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Muscarinic cholinergic receptor in the human heart evidenced under physiological conditions by positron emission tomography

(acetylcholine receptor/binding kinetics/receptor model)

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ABSTRACT **The muscarinic receptor was studied *in vivo* in the human heart by a noninvasive method, positron emission tomography (PET). The study showed that the binding sites of ^{11}C-labeled methiodide quinuclidinyl benzilate ([^{11}C]MQNB), a muscarinic antagonist, were mainly distributed in the ventricular septum (98 pmol/cm^3 of heart) and in the left ventricular wall (89 pmol/cm^3), while the atria were not visualized. A few minutes after a bolus intravenous injection, the concentration of [^{11}C]MQNB in blood fell to a negligible level (<100th of the concentration measured in the ventricular septum). When injected at high specific radioactivity, the concentration of [^{11}C]MQNB in the septum rapidly increased and then remained constant with time. This result was explained by rebinding of the ligand to receptors. It was the major difference observed between the kinetics of binding of [^{11}C]MQNB to receptor sites after intravenous injection *in vivo* and that of [^{3}H]MQNB to heart homogenates *in vitro*. The MQNB concentrations in the ventricular septum of different individuals were found to be highest when the heart rate at the time of injection was slow. This result suggests that the antagonist binding site is related to a low-affinity conformational state of the receptor under predominant vagal stimulation. Thus, positron emission tomography might be the ideal method to study the physiologically active form of the muscarinic acetylcholine receptor in man.**

A population of specific, saturable, high-affinity muscarinic receptors in the mammalian heart was identified *in vitro* by using the potent muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) (1). However, the disadvantage of *in vitro* binding studies is that they cannot distinguish between ligand-binding sites and effector-coupled receptors; in particular, the correlation between the affinity for receptor subtypes and the biological effect of agonists is difficult to demonstrate. Furthermore, the conditions of *in vitro* binding studies are quite different from those governing the receptor during physiological transmission (2). The properties of muscarinic receptors studied in homogenates or after solubilization may be altered or may correspond to selective fractions of the total population (3). The accessibility of receptor sites to acetylcholine in the intact organ and in homogenates is also different. It appears that the range of acetylcholine concentrations used for *in vitro* binding studies of the nicotinic receptor (approximately micromolar) is much smaller than that of physiologically active concentrations in the synaptic cleft (approximately millimolar) (2). Since binding studies are performed at equilibrium, the ligand is incubated with the receptor preparation for a much longer time (minutes or hours) than that corresponding to the channel lifetime (4). Thus, the correlation between the active conformation of the receptor leading to the response (opening of ions channels) and the conformational transitions identified *in vitro* is difficult to establish (5).

In this communication we report quantitative data obtained on the dynamics of the myocardial acetylcholine receptor in man by a safe, noninvasive technique. Positron emission tomography (PET) is a visualization technique that gives an accurate and quantitative representation of the spatial distribution of a positron-emitting radionuclide in any desired transverse section of the body. Carbon-11, a short-lived positron emitter (20.4 min) can be incorporated into ligands for receptors. Owing to its short half-life, relatively large amounts of ^{11}C-labeled ligand can be intravenously injected at a high specific activity (10–100 times higher than that of a ^{3}H-labeled ligand) with a low radiation dose to the patient. The PET approach may be compared to quantitative autoradiography with the added advantage of allowing *in vivo* studies in man under normal and pathological conditions.

Methiodide quinuclidinyl benzilate (MQNB) was chosen instead of QNB for *in vivo* PET studies of the properties of muscarinic acetylcholine receptors in the human heart because MQNB is a hydrophilic nontoxic molecule that cannot cross the blood–brain barrier and is not extracted by the lungs. Large accumulation in the heart, low nonspecific binding, and high affinity and specificity for the muscarinic receptor in rat, guinea pig, and rabbit suggest that MQNB could be an ideal radioligand for *in vivo* studies (6). Using [^{11}C]MQNB, Mazière *et al.* showed that the *in vivo* [^{11}C]MQNB uptake in the baboon heart corresponds to specific binding to acetylcholine muscarinic receptors (7).

In the present communication, we show that by using PET it is possible to study the localization of the muscarinic cholinergic receptor in the human heart under physiological conditions and to study the relationship between the conformational state of the receptor and the physiological response.

MATERIALS AND METHODS

Thirty-four studies were performed on 33 subjects with their informed consent. Twenty-two subjects showed no detectable sign of heart disease; the other cases included five old myocardial infarctions and six mitral or aortic valve diseases.

[^{11}C]MQNB Synthesis. [^{11}C]MQNB was synthesized by the method of Mazière *et al.* (7), whereby the ^{11}C produced as $^{11}CO_2$ in a cyclotron was converted into [^{11}C]methyl iodide, which reacted with QNB. High-performance liquid chromatography purification led to a radiochemically pure material with a specific radioactivity varying from 400 to 800 Ci/mmol (1 Ci = 37 GBq) at the time of administration to the patient.

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Abbreviations: QNB, quinuclidinyl benzilate; MQNB, methiodide quinuclidinyl benzilate; PET, positron emission tomography.

Regional Measurement of [^{11}C]MQNB Concentration in the Human Heart. Cross-sectional imaging of the heart was obtained with a single-slice positron tomographic system (ECAT II, ORTEC, Oak Ridge, TN) with a transverse spatial resolution of 17 mm and a slice thickness of 19 mm. Scans were started immediately after intravenous injection of 10–25 mCi (370–925 MBq) of [^{11}C]MQNB. Thirty-five to 40 images were recorded at a single level from 0 to 70 or 120 min after injection. The first 10 images were obtained in 1 min each; the acquisition time then was increased for the others so that at least 300,000 cpm were accumulated for each section. Quantitative images were corrected for 511-keV γ-ray attenuation and radioactive decay. Regions of interest were selected over the ventricles and the septum to measure the regional tracer concentration. The loss in cpm recovery related to the small size of the ventricular septum was corrected by using echocardiography (8). From a calibration factor measured experimentally with a standard solution of a positron emitter, the results were expressed as the percentage of injected dose per ml of tissue, or, after multiplication, by the injected amount of MQNB in pmol/ml of tissue. One of the patients was studied with a new photon time-of-flight-assisted PET system (LETI, Commissariat à l'Energie Atomique, Grenoble, France) providing five slices simultaneously with transverse and axial spatial resolutions of 12 and 13 mm, respectively.

Blood radioactivity also was measured in venous samples at various times after [^{11}C]MQNB injection. Twenty-one of the 34 subjects received an atropine injection (1.4–28 nmol/kg) 20, 42, or 105 min after the [^{11}C]MQNB injection in order to study the displacement of bound tracer.

Binding of [^{3}H]MQNB on Rat Heart Homogenates. Freshly dissected rat heart tissues were placed in 19 vol of 0.25 M sucrose (pH 7.4), homogenized, and centrifuged (1000 × *g*). The membrane fraction obtained after centrifugation (20,000 × *g*) of the supernatant was resuspended in 0.05 M Na/K phosphate buffer at pH 7.4 and stored at −25°C. For binding assays, aliquots of the membrane fraction (0.1–0.5 mg of protein) were incubated at 37°C with tritium-labeled methyl-QNB (Service des Molécules Marquées, Commissariat à l'Energie Atomique, Saclay, France) in 1 ml of phosphate buffer, both with and without 0.1 M atropine. After a 60-min incubation (or less for the determination of the rate constant of association), the samples were vacuum-filtered through Whatman GF/C filters and washed with 15 ml of cold buffer.

RESULTS

Regional Distribution of [^{11}C]MQNB Binding Sites. Only the ventricular septum and the left ventricle contained significant concentrations of [^{11}C]MQNB. The radioactivity in the right ventricle was very low, and the atria were never visualized (Fig. 1). The ratio between septum and ventricular wall [^{11}C]MQNB concentrations was 1.10 ± 0.02 (mean ± SEM, n = 34).

Relationship Between [^{11}C]MQNB Specific Activity and the Pattern of [^{11}C]MQNB Activity-vs.-Time Curves. After a bolus injection of [^{11}C]MQNB at a high specific activity in a brachial vein, the [^{11}C]MQNB blood concentration fell very rapidly to a negligible value a few minutes after intravenous injection (Fig. 2 *Upper*). In contrast, the [^{11}C]MQNB concentration increased rapidly in the myocardium to reach a maximum in 1–5 min and then remained constant for 70 min; under these conditions the [^{11}C]MQNB concentration was >100 times higher in heart than in blood (Fig. 2 *Upper*).

In other patients the radioligand specific activity in the injectate was decreased by addition of various amounts of unlabeled MQNB (between 21 and 508 nmol/m^2 of body surface area; Fig. 2 *Lower*). The activity-vs.-time curves in the patients injected with a dose larger than 100 nmol/m^2 showed a maximum during the first minute, followed by a decrease. The larger the amount of MQNB injected in a patient, the faster the myocardial radioactivity decreased (Fig. 2 *Lower*).

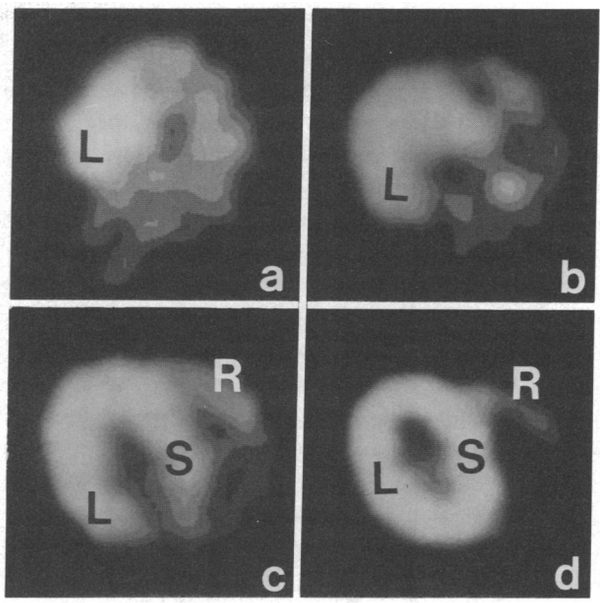

FIG. 1. Tomographic, transaxial cross-sectional images of the heart recorded 10 min after rapid intravenous injection of [^{11}C]MQNB from a normal subject with a time-of-flight PET system. Four contiguous images are shown. Each slice is 13 mm thick. Images: *a* and *b*, upper part of the left ventricle (L); *c*, the body of left ventricle (L), the ventricular septum (S), and the right ventricle (R); *d*, the apex of the left ventricle. [^{11}C]MQNB binding sites are located mainly in the left ventricle and septum and to a lesser degree in the right ventricle. The atria are not visualized.

[^{11}C]MQNB Displacement by Unlabeled Atropine. The rapid intravenous injection of unlabeled atropine led to a rapid decrease (lasting a few minutes) in the septal [^{11}C]MQNB concentration (Fig. 2 *Upper*). The percentage of radioligand displaced 30 min after atropine injection was a curvilinear function of the amount of injected atropine. The curve tended asymptotically to a value of 75%. For each patient given a dose of atropine high enough (>19 nmol/kg of body weight) to saturate all receptor sites, the dissociation rate constant, k_{-1}, was calculated from the initial slope of the [^{11}C]MQNB activity-vs.-time curve obtained after atropine injection and was found to be 0.15 ± 0.02 min^{-1} (mean ± SEM, n = 12). For a given amount of atropine, the time of its injection relative to that of [^{11}C]MQNB had no effect on the percentage of radioligand displaced.

Effect of the Injected Quantities of MQNB on the Binding of [^{11}C]MQNB to Muscarinic Receptors. In each individual, the curve of the concentration of [^{11}C]MQNB in the septum as a function of time was extrapolated to time zero. Thus, the concentration values in the ventricular septum (expressed as pmol/cm^3 of septum) calculated for each of the 34 experiments were plotted as a function of the amount of MQNB injected in blood. These individual values were proportional to the injected amount if it did not exceed 200 nmol/m^2 of body surface (Fig. 3). Above 300 nmol·m^{-2}, the concentration became constant at 98 pmol per cm^3 of septum.

Relationship Between Receptor Occupancy and Heart Rate. Fig. 4 demonstrates a relationship between the percentage of MQNB found in 1 cm^3 of septum after rapid intravenous injection in 12 subjects injected with comparable amounts of MQNB (between 1 and 2 nmol/kg of body weight) and the heart rate value recorded at the time of injection. Fig. 4 shows that, for a given subject, the MQNB concentration in

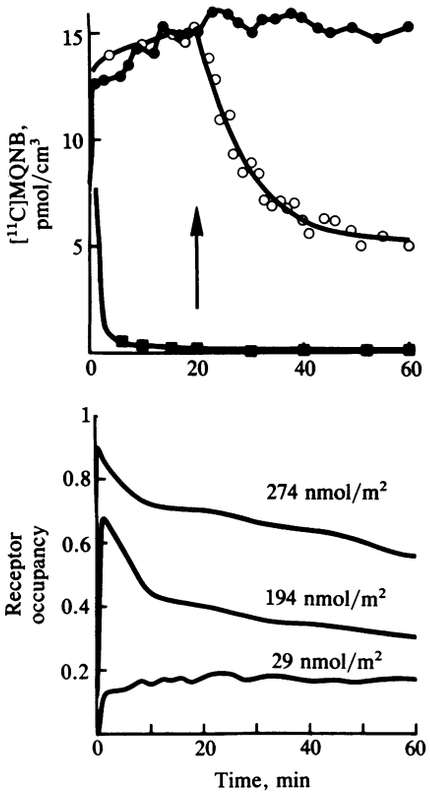

FIG. 2. (*Upper*) [^{11}C]MQNB radioactive concentration-vs.-time curves. After a bolus intravenous injection of 26 mCi of [^{11}C]MQNB at a high specific activity (639 Ci/mmol) in a normal subject, the tracer is rapidly cleared from the blood, and the MQNB concentration is around 0.1 pmol/cm^3 (■). By contrast, the activity rises almost immediately in the ventricular septum (●) and reaches a mean value around 15 pmol per cm^3 of tissue. In another subject (○), a similar dose of [^{11}C]MQNB was intravenously injected, but 20 min later (arrow) an additional intravenous injection of unlabeled atropine (18.7 nmol/kg) was performed. The injection of the competitive inhibitor results in a displacement of [^{11}C]MQNB from its binding sites (○). The displacement calculated 30 min after the injection of atropine is equal to 64%. The value of the dissociation rate constant calculated from the initial part of the descending curve is equal to 0.12 min^{-1}. (*Lower*) Time course of fractional occupancy of acetylcholine receptor sites in the ventricular septum recorded from three different subjects. Each subject was intravenously injected with different amounts of MQNB added to [^{11}C]MQNB in the same syringe. For each subject the activity-vs.-time curve was calculated in a region of interest selected in the ventricular septum; after correction for the partial volume effect, the MQNB concentration (pmol/cm^3 of tissue) was divided by the total concentration of binding sites (98 pmol/cm^3; see Fig. 3) to obtain the fractional receptor occupancy. For the lowest amounts of MQNB (29 nmol/m^2 body surface area), the receptor occupancy is low and remains constant (same patient as in *Upper*, ●). For higher amounts (194 and 274 nmol/m^2), after an initial peak following the bolus intravenous injection, the receptor occupancy decreases rapidly for a few minutes and then slowly. Thus, this figure shows that the dissociation rate of ligand depends on the receptor occupation at a given time.

the ventricular septum is higher when the heart rate is lower.

***In Vitro* Saturability of [^{3}H]MQNB Binding in Rat Heart Homogenates.** Incubation of the membrane fraction of rat heart with various concentrations of [^{3}H]MQNB (0.05–1.50 nM), revealed a saturable binding. The nonspecific binding measured in the presence of 1 μM atropine was linear and accounted for <10% of the total binding at half saturation. Scatchard analysis of the specific [^{3}H]MQNB binding indicated a single high-affinity binding site. The equilibrium dissociation constant value at "infinitely low" receptor concentration (K_d) was 3.2×10^{-8} M; the receptor density was 228 fmol/mg of protein. The value of the Hill coefficient was 1.02.

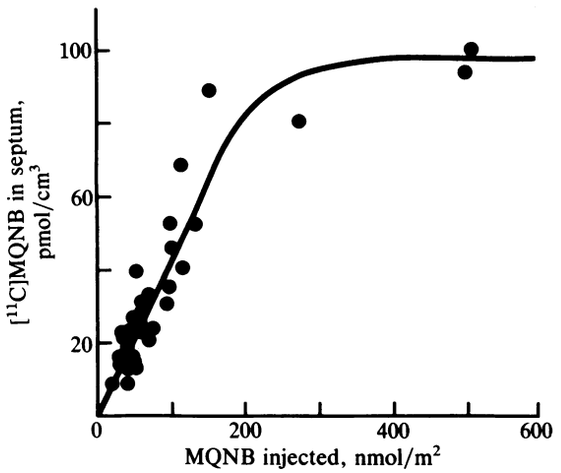

FIG. 3. [^{11}C]MQNB concentration in the ventricular septum (pmol/cm^3 of septum) as a function of the amount of MQNB injected (nmol/m^2 of body surface area). The concentration values measured in 34 separate experiments were plotted against the amount of MQNB injected as a bolus in a brachial vein. Above 300 nmol/m^2 of body surface area, the concentration in the ventricular septum becomes independent of the injected amount; it represents the total concentration of MQNB binding sites (98 pmol/cm^3 of septum).

***In Vitro* Kinetics of Specific [^{3}H]MQNB Binding.** The maximum specific binding was obtained after a 10-min incubation. The dissociation rate constant measured either by dilution (1:200) or by addition of 1 μM atropine was 0.81 min^{-1}. The association rate constant was 2.73×10^9 M^{-1}·min^{-1}. The ratio of these rate constants yields a K_d of 3.0×10^{-8} M.

DISCUSSION

The present study shows that cardiac muscarinic cholinergic receptors may be identified and characterized noninvasively in man by the use of [^{11}C]MQNB, a hydrophilic antagonist labeled with a positron emitter, and PET. *In vitro* the properties of [^{3}H]MQNB were not very different from those of the most widely used ligand, [^{3}H]QNB, a potent lipophilic antagonist (1). Binding of [^{3}H]MQNB to rat heart membranes was

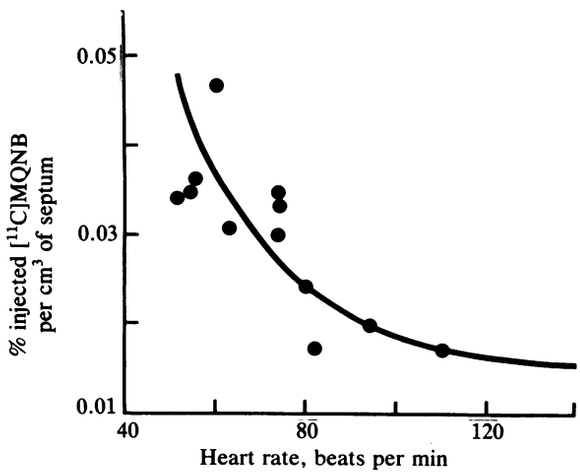

FIG. 4. Dependence of [^{11}C]MQNB concentration in the ventricular septum on heart rate. In 12 individuals, the percentage of the injected dose present in 1 cm^3 of septum is plotted as a function of heart rate (expressed in beats per min) recorded at the time of [^{11}C]MQNB injection.

saturable and of high affinity ($K_d = 3.2 \times 10^{-8}$ M). The dissociation rate constant of [^{3}H]MQNB bound to rat heart membranes (k_{-1}) was 0.81 min^{-1} and was of the same order of magnitude as the value found with [^{3}H]QNB in intact chicken heart (0.33 min^{-1}) and in embryonic chicken heart cell cultures (0.27 min^{-1}) (9, 10). However, an isomerization of the receptor antagonist complex was demonstrated with [^{3}H]QNB but not with [^{3}H]MQNB (9–12). This discrepancy could be due to the difference in liposolubility between QNB and its *N*-methyl quaternary salt, MQNB (13), since it has been suggested that hydrophobic interactions within the receptor or between the receptor and the membrane lipids are necessary for the conformational change to occur (10, 14).

The regional distribution of receptor densities for *in vivo* [^{11}C]MQNB binding in the human heart was found to differ somewhat from that reported by Fields *et al.* in rabbit, rat, and guinea pig heart homogenates with [^{3}H]QNB (1). In our *in vivo* human studies, the highest concentrations were obtained in the septum (98 pmol/cm^3 of heart) and in the left ventricle (89 pmol/cm^3), whereas they were found in the left and right atria with [^{3}H]QNB in rat heart homogenates (1). However, although rat septal and ventricular tissue contained a lower concentration of receptors per g of protein, because of their weight, these regions contained a higher percentage of acetylcholine receptors than in the atria (1).

In PET studies one must take into account the partial volume effects that lead to a loss in cpm recovery when the object size is smaller than the spatial resolution of the camera. However, even in the patient examined with the time-of-flight-assisted PET system, the atria were not visualized; thus, it is likely that the regional myocardial distribution of muscarinic receptors is different in man and in rat. Indeed, a species-dependent regional distribution of cholinergic receptors has been observed in different animals: the receptor site density was highest in the right and left atria in rat and rabbit heart, whereas it was diffuse in guinea pig and dog, where 75% of the total cardiac receptors were found in the ventricles (15). Although the existence of an abundant parasympathetic innervation of the atria is well known, that of the ventricles has been a subject of considerable controversy. Both histological and physiological data were generally considered to indicate an absence of ventricular parasympathetic innervation (16), but in the past decade numerous data, both chemical and physiological, have proved the existence of a direct parasympathetic innervation of the mammalian ventricle (see ref. 17 for review). The ventricular muscarinic receptors are localized to sarcolemna in dog (18); their precise role in the modulation of biochemical and electrophysiological events at the cellular level remains a subject of considerable interest.

We showed in a recent paper that the quantitative results obtained in man when [^{11}C]MQNB was intravenously injected were different from those obtained *in vitro* when [^{3}H]QNB was incubated with heart tissue homogenates (19). When trace amounts of [^{11}C]MQNB were intravenously injected, the blood activity became negligible in a few minutes because the tracer was extracted from blood not only by the heart but also by the liver where the binding is not specific (7). Although this experiment can be compared with those performed *in vitro* at infinite dilution, [^{11}C]MQNB blood concentration being 1/100th of heart concentration after 4 or 5 min, the septum radioactivity remained constant with time. However, the rapid intravenous injection of a larger amount of unlabeled atropine resulted in a rapid decrease in cardiac radioactivity as is found in *in vitro* saturation experiments, the acetylcholine receptors being blocked by the unlabeled drug. We have shown in this study that *in vitro* [^{3}H]MQNB dissociated under dilution or saturation conditions with the same mono-exponential decay characterized by the dissociation rate constant k_{-1}. Therefore, the results obtained *in vivo* in man after a bolus intravenous injection of [^{11}C]MQNB have been explained by suggesting that, although the binding follows a bimolecular reaction as in the *in vitro* situation, a boundary layer exists in the immediate vicinity of the binding sites (19).

A simple mathematical model was developed. The main result was that the higher the concentration of receptor-bound ligand or the fractional receptor occupancy, the larger the ligand fraction that escapes from the boundary layer (19). In the present study, the rate constant for the dissociation of the MQNB complex was calculated to be 0.15 min^{-1} according to this model. The same value was obtained whether atropine was injected at minute 20 or 105 after [^{11}C]MQNB injection, which means that the affinity of the binding sites was independent of the duration of MQNB "incubation" with the receptor and thus that, in contrast to QNB, there was no evidence of isomerization of the MQNB–receptor complex *in vivo* nor *in vitro*. The k_{-1} value for the dissociation of MQNB complex measured *in vitro* on rat heart homogenates (0.81 min^{-1}) was of the same order of magnitude. These results suggest that the main difference between MQNB binding to acetylcholine receptor *in vivo* and *in vitro* is the presence of a boundary layer in intact tissue that contains the ligand at a finite concentration in the vicinity of the receptor sites, although its concentration in interstitial tissue and blood is close to zero.

The plots of the individual MQNB septum concentrations extrapolated at $t = 0$ vs. the amount of MQNB (Fig. 3) seem to be similar to those of bound ligand vs. total ligand concentration obtained *in vitro* (20). In both cases the plateau corresponds to the total receptor concentration. However, the linear ascending part of the curve does not represent the inverse of the total receptor concentration ($1/|R_T|$) as in *in vitro* equilibrium saturation experiments when $|R_T|$ is much larger than the equilibrium dissociation constant K_d (20). Since [^{11}C]MQNB was intravenously injected as a bolus and very rapidly extracted from blood to reach a stable level in myocardium, its fractional uptake must have been equal to the myocardial blood flow fraction of the cardiac output (21). Thus, the slope of the regression line in Fig. 3 represents the ratio of the coronary blood flow per cm^3 of myocardium to the cardiac index (expressed as ml·min^{-1}/m^2 body surface area). The mean value calculated from 30 points corresponding to 29 different subjects was 0.417×10^{-3} m^2 per cm^3 of septum. By using a cardiac index value of 3500 ml·min^{-1} per m^2, the mean coronary blood flow comes out to be 1.46 ml·min^{-1} per cm^3 of myocardium, which is at the upper limit of normal values (22).

Since PET allows an *in vivo* analysis of muscarinic receptors in the human heart under sympathetic and parasympathetic physiological control, it is worth considering that this method might represent the ideal approach to research on the physiologically active form of the acetylcholine receptor. This hypothesis is supported by the finding of a relationship between heart rate at injection time and the percentage of the amount of MQNB intravenously injected that is found in 1 cm^3 of septum. The interesting result was that the lower the heart rate in an individual, the higher the receptor occupancy by the antagonist. It has been suggested from *in vitro* data that the low-affinity form of the cardiac muscarinic receptor is the active form (23–26) because it mediates the inhibition of adenylate cyclase (24) and the negative chronotropic response in embryonic chick atria (27). Complex sympathetic–parasympathetic interactions occur at the heart level (the force and frequency of contraction are increased by noradrenaline and decreased by acetylcholine), and at the molecular level these transmitters act upon various effector systems. Nevertheless, a low frequency is related to a predominant vagal influence. The greater [^{11}C]MQNB binding in the septum linked to vagal stimulation could be explained by an

increase in either the number or the affinity of antagonist binding sites. In the physiologically active state, the agonist is released from the receptor in a low-affinity form, and more sites are available for [^{11}C]MQNB binding (28). According to this hypothesis, vagal stimulation would elicit a selective increase in the [^{11}C]MQNB binding capacity of the receptor without changing the affinity of the antagonist for the receptor. The presence of two interconvertible forms of the muscarinic cholinergic receptor respectively favored by agonists and antagonists and displaying high-agonist/low-antagonist and low-agonist/high-antagonist affinities, respectively, has been demonstrated (29). According to this hypothesis, vagal stimulation would be characterized by a conversion to the low-agonist/high-antagonist affinity form of the muscarinic receptor.

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