Peripheral-type benzodiazepine receptors in the living heart characterized by positron emission tomography

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ABSTRACT The presence of specific benzodiazepine binding sites in the hearts of dogs and human beings was demonstrated in vivo by a noninvasive method, positron emission tomography (PET). An antagonist of the peripheral-type benzodiazepine binding site, PK 11195, was labeled with carbon-11, a short-lived positron emitter. When injected at high specific activity, $^{11}$C–PK 11195 was concentrated in the myocardium. As increasing amounts of unlabeled PK 11195 were added to the radioactive ligand, the myocardial ligand concentration was proportional to myocardial regional perfusion up to quantities of 40 nmoI/kg body weight. Above 40 nmoI/kg the ligand concentration reached a maximum value (6000 pmol/cm$^3$), which could be considered as the total number of binding sites per unit heart volume. The specificity of $^{11}$C–PK 11195 binding to canine heart was demonstrated from a study on the inhibition of binding for radioligand by an excess of several agonists or antagonists of benzodiazepine receptor. The distribution and specificity of $^{11}$C–PK 11195 was similar in dogs and in human beings. PET thus opens the way to the investigation of the peripheral-type benzodiazepine receptor in a clinical situation, since it has recently been shown that this receptor could be coupled to the calcium channel in the heart.


SHORTLY AFTER the discovery of benzodiazepine binding sites in the central nervous system,1–4 specific high-affinity binding sites were also demonstrated in peripheral organs such as the kidney, liver, skeletal and ileal muscle, lungs, and heart.5–7 However, the ligand specificity and affinity for the peripheral-type binding site is completely different from that of the central-type site.8, 9 It was found recently that a compound with a nonbenzodiazepine structure, 1-(2-chlorophenyl)-N-methyl N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195), is very potent in the binding inhibition of ligands such as $^3$H–RO 5-4864, known to bind with high affinity to peripheral-type sites.5, 10–12 In the heart, in vitro and in vivo, PK 11195 was even more potent than RO 5-4864. Furthermore, it has been shown that peripheral-type benzodiazepine binding sites are pharmacologic receptors coupled to calcium channels in guinea pig papillary muscle13 and that PK 11195, a compound devoid of activity in heart muscle, is an antagonist of the peripheral-type benzodiazepine receptor.

It thus seemed interesting to characterize the peripheral-type benzodiazepine receptor noninvasively in vivo in the canine and human heart with PK 11195 as ligand. Noninvasive investigation of cardiac receptors was impossible until the introduction of labeling methods using carbon-11, a short-lived cyclotron isotope, and of positron emission tomography (PET). PET is a visualization technique that permits quantitative measurements in vivo of local perfusion, metabolism, pharmacology, and biochemistry in the heart by external detection.14, 15 It thus offers the possibility of characterizing changes in receptor binding properties as well as regional receptor density in the hearts of intact animals and ultimately in human cardiac diseases.16, 17 We report here a first attempt to characterize the peripheral-type benzodiazepine receptor in the canine and human heart by PET and $^{11}$C–PK 11195.

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Methods

Preparation of $^{11}$C–PK 11195. The $^{11}$C–PK 11195 synthesis method has been described elsewhere. $^{11}$CO$_2$ was produced in a medical cyclotron and $^{11}$C-methyl iodide used as the labeled precursor. N-$^{11}$C-methyl, N-$^{11}$C-methyl-$^{1}$-propyl, 2 (chloro-2-phenyl)-1 isoquinolinecarboxamide -3 was produced in about 45 min at high specific activity (around 800 Ci/mm). The radiochemical purity, as assayed by high-pressure liquid chromatography, was better than 99%. Sterility and aipyrogenicity were checked.

Animal studies. Twenty-four experiments were carried out on 10 beagles (eight females, two males) weighing 10 to 18 kg. Each animal was anesthetized with sodium pentobarbital (25 mg/kg) 2 hr before the PET study, curarized with pancuronium sulfate (0.0025 mg/kg/min), intubated, and ventilated with room air. For the curare, agonist and antagonist injections, and withdrawal of blood samples a catheter was advanced through the external jugular vein into the right atrium. To monitor arterial blood pressure in three experiments a catheter was placed in the femoral artery and attached to a Statham P23Db pressure transducer. The electrocardiogram was recorded continuously during each study.

After instrumentation each dog was positioned within the ring of a single-slice positron tomographic system (ECAT II, ORTEC, Oak Ridge, TN) or a seven-slice time-of-flight assisted PET system (LETI, Commissariat à l’Energie Atomique, Grenoble, France). Two centimeter (ECAT II) or 1 cm (LETI) thick transverse sections of the heart were examined. Before each experiment, transmission scans were collected by means of a germanium-68 ring source to correct emission data for 511 keV photon attenuation through the thorax. A mid left ventricular position was selected for serial transaxial emission tomography. Emission scans were started immediately after injection of 3.6 to 34.4 mCi (133 to 1270 MBq) of $^{11}$C–PK 11195 into the right atrium. Specific activities at the time of injection ranged from 4 to 3000 mCi/μmol. With the ECAT II system the images were recorded at a single level as a function of time, from 0 to 75 or 120 min after injection. With the LETI time-of-flight system, seven slices were obtained simultaneously. At each level 40 to 42 images were recorded and the acquisition time varied from 1 min each for the first images to 5 min each for the last ones to accumulate at least 500,000 counts for each slice. Use of a medium resolution filter yielded a spatial resolution of 17 mm for the ECAT II system and 12 mm for the LETI time-of-flight system.

Three regions of interest (septum, lateral wall of left ventricle, and whole left ventricle) were selected in significant areas of the imaging plane that crossed the ventricular myocardium. $^{11}$C–PK 11195 concentrations were measured in the different regions of interest for all sections. Calibration was performed every week for each system by the use of a cylindrical phantom containing a uniform source of germanium-68. Venous blood samples were collected at different times during PET imaging and the $^{11}$C radioactivity was measured in a gamma counting system. The PET data were corrected for the loss in count recovery because of the small size of the heart wall compared with the spatial resolution of PET. The thickness of the ventricular septum and lateral wall was measured after death in dogs and was found to be 10 ± 0.2 mm; the thickness was measured by bimedimensional echocardiography in human subjects in the same transverse section as that used for PET. From a calibration factor measured experimentally on a phantom with a standard solution of a positron emitter, the results were expressed as the percentage of injected dose per milliliter of tissue, or, after multiplication by the amount of PK 11195 injected, in picomoles per milliliter of tissue.

In four experiments 50, 100, 200, and 2500 μg/kg unlabeled PK 11195 was rapidly injected 30 min after the injection of $^{11}$C–PK 11195 to inhibit the binding of the labeled ligand from its specific binding sites. In one experiment 200 μg/kg PK 11195 was injected 110 min after the $^{11}$C–PK 11195 injection. The benzodiazepine binding sites were further characterized in nine experiments by injection of various amounts of unlabeled agonist or antagonist 30 min after that of $^{11}$C–PK 11195. The same protocol was used in two other experiments to study the effect of $^{11}$C–PK 11195 solvent and the effect of the norderivative (PK 13042) on the binding of $^{11}$C–PK 11195. In five other experiments, the effect of specific activity variations was studied by addition of unlabeled PK 11195 (100, 160, 250, 300, and 500 nmol/kg) to $^{11}$C–PK 11195.

Human studies. Seven normal male volunteers (22 to 35 years old) were positioned in the ECAT II camera and an 18-gauge needle was placed in an antecubital vein. Transmission scans were recorded for later attenuation correction of emission data. Seven to 21 mCi (259 to 777 MBq) of $^{11}$C–PK 11195 was injected rapidly. Specific activities at the time of injection ranged from 41 to 580 mCi/μmol. Myocardial scans were obtained as described above. To study the binding inhibition of $^{11}$C–PK 11195 on its specific binding sites, 100, 200, or 500 μg/kg PK 11195 was rapidly injected in four experiments 30 min after the $^{11}$C–PK 11195 injection. The electrocardiogram was monitored continuously in each subject. Radioactive concentrations were calculated in three regions of interest for each scan as described above.

Results

Animal studies. Lung and heart images obtained at different times after injection of $^{11}$C–PK 11195 in a dog (No. PKC 05) are shown in figure 1. During the first minutes the activity was concentrated in the lungs, but after 5 min activity in the myocardium became higher than that in the lung (figure 1, 2). Blood activity was soon negligible and the blood pool was never visualized except during the first pass. Thirty minutes after injection, activity in the heart remained fairly constant and that in the lung was undetectable (figure 1, 3). In the same dog, a large dose of unlabeled PK 11195 (200 μg/kg) was rapidly injected at 31 min. Competition between labeled and unlabeled ligand for the same benzodiazepine binding sites led to a fall in the myocardium $^{11}$C–PK 11195 activity (figure 1, 4).

The concentration of PK 11195 in the heart (expressed as pmol/cm$^3$) measured 20 min after injection was proportional to the amount of PK 11195 injected intravenously up to doses of 40 nmol/kg body weight (figure 2, inset) ($y = 18.96x - 3.01$, $r = .999$). Above this dose the concentration in the heart increased nonlinearly with the amount of ligand injected to reach a maximum of about 6000 pmol/cm$^3$ (figure 2); the $^{11}$C–PK 11195 concentration then became independent of the amount of ligand injected.

The kinetics of $^{11}$C–PK 11195 binding inhibition on its binding sites by unlabeled PK 11195 is shown in figure 3. After a bolus injection of tracer at high specif-
FIGURE 1. PET images of a dog chest after intravenous injection of $^{11}$C–PK 11195. All four cross-sectional images are taken through the body of the left ventricle. 1, PET scan obtained 2 min after injection of $^{11}$C–PK 11195 (20.9 mCi, 3000 mCi/μmol) shows a high activity in the lungs and no activity in the heart. 2, The same PET section 11 min after injection shows a high accumulation of activity in the myocardium with a decreased concentration in the lungs. 3, Thirty minutes after injection only the heart is visualized. 4, Thirty-one minutes after tracer injection excess unlabeled PK 1195 (200 μg/kg) was injected rapidly. Compared with panel 3, the image shows a decreased radioactivity in the heart, indicating a binding inhibition of radioactive ligand by excess unlabeled ligand, which competes for binding to the receptors.

FIGURE 2. $^{11}$C–PK 11195 concentration in the left ventricle (pmol/cm$^3$ of myocardium) vs amount of PK 11195 injected (nmol/kg body weight). The values measured 20 min after injection in 21 separate experiments are plotted against the amount of PK 11195 injected as a bolus in the right atrium. Above 40 nmol/kg body weight the concentration in the myocardium increased nonlinearly with the amount of ligand injected to reach a maximum value of about 6000 pmol/cm$^3$. Inset, The first part of the curve described above is enlarged. The cardiac concentration of PK 11195 is proportional to the amount injected for doses below 40 nmol/kg body weight ($y = 18.96 x - 3.01; n = 16; r = .999$). The slope of the regression line ($\times 10^{-3}$) is equal to the ratio of myocardial blood flow to cardiac output (see Discussion).
ic activity, the tracer blood concentration fell very rapidly to a low value in a few minutes after intravenous injection, whereas the $^{11}$C–PK 11195 concentration increased rapidly in the myocardium to reach a maximum in 4 to 15 min and then remained constant for about 30 min before decreasing slowly. The injection of excess unlabeled PK 11195 (doses varying from 50 to 2500 $\mu$g/kg) 30 min after injection of the radioactive ligand led to a very rapid fall (lasting a few minutes) in the myocardial $^{11}$C–PK 11195 concentration. The percentage of radioligand displaced 5 min after injection of cold PK 11195 was related to the dose injected (table 1); the maximum binding inhibition (68%) was observed with PK 11195 doses of 200 $\mu$g/kg or more. For these doses, high enough to saturate all receptor sites, the dissociation rate constant $k_{-1}$ was calculated from the initial slope of the $^{11}$C–PK 11195 activity-time curve obtained after injection of PK 11195 according to a mathematical model described previously. The mean value calculated over four experiments was 0.15 ± 0.02 min$^{-1}$ (mean ± SEM). For the same amount of PK 11195, the percentage of radioligand displaced was the same whenever the injection time (30 or 110 min after the tracer injection). No inhibition was observed when the solvent or a large dose of the norderivative (200 $\mu$g/kg PK 13042) was injected.

The specificity of the PK 11195 interaction with myocardial benzodiazepine binding sites was investigated in a study on the inhibition of radioligand binding by an excess of several agonists or antagonists of the benzodiazepine receptor: diazepam, RO 5-4864, clonazepam, and RO 15 1788. Clonazepam, a central agonist, and RO 15-1788, a central antagonist, were unable to inhibit $^{11}$C–PK 11195. However, ligands known to bind to peripheral-type benzodiazepine sites inhibited the radioligand (figure 4). At the same dose, RO 5-4864 (a peripheral inverse agonist) was found to be a more potent competitive agent than diazepam (a peripheral and central agonist) but less effective than PK 11195. A 50% inhibition was obtained with the

![FIGURE 3. $^{11}$C–PK 11195 radioactivity concentration-time curves. Concentrations are expressed as a percentage of the injected dose per cubic centimeter of tissues multiplied by 10$^3$. After a bolus injection of $^{11}$C–PK 11195 (3.6 to 34.4 mCi) at high specific activity (800 to 1200 mCi/µmol) in five separate experiments, the labeled ligand is rapidly cleared from the blood. By contrast, the activity rises almost immediately in the left ventricle and reaches a plateau. In the control curve the radioactivity concentration decreases slowly. The injection of various doses of unlabeled PK 11195 30 min later (arrow) results in a binding inhibition of $^{11}$C–PK 11195 on its binding sites. The displacement calculated 5 min after injection of unlabeled PK 11195 is related to the dose injected and reaches a maximum value (68%) for doses of 200 and 2500 $\mu$g/kg. The value of the dissociation rate constant calculated from the initial part of the descending portion of the curve is 0.15 ± 0.02 min$^{-1}$.](http://circ.ahajournals.org/)

![FIGURE 4. Percent inhibition of $^{11}$C–PK 11195 binding after injection of excess PK 11195 (1000 $\mu$g/kg), RO 5-4864 (1000 $\mu$g/kg), diazepam (1000 $\mu$g/kg), clonazepam (1000 $\mu$g/kg), and RO 15-1788 (1000 $\mu$g/kg). In each experiment the agonists and antagonists were injected 30 min after injection of $^{11}$C–PK 11195. RO 5-4864 proved to be a more potent displacing agent than diazepam. Clonazepam and RO 15-1788, which are central antagonists, were unable to compete with the labeled ligand.](http://circ.ahajournals.org/)
injection of 100 μg/kg PK 11195, 1000 μg/kg RO 5-4864, and 10,000 μg/kg Diazepam; that is, PK 11195 was about 10 times more active than RO 5-4864 and 100 times more active than diazepam.

No significant change in heart rate was observed after injection of 11C-PK 11195 or of excess unlabeled PK 11195 whatever the amount injected. However, when RO 5-4864 was used to displace 11C-PK 11195 from its myocardial binding sites, a pharmacologic effect was shown. A significant drop in the heart rate (11.2 ± 1.7%, n = 3) was seen when 200 μg/kg or more of RO 5-4864 was injected 30 min after injection after a trace dose of 11C-PK 11195. The pharmacologic effect and the binding inhibition of radioligand were synchronous.

Human studies. Figure 5 shows the cardiac image obtained after injection of 12.3 mCi of 11C-PK 11195 (specific activity 484 mCi/μmol) in a volunteer (PKH No. 01). The radioactivity was concentrated in the lung for a longer time in human beings than in dogs (10 min), but the myocardium was visualized earlier (at 2 min). The activity decreased for 20 min and then remained fairly constant until the end of the acquisition time at 75 min.

Percentages of 11C-PK 11195 binding inhibition on the myocardium by unlabeled PK 11195 are given in table 2. The injection of various amounts of PK 11195 (100, 200, or 500 μg/kg body weight) 30 min after injection of the labeled ligand led to a rapid fall in radioactivity in the heart. As in dogs, the percentage of 11C-PK 11195 binding inhibition in the heart was related to the amount of unlabeled ligand injected. The maximum value (48%) obtained with 500 μg/kg PK 11195 as competitive agent was less than that observed in dogs with a smaller amount. A linear correlation was demonstrated between the concentration of PK 11195 in the heart (expressed as pmol/cm³) and the amount of PK 11195 injected for doses below 3 nmol/kg (r = .991). For the same injected doses of 11C-PK 11195 (range 0.3 to 2.50 nmol/kg body weight), the concentration of PK 11195 in the heart was similar in dogs and human beings (range 5 to 55 pmol/cm³).

Discussion
The existence of specific benzodiazepine binding sites in the canine and human heart is demonstrated in this study in vivo using PET, a noninvasive imaging technique that provides information analogous to that obtained by autoradiography in vitro, with the added advantage that myocardial receptors may be studied under physiologic conditions.16, 17, 19

The demonstration of peripheral-type benzodiazepine binding sites was first made in vitro with 3H-diazepam.6 Specific high-affinity 3H-diazepam binding was shown in peripheral organs such as the kidney, lung, liver, heart, skeletal and ileal muscle, mast cells, spinal cord, and human circulating lymphocytes.5,7, 20 The peripheral-type site has also been shown to predominate in various cultured neural cells.8 New ligands that bind only to peripheral-type sites and not to the classic central-type have been synthesized. RO 5-4864 and PK 11195 are almost inactive in binding
inhibition of $^3$H-diazepam on its sites in the brain but have a very high affinity for peripheral sites. $^{10-12}$ In vitro the PK 11195 binding sites in rat cardiac membranes are specific, saturable with a $K_d$ of 1.41 nM and a $B_{\text{max}}$ of 2250 pmol/g of protein. $^{10,12}$ Although RO 5-4864 displays the same affinity for peripheral-type sites ($K_D = 3.18$ nM, $B_{\text{max}} = 1964$ pmol/g of protein), the two ligands seem to act differently; $^3$H–PK 11195 binding was entropy-driven, whereas $^3$H–RO 54864 was enthalpy-driven. $^{12}$ Furthermore, PK 11195 reversed the action of RO 5-4864 on the guinea pig papillary muscle; the shorter duration of intracellular action potential and muscle contractility induced by RO 5-4864 were antagonized by PK 11195 but not by the selective antagonist of the central-type benzodiazepine receptor, RO 15-1788. $^{13}$ The guinea pig cardiac benzodiazepine binding sites of the peripheral type were shown to be pharmacologic receptors, $^{13}$ which were coupled to calcium channels in the papillary muscle. $^{21}$

PK 11195, a potent antagonist of the benzodiazepine receptor in the heart, could therefore be an ideal ligand to characterize this receptor in vivo by external detection with PET. PK 11195 was thus labeled with carbon-11 at very high specific activity and injected intravenously in dogs and human beings. An initial uptake of $^{11}$C–PK 11195 was seen in the lung, followed by a high uptake in the heart. Benzodiazepine binding sites were uniformly distributed in the canine and human hearts. A few minutes after injection, $^{11}$C–PK 11195 distribution in the heart was similar to that of regional myocardial blood flow. The amount of PK 11195 found in the heart was proportional to the quantity injected at values below 40 nmol/kg. The slope of the regression line ($\times 10^{-3}$) is equal to the ratio of myocardial blood flow per cubic centimeter of tissue to the cardiac output per kilogram of body weight. $^{17}$ The value calculated from 16 experiments in different dogs, $18.96 \times 10^{-3}$ of myocardium, was higher than but of the same order of magnitude as that measured directly by Seroussi et al. $^{22}$ in dogs: $8.5 \times 10^{-3}$. This overestimation was probably caused by correction for the loss in count recovery, obtained from static measurements in phantoms and neglecting the motion of the heart.

Above 40 nmol/kg, however, the curve showed a plateau because of saturation of the benzodiazepine binding sites. This result agrees with the mathematical model of a ligand receptor interaction studied in vivo, $^{16}$ and a similar curve was also obtained in human beings from a PET study on the muscarinic acetylcholine receptor. $^{17}$ From the PK 11195 concentration, the number of benzodiazepine binding sites in the dog ventricular myocardium ($R_T$) was found to be around 6000 pmol/cm$^2$ of tissue. This value is probably overestimated, since one fraction of PK 11195 corresponded to nonspecific binding and another to circulating PK 11195. This latter fraction could be estimated at 150 pmol/cm$^2$ of tissue from the $^{11}$C–PK 11195 blood concentrations and from an estimation of the fractional volume of blood in the heart. An additional cause of overestimation of $R_T$ was the factor used to correct for the partial volume effect as discussed above. The receptor density found in vivo in dogs could be compared with that found in heart homogenates of rats (4420 pmol/g protein), dogs (3125), and baboons (11,330) (B. Mazière, unpublished data).

Several criteria, in addition to the specific regional distribution of binding sites, must be met for identification of the ligand receptor interaction by PET. Saturability of the ligand receptor complex was demonstrated by two kinds of experiments. In the binding inhibition experiments, excess cold agonist or antagonist was injected intravenously 30 min after injection of the labeled ligand. The radioactivity then decreased rapidly with time because of the competitive inhibition between tracer and excess unlabeled ligand. The dissociation rate constant of the ligand-receptor complex $k_{-1}$ was calculated from the initial part of the “displacement curve” as previously described $^{16}$ and found to be 0.15 min$^{-1}$. The $k_{-1}$ value for the dissociation of $^3$H–PK 11195 measured in vitro on rat heart homogenates (0.49 min$^{-1}$) was very much the same. $^{12}$ $^{11}$C–PK 11195 binding was inhibited by unlabeled PK 11195 and by other ligands that compete for peripheral-type sites, but not by those that bind only to brain-type sites such as RO 15-1788. Furthermore, the order of potency in the $^{11}$C–PK 11195 inhibition was similar to that found in rat heart with $^3$H–PK 11195. $^{20}$ The maximum binding inhibition, 68%, was measured 5 min after injection of the unlabeled compound. The fraction of labeled ligand inhibited by unlabeled ligand was shown to decrease very rapidly during the first minutes and then slowly. $^{16}$ A fraction of “non-displaceable” binding, as measured 5 min after injection of excess unlabeled ligand, thus corresponded to specific binding and another fraction to nonspecific binding. Specific and nonspecific binding in the heart are impossible to distinguish by PET, and our results simply indicate that the $^{11}$C–PK 11195 fraction nonspecifically bound to the heart was less than 30%.

The saturation of the receptor was also demonstrated by the simultaneous injection of unlabeled PK 11195 and $^{11}$C–PK 11195. The tracer radioactive concentra-
tion in the myocardium was lower than that measured in the absence of unlabeled PK 11195. Stereosel ectivity also provides strong proof of receptor binding. Unfortunately, a stereoisomer of PK 11195 could not be synthesized and this experiment was not possible.

Correlation between binding and biological effect is essential because a competitive binding site with no signal transmission is to be distinguished from a receptor binding site related to physiologic response. Mestre et al. recently showed that PK 11195 antagonized the effects of several calcium-channel blockers (diltiazem, nitrrendipine, verapamil) and of a calcium-channel agonist (BAY K 8644) in a guinea pig papillary muscle preparation. PK 11195 did not depress the duration of the cardiac action potential and thus the contractile force. A PET study of these receptors in man could thus be interesting in clinical situations. During the last 20 years Abel and Chai have described the cardiovascular effect of diazepam. These first reports were contradictory: an increase in myocardial contractility with a systemic vasodilatation was found by Abel, whereas Chai described a reduction of the resting blood pressure, heart rate, and cardiac contractile force with systemic vasodilatation. It seems today that the finding of an increase in myocardial contractility was due to the systemic vasodilatation. Thus the pharmacologic effects of diazepam on myocardial contractility could be due to a conformational change in the calcium channel induced by the binding of diazepam on peripheral-type benzodiazepine receptors. Le Fur and associates recently demonstrated that PK 11195 inhibits arrhythmias induced by ischemia and abnormalities after reperfusion in the canine heart (personal communication).

These results suggest that the investigation of benzodiazepine receptors with $^1$C–PK 11195 and PET might be very useful for the investigation of cardiac ischemia in human beings.

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