Successful protocol of anaesthesia for measuring transepithelial nasal potential difference in spontaneously breathing mice

T Leal¹, J Lebacq², R Vanbinst¹, Ch Lederman³, M De Kock³ and P Wallemacq¹

¹Department of Clinical Chemistry; ²Department of Physiology; ³Department of Anaesthesiology and Pain Clinic, Université Catholique de Louvain, Brussels, Belgium

Summary

Numerous difficulties arise during in vivo measurements of transepithelial nasal potential difference (PD) in mice, such as inadequate duration and depth of anaesthesia, bronchoaspiration of solutions perfused in the nose, and respiratory and/or cardiovascular depression. Anaesthesia was induced in adult C57 mice with intraperitoneal injection of a combination of fentanyl, droperidol and medetomidine, each of these at either a small dose (0.20, 10 and 0.33 mg/kg, respectively) or at a large dose (0.40, 20 and 0.40 mg/kg, respectively), combined with a fixed dose of 0.375 mg clonidine. In order to establish a pharmacokinetic–pharmacodynamic relationship, blood concentrations of the first three drugs were measured in 24 animals by liquid-chromatography tandem mass spectrometry. At the end of the experiment, naloxone, a competitive morphinic antagonist, and atipamezole, an α-2 adrenergic antagonist, were administered. Bronchoaspiration was prevented by tilting the animal head downwards and by absorbing the excess fluid from the opposite nostril and from the oral cavity. Optimal assessment of anaesthesia associated with regular respiration, loss of blink, pupillary and pedal withdrawal reflexes was obtained with doses of fentanyl, droperidol and medetomidine corresponding to 0.20, 20 and 0.40 mg/kg, respectively. Blood concentrations of fentanyl around 17 ng/mL induced loss of respiratory efforts and were followed by death during the experiment. Integrity of ion transport was demonstrated under continuous perfusion by successive depolarization after amiloride and repolarization after chloride-free solution.

The combination investigated in this study lead to adequate surgical anaesthesia (stage III, plane 2) for prolonged nasal PD measurements in spontaneously breathing mice.

Keywords  Nasal potential difference; cystic fibrosis; anaesthetics, combined; mass spectrometry; mice

Measurement of the electrical potential difference (PD) across the nasal mucosa following a specific protocol under continuous perfusion of solutions is a non-invasive test in humans (Leal et al. 2003a) not requiring anaesthesia. Its clinical applications include differential diagnosis of atypical cystic fibrosis (CF) disease, assessment of residual chloride conductance, CF transmembrane conductance regulator (CFTR) genotype-phenotype studies, and studies on therapeutic modulation of CF basic defects (Middleton et al. 1994, Knowles et al. 1995, Egan et al. 2004). The technique

Correspondence: T Leal, Department of Clinical Chemistry, St Luc University Hospital, 10 Av Hippocrate, B-1200 Brussels, Belgium. Email: teresinha.leal@clin.ucl.ac.be

Accepted 1 April 2005
is important to improve understanding of the pathophysiology of CF, to examine \textit{in vivo} transepithelial ion transport, and to follow attempts to deliver gene therapy in transgenic animals (Grubb \textit{et al.} 1994, Smith \textit{et al.} 1999). In this framework, optimizing experimental conditions for measuring nasal PD in spontaneously breathing mice, with subsequent recovery, is becoming mandatory. Numerous problems arise during nasal PD measurements in mice: duration of anaesthesia too short for completion of the required experimental protocol, depth of anaesthesia insufficient for keeping a nasal catheter \textit{in situ} with continuous perfusion, bronchoaspiration of solutions perfused in the nose, and respiratory and/or cardiovascular depression (Van Doorninck \textit{et al.} 1995, Ghosal \textit{et al.} 1996, Hardiman \textit{et al.} 2001). Adequate depth of anaesthesia appears indeed particularly difficult to reach when operating in the nasal region in rodents, probably related to the essential role of sensory input from the nasofacial region in the control of behaviour \textit{vis-à-vis} an external situation, including a threat. In some studies (Ghosal \textit{et al.} 1996, 2000), nasal PD test in mice was performed by administration of drugs following nebulization instead of continuous perfusion. This alternative method requires intermittent withdrawals and reinsertions of the nasal recording bridge, and the results obtained are difficult to interpret due to questionable repeatability. This study is aimed at investigating and improving relevant technical aspects, including the duration and the quality of the anaesthesia necessary to obtain successful prolonged nasal PD measurements in spontaneously breathing mice.

\textbf{Materials and methods}

\textbf{Animals}

\textit{C57} mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The mice used in this study were adults of either sex weighing from 27 to 44 g. They were maintained on alternating 12 h/12 h light/dark cycles, housed at 22°C (45% relative humidity), with free access to water and standard food from Pavan Sevice Carfil Quality, Oud-Turnhout, Belgium. They were treated in accordance with the guidelines of the Bill of Animal Protection and Well-being of the Belgian Parliament (14 August 1986). The project was approved by our Institutional Ethical Animal Care and Use Committee.

\textbf{Anaesthesia and nasal PD measurements}

Mice were anaesthetized by a single intraperitoneal injection of a mixture of fentanyl [\textit{Fentanyl-Janssen}® 0.05 mg/mL, Janssen Cilag, Berchem, Belgium], medetomidine [\textit{Domitor}® 1 mg/mL, Orion Pharma, Espoo, Finland], droperidol [\textit{Dehydrobenzperidol}® 2.5 mg/mL, Janssen Cilag, Berchem, Belgium], and clonidine [\textit{Catapressan}® 0.15 mg/mL, Boehringer Ingelheim Pharma KG, Ingelheim am Rhein, Germany]. When necessary, stock solutions were diluted to accurately dose animals. Clonidine was administered in a fixed dose of 0.375 μg. In order to find the optimal dosage regimen, different combinations of drugs were tested. Fentanyl, droperidol, and medetomidine were each used at small (0.20, 10, and 0.33 mg/kg, respectively) and large (0.40, 20, and 0.40 mg/kg, respectively) doses. These doses correspond to those usually administered in rodents: 0.2–0.5 mg/kg fentanyl (Vuckovic \textit{et al.} 1998), 3–12 mg/kg droperidol (Freye & Kuschinsky 1976), and 0.4–0.8 mg/kg medetomidine (Hellebrekers \textit{et al.} 1997). Assessment of anaesthesia was categorized in stages and planes. Stage III was initially achieved for all anaesthetic combinations used. At plane 1 (stage III), animals had regular respiration and pedal withdrawal reflex was absent when slightly pinching the toe, but some pain response was present when inserting a catheter in the subcutaneous space in a hind leg. Animals still had blink reflexes. At plane 2 (stage III), the animals lost blink reflexes and pupils became fixed. Pedal withdrawal reflex was abolished and the insertion of a catheter in the subcutaneous space was not followed by any painful reaction. Respiration was regular. At plane 4...
stage III, the animals started losing the ability to use the respiratory muscles and breathing became shallower and finally stopped. This plane was followed into stage IV, resulting in the death of the animal during the test. Heart and respiratory rates were monitored in 12 mice anaesthetized for obtaining either plane 2 or plane 4 (stage III) of anaesthesia. Respiratory rate was monitored every 5 min from the time of injection of drugs throughout the experiment. For monitoring the heart rate, a fine needle [0.4 × 20 mm, Terumo®, Brico Medical Supplies, Metuchen, NJ, USA] was inserted in the subcutaneous space at each foreleg and at the left hind leg, and wire electrodes were connected to an electrocardiograph (Helige 23 502 704, Servomed, Haasrode, Belgium). An electrocardiogram tracing [lead II] was printed out at 50 mm/s every 5 min from the beginning of the experiment.

Nasal PD measurements were performed in vivo as previously described by Smith et al. (1999) with some modifications, and using a data memory high-impedance (> 10¹² Ω) voltmeter (Knick Portamess® 913, Elektronische Meßgeräte, Berlin, Germany). Briefly, mice were placed on their backs on a heating pad until full recovery, which usually occurred 3–4 h afterwards.

Blood sample collection

Blood samples were obtained from 24 animals for quantitative analysis of the pharmacological agents by liquid-chromatography tandem mass spectrometry (LC-MS/MS). Intracardiac puncture was used in 12 animals that died during the experiment (30–45 min after administration of drugs). For the others, a venous blood sample was drawn from the medial saphenous vein (Hem et al. 1998) without killing the animal, 45 min after injection of drugs, and before administration of the antidotes. One hind leg was extended and fixed by holding the fold of skin between the tail and thigh. The leg was fixed and depilated by using a commercially available depilatory cream (Veet®, Reckitt Benckiser, Belgium) and scratching with a spatula. A 23-gauge needle was used to puncture the vein, and 50–100 µL blood was collected into heparinized tubes (Hemato-Clad, Drummond®, Broomall, PA, USA). Samples were stored refrigerated until analysis.
Quantitative analysis of the drugs by LC-MS/MS

A Quattro micro™ tandem mass spectrometer (Waters-Micromass Ltd, Manchester, UK) fitted with a Z-Spray™ ion source was used for analyses of fentanyl, droperidol and medetomidine. The instrument was operated in electrospray-positive ionization mode, and was coupled to a Waters 2795 Alliance HT HPLC system. All aspects of system operation and data acquisition were controlled using MassLynx NT™ v3.5 software. Rapid separation was obtained using a column X Terra® C18 (2.1 × 50 mm, 3.5 µm, Waters), maintained at 50°C under a flow rate of 0.2 mL/min of a mobile phase consisting of 80% acetonitrile and 20% water with 10 mmol/L ammonium acetate. Drugs were monitored by detecting specific product ions resulting from the fragmentation of their precursor ions using the multiple reaction monitoring (MRM) acquisition mode. The first quadrupole was set to select the precursor ions \([M+H]^+\) of fentanyl \((m/z 337)\), droperidol \((m/z 379.9)\), and medetomidine \((m/z 200.9)\), and the second was used to select and quantify the characteristic and intense product ions of these drugs \((m/z 104.9, 165.0, \text{and} 94.9, \text{respectively})\). The analytical methods were validated for their linearity, sensitivity, and reproducibility according to a protocol described elsewhere (Wallemacq et al. 2003).

Chemicals

All chemicals were of the highest available grade from Sigma Chemical Co, St Louis, MO, USA. Amiloride was a generous gift from Merck Research Laboratories (Rahway, NJ, USA). Fentanyl, droperidol, and medetomidine were obtained from the pharmaceutical companies. Acetonitrile was of HPLC grade, from Riedel-de-Haën (Seelse, Germany). Ammonium acetate and zinc sulphate were of analytical reagent grade from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA). The calibrators and in-house controls were prepared in drug-free whole blood obtained from non-treated animals. Standard curves for fentanyl and medetomidine included the following concentrations: 2.5, 5, 10, 25, 50, and 100 ng/mL, and for droperidol: 100, 500, 1000, 2000, and 3000 ng/mL.

Sample preparation

A high-precision positive displacement pipette (Microman®, Gilson, Middleton, WI, USA) was used to transfer 10 µL whole blood or calibrator samples to 1.5 mL polypropylene tubes (Eppendorf, Hamburg, Germany). A volume of 10 µL of zinc sulphate (100 mmol/L) was then added and the samples were briefly mixed. Proteins were precipitated by adding 50 µL acetonitrile. The tubes were vortex-mixed vigorously for 5 s, and then centrifuged for 5 min at 10,500 g (Heraeus Labofuge, Hanau, Germany). The clear and colourless supernatant (10 µL) was injected into the system.

Statistical analysis

Results are expressed as means ± standard deviation (SD), with the number of mice shown \((n)\). Statistical comparisons of data were performed using the one-way analysis (ANOVA) for multiple means comparisons (SAS-JMP software) and a general linear random coefficients model for repeated measures data (SPSS software). Null hypothesis was rejected at \(P<0.05\).

Results

Quantitative analysis of drugs and depth of anaesthesia

Under the analytical conditions described above, retention times of fentanyl, medetomidine, and droperidol were 1.32, 1.30, and 1.27 min, respectively. Linearity was demonstrated for fentanyl and medetomidine from 2.5 to 100 ng/mL, and for droperidol from 100 to 3000 ng/mL. Limits of quantification (LOQ) were 2.0, 3.0, and 75 ng/mL for fentanyl, medetomidine, and droperidol, respectively. Coefficients of variation were kept below 7.9% within the analytical range and below 10.5% at the
LOQ. At small dosage regimen \( n = 12 \), concentrations of fentanyl averaged \( 4.8 \pm 0.8 \) (\( \pm SD \)) ng/mL and at large dosage regimen \( n = 12 \) the corresponding values were consistently larger \( 17.8 \pm 1.4; P < 0.0001 \).

Concentrations of droperidol obtained after 20 mg/kg \( 1326 \pm 334 \) ng/mL; \( n = 12 \) were slightly, though not significantly \( P = 0.06 \), larger than those obtained after 10 mg/kg \( 1104 \pm 202 \) ng/mL; \( n = 12 \). Concentrations of medetomidine were significantly larger in animals receiving 0.40 mg/kg when compared with those receiving 0.33 mg/kg \( 39.8 \pm 4.2 \) and \( 19.1 \pm 3.4 \) ng/mL; \( P < 0.0001 ; n = 12 \) for each group).

The pharmacokinetic–pharmacodynamic relationship between blood concentrations of these drugs and the obtained anaesthetic level is shown in Table 1. Stage III, plane 1 obtained in six mice \( \text{numbers 1–6} \) was associated with insufficient depth and duration of anaesthesia for performing continuous perfusion of the nasal cavity. In those animals, nasal PD recordings were interrupted during the experiment because of persistence of muscle movements. Such movements could either cause the expulsion of the intranasal catheter or introduce artefacts in the PD records, together with an increased risk of bronchoaspiration of solutions perfused in the nose. These animals have received small doses of fentanyl, droperidol, and medetomidine. Blood concentrations of fentanyl were \( 5.1 \pm 1.0 \) ng/mL, and those of droperidol and medetomidine \( 1067 \pm 267 \) and \( 17.5 \pm 2.1 \) ng/mL, respectively. Following increasing the dose of fentanyl \( \text{numbers 13–24} \), stage III, plane 4 of anaesthesia was reached for all permutations of droperidol and medetomidine. Consistently larger blood concentrations of fentanyl \( 17.8 \pm 1.4 \) were found in these animals. As anaesthesia deepened, breathing became shallower and more predominantly abdominal. The animals died during the experiment. Finally, following association of a small dose of fentanyl and high doses of droperidol and medetomidine

Table 1  Pharmacokinetic/pharmacodynamic relationship between blood concentrations of fentanyl, droperidol, and medetomidine during general anaesthesia for measuring nasal potential difference (PD) in mice and the obtained anaesthetic plane

<table>
<thead>
<tr>
<th>Animal</th>
<th>Fentanyl Dose (mg/kg)</th>
<th>Blood Concentration (ng/mL)</th>
<th>Droperidol Dose (mg/kg)</th>
<th>Blood Concentration (ng/mL)</th>
<th>Medetomidine Dose (mg/kg)</th>
<th>Blood Concentration (ng/mL)</th>
<th>Anaesthetic Level (Stage III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.20</td>
<td>4.3</td>
<td>10</td>
<td>712</td>
<td>0.33</td>
<td>15.1</td>
<td>Plane 1</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>5.9</td>
<td>10</td>
<td>860</td>
<td>0.33</td>
<td>14.8</td>
<td>Plane 1</td>
</tr>
<tr>
<td>3</td>
<td>0.20</td>
<td>3.9</td>
<td>10</td>
<td>1013</td>
<td>0.33</td>
<td>19.4</td>
<td>Plane 1</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>6.6</td>
<td>10</td>
<td>1374</td>
<td>0.33</td>
<td>19.1</td>
<td>Plane 1</td>
</tr>
<tr>
<td>5</td>
<td>0.20</td>
<td>4.8</td>
<td>10</td>
<td>1077</td>
<td>0.33</td>
<td>18.9</td>
<td>Plane 1</td>
</tr>
<tr>
<td>6</td>
<td>0.20</td>
<td>5.1</td>
<td>10</td>
<td>1368</td>
<td>0.33</td>
<td>17.5</td>
<td>Plane 1</td>
</tr>
<tr>
<td>7</td>
<td>0.20</td>
<td>4.6</td>
<td>20</td>
<td>1019</td>
<td>0.40</td>
<td>38.2</td>
<td>Plane 2</td>
</tr>
<tr>
<td>8</td>
<td>0.20</td>
<td>4.6</td>
<td>20</td>
<td>1262</td>
<td>0.40</td>
<td>30.8</td>
<td>Plane 2</td>
</tr>
<tr>
<td>9</td>
<td>0.20</td>
<td>4.1</td>
<td>20</td>
<td>1455</td>
<td>0.40</td>
<td>47.2</td>
<td>Plane 2</td>
</tr>
<tr>
<td>10</td>
<td>0.20</td>
<td>4.4</td>
<td>20</td>
<td>1171</td>
<td>0.40</td>
<td>38.0</td>
<td>Plane 2</td>
</tr>
<tr>
<td>11</td>
<td>0.20</td>
<td>4.9</td>
<td>20</td>
<td>1235</td>
<td>0.40</td>
<td>38.3</td>
<td>Plane 2</td>
</tr>
<tr>
<td>12</td>
<td>0.20</td>
<td>4.8</td>
<td>20</td>
<td>1003</td>
<td>0.40</td>
<td>39.5</td>
<td>Plane 2</td>
</tr>
<tr>
<td>13</td>
<td>0.40</td>
<td>18.1</td>
<td>20</td>
<td>2018</td>
<td>0.33</td>
<td>17.7</td>
<td>Plane 4</td>
</tr>
<tr>
<td>14</td>
<td>0.40</td>
<td>17.2</td>
<td>20</td>
<td>1030</td>
<td>0.33</td>
<td>18.6</td>
<td>Plane 4</td>
</tr>
<tr>
<td>15</td>
<td>0.40</td>
<td>16.8</td>
<td>20</td>
<td>1060</td>
<td>0.33</td>
<td>17.9</td>
<td>Plane 4</td>
</tr>
<tr>
<td>16</td>
<td>0.40</td>
<td>16.9</td>
<td>10</td>
<td>972</td>
<td>0.33</td>
<td>27.7</td>
<td>Plane 4</td>
</tr>
<tr>
<td>17</td>
<td>0.40</td>
<td>19.2</td>
<td>10</td>
<td>1215</td>
<td>0.33</td>
<td>20.3</td>
<td>Plane 4</td>
</tr>
<tr>
<td>18</td>
<td>0.40</td>
<td>18.5</td>
<td>10</td>
<td>1264</td>
<td>0.33</td>
<td>22.3</td>
<td>Plane 4</td>
</tr>
<tr>
<td>19</td>
<td>0.40</td>
<td>15.3</td>
<td>20</td>
<td>1872</td>
<td>0.40</td>
<td>42.8</td>
<td>Plane 4</td>
</tr>
<tr>
<td>20</td>
<td>0.40</td>
<td>16.1</td>
<td>20</td>
<td>1510</td>
<td>0.40</td>
<td>40.7</td>
<td>Plane 4</td>
</tr>
<tr>
<td>21</td>
<td>0.40</td>
<td>18.3</td>
<td>20</td>
<td>1274</td>
<td>0.40</td>
<td>38.8</td>
<td>Plane 4</td>
</tr>
<tr>
<td>22</td>
<td>0.40</td>
<td>17.9</td>
<td>10</td>
<td>988</td>
<td>0.40</td>
<td>36.6</td>
<td>Plane 4</td>
</tr>
<tr>
<td>23</td>
<td>0.40</td>
<td>18.6</td>
<td>10</td>
<td>1170</td>
<td>0.40</td>
<td>45.4</td>
<td>Plane 4</td>
</tr>
<tr>
<td>24</td>
<td>0.40</td>
<td>20.2</td>
<td>10</td>
<td>923</td>
<td>0.40</td>
<td>40.8</td>
<td>Plane 4</td>
</tr>
</tbody>
</table>
[numbers 7–12], a stage III, plane 2 of anaesthesia was reached. The animals had completely lost blink, pupillary, and withdrawal reflexes, and breathing was regular, with equal distributions from the chest and abdomen. This optimal surgical anaesthetic level allowed performing a complete and prolonged nasal PD test, followed by full recovery. Concentrations of fentanyl, droperidol, and medetomidine were 4.6 ± 0.3, 1191 ± 169, and 38.7 ± 5.2 ng/mL, respectively.

Respiratory rates
At the time of injection of anaesthetics, the respiratory rate in restrained animals ranged from 120 to 168 breaths/min, with a mean value of 146 ± 14 (n = 12). Compared with the corresponding initial values, a decrease in respiratory rate was observed during the first 25 min after administration of a small dose of fentanyl (Figure 1). When compared with animals receiving a small dose of fentanyl, those displaying stage III, plane 4 of anaesthesia showed a higher degree of respiratory depression throughout the experiment (P: 0.0002).

Heart rates
No significant decrease in heart rate was seen throughout the experiment independently of the doses of drugs administered (Figure 2).

Measurements of nasal PD
With adequate doses, a good and stable level (stage III, plane 2) of anaesthesia was obtained 4–6 min after administration of the drugs. The mean duration of deep anaesthesia was 45 min, allowing completion of the test without interruption and full subsequent recovery. C57 mice had baseline values of nasal PD, similar to those recorded in healthy human subjects (Leal et al. 2003b). In the example of record of nasal PD shown in Figure 3, successive phases of blockage of apical Na⁺ entry by addition of amiloride (10⁻⁴ mol/L) and of stimulation of Cl⁻ efflux by perfusing the nasal mucosa with a chloride-free solution were then alternated in order to demonstrate that prolonged measurements could be performed during nasal perfusion in mice under suitable anaesthesia. Depolarization after amiloride and marked

Figure 1  Respiratory rates after anaesthetic administration in mice. ■: stage III, plane 2 of anaesthesia obtained in six animals after administration of 0.20, 10, and 0.33 mg/kg of fentanyl, droperidol, and medetomidine, respectively; ◆: stage III, plane 4 of anaesthesia after administration of 0.40, 20, and 0.40 mg/kg of fentanyl, droperidol, and medetomidine, respectively. Experiments were interrupted due to respiratory arrest (◆). Values are means ± standard deviation (SD) from the indicated number of mice. *P < 0.05; **P < 0.001 when compared with the corresponding values obtained at the time of injection of drugs in restrained animals.
repolarization following the use of chloride-free solution indicated the integrity of the transepithelial transport of both sodium and chloride.

Figure 2 Heart rates after anaesthetic administration in mice. ■: stage III, plane 2 of anaesthesia obtained in six animals after administration of 0.20, 10, and 0.33 mg/kg of fentanyl, droperidol, and medetomidine, respectively; ◆: stage III, plane 4 of anaesthesia after administration of 0.40, 20, and 0.40 mg/kg of fentanyl, droperidol, and medetomidine, respectively. Experiments were interrupted due to respiratory arrest (◆). Values are means ± standard deviation (SD) from the indicated number of mice.

Figure 3 Example of record of nasal potential difference (PD) obtained in a C57 mouse with stage III, plane 2 of anaesthesia, obtained by the association of a low dose of fentanyl and large doses of droperidol and medetomidine, in response to continuous perfusion of the nasal mucosa successively with amiloride (10^{-4} mol/L; down arrows), in order to block the apical sodium entry, and with a chloride-free solution (up arrows), in order to induce chloride efflux. Arrows show changes of solutions.

Discussion

The present study describes an adequate anaesthetic assessment and management for measuring the nasal PD under continuous perfusion of solutions in spontaneously breathing mice for a length of time required by the protocol. It leads to a complete recovery of animals and to a reduction in the number of animals to be used. Proper choice of anaesthetics is of paramount importance for reliable measurements and for survival of the animal, allowing further experiments with the same animal later on. Ketamine, usually used during general anaesthesia in veterinary medicine, has some degree of sympathomimetic effect (Hill et al. 1999). This was considered a major drawback in this work, since the effect of isoproterenol is commonly searched for in functional studies on transepithelial transport of chloride (Middleton et al. 1994, Knowles et al. 1995, Egan et al. 2004). Instead, a modified neuroleptanalgesia, comprising a combination of an opioid with a neuroleptic, was tested. Fentanyl, when applied at large doses in association with small and/or large doses of droperidol and medetomidine, was invariably toxic. Optimal concentrations of fentanyl were found to be between 3.9 and 6.6 ng/mL corresponding to a small dose of...
0.20 mg/kg. Concentrations of fentanyl in the blood appeared similar in animals displaying stage III, either plane 1 or 2 of anaesthesia, but quite different from those displaying stage III, plane 4 (around 17 ng/mL). It is interesting to note that, in humans, fatality has been reported with blood concentrations of fentanyl above 27 ng/mL, in a case of self-administration of fentanyl alone (Chaturvedi et al. 1990). In order to potentiate the analgesic effect of fentanyl without increasing the risk of respiratory depression (Meert & De Kock 1994), two α-2 adrenergic drugs have been added: medetomidine, which is selective for α-2 receptors, and clonidine, which also acts on α-1 receptors, thereby avoiding a large secondary cardiovascular depression caused by α-2 stimulation (Virtanen 1989). After injection of anaesthetics, no significant degree of primary cardiac depression was observed in association of drugs used. However, when comparing with values obtained in restrained animals at the time of injection of drugs, some degree of respiratory depression was seen during the first 25 min following anaesthesia even in association with small doses of fentanyl.

We found the combination of a small dose of fentanyl with a large dose of droperidol and medetomidine and with a fixed dose of clonidine to be optimal, and the drugs are not known to influence transepithelial transport of chloride and sodium. In this work, successive depolarization responses were seen after addition of amiloride. The integrity of the airway epithelium to secrete chloride was demonstrated by the marked repolarization in response to a chloride-free solution in the presence of amiloride. This response is reduced or abolished in CF (Middleton et al. 1994, Knowles et al. 1995, Leal et al. 2003a). When nasal PD was performed by nebulization of drugs, instead of a continuously perfused method, no significant PD changes could be observed with a low chloride vehicle nebulized after a sodium channel blocker (Ghosal et al. 2000). The response after induction of a gradient of chloride favourable to efflux of this ion is brief and quickly reversible after interruption of the stimulation. Another critical aspect that could explain the absence of response to a low-chloride solution under nebulization is the difficulty in replacing the nasal catheter at the same previous site of measurement, following intermittent withdrawals and reinsertions needed for administration of drugs. It is known that regional differences in PD values are related to the morphological epithelial composition and particularly to the density of respiratory ciliated cells (Knowles et al. 1981). In the mouse, the nasal membrane contains four types of epithelia: respiratory, olfactory, transitional, and squamous (Mery et al. 2000). Differential location of each epithelial type must therefore be taken into account when performing studies in the nasal cavity of mice. A fine (≤0.3 mm) double lumen catheter placed continuously in the nostril throughout the experiment, without interruption of recording, is undoubtedly a better method for performing the nasal PD test.

Administration of antidotes of anaesthetic drugs at the end of the experiment improves recovery without after-effects. Interestingly, at the dosage range used in this study, neither fentanyl nor medetomidine concentrations seemed to follow linear kinetics. Indeed, blood concentrations increased to a larger extent than the increase of their respective doses, suggesting a saturation process of the metabolizing enzymes in mice. Blood concentrations of medetomidine were smaller than reported therapeutic concentrations in humans (Flanagan 1998), but needed to be over 30 ng/mL to maintain adequate anaesthesia.

The central action of medetomidine could lead to some characteristic pattern of pharmacodynamic responses, including, e.g. hypotension and hypothermia (Virtanen 1989), which should be reversed by administration of atipamezole. Hypothermia was prevented by laying the animal on a heating pad during the experiment and after operation. Droperidol, a neuroleptic compound retaining myorelaxant properties, did not appear critical for cardiovascular toxicity at the doses used. A combination of droperidol could be useful to attenuate airway smooth muscle contractility by
inhibiting contraction of tracheal rings [Sato et al. 1996, Shibata et al. 2003] and possibly by helping to avoid bronchoaspiration of solutions perfused in the nose. Bronchoaspiration of solutions was also prevented by tilting the animal head downwards and by absorbing the excess liquid from the opposite nostril and from the oral cavity.

We conclude that, with the protocol proposed above, it is possible to obtain a safe and reversible level of anaesthesia for operating during at least 45 min in the nasal cavity in mice. Such conditions are suitable for functional in vivo studies on transepithelial ion conductances and those testing pharmacological modulation of drugs acting on the level of Na$^+$ and Cl$^-$ transport across the airway epithelium in mouse. The drug combination described here could also be suitable for other studies in mice in which recovery from anaesthesia is needed.

Acknowledgements The scientific assistance of Professor E Alton and Dr S Smith, National Heart and Lung Institute, London, UK, is gratefully acknowledged in the refinement of the nasal PD technique in mice. This work was supported by the French CF Association Vaincre la Mucoviscidose.

References


