corresponds even more closely to our value of 1.2% area expansion. Data for the volatile anesthetics was obtained by Seeman from the protection they afforded against hypotonic lysis. The estimated expansion for halothane, for example, at a concentration of 2.2 × 10⁻⁴ M was 0.4% compared with 0.22% for our method at the same concentration.

The degree of correlation is good considering the disparate methods of measurement employed. In previous work, the measured area expansion was thought to be three to ten times more than would be expected from the calculated molecular volume of anesthetic absorbed by the membrane. Considerable effort has therefore been expended in an endeavor to explain this discrepancy.5-7 Since the expansion itself is comparable, the question arises whether the disparity may be due to the method of calculating the volume occupied by the
The expansion produced by an anesthetic entering a membrane from aqueous solution can be calculated from the equation of Kita, Bennett, and Miller:

$$E^{50} = \bar{V}_2 \cdot \lambda_{4/3} \cdot C_a^{50} / V_m$$

where $E^{50}$ is the fractional expansion at a dose which anesthetizes 50% of a group of aquatic animals; $\bar{V}_2$ is the partial molar volume of the anesthetic; $\lambda_{4/3}$ is the partition coefficient of the anesthetic between lipid and water; $C_a^{50}$ is the concentration of anesthetic in water corresponding to $E^{50}$; and $V_m$ is the site volume where the anesthetic acts.

The concentration $C_a^{50}$ is accurately known so that any error in $E^{50}$ revolves about the factors $\bar{V}_2$, $\lambda_{4/3}$, and $V_m$. Kita, Bennett, and Miller have recently used a novel design of excess volume dilatometer to measure the partial molar volume of benzyl alcohol, halothane, and methoxyflurane in synthetic membranes, while values for the 1-alkanols have been obtained using ultrasensitive densitometry. Their work shows that the partial molar volume in representative lipid bilayers is quite close to the actual molar volume. Seeman had assumed, on the information then available, that it would be somewhat less than the molar volume. Thus, the value taken for $\bar{V}_2$ can account for some of the discrepancy between the calculated and measured value of expansion. The partition coefficient $\lambda_{4/3}$ has much greater potential for introducing error than the partial molar volume $\bar{V}_2$. A wide range for $\lambda_{4/3}$ is available in the literature, depending on the type of membrane or bilayer used for measurement and whether intact membranes or their components were studied. The higher values for the partition coefficient eliminate the discrepancy entirely, and in some cases, would account for expansion larger than that actually observed.

The value for the site volume $V_m$ (the fraction of the volume of the membrane actively participating in the expansion by the anesthetic) is not known and may vary for different anesthetics in the same membrane. Data on the partition coefficients cited in table 1 assume that $V_m$ is unity, but variation from this value could account for most of the remaining discrepancy. Finally, different values must be considered for the expected expansion if the membrane expands isotropically than if it expands anisotropically.

Using the accurately measured partial molar volume for halothane and benzyl alcohol, the partition coefficients into egg lecithin cholesterol, and assuming anisotropic expansion at constant membrane thickness, the values for the expected expansion are very close to the measured expansion.

If better data for the partial molar volume, and the partition coefficient between buffer and that portion of the cell membrane which is expanding, were available for all the anesthetic substances listed in table 1, it is possible that very close correlation would always occur between the calculated and measured values. If this proves to be so, theories for the mechanism of action of anesthetics built around the previous discrepancies will have lost their support, and attention can be directed to other more meaningful phenomena associated with the onset of anesthesia.

The observation that the nonanesthetic alcohols tridecanol, tetradecanol, and hexadecanol caused expansion similar to the alcohols which are known to have anesthetic properties, suggests that expansion is only a coincidental effect in the presence of an anesthetic, and is not the mechanism by which it blocks passage of impulses or causes unconsciousness. These inferences are drawn from the red blood cell membrane, which is not an excitable membrane, but this study indicates that it may be important to consider reasons other than membrane expansion for the mechanism of action of anesthetics.

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