Studies on the binding of [3H]amethocaine to rat cerebrocortical membranes

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Summary
We have examined the binding of the local anaesthetic agent [3H]amethocaine to rat cerebrocortical membranes. All studies were performed in Tris buffer 50 mmol litre\(^{-1}\) at pH 7.4. Bound and free radioligand were separated by rapid vacuum filtration. [3H]Amethocaine binding at room temperature was dose-dependent and saturable, with mean \(K_d\) and \(B_{\text{max}}\) values of 153 (SEM 18) nmol litre\(^{-1}\) and 9.4 (1.6) pmol/mg protein, respectively. [3H]Amethocaine binding was displaced in a dose-dependent manner (plC \(_50\)) by unlabelled amethocaine (6.89), procaine (5.20), lignocaine (3.46) and prilocaine (2.81). Ropivacaine and bupivacaine did not produce 50% displacement at the highest concentrations used (10\(^{-4}\) and 10\(^{-3}\) mol litre\(^{-1}\), respectively). We examined the nature of the binding site further with a range of ion channel antagonists (nifedipine, verapamil, diltiazem, \(\omega\)-conotoxin, tetrodotoxin, tetraethylammonium and 4-aminopyridine) and ion channel coupled receptor ligands (L-glutamate, MK801, GABA, glycine and nicotine). With the exception of tetraethylammonium (plC \(_{50}\) 3.07) and 4-aminopyridine (plC \(_{50}\) 3.68), all non-anaesthetic agents failed to displace [3H]amethocaine. Collectively our data suggest that it is unlikely that there is a single target site for all local anaesthetic agents.

Materials and methods

MEMBRANE PREPARATION
All studies were performed in rat cerebrocortical membranes resuspended in Tris HCl 50 mmol litre\(^{-1}\), pH 7.4 (prepared as described previously) by homogenization and centrifugation.\(^9\)\(^10\)

[3H]AMETHOCaine BINDING ASSAY
In order to determine the maximum binding capacity or total number of binding sites (\(B_{\text{max}}\)) and the equilibrium dissociation constant for [3H]amethocaine (\(K_d\)), membranes (approximately 50 \(\mu\)g of membrane protein) were incubated in 150-\(\mu\)l volumes with increasing concentrations (approximately 3–600 nmol litre\(^{-1}\)) of [3H]amethocaine (>95% pure, 43 Ci mmol\(^{-1}\), Amersham International, UK). In addition, we attempted to determine the \(K_d\) kinetically by measuring the ratio of the dissociation rate constant (\(K_1\)) to the association rate constant (\(K_{-1}\)). In these studies, membranes (approximately 750 \(\mu\)g) were incubated in 1-ml volumes with approximately 1 nmol litre\(^{-1}\) of [3H]amethocaine to equilibrium (>15 min at room temperature and >2.5 h on ice). At 60 min (room temperature) or 3 h (ice), dissociation was initiated by the addition of unlabelled amethocaine 1 mmol litre\(^{-1}\).

For displacement studies the assay volume was 1 ml and a fixed concentration of [3H]amethocaine (approximately 1 nmol litre\(^{-1}\)) was used. Agents that displace [3H]amethocaine are likely to interact at the same target site or to be coupled allosterically to the [3H]amethocaine binding site. Increasing concentrations of unlabelled amethocaine, lignocaine, procaine, prilocaine, bupivacaine and R(+)/S(−)ropivacaine were added and the dose-dependent and saturable binding of [3H]amethocaine to rat cerebrocortical membranes and examine the effects of a range of local anaesthetic agents and transmitters/channel blockers with this binding site.
concentration producing 50% displacement (IC₅₀) determined. In addition, displacement studies were performed with the following ion channel blockers/transmitters in order to examine the site(s) of interaction: nifedipine, verapamil, diltiazem (L-type VSCC blockers), ω-conotoxin G Via (N-type VSCC blocker), tetrodotoxin (Na⁺ channel blocker), tetraethylammonium (K⁺ channel blocker), L-glutamate, MK801 (NMDA receptor ion channel blocker), GABA, glycine and nicotine. In all experiments non-specific binding (NSB) was defined in the presence of excess (1 mmol litre⁻¹) unlabelled amethocaine. Saturation, kinetic and amethocaine isotope dilution studies were performed at either room temperature (approximately 23 °C) or on ice (approximately 0 °C to limit ligand dissociation). Bound and free radioligand were separated by rapid vacuum filtration.

DATA ANALYSIS
All data are presented as mean (SEM) (n). Saturation data were analysed according to Scatchard.¹¹ Observed association (Kₘ) and dissociation (K₋₁) rate constants were estimated from non-linear, one-phase exponential association and dissociation equations using Graphpad-Prizm. Kₘ was corrected for K₋₁ and the concentration of [³H]amethocaine to yield K₊₁. Kₘ was then calculated using K₋₁/Κ₊₁. The concentration of displacing drug producing 50% displacement of specific binding (pIC₅₀) was estimated from individual curves by non-linear regression (Graphpad-Prizm). In some experiments 100% displacement was not achieved and data were analysed based on an assumed 100% displacement.

Results
Binding of [³H]amethocaine was dose-dependent and saturable at room temperature (fig. 1A, B) and on ice (data not shown). Scatchard analysis of the specific binding data on ice and at room temperature yielded Kₘ and B₀ values of 169 (20) nmol litre⁻¹, 11.0 (1.6) pmol/mg protein and 153 (18) nmol litre⁻¹, 9.4 (1.6) pmol/mg protein, respectively. At the radioligand Kₘ specific binding was in excess of 50%. Unlabelled amethocaine 1 mmol litre⁻¹ was added to membranes labelled for 60 min at room temperature (fig. 1C) and 3 h on ice (data not shown) in an attempt to initiate ligand dissociation.

Table 1 pIC₅₀ values (mean (SEM)) for local anaesthetic displacement of [³H] amethocaine binding to rat cerebrocortical membranes. *Determined by isotope dilution

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Maximum dose used</th>
<th>n</th>
<th>% Displacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifedipine</td>
<td>10⁻⁵</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Verapamil</td>
<td>10⁻⁵</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>10⁻⁵</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>ω-conotoxin</td>
<td>3 x 10⁻⁷</td>
<td>6</td>
<td>14.1 (3.0)</td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td>3 x 10⁻⁴</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>10⁻³</td>
<td>5</td>
<td>50.4 (1.9)</td>
</tr>
<tr>
<td>L-glutamate</td>
<td>10⁻³</td>
<td>6</td>
<td>75.0 (1.5)</td>
</tr>
<tr>
<td>MK801</td>
<td>10⁻⁴</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>GABA</td>
<td>10⁻⁴</td>
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</tr>
<tr>
<td>Glycine</td>
<td>10⁻⁵</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Nicotine</td>
<td>10⁻⁵</td>
<td>6</td>
<td>29.1 (2.6)</td>
</tr>
</tbody>
</table>

Figure 1 [³H]Amethocaine binding (total, non-specific (NSB) and specific) to rat cerebrocortical membranes incubated at room temperature was dose-dependent and saturable (λ) and displaced in a dose-dependent manner by unlabelled amethocaine (β). Association and dissociation time courses for [³H]amethocaine (approximately 1 nmol litre⁻¹) binding are shown in (c). In (c), ligand dissociation was initiated by addition of excess unlabelled (1 mmol litre⁻¹, arrow) amethocaine and data are expressed relative to the r = 60 time point normalized to 100%. Data are from a single representative experiment (from n = 15) in (λ), and mean (±SEM) (n = 11) in (β) and (n = 6) in (c).
At room temperature, <20 % of ligand had dissociated by 15 s and there was no appreciable dissociation on ice. Kinetic analysis yielded $K_{d}$ and $K_{-1}$ values on ice and at room temperature of 0.023 min$^{-1}$ and 0.001 min$^{-1}$ ($n = 2$) and 0.307 (0.026) min$^{-1}$ and 0.299 (0.016) min$^{-1}$ ($n = 6$), respectively. As $K_{d}$ was so close numerically to $K_{-1}$, a dissociation constant value could not be calculated. In order to confirm the $K_{d}$ value obtained in saturation studies, $K_{d}$ was estimated in a series of isotope dilution studies where $[^{3}H]$amethocaine was displaced by unlabelled amethocaine (fig. 1b). $pIC_{50}$ (approximate $K_{d}$) values of 6.97 (0.05) (107 nmol litre$^{-1}$) and 6.89 (0.02) (129 nmol litre$^{-1}$) were obtained on ice and at room temperature, respectively.

A range of unlabelled local anaesthetics displaced $[^{3}H]$amethocaine binding in a dose-dependent manner, with $pIC_{50}$ values (as concentration of radiolabel was negligible compared with the $K_{d}$, $IC_{50}$ values are presented uncorrected for the competing mass of $[^{3}H]$amethocaine) shown in table 1. The rank order potency was amethocaine > procaine > lignocaine > prilocaine. Hill numbers for procaine, prilocaine and lignocaine were 0.65, 0.72 and 0.81, respectively, perhaps suggesting interaction with more than one site. However, these Hill numbers should be interpreted with caution because of the lack of complete displacement by these agents. Further discriminatory analyses were not performed. At bupivacaine 1 mmol litre$^{-1}$, R($+$)ropivacaine 10$^{-4}$ mol litre$^{-1}$ and S($-$)ropivacaine 10$^{-4}$ mol litre$^{-1}$, 15.7 (1.7) %, 27.4 (1.5) % and 26.1 (1.0) % displacement was observed. There was no significant correlation between lipid solubility (measured as octanol:buffer partition coefficient$^{12}$) and the $pIC_{50}$ for displacement of $[^{3}H]$amethocaine binding ($r^{2} = 0.47$, $P = 0.31$). Of the non-local anaesthetic agents tested, only 4-AP and tetraethylammonium produced greater than 50 % displacement of specific binding with estimated $pIC_{50}$ values of 3.68 (0.02) (209 µmol litre$^{-1}$) and 3.07 (0.05) (851 µmol litre$^{-1}$), respectively (table 2).

**Discussion**

We have demonstrated dose-dependent and saturable binding of $[^{3}H]$amethocaine to rat cerebrocortical membranes. The binding was displaced by a range of unlabelled local anaesthetic agents but there was no correlation with lipid solubility, and bupivacaine and ropivacaine failed to produce 50 % displacement at the maximum concentrations used. In addition, binding of $[^{3}H]$amethocaine was displaced by the K$^{+}$ channel blockers, 4-AP and tetraethylammonium. The $K_{d}$ values calculated by Scatchard analysis (153 nmol litre$^{-1}$ at room temperature, 169 nmol litre$^{-1}$ on ice) and by isotope dilution (129 nmol litre$^{-1}$ at room temperature, 107 nmol litre$^{-1}$ on ice) compare favourably with the values quoted by Grima and colleagues$^{13}$ and Reith, Kim and Lajtha$^{14}$ of approximately 170–180 nmol litre$^{-1}$ in synaptosomes (an excitable preparation) analysed according to Scatchard.$^{11}$ $B_{max}$ values quoted in this study (approximately 13 pmol/mg protein) compare well with our data. In addition, Voight and Mannhold have reported binding of $[^{3}H]$lignocaine in a range of predominantly peripheral tissues concluding that the pharmacological significance of the binding site remains to be determined.$^{15}$ It should be noted that in these studies the emphasis was to examine binding to Na$^{+}$ channels rather than to use the radioactively labelled local anaesthetic to examine “anaesthetic binding sites”.

Binding of $[^{3}H]$amethocaine was displaced in a dose-dependent manner by amethocaine, procaine, lignocaine and prilocaine. There was no correlation between displacement of $[^{3}H]$amethocaine binding ($pIC_{50}$) and lipid solubility or anaesthetic potency. In addition, ropivacaine and bupivacaine produced only approximately 25 % displacement at the highest doses used. The lack of an interaction with all local anaesthetic agents implies that the binding site for $[^{3}H]$amethocaine in rat cortical membranes does not represent a common target site for local anaesthesia. However, it is possible that if soluble, and available at higher concentrations, bupivacaine and ropivacaine may have been able to produce 50 % displacement but the $pIC_{50}$ values would be very weak. In our study amino-ester local anaesthetics (amethocaine and procaine) displaced with highest affinity while amino-amide local anaesthetic agents (lignocaine, prilocaine, bupivacaine and ropivacaine) displaced with the weakest affinity.

L-channel (nifedipine, diltiazem and verapamil) and N-channel (ω-conotoxin) antagonists failed to displace $[^{3}H]$amethocaine from its binding site, implying a lack of interaction with L- and N-type voltage-sensitive Ca$^{2+}$ channels. However, these studies report a lack of “direct” channel interaction and possibly the absence of an allosteric interaction between the displacer and $[^{3}H]$amethocaine binding sites. It is still conceivable that the displacing agent could interact at another binding site(s) not coupled to the $[^{3}H]$amethocaine binding site. Our previous study of the effects of local anaesthetic agents with $[^{3}H]$PN200-110 binding sites (L channels) showed a significant interaction but we did not examine if the interaction was allosteric.$^{16}$ However, Bolger and colleagues have shown such an allosteric interaction with $[^{3}H]$nitrendipine binding sites in rat brain and cardiac membranes.$^{17}$

L-glutamate and MK801 (agents that interact with glutamate receptors), GABA, glycine and nicotine failed to displace $[^{3}H]$amethocaine binding. We were unable to displace $[^{3}H]$amethocaine binding with the Na$^{+}$ channel blocker, tetrodotoxin. This is in agreement with the work of Grima and colleagues$^{13}$ although they reported displacement with amiloride (and analogues), an agent that also binds to voltage-dependent Na$^{+}$ channels.$^{16,17}$ 4-AP and tetraethylammonium, agents that interact with K$^{+}$ channels, produced dose-dependent displacement of $[^{3}H]$amethocaine binding, which for 4-AP resulted in an $IC_{50}$ of approximately 209 µmol litre$^{-1}$. However, it is not possible to state that local anaesthetic agents interact with K$^{+}$ channels based on these data alone.

In summary, we have reported dose-dependent and saturable binding of $[^{3}H]$amethocaine to rat...
cerebrocortical membranes that was displaced in a dose-dependent manner by a range of unlabelled local anaesthetic agents. Amino-ester agents were more potent than amino-amide anaesthetics, perhaps implying that a single common binding site for all local anaesthetic agents is unlikely. There did not appear to be a direct interaction with known ligand binding sites on L- or N-type VSCC, GABA, glutamate, glycine or nicotinic receptors. Our data suggest a possible interaction with K⁺ channels. However, at present, a non-specific binding site cannot be excluded.

References

8. Browne T, Hirota K, Lambert DG. Is the neuronal dihydropyridine binding sites on L-type Ca\(^{2+}\) channels a target for local anaesthetic agents? British Journal of Anaesthesia 1997; 77: 290P.