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Down-Regulation of Cardiac Muscarinic Receptors Induced by Di-Isopropylfluorophosphate

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The feasability of PET determination of myocardial muscarinic acetylcholine receptor (mAChR) density has been demonstrated in dogs and humans. The results of the PET method, however, were not validated by a direct comparison with the in vitro determination of mAChR density. **Methods:** Left ventricular mAChR concentrations were studied in beagle dogs at baseline and after a 5- or a 11-day treatment with the irreversible acetylcholinesterase inhibitor di-isopropylfluorophosphate (DFP). The determination of mAChR densities were performed in vivo using PET, ^{11}C-MQNB, the three-injection protocol and the compartmental model previously described. In a parallel group of dogs, determination of mAChR density was performed in vitro using ^{3}H-(-)-MQNB. **Results:** In control dogs (n = 4), PET left ventricular density of mAChR was 61.1 ± 8.1 pmol/ml tissue. In the 5-day DFP-treated animals (n = 3), Bmax decreased to 38.2 ± 8.3 pmol/ml tissue (−38%; p = 0.005 versus control). In the 11-day DFP-treated animals (n = 3), Bmax was 34.7 ± 5.5 pmol/ml tissue (−43%; p = 0.003). There was no change in the affinity constant either at 5 or 11 days. In control dogs, Bmax, measured in vitro, was 9.53 ± 0.93 pmol/g tissue. In the 5-day DFP-treated animals, Bmax decreased to 6.2 ± 0.9 pmol/g tissue (−35%; p = 0.003). In the 11-day DFP-treated animals, Bmax was 5.1 ± 0.6 pmol/g tissue (−47%; p = 0.003 versus control). At that time, there was no change in affinity constant. On the fifth and 11th days, myocardial acetylcholinesterase activity was reduced by 88% and 90%, respectively. **Conclusion:** The in vivo and in vitro methods showed a similar decrease in mAChR density while for both methods affinity constant remained unchanged. This study validates the ability of PET and of the compartmental model to in vivo quantify changes in mAChR density.

Key Words: muscarinic acetylcholine receptor; PET; methyl-quinuclidinyl-benzilate; di-isopropylfluorophosphate; down-regulation; heart

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The neurotransmitter of the postganglionic parasympathetic system, acetylcholine, interacts with the heart through the action of the muscarinic acetylcholine receptors (mAChR). The in vivo myocardial characterization and quantification of these receptors were greatly facilitated by the development of ^{11}C-radiolabeled methyl-quinuclidinyl-benzylate (MQNB; *1–3*) and by that of compartmental analysis (*4–5*). The main goal of this study was to investigate the ability of PET and of compartmental analysis to assess changes in density of myocardial mAChR. Therefore, the changes in the PET values of mAChR density and of those of the in vitro measurements were compared. In experimental cardiac disease in large animals like dogs, changes

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in the density of myocardial mAChRs detectible with PET (at least 20%) remain to be described (*6*). In order to obtain large changes in myocardial mAChR density, we chose, as an experimental model, in vivo treatment of dogs with di-isopropylfluorophosphate (DFP), an irreversible acetylcholinesterase inhibitor.

MATERIALS AND METHODS

Animal Treatment

The animals used in the study were maintained in accordance with the guidelines of the committee on care and use of laboratory animals of the Institute of Laboratory Animal Resources, National Research Council.

A group of four female beagle dogs (mean weight: 10 kg) was used for in vivo baseline measurements of mAChR density. Two groups of six female beagle dogs (mean weight: 10 kg) were used for experiments with DFP. One group was used for in vivo measurement of mAChR density, the other one for in vitro determination of mAChR density. Dogs received one subcutaneous injection of 2 mg/kg DFP (dissolved in peanut oil) followed by a maintenance dose of 1 mg/kg every day for a total duration of 5 days (n = 6) or 11 days (n = 6) from the first injection. Dogs were killed with an overdose of pentobarbital after completion of the treatment (in vitro measurement) or after the PET experiment (in vivo measurement). For the latter group, this was mandatory because of the unavoidable development of organophosphorous inorganic delayed neuropathy after treatment with DFP at this dosage (*personal communication*).

PET Determination of mAChR Density

Radiosynthesis of Carbon-11-MQNB. MQNB was labeled with high-specific radioactivity using ^{11}C by methylation of QNB with ^{11}C-methyliodide (*1*). Labeled compound, with a specific radioactivity ranging from 600-2000 mCi/μM at the moment of the injection, was purified using HPLC.

PET Data Acquisition and PET Experimental Protocol. Dogs were anaesthetized with pentobarbital, intubated and artificially respired. Blood samples were obtained from the femoral artery. Dogs were positioned in the TTV01 time-of-flight PET scanner (LETI, CEA, Grenoble, France). Each slice was 13 mm thick and spatial transverse resolution was 12 mm. Transmission scans were obtained with a rotating ^{68}Ge source and used for attenuation correction of the emission scans.

The protocol included three injections (*4*). A first dose of ^{11}C-MQNB (2–4 nmol) was intravenously injected. Thirty minutes later, an excess of unlabeled MQNB (0.5 μmol) was injected. Forty minutes later, a mixture of labeled (4–8 nmol) and unlabeled (1.25 μmol) MQNB was injected. The PET examination lasted 120 min. The scanning protocol consisted of 82 images (12 × 10 sec, 8 × 1 min, 10 × 2 min, 8 × 1 min, 16 × 2 min, 12 × 10 sec, 8 × 1 min, 8 × 5 min images). Since the identification model parameters requires the knowledge of the plasma time radioactivity curve as input function, 64 arterial blood samples (0.5 ml) were collected at designated times. The blood ^{11}C radioactivity was measured in a gamma counting system (CG4000, Kontron) and the blood time-activity curves were corrected for the decay of ^{11}C from the time of the first injection.

PET Data Processing. Myocardial time-concentration curves were measured from a ROI encompassing the left ventricular myocardium. Carbon-11-MQNB concentrations were obtained after correction for ^{11}C decay and expressed as pmol/ml after normalization using the specific radioactivity measured at the beginning of the PET experiment. Data were corrected for partial volume effect using postmortem measurements of left ventricular wall thickness (four dogs) and a recovery factor measured on a heart phantom. In beagle dogs of this size (10 kg), the thickness of the septum as well as the thickness of the lateral wall is equal to 12 mm. The ratio of true-to-measured concentration was equal to 0.45 for a 12-mm thickness in the phantom calibration experiment performed on the TTV01 PET system. Therefore, true concentrations were obtained by dividing the measured concentration values by this 0.45 recovery coefficient.

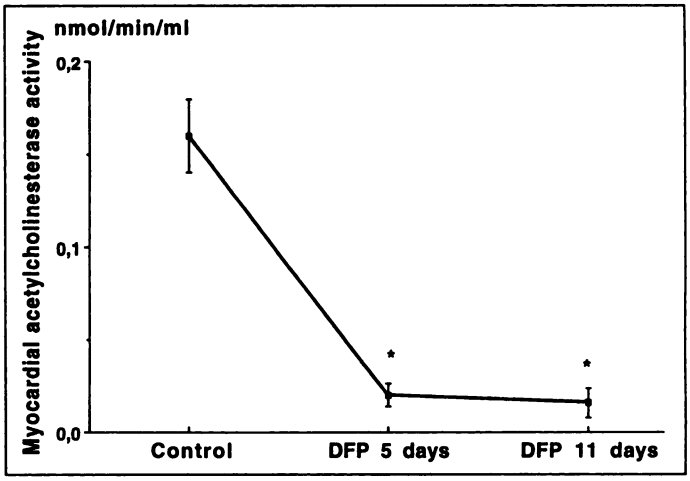

FIGURE 1. Changes in myocardial acetylcholinesterase activity in control dogs and after DFP treatment. A * denotes a statistically significant change in acetylcholinesterase activity.

Calculation of mAChR Density. The compartmental model used is a nonequilibrium nonlinear one, justified by the properties of the MQNB kinetics (*4*). It included two steps: first, a transport of the ligand from blood to a free ligand compartment; and second, a classical ligand-receptor interaction. The rate constant p characterized the transfer of ligand from blood to tissue. K characterized the transfer from tissue to blood and V_R is defined as the fraction of the ROI delineated by PET in which the ligand can react with receptors. The product pV_R is the clearance of the ligand. The model parameters introduced in the ligand-receptor interactions were similar to that used in in vitro studies: the concentration of available receptors (B'max) and the association and dissociation rate constants (k_{+1} and k_{-1}, respectively). By fitting the mathematical model to time-concentration curves, it was possible to obtain estimates of parameters $p.V_R$, k, B'max, k_{+1}/V_R , k_{-1}. The volume of reaction V_R was deduced by assuming that the transport between blood and tissue was passive and the two parameters p and k had the same value. Thus, V_R could be estimated from the $p.V_R/k$ ratio.

In Vitro Determination of mAChR Density

Four control dogs underwent the PET experiment. Two weeks later, a period enough long to allow the elimination of MQNB injected during the PET experiment, the dogs were killed with an overdose of pentobarbital. Six other dogs were treated with DFP as mentioned above. On the fifth (n = 3) or on the 11th days (n = 3), the dogs were killed with an overdose of pentobarbital. The heart was rapidly removed, the left ventricle dissected and minced with scissors. Samples were placed in 19 vol of 0.25 *M* sucrose at pH 7.4, homogeneized and centrifugated (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 −80°C. For binding assays, aliquots of the membrane fraction (0.1–0.5 mg of protein) were incubated at 37°C with ^3H-(-)-MQNB 0.05–1.5 n*M* (Du Pont de Nemours, France) in 1 ml phosphate buffer. After a 60-min incubation, samples were vacuum-filtered through Whatman GF/C filters and washed with 15 ml of cold buffer. The nonspecific binding measured in presence of

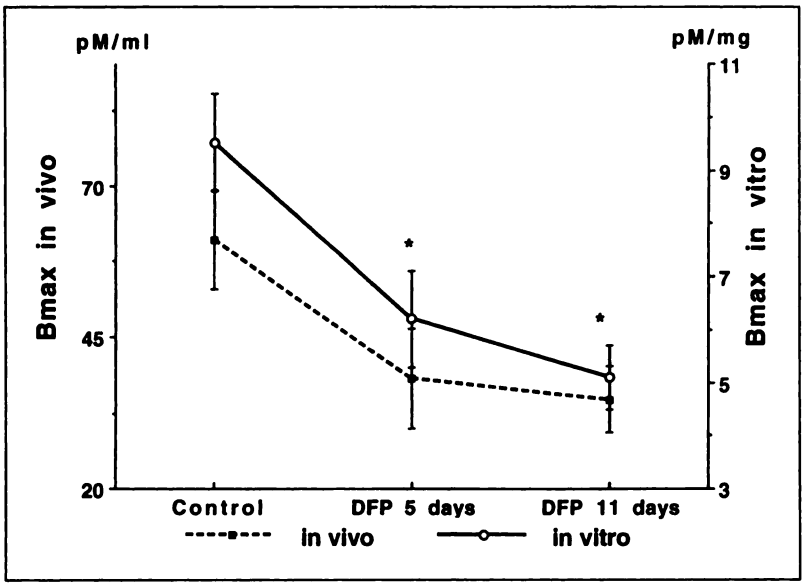

FIGURE 2. Changes in mAChR density, measured in vivo and in vitro, in control dogs and after DFP treatment. A* denotes a statistically significant change in Bmax value.

1 μM atropine accounted for less than 10% of total binding at half saturation. The dissociation rate constant was measured by dilution (1/200). Protein content was measured according to Lowry's method (*7*). Mean value for left ventricular protein content was found to be 85 ± 9 mg/g of tissue.

Myocardial Acetylcholinesterase Activity

Myocardial acetylcholinesterase activity was quantitated in fresh heart homogenates using a spectrophotometric method (*8*). The incubation mixture consisted of 50 μl of myocardial homogenate, 50 μl of 1 m*M* 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 0.75 m*M* acetylthiocholine and 0.1 *M* phosphate buffer (pH 8). ISO-OMPA (10^{-5} *M*) was used as inhibitor of butyrilcholinesterase. After an initial reading at 412 nm, a second reading was made 30 min later (incubation at 27°C). The difference in absorbance between the second and the first reading is proportional to the quantity of thiocholine released for reaction with DTNB.

Statistical Analysis

Results are expressed as mean values ±s.d. Analysis of variance and Bonferroni's test were used to compare the effect of DFP on mAChR density and acetylcholinesterase activity. A correlation between in vivo and in vitro data could not be assessed for the dogs treated with DFP because the same dog could not be used for the two methods of determination of Bmax. A p value of <0.05 was considered statistically significant.

RESULTS

Myocardial Acetylcholinesterase Activity

In control dogs, myocardial acetylcholinesterase activity was 0.16 ± 0.02 nmol/min/ml tissue (Fig. 1). In dogs treated with DFP, myocardial acetylcholinesterase activities were 0.02 ± 0.006 and 0.016 ± 0.008 nmol/min/ml tissue for the 5- and the 11-day treated animals, respectively ($p < 0.05$ for both values versus baseline).

In Vivo mAChR Density

In control dogs, a left ventricular density of mAChR was 61.1 ± 8.1 pmol/ml tissue (Fig. 2). KdV_R was 0.07 ± 0.017 pmol/ml. The rate of clearance of the ligand from the circulation (pV_R) was 0.67 ± 0.1 min^{-1}. In the 5-day DFP-treated animals, Bmax decreased to 38.2 ± 8.3 pmol/ml tissue ($p < 0.01$) and KdV_R was 0.06 ± 0.02 pmol/ml ($p = 0.2$). The pV_R value was 0.64 ± 0.09 min^{-1} ($p = 0.3$). In the 11-day DFP-treated animals, Bmax was equal to 34.7 ± 5.5 pmol/ml tissue ($p < 0.01$ versus control dogs), KdV_R to 0.05 ± 0.02 pmol/ml ($p = 0.2$) and pV_R to 0.69 ± 0.1 min^{-1} ($p = 0.3$). Bmax, KdV_R and pV_R values were not statistically different on the fifth and the 11th days.

In Vitro mAChR Density

In control dogs, left ventricular density of mAChR was 104.9 ± 10.2 fmol/mg protein (9.53 ± 0.93 pmol/mg tissue; Fig. 2). Kd was 0.47 ± 0.04 nmol. After a 5-day DFP treatment, Bmax was 68.3 ± 10 fmol/mg protein (6.2 ± 0.9 pmol/mg tissue; $p = 0.003$) and Kd was 0.44 ± 0.04 pmol/ml ($p = 0.2$). After a 11-day DFP treatment, Bmax was 55.7 ± 6.5 fmol/mg protein (5.1 ± 0.6 pmol/mg tissue; $p = 0.003$) and Kd was 0.47 ± 0.04 pmol/ml ($p = 0.2$). As for the in vivo data, Bmax and Kd were not statiscally different for the measurements performed on the fifth and 11th days.

DISCUSSION

In this study, we have compared the in vivo and in vitro measured changes in left ventricular mAChR density in dogs after administration of an irreversible acetylcholinesterase inhibitor. The large decrease in acetylcholinesterase activity demonstrated the strong (about 90%) inhibition of this enzyme by DFP. This amount of change is in accordance with findings of other authors (*9*). The inhibition of acetylcholinesterase was similar on the fifth and 11th days with the dosage and the regimen of administration of DFP we have used. On the fifth day, there was a clear down-regulation of left ventricular mAChR. Between the fifth and the 11th days, mAChR density still decreased slightly, this decrease being not statistically significant. mAChR density values obtained in vivo are the same order of magnitude than those obtained with the in vitro method. Furthermore, the mean changes in mAChR density after DFP were similar for both methods.

Methodoligical Considerations

The PET Protocol. The three-injection PET protocol we have used is a complex one. As shown previously (*5*), the three-injection (injection, displacement and co-injection) and the two-injection (injection and co-injection) protocols gave similar values for Bmax. However, the drawback of the two-injection protocol for nonroutine investigations is to lead to a poorer estimation of all other parameters of the model. Since clinical studies mainly deal with measurement of Bmax, the extrapolation of the present results to a simplified protocol seems to be

justified and, therefore, this study is of clinical relevance. The large s.d. of the PET Bmax values is due to both the few number of dogs and to the standard errors in the measured mAChR density that are about 10%, even using the three-injection protocol (*4*).

The PET Ligand

MQNB is not specific of one subtype of mAChR. However, the left ventricular myocardium contains only the M_2 subtype as indicated by radioligand binding (*10*) and mRNA in situ hybridization techniques (*11*).

Carbon-11-MQNB is a racemic mixture. In vitro measurements demonstrated that both enantiomers has similar association (4.3×10^6 and 9.7×10^6 M^{-1}. sec^{-1}, respectively) and dissociation (1.8×10^{-3} and 4.2×10^{-3} sec^{-1}, respectively) rate constants in cardiac homogenates (*12*).

The binding of MQNB is mainly flow dependent. Administration of an acetylcholinesterase inhibitor could lead, potentially, to an increased myocardial blood flow (MBF) since acetylcholine is a potent vasodilator of normal coronary arteries. From PET pV_R value and from extraction coefficient of ^{3}HMQNB measured in vitro (*5*), the flow rate of fluid containing ^{11}C-MQNB can be calculated. Therefore, MBF can be estimated because MQNB rapidly stabilized between plasma and red blood cell water space. Using the value of 0.93 for the fractional volume of water in plasma (*5*) and 0.45–0.5 for the hematocrite, calculated MBF values were 129 $ml.min^{-1}$/100 ml tissue, 125 $ml.min^{-1}$/100 ml tissue and 136 $ml.min^{-1}$/100 ml tissue in control, in 5- and 11-day DFP-treated dogs, respectively ($p > 0.1$). Therefore, although coronary blood flow was not specifically measured in this study, it is likely that the above calculated changes in MBF do not significantly influence our findings.

Study Design

We could not use the same dog pretreated with DFP to compare the in vivo and in vitro measurements of mAChR density because, after the PET experiment with the present protocol (injection of a total of 1.75 μmol of cold MQNB), more than 90% of mAChRs are occupied by the ligand (*4*). With such a dose, in vitro binding study cannot be performed immediately after the PET experiment. Since the duration of elimination of MQNB is unknown, a parallel group of dogs had to be used for in vitro measurements.

In spite of the above limitations, the close relationship between receptor density obtained with both methods demonstrated the ability of PET and of the compartmental modeling approach to detect such changes. Furthermore, with both methods no changes in affinity constants were observed.

Possible Mechanisms of mAchR Down-Regulation in the DFP Model

A rapid (few hours) down-regulation of cardiac muscarinic receptor density has been reported with exogenous muscarinic agonists either in ovo or in vitro (*13–14*). In vivo, a slower (3 days) time-course down-regulation was observed in the rat heart using another irreversible acetylcholinesterase inhibitor paraoxon (*15*) or a muscarinic agonist (metacholine: 10 days; *16*). In this study, the 5-day administration of DFP induced a high degree of inhibition of acetylcholinesterase activity and a subsequent down-regulation of mAChR. The results of the present studies are in accordance with results obtained in rat brain after DFP administration (*9*). Eleven-day treatment did not induce further significant change in acetylcholinesterase activity or in mAChR density.

With organophosphorous inorganic agents, this down-regulation of mAChRs may result from an increasing amount of endogenous acetylcholine available for mAChR activation. The down-regulation of mAChR also has been ascribed to a more efficient sequestration from the cellular surface (because of its hydrophilic characteristics, MQNB binds only to externalized receptors) and/or to changes in the rate of phosphorylation of receptors. An alternative hypothesis is that mAChR down-regulation may be due to an increased rate of receptor degradation (*17*). A decrease in mRNA encoding for cardiac mAChRs also has been described in rat heart (*17*). Finally, a possible affinity of the organophosphorous compound for the mAChRs cannot be ruled out as evidenced for other compounds of this family (*18*).

CONCLUSION

The close relationship between the changes in mAChR densities, measured in vitro and in vivo, validates the use of ^{11}C-MQNB and that of compartmental analysis for the PET determination of changes in left ventricular mAChR density.

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