Noninvasive Quantification of Muscarinic Receptors In Vivo With Positron Emission Tomography in the Dog Heart

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The in vivo quantification of myocardial muscarinic receptors has been obtained in six closed-chest dogs by using positron emission tomography. The dogs were injected with a trace amount of \[^{11}\mathrm{C}\]labeled methylquinuclidinyl benzilate (MQNB), a nonmetabolized antagonist of the muscarinic receptor. This was followed 30 minutes later by an injection of an excess of unlabeled MQNB (displacement experiment). Two additional injections of unlabeled MQNB with \[^{11}\mathrm{C}\]MQNB (coinjection experiment) and without \[^{11}\mathrm{C}\]MQNB (second displacement experiment) were administered after 70 and 120 minutes, respectively. This protocol allowed a separate evaluation of the quantity of available receptors (B\(_{\text{max}}\)) as well as the association and dissociation rate constants (k\(_{+1}\) and k\(_{-1}\)) in each dog. The parameters were calculated by using a nonlinear mathematical model in regions of interest over the left ventricle and the interventricular septum. The average value of B\(_{\text{max}}\) was 42±11 pmol/ml tissue, the rate constants k\(_{+1}\), k\(_{-1}\), and K\(_d\) were 0.6±0.1 ml·pmol\(^{-1}\)·min\(^{-1}\), 0.27±0.03 ml·pmol\(^{-1}\)·min\(^{-1}\), and 0.49±0.14 pmol·ml\(^{-1}\), respectively, taking into account the MQNB reaction volume estimated to 0.15 ml/ml tissue. Although \[^{11}\mathrm{C}\]MQNB binding would appear irreversible, our findings indicate that the association of the antagonist is very rapid and that the dissociation is far from negligible. The dissociated ligand, however, has a high probability of rebounding to a free receptor site instead of escaping into the microcirculation. We deduce that the positron emission tomographic images obtained after injecting a trace amount of \[^{11}\mathrm{C}\]MQNB are more representative of blood flow than of receptor density or affinity. We also suggest a simplified protocol consisting of a tracer injection of \[^{11}\mathrm{C}\]MQNB and a second injection of an excess of cold MQNB, which is sufficient to measure B\(_{\text{max}}\) and K\(_d\) in humans. (Circulation 1990;82:1494-1504)

Muscarinic cholinergic receptors play a key role in the regulation of the rate and force of contraction of the heart, and various changes in receptor number and affinity occur in various physiological, pharmacological, and clinical conditions in animals and humans.\(^1\) The development and use of radioligands with high affinity and specificity to the muscarinic receptor has greatly contributed to our knowledge of the receptor biochemistry.\(^9\) Various tritium-labeled antagonists such as quinuclidinyl benzilate (QNB), atropine, dexetimide, N-methylscopolamine (NMS), or N-methylatropine have been shown to bind to an equal number of sites with Hill coefficients close to unity. This indicates that these antagonists bind to a single class of high affinity binding sites.\(^10\)–\(^13\)

Such measurements have been obtained from tissue homogenates or samples collected during surgery or at autopsy by using tritiated ligands in the cases of patients with heart disease. To investigate possible changes in receptor structure (for example after pharmacological intervention or during the evolution of a disease), however, the quantification of muscarinic receptor in man must be obtained noninvasively. Positron emission tomography (PET) now makes this possible. It can measure the concentration of a labeled ligand within the myocardium and can follow its variations for at least 1 or 2 hours.\(^14\)–\(^15\) Measurements were made by using a specifically synthesized
radioligand, in most cases a selective receptor antagonist labeled with a positron-emitting isotope such as carbon-11 or fluorine-18.

In previous studies, we characterized the muscarinic receptor in the baboon, the dog, and humans with PET by using \[^{11}C\]methyl-QNB (MQNB) as a ligand.\(^{16,17}\) MQNB, the quaternary derivative of QNB, is a particularly hydrophilic antagonist that is not extracted by the lungs and displays a high affinity and specificity for cholinergic receptors in rat heart homogenates. The highest concentrations were found in the ventricular septum and the left ventricle.\(^{17}\) The binding of MQNB was saturable and the percentage of \[^{11}C\]MQNB that could be displaced in a dog by an excess of unlabeled atropine was 94%, showing that less than 6% of binding was nonspecific.\(^{15,16}\) This binding was also stereospecific because the pharmacologically active isomer dextemide displaced \[^{11}C\]MQNB from its binding sites; whereas the inactive isomer levetemide remained inactive.\(^{16}\) Finally, a correlation between receptor occupancy and a physiological effect was demonstrated.\(^{17}\) Previously, we had shown that it was possible with some hypotheses to calculate a lumped constant for the ligand-receptor interaction in a single experiment by using PET. Dogs were injected with \[^{11}C\]MQNB at high specific activity followed 20 or 30 minutes later by an injection of an excess of unlabeled MQNB.\(^{18}\)

The present results show that it is possible to estimate the muscarinic receptor density, and the association and dissociation rate constants in the live dog noninvasively. These findings are based on the analysis of plasma and myocardium time-activity curves obtained from arterial samples and PET regions of interest by means of a nonlinear mathematical model including four compartments and seven parameters.

**Methods**

**Animal Preparation**

Six male or female beagle dogs weighing 10–12 kg were fasted overnight before the experiment. They were anesthetized first with a bolus of sodium pentothal (25 mg/kg) followed by a continuous injection at the rate of 5 mg/kg/hr. All dogs had undergone an endotracheal intubation and were ventilated with room air by a respirator (Monnal D, Air Liquide, Paris). A catheter to inject ligand was inserted in the right atrium through the jugular vein, whereas a second catheter to withdraw arterial blood was inserted into the ascending aorta through a femoral artery. The dogs were monitored continuously by an electrocardiogram.

**Preparation of \[^{11}C\]MQNB**

MQNB was labeled with high specific radioactivity using carbon-11 by methylation of QNB with \[^{11}C\]methyliodide.\(^{16}\) Labeled material with a specific radioactivity varying from 200 to 1,100 mCi/\(\mu\)M at the time of injection was purified by using high-performance liquid chromatography.

**Positron Emission Tomographic Measurements**

PET studies were performed by using the seven-slice time-of-flight assisted positron camera\(^{19}\) (LETI TTV01, Commissariat à l’Energie Atomique, Grenoble, France). Each slice was 13 mm thick, and spatial transverse resolution was approximately 12 mm. Transmission scans were performed with a rotating \(^{68}\)Ge source to correct emission scans for the attenuation of 511 KeV photon rays through the thorax. Emission data were recorded in list mode starting with the first injection of \[^{11}C\]MQNB until the end of the experiment. One hundred ten sequential images using one of the seven cross-sections were reconstructed according to the experimental protocol for each dog.

**Experimental Protocol**

A PET study was performed on six dogs. The protocol included three or four injections of \[^{11}C\]MQNB, MQNB, or both. At the beginning of the experiment (time \(T_0\)), approximately 4 mCi (148 MBq) of \[^{11}C\]MQNB were intravenously injected in approximately 1 minute. The corresponding dose of radioactive tracer was denoted by \(J_0\). At time \(T_0\), an additional intravenous injection of an excess of unlabeled ligand (amount \(J_1\)) was given (displacement experiment). At time \(T_2\), a third injection, a mixture of labeled and unlabeled MQNB in the same syringe, was performed ("coinjection" experiment). The injected labeled \[^{11}C\]MQNB and unlabeled ligand quantities are denoted by \(J_2\) and \(J_3\) respectively. In four of the six experiments, a fourth injection consisting of a much larger amount of unlabeled MQNB (second "displacement") was performed at time \(T_3\) (amount \(J_3\)). Timing and doses administered are given in Table 1.

**Image Processing**

Sequential images corresponding to one of the seven cross-sections that intersected the ventricular septum were selected for analysis. Outer myocardial boundaries were automatically defined with an isocontour plotting routine. The 80% isocontour that was selected on a 10-minute image included the septum and the left ventricular wall. The size of the regions of interest was about 10 cm\(^2\).

List mode acquisition allowed the time-of-flight confidence weighted reconstruction of 4-second images during the first 2 minutes after time \(T_0\) and time \(T_2\), and longer duration images (up to 5 minutes) when radioactivity level was reduced. Radioactivity was measured in each region of interest after correction for \(^{11}C\) decay and expressed as picomoles per milliliter after normalizing by the specific radioactivity measured at time \(T_0\). \[^{11}C\]MQNB injected at \(T_0\) and \(T_2\) was produced by the same synthesis so that the specific radioactivity measured at \(T_0\) was identical in both injections.

Calibration was performed with a cylindrical phantom containing a uniform source of \(^{68}\)Ge. Myocardial
wall thickness was measured postmortem and PET data corrected for the loss in count recovery. This was due to the small size of the heart wall compared with the spatial resolution of PET. The correction was performed by using a recovery factor measured experimentally on a heart phantom with the same PET system. The thickness of the ventricular septum and lateral wall of the left ventricle was measured after death and was found to be 10±0.2 mm.20 The ratio of true-to-measured concentration was equal to 0.45 for a 10-mm thickness in our phantom calibration experiments. True $^{[1]}$C MQNB concentrations were obtained by dividing the measured concentration values by this 0.45 recovery coefficient. Spillover from blood to myocardium was not corrected but was accounted for through a vascular fraction $F_v$, see the ligand-receptor model section) that was fitted.

**Plasma Radioactive Concentration Measurements**

The identification of model parameters required the knowledge of the plasma time-activity curve $C_a(t)$, which was used as the input function in subsequent modeling. Sixty-four arterial blood samples (approximately 1 ml) were collected from the ascending aorta. The time interval between each sample was variable to improve the accuracy of the parameter identification. Samples were taken every 5 seconds for 2 minutes after the 2 injections of labeled MQNB. The sampling interval was increased to 10 minutes when blood radioactivity slowed down. The $^{11}$C radioactivity was measured after rapid centrifugation in a gamma counting system (CG 4000, Kontron, Plaisir, France), and the time-activity curves were corrected for physical decay of $^{11}$C activity from time $T_p$. Plasma concentrations were expressed as picomoles per milliliter after division by the specific radioactivity.

The possible presence of labeled metabolites was checked in dog blood samples 2, 10, and 30 minutes after injection of $^{[1]}$C MQNB. After the precipitation of plasma protein in methanol, the concentrated supernatant was analyzed by thin-layer chromatography, and the radioactivity was measured by using a static radiochromatogram analyzer (Chromecel Num-elec, Les Essarts Le Roi, France).

**The Ligand-Receptor Model**

The compartmental model used in this study and shown in Figure 1 is a nonequilibrium nonlinear model.18 The flux of nonmetabolized free ligand from compartment 1 to compartment 2 is given by $pV_R C_a(t)$ (as pmol·min$^{-1}$/ml tissue). $C_a(t)$ is the radioactive plasma concentration at time $t$, $p$ is a rate constant (as ml·min$^{-1}$/ml), and $V_R$ (as ml/ml) is defined as the fraction of the region of interest delineated by PET in which the ligand can react with receptors. With a hydrophilic ligand, $V_R$ should correspond approximately to the fraction of extracellular fluid. It should be noted that the size of compartment 1 could be larger than that of the vascular volume if the myocardial permeability for MQNB is high. The quantity of radioactive free ligand present in the extravascular extracellular volume (compartment 2) (/ml tissue) is $M_f$, which could be merely restricted to a boundary layer.17 The free ligand could bind directly to a free receptor site, or bind irreversibly to a non-specific site or escape with rate constant $k$. The specific binding probability depends on the bimolecular association rate constant ($k_{a}$) and on the local concentration of free receptor sites, which is equal to $[B_{max} - M_o(t)]$. $B_{max}$ is the unknown quantity of receptor sites available for binding, and $M_o(t)$ is the quantity of labeled ligand bound to receptors in 1 ml of myocardium. The irreversible
nonspecific binding probability of the free ligand is denoted by $k_{ns}$ and the rate constant for the dissociation of the bound ligand by $k_{-1}$.

Thus, the model has the following four compartments: a compartment in which ligand concentration is equal to plasma concentration (compartment 1), an extravascular extracellular space occupied by free ligand (compartment 2), a compartment corresponding to specifically bound ligand (compartment 3), and a compartment consisting of nonspecifically and irreversibly bound ligand (compartment 4).

This model contains six parameters, the two most important being the concentration of receptor sites available for binding $B'_{max}$ and the ratio of $k_{-1}$ to $k_{+1}$, that defines the equilibrium dissociation constant $K_p$.

The protocol described in the previous section includes injections of unlabeled ligand during displacement and coinjection experiments. Thus, the kinetics of the unlabeled ligand affect the local concentration of free receptors and must therefore be considered. Free unlabeled ligand is denoted by $M_f(t)$, specifically bound unlabeled ligand by $M_b(t)$, and nonspecifically bound unlabeled ligand by $M_{ns}(t)$. They are expressed as picomoles per milliliter of tissue.

Parameter identification and simulations of labeled and unlabeled ligand kinetics were performed by using the following equation system (which corresponds to the model diagram of Figure 1):

\[
\frac{dM_{f}^{*}(t)}{dt} = pV_{R}C_{a}^{*}(t) - (k + k_{ns})M_{f}^{*}(t) - \frac{k_{+1}}{V_{R}}[B'_{max} - M_{b}^{*} - M_{b}(t)]M_{f}^{*}(t) + k_{-1}M_{ns}^{*}(t)
\]

\[
\frac{dM_{ns}^{*}(t)}{dt} = \frac{k_{+1}}{V_{R}}[B'_{max} - M_{b}^{*} - M_{b}(t)]M_{f}^{*}(t) - k_{-1}M_{ns}^{*}(t)
\]
Table 2. Model Parameters Identified From MQNB Data Obtained From the Protocols Described in Table 1

<table>
<thead>
<tr>
<th>Parameters (units)</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Parameter estimates±SEE†</th>
<th>Exp 3</th>
<th>Exp 4</th>
<th>Exp 5</th>
<th>Exp 6</th>
<th>Average ± SD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVR (ml/min⁻¹)</td>
<td>0.683±0.014</td>
<td>0.630±0.008</td>
<td>0.809±0.014</td>
<td>0.611±0.011</td>
<td>0.587±0.025</td>
<td>0.545±0.017</td>
<td>0.65±0.08</td>
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<tr>
<td>k (min⁻¹)</td>
<td>5.41±0.87</td>
<td>3.53±0.26</td>
<td>2.97±0.55</td>
<td>6.11±1.93</td>
<td>4.52±1.04</td>
<td>2.35±0.29</td>
<td>4.3±1.2</td>
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</tr>
<tr>
<td>k⁺/VR (pmol/ml)</td>
<td>2.97±0.56</td>
<td>2.69±0.32</td>
<td>4.24±0.77</td>
<td>5.15±1.93</td>
<td>4.67±2.00</td>
<td>4.63±1.09</td>
<td>4.0±0.9</td>
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<tr>
<td>k⁻ (min⁻¹)</td>
<td>0.286±0.010</td>
<td>0.289±0.006</td>
<td>0.286±0.012</td>
<td>0.288±0.016</td>
<td>0.212±0.026</td>
<td>0.292±0.028</td>
<td>0.27±0.03</td>
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</tr>
<tr>
<td>Bmax (pmol/ml)</td>
<td>63.3±7.1</td>
<td>38.8±2.9</td>
<td>30.9±1.1</td>
<td>42.6±1.1</td>
<td>29.7±1.8</td>
<td>44.6±2.5</td>
<td>42±11</td>
<td></td>
</tr>
<tr>
<td>kₙ (min⁻¹)</td>
<td>0.046±0.005</td>
<td>0.046±0.005</td>
<td>0.034±0.005</td>
<td>0.064±0.020</td>
<td>0.043±0.010</td>
<td>0.041±0.005</td>
<td>0.046±0.009</td>
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</tr>
<tr>
<td>Fv</td>
<td>0.351±0.035</td>
<td>0.226±0.018</td>
<td>0.408±0.039</td>
<td>0.517±0.046</td>
<td>0.508±0.051</td>
<td>0.414±0.053</td>
<td>0.40±0.10</td>
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</tr>
<tr>
<td>k₃⁺ (min⁻¹)</td>
<td>0.095±0.015</td>
<td>0.106±0.013</td>
<td>0.066±0.013</td>
<td>0.055±0.017</td>
<td>0.046±0.015</td>
<td>0.063±0.012</td>
<td>0.072±0.021</td>
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<tr>
<td>k₃⁻ (min⁻¹)</td>
<td>38.8±2.9</td>
<td>30.9±1.1</td>
<td>42.6±1.1</td>
<td>29.7±1.8</td>
<td>44.6±2.5</td>
<td>42±11</td>
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</tr>
<tr>
<td>k₄ (min⁻¹)</td>
<td>5.15±0.034</td>
<td>30.9±1.1</td>
<td>42.6±1.1</td>
<td>29.7±1.8</td>
<td>44.6±2.5</td>
<td>42±11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k₅ (min⁻¹)</td>
<td>0.65±0.08</td>
<td>4.3±1.2</td>
<td>4.0±0.9</td>
<td>4.0±0.9</td>
<td>4.0±0.9</td>
<td>4.0±0.9</td>
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</tr>
</tbody>
</table>

Exp, experiment.

†Standard errors corresponding to the parameter estimates calculated by the error analysis described in the text.

‡The parameter kₙ was inaccurately identified from the curves obtained during the first two experiments, and the presented results concerning these experiments have been obtained by setting the value of kₙ to 0.046 min⁻¹ (the average of the values identified from the last four experiments).

\[ kₙ = k₊/k₋. \]

\[ j₃ = \frac{k₊}{R} \cdot B'_{max}. \]

\[
\frac{dM_{ns}(t)}{dt} = k_{ns}M_R(t)
\]

\[
\frac{dM_I(t)}{dt} = pVRCA(t) - (k + k_{ns})M_I(t) - \frac{k₊}{V_R}B'_{max} - M_B(t)M_I(t) + k₋M_B(t)
\]

\[
\frac{dM_B(t)}{dt} = \frac{k₊}{V_R}B'_{max} - M_B(t)M_I(t) - k₋M_B(t)
\]

where B'_{max}, pVR, k, k₊/V_R, k₋, and kₙ are the unknown parameters. Units are given in Table 2.

The unlabeled ligand kinetics were assumed to be similar to that of the labeled ligand. To estimate the plasma concentration of unlabeled ligand \( [Cₐ(t)] \), we considered the curve \( Cₐ'(t) \), which was identical to \( Cₐ(t) \) for the first \( T₁ \) minutes and which was extrapolated after this time by using an exponential function. The arterial concentration \( Cₐ(t) \) of the unlabeled ligand was then deduced from \( Cₐ'(t) \) by the following equation:

\[
Cₐ(t) = \sum_{k=1}^{3} \muₖ(t) \int_{Tₖ}^{t} Cₐ'(t) dt
\]

where \( k \) refers to injection \( k \) for a given experiment, \( \muₖ(t) = 0 \) if \( t < Tₖ \), and \( \muₖ(t) = 1 \) if \( t > Tₖ \).

In PET studies, experimental data acquired between time \( t_{l-1} \) and time \( t_l \) is given by the following integral relation:

\[
M_I^*(t_l) = \frac{1}{t_l - t_{l-1}} \int_{t_{l-1}}^{t_l} [M_B^*(t) + M_I^*(t) + F_VC^*(t)] dt
\]

where \( C^*(t) \) is the arterial blood time-concentration curve and where \( F_V \) is a seventh parameter identified at the same time as the other model parameters. \( F_V \) represents the fractional volume including both the fraction of blood present in the tissue volume and the effect of spillover from blood cavity to myocardium.

Results

Myocardial and Plasma Time-Activity Curves

After the first injection of high specific activity \([¹⁴C]MQNB\) into the right atrium, \([¹¹C]MQNB\) plasma concentration decreased rapidly to a low value within a few minutes after injection (Figure 2). In contrast, the myocardial \([¹¹C]MQNB\) concentration increased very rapidly and then remained constant until time \( T₁ \) (Figure 3). The injection of a large amount of unlabeled MQNB at time \( T₁ \) resulted in a rapid decrease in \([¹¹C]MQNB\) myocardial concentration showing a fast dissociation of labeled bound ligand and a slight increase in \([¹¹C]MQNB\) plasma concentration due to the reappearance of this dissociated labeled ligand in the blood circulation.

The coinjection of \([¹¹C]MQNB\) and cold MQNB at time \( T₂ \) produced a high peak of activity in the myocardium and in the plasma that lasted a few minutes. This peak contrasted with the plateau observed when \([¹¹C]MQNB\) was injected alone. In the four dogs in which it was performed, the fourth injection at time \( T₁ \), using cold MQNB again produced a displacement of bound \([¹¹C]MQNB\) followed by a plateau. This second displacement was used to inhibit the rebinding of the labeled ligand to receptor sites and thus allowed us to estimate the irreversible
nonspecific binding. The displacement observed in experiments 3, 4, 5, and 6 was 36%, 41%, 37%, and 33%, respectively. This second displacement was therefore much lower than in the first displacement performed at T1. The last part of the myocardial time-activity curve corresponding to nondisplaceable binding was thus considered to represent mainly the [11C]MQNB irreversible nonspecific binding in the dog heart. Nonspecific binding was also determined in vitro by incubation of dog heart homogenates with 1 µM atropine and amounted to 7.2% of total binding at the Kd concentration of [3H]MQNB.

Results of thin-layer chromatography performed in arterial blood in the dog have shown that unchanged [11C]MQNB represented more than 97%, 96%, and 90% of the total plasma radioactivity 2, 10, and 30 minutes after injection, respectively. The plasma-to-whole blood concentration ratio was found to be constant and equal to 1.7, and the arterial blood hematocrit in the same dog was 0.52. It can be deduced that approximately 80% of blood radioactivity was in plasma and 20% was in red blood cells.

No significant change in dog heart rate was observed during continuous monitoring even for the highest amounts of cold MQNB.

The part of the myocardial time-activity curve corresponding to the tracer injection (before time T1) was plotted against a "normalized time" defined as the integral of plasma activity from time 0 to t divided by the plasma activity at time t. The normalized graphical curves that were drawn with the six sets of MQNB data obtained before the displacement time T1 finally led to asymptotic straight lines (Figure 5 gives the example of experiment 3).

Parameter Identification

Fitting the complete mathematical model to experimental data provided values for kinetic rate constants and receptor densities as shown in Table 2. The final quality of the fits was satisfactory, as can be seen in the Figure 3 example.

An estimate of standard error was obtained for each identified parameter. This value corresponds
FIGURE 5. Example of normalized graphic plot used in analysis of myocardial tissue data obtained after intravenous injection of $^{11}$C-labeled methylquinuclidinyl benzilate ($[^{11}\text{C}]$MQNB). (Data from 0 to 30 minutes of experiment 3). The ratio of tissue to plasma activity was plotted versus the “normalized time” defined as the integral of plasma activity from time 0 to t divided by the plasma activity at time t according the method first proposed by Patlak.8 The asymptotic slope of this curve (shown with a straight line), is often used to obtain an order of magnitude of the binding probability ($k$). In the $[^{11}\text{C}]$MQNB case, however, comparison with the parameters obtained from the model approach showed that this slope corresponds to the order of magnitude of the transfer probability from arterial compartment to the free ligand compartment. The positron emission tomographic images obtained after injection of a trace amount of $[^{11}\text{C}]$MQNB therefore reflect more blood flow than receptor density or affinity (see “Discussion”).

only to the error estimates resulting from the difference between the model-predicted values and the experimental data. It may be noted that the relative standard errors were quite different depending on the parameters. Standard errors were small for some parameters (e.g., approximately 2% for the parameter $pV_R$) and acceptable for the others (e.g., 3-16% for $B_{\text{max}}$ and 2-11% for $k_{-1}$), but they reached up to 20% or 30% for $k_{-1}V_R$ and for $k_{1}/V_R$.

Average parameter values and standard deviations were calculated for the six dogs. The receptor density was 42±1 pmol/ml tissue, and the product $k_{-1}V_R$ was 0.072±0.021 pmol/ml. The relative standard deviations were in the range of 20-30% for all parameters except for parameters $k_{-1}$ (11%) and $pV_R$ (12%). These standard deviations were larger than the standard errors because they included the biological variability as well as other numerical errors such as uncertainty on the specific activity, which can reach 10%.

Computer Simulations

Myocardial activity-versus-time curves for the free, bound, and total ligand were calculated for the six experiments by using the parameter values shown in Table 2. Figure 4 shows the result of the simulation obtained with the parameters estimated from experiment 3. The concentration measured with PET is the sum of $[^{11}\text{C}]$MQNB concentrations in compartments 2, 3, and 4 and in the vascular fraction of the myocardium in the region of interest. Computer simulations showed that most of the radioligand was specifically bound to receptor after injection at high specific activity (from $T_0$ to $T_1$). The percentage of free ligand was equal to 0.6% at 1 minute and decreased to 0.2% at 30 minutes. The percentage corresponding to nonspecifically bound ligand was equal to 0.03% at 1 minute and increased to 0.3% at 20 minutes. After the first displacement, the concentration of specifically bound ligand remained higher than that of other compartments.

The dissociation rate constant $k_{-1}$ was the limiting parameter in the dissociation of bound $[^{11}\text{C}]$MQNB during the displacement between $T_1$ and $T_2$. Just before the coinjection of $[^{11}\text{C}]$MQNB and MQNB at time $T_2$ (70 minutes), the percentage of free ligand was equal to 2.9% and the percentage of nonspecifically bound ligand increased to 22%. Rapidly after the coinjection, all receptors were occupied by unlabeled ligand, so the concentration of free labeled ligand became preponderant for a short time. A large increase of nonspecifically bound ligand was simultaneously observed; it is multiplied by 3 between 70 and 80 minutes. Just before the second displacement ($T_3=120$ minutes), 53% of the ligand corresponded to nonspecifically bound ligand, 37% to specifically bound ligand, and 4% to free ligand. Ten minutes later, these percentages were equal to 79%, 15%, and 4%, respectively.

Discussion

Until recently, investigation of human diseases involving cardiac receptors for neurotransmitters has been limited to postmortem studies or to biopsy samples obtained during surgery. It is now possible to characterize various cardiac receptors by using antagonists of muscarinic, β-adrenergic, or peripheral-type benzodiazepine receptors labeled with positron-emitting isotopes.14,17,20 A method for quantitatively measuring these receptors, however, was required to investigate possible alterations in diseases. The present article introduces a mathematical model describing in vivo binding of a specific antagonist to muscarinic cholinergic receptors in the dog myocardium. The nonlinear model predicts the rates of association and dissociation ($k_{+1}$ and $k_{-1}$) of the ligand to its receptor separately of the receptor density ($B'_{\text{max}}$). In all previous studies, it has been assumed that the ligand occupies a negligible part of the available receptors and that the level of free receptors is not significantly altered during the study [i.e., the quantities of bound ligand $M^*_a(t)$ and $M_b(t)$ are much lower than $B'_{\text{max}}$].21-26 While this assumption simplifies the models, $k_{+1}$ and $B'_{\text{max}}$ cannot be identified independently in such cases, leaving only the composite value of the transfer coefficient describing the flux of ligand into the pool of specifically bound ligand defined by $k_{1}=k_{-1}B'_{\text{max}}/V_R$ to be determined.

An irreversible and nonspecific binding was included in the model because the last part of the
myocardial PET curves showed that a part of the binding was not displaced by a large excess of cold MQNB and appeared irreversible during the duration of the experiments (see example in Figure 3). The modeling approach from PET data is not sensitive enough to detect a possible nonspecific and reversible binding reaction with association-dissociation kinetic rate constants much larger than the other compartmental rate constants. Such nonspecific binding is usually located in compartment 2.21,22

Parameter Values

The quantity of receptor sites available for in vivo binding estimated by the model, \( B_{\text{max}} \), was found to be 42±11 pmol/ml tissue. The receptor concentration was also measured in vitro with \([^{3}H]MQNB \) and found to be 8.6±0.9 pmol/g tissue or 105±5 pmol/g protein. This result is similar to those obtained in vitro with \([^{3}H]QNB \) in dog ventricle, that is, 11 pmol/g tissue11 and 242 pmol/g protein.27 When comparing these results, the \( B_{\text{max}} \) found in vivo by the PET modeling approach is the same order of magnitude although larger than those obtained by in vitro methods.

The \( K_{d} \cdot V_{R} \) value determined by the model was 0.072±0.021 pmol/ml tissue, whereas the \( K_{d} \) value obtained by the in vitro method was 0.49±0.06 pmol/ml. Agreement between the \( K_{d} \) value measured with \([^{3}C]MQNB \) and PET and the value obtained in vitro with \([^{3}H]MQNB \) could be obtained if the fractional volume of reaction \( V_{R} \) would be equal to approximately 0.15 ml/ml tissue. This \( V_{R} \) value is close to that of the extracellular space,28 which confirms the fact that MQNB, a very hydrophilic molecule, binds to cell membrane receptors. It has been shown recently that \([^{3}H]QNB \) labels significantly more sites than two other hydrophilic muscarinic antagonists \([^{3}H]NMS \) and \([^{3}H]MQNB \).29 It has also been suggested that while the subset of receptors detected only by \([^{3}H]QNB \) are also muscarinic, they are not likely to influence the physiologic response, perhaps because they are sequestered in a hydrophobic compartment within the cell membrane. Furthermore, when \([^{3}H]QNB \) was used to label intact cells instead of membrane preparations, a higher nonspecific binding suggested a trapping of the ligand within the cells, presumably within lysosomes.20 Thus, the hydrophilicity of MQNB may explain the low nonspecific binding observed here in vivo in dogs and humans.17

The association rate constant was 0.6±0.1 ml·pmol\(^{-1}\)·min\(^{-1}\) if \( V_{R} \) is assumed to be 0.15 ml/ml tissue. The only available in vitro result is that obtained in rat myocardium \( (k_{4}=2.7 \text{ ml·pmol}^{-1}·\text{min}^{-1}) \).17

The transfer coefficient \( \frac{k_{+1}B_{\text{max}}}{V_{R}} \), denoted by \( k_{3} \), was calculated as 164 min\(^{-1}\), which implies a very rapid binding. The probability of specific \([^{3}C]MQNB \) binding to free receptor sites is \( k_{3}/(k_{4}+k+k_{m}) \). After the first labeled injection but before the displacement time \( T_{1} \), this probability was equal to 98% and the mean residence time in the free ligand compartment was approximately 0.4 seconds. After the first displacement experiment, most of the receptor sites were occupied, but the small part of unoccupied sites still resulted in significant binding. Simulations showed that 10 minutes after the displacement, the probability of binding of the free ligand was still equal to approximately 30%, although only 4% of receptors were free.

The dissociation rate constant (parameter \( k_{-1} \)) equaled 0.27±0.03 min\(^{-1}\), a value similar to the dissociation rate constant value found in intact chicken heart (0.33 min\(^{-1}\))31 and in embryonic chicken heart cell cultures with \([^{3}H]MQNB \) (0.27 min\(^{-1}\))32; it was of the same order of magnitude as the value obtained with rat heart membranes and \([^{3}H]QNB \) (0.81 min\(^{-1}\)).17 This value indicates that at any given time approximately 27% of specifically bound ligand dissociated each minute, a far from negligible amount.

Simple visual inspection of the uptake curves showed a plateau a few minutes after injection of \([^{1}C]MQNB \). This seemed to indicate that \([^{1}C]MQNB \) irreversibly bound to muscarinic receptor, so that \( k_{-1} \) could be disregarded in the model. Such an assumption has been made in a number of studies of dopamine brain receptors with specific high affinity ligands.21–24 Our results, however, show that although MQNB binds with a very high affinity to the muscarinic receptor, it can clearly dissociate from the receptor sites, and once in the free compartment, there is a much higher probability of the ligand rebinding to the same or another free receptor site than escaping into capillary blood. In the present study, only 0.5% of bound ligand reappeared in blood every minute. This high probability of rebinding could be due to the distribution of receptors in clusters at synapses.18 Only in vivo PET measurements allow the direct measurement of binding parameters in these physiological conditions.

The rate of clearance of the ligand from the circulation (\( pV_{R} \)) is equal to \( E\), \( E \) being the extraction fraction and \( F \) the flow rate of fluid containing ligand per unit volume.33 The extraction of \([^{3}H]MQNB \) measured in the isolated perfused rat heart preparation by using the paired injection dilution technique was 60–65%.34 From the \( pV_{R} \) value (65 ml·min\(^{-1}\)/100 ml tissue), the flow rate of fluid containing ligand \( F \) is then approximately 105 ml·min\(^{-1}\)/100 ml tissue. Myocardial blood flow can then be estimated because MQNB rapidly stabilizes between plasma and a fraction of the red blood cell water space. By using a value of 0.93 for the fractional volume of water in plasma35,36 and 0.52 for the hematocrit, we found that the left ventricular average myocardial blood flow was about 130 ml·min\(^{-1}\)/100 g tissue, which corresponds to the 111–169 ml·min\(^{-1}\)/100 g tissue found by Domenech et al.37

Normalized Graphic Method

We then compared the parameter values obtained with our method with those obtained with the nor-
ormalized graphic method, also known as the ratio method. This method was first proposed by Patlak et al.\textsuperscript{18} to analyze tissue deoxyglucose uptake, which is considered irreversible. More recently, it has been suggested for analyzing in vivo ligand-receptor interaction with PET.\textsuperscript{24}

The linearity of the curve obtained by the normalized graphic method is generally considered as the test for binding irreversibility.\textsuperscript{24} From the model described in Figure 1, and assuming that $k_{-1}=0$ (justified by the linearity of the graphic curve), the slope of this line corresponds to $(pV_{R}k_{3})/(k+k_{3}+k_{n})$ where $k_{3}$ is the classical notation of $-B'_{max}/V_{R}$.

Assuming also that $k_{n}=0$, $k_{3}<k$, and $pV_{R}=k$, some authors used this slope to identify parameter $k_{3}$.\textsuperscript{21,25} In the MQNB case, however, it was easy to see that the order of magnitude of the final slope of the curve shown in Figure 5 (0.6 min$^{-1}$) did not correspond to the order of magnitude of parameter $k_{3}$ (130 min$^{-1}$) but corresponded to that of $pV_{R}$ (0.65 min$^{-1}$). It was not surprising that the slope did not correspond to the parameter $k_{3}$ because the above assumptions were not valid for MQNB; in particular, we found $k<<k_{3}$ and not the opposite, $k_{-1}$ was not negligible, and $(pV_{R})/k=0.14$, which was far from unity.

The plateau of $[^{14}C]$MQNB concentration observed in the dog myocardium when the ligand is injected at high specific activity is thus due to a high probability of rebinding and not to an irreversible binding.\textsuperscript{18} These findings must be applied cautiously when interpreting PET images if the used ligand displays a high affinity for specific binding sites. Myocardial PET images obtained after a single injection of a high affinity ligand reflect the myocardial blood flow more than the receptor density. The normalized graphic plot and the computer simulations showed that the binding of the labeled ligand is not limited by the receptor concentration but by the input into the compartment of free ligand (2) given by $pV_{R}C_{a}^{3}(t)$. The possible changes of concentration we observed on the images are mainly the consequence of the local changes of blood flow, which alter the corresponding value of the clearance rate, $pV_{R}$, also equal to $EF$. To estimate the distribution of receptor sites in the heart, it is thus necessary to use a model and an experimental protocol including at least two injections, one at high specific activity, the other at low specific activity (or of cold ligand). Obviously, this is only possible if the administration of a high amount of ligand does not induce pharmacological effect.

Model Identifiability and Clinical Experimental Protocol

This study has shown that the model approach is useful for the study of ligand-receptor interactions in vivo in animals. The main problem seems to be the need to maintain a balance between the respective complexity of the model structure and the experimental protocol. If the number of parameters is too large in comparison with the available experimental data, two difficulties may appear. First, the uncertainties of parameters may be so large that the identified values are meaningless.\textsuperscript{39,40} Second, the identification problem may have several distinct solutions, that is, several very distinct sets of parameter values may lead to similar theoretical curves.\textsuperscript{41,42} In both cases, the identification procedure does not lead to a valid solution. Such a risk cannot be ignored in PET ligand-receptor data analysis because the models used in this field have four to eight parameters.\textsuperscript{42}

In this study, we have used a nonlinear ligand-receptor model including six parameters for which we have previously studied its identifiability.\textsuperscript{40,42} The present protocol included four injections combining labeled, unlabeled, or labeled and unlabeled ligand, and each injection allowed us to follow ligand-receptor interaction in a particular situation. The first injection of radioligand in a trace amount mainly reflects the input kinetics, the first displacement reveals the dissociation and reassociation kinetics, the coinjection allows the investigation of the input-output kinetics when all receptors are occupied, and finally, the second displacement allows measurement of the nonspecific binding rate constant.

The complexity of the experimental protocol is inconvenient. This approach, however, is now available for clinical application with a simplified protocol. A mean value for the low nondisplaceable nonspecific binding can be used without leading to major errors. As previously shown by Delforge et al.,\textsuperscript{42} the suppression of the third injection of $[^{14}C]$MQNB and MQNB leads to two different sets of parameter values, which can be easily distinguished depending on either $k_{-1}>k$ or the opposite. This study, however, shows that the solution with a high $k$ value and a low $k_{-1}$ value is the correct solution. This method has been tested by using the data available in this study. From the data corresponding only to the first two injections (from $T_{0}$ to $T_{2}$), we obtained identified parameters similar to the parameters identified from the complete data. The receptor density, $B_{\text{max}}$, was found equal to $41\pm14$ pmol/ml instead of $42\pm11$ pmol/ml by using the complete protocol, and $K_{n}V_{R}$ was $0.089\pm0.049$ pmol/ml instead of $0.072\pm0.021$ pmol/ml. Therefore, it now seems possible to quantitatively distinguish the myocardial muscarinic receptor in humans by using PET and a simplified protocol starting with a tracer injection of $[^{14}C]$MQNB followed 20 or 30 minutes later by an injection of a much higher amount of unlabeled MQNB.

Appendix: Mathematical Methods

Parameter Identification

To identify the model parameters, we define a cost function allowing to measure for a given parameter set the differences between the experimental data and the corresponding values predicted by the model. Parameter identification is performed by choosing
those parameter values that minimize the cost function. In this study, model parameters are identified by means of minimization of a usual weighted least-square cost function defined by

$$D(P) = \sum_{i=1}^{m} w_i [y(t_i) - Y(t_i, P)]^2$$

where \(\{t_i\}_{i=1,2,..,m}\) are the data sampling times, \(\{w_i\}\) are the weights associated with the decay-corrected measures \(\{y(t_i)\}\), \(P = \{p_1, p_2, \ldots, p_L\}\) represents the model parameters, and \(Y(t_i, P)\) are the values predicted by the model from parameter P and from the experimental protocol. The simulated curves \(Y(t_i, P)\) are calculated by using a fourth-order Runge-Kutta method. The cost function was minimized by using a Marquardt algorithm. Parameter P corresponding to the minimum of \(D(P)\) is the best estimate and is denoted by \(P\).

**Error Analysis**

The evaluation of the parameter uncertainties is essential in any complete identification procedure. We have used the classical approach where the information matrix and the model residual differences between experimental data and model predicted values allow to generate an approximate parameter covariance matrix and to deduce an estimation of the standard error of each parameter.

Sensitivity functions are the derivatives of the theoretical function \(Y(t_i, P)\) with respect to the parameters and could always be calculated by using a numerical procedure. Let \(s_k(t_i, P)\) be the sensitivity function corresponding to a parameter \(p_k\), and S the matrix called the sensitivity matrix and defined by

$$S_{ik} = s_k(t_i, P)$$

Let \(W\) be the diagonal matrix composed with the elements \(\{w_i\}\) and called the weighting matrix. The matrix \(S'W S\) is called the *information matrix* and will be denoted by \(M\). If the data variances are unknown and if the weighting matrix is correctly justified, an approximation of the covariance matrix can be constructed as follows:

$$\text{COV}(P) \approx s^2 M^{-1}$$

where

$$s^2 = D(P)/(m-r)$$

with \(D(P)\) being the residual value of the cost function, \(m\) and \(r\) being the number of measurements and the number of model parameters, respectively.

Let \(\text{COV}(P)\) be the parameter covariance matrix. Assuming that the sampling distribution of the estimate \(P\) is approximately gaussian, an estimate of the standard error \(\Delta p_k\) of parameter \(p_k\) is given by the \(k^{th}\) diagonal element of \(\text{COV}(P)\) denoted by \(\text{COV}(P)_{kk}\) (see References 43 and 47). For a specified probability level \(1-\alpha\), the asymptotic \(100(1-\alpha)\%\) confidence interval for parameter \(p_k\) is defined as follows:

$$p_k \in \left[ p_k - T_{1-\alpha/2} \Delta p_k, p_k + T_{1-\alpha/2} \Delta p_k \right]$$

where

$$\Delta p_k = \sqrt{\text{COV}(P)_{kk}}$$

and where \(T_{1-\alpha/2}\) is the indicated value of the T distribution for \(m-r\) degree of freedom (Student law). It is common to use a 95% confidence interval (\(\alpha=0.05\)) and a very large number of freedom degrees (\(>30\)).

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