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Evaluation of (−)[^{18}F]Fluoroethoxybenzovesamicol as a New PET Tracer of Cholinergic Neurons of the Heart

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^{18}F-labeled (−)fluoroethoxybenzovesamicol [(−)FEOBV] is a novel PET tracer which binds to the vesicular acetylcholine transporter of cholinergic neurons. To evaluate the *in vivo* binding specificity and kinetic properties of (−)FEOBV, studies were performed in isolated working rat hearts. External γ,γ-coincidence monitoring of hearts indicated high extraction of radiotracer by the myocardium and rapid wash-out of unbound tracer (>90% of maximal accumulation) within 5 min. Inclusion of (−)vesamicol (10 μM) throughout perfusion decreased the retention of (−)FEOBV by 71% ($P < 0.005$) and 76% ($P < 0.005$) in atria and ventricles, respectively. However, the initial uptake rate of the tracer was unaffected. Additional experiments showed the inactive stereoisomer, (+)FEOBV, to have a lower retention than the (−)FEOBV isomer in ventricles, indicating stereospecificity of the binding process that is consistent with structure–activity relationships of vesamicol congeners. The results indicate (−)FEOBV to be a moderately specific probe of vesamicol-sensitive binding in cholinergic neurons of the heart in experimental conditions that assure adequate washout of unbound tracer. However, the utility of the radiotracer for *in vivo* studies with PET is likely to be limited by the low rate of specific binding in myocardium consistent with the low density of cholinergic neurons in the heart.

Introduction

The sympathetic and parasympathetic nervous systems are involved in important control functions of the heart. While the entire heart is densely innervated by sympathetic nerves, parasympathetic innervation is diffuse and concentrated in the atria, providing regulation of heart rate and to a lesser extent the myocardial contractile state (Higgins *et al.*, 1973; Binkley *et al.*, 1991). With the advent of positron emission tomography (PET) and the development of positron-labeled catecholamines analogs, the sympathetic innervation of the human heart has been recently probed (Goldstein *et al.*, 1990; Schwaiger *et al.*, 1990). Vesamicol [(−)trans-2-(4-phenylpiperidino)cyclohexanol; AH5183] is a synthetic molecule that binds to the vesicular acetylcholine transporter of cholinergic neurons (Brittain *et al.*, 1969; Marshall and Parsons, 1987; Marien *et al.*, 1987). A subclass of analogs called benzovesamicols by their developers (Rogers *et al.*, 1989) appear to be particularly active in this regard. Radiolabeled analogs of vesamicol may allow the non-invasive assessment of cholinergic neurons of the parasympathetic nervous system (Jung *et al.*, 1990; Kilbourn *et al.*, 1990; Widen *et al.*, 1992).

Several ^{18}F-labeled benzovesamicols have been recently evaluated for PET imaging of cholinergic neurons (Widen *et al.*, 1992; Rogers *et al.*, 1993; Mulholland *et al.*, 1993a, b). A simple, one-step synthesis procedure for (−)[^{18}F]fluoroethoxybenzovesamicol (FEOBV) has been developed (Mulholland *et al.*, 1993a). Rodent data show FEOBV to specifically localize in regions in brain that are dense with cholinergic neurons (Mulholland *et al.*, 1993b). We now extend our observations to the heart and evaluate FEOBV as a PET tracer of the parasympathetic innervation in the myocardium.

Methods

Synthesis of radiotracer

(−)- and (+)FEOBV were synthesized as previously described (Mulholland *et al.*, 1993a). Radiospecific activity of this tracer was >74 GBq/μmol. (−)Vesamicol–HCl was purchased from Research Biochemicals Inc. (Natick, Mass.).

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Perfusion of isolated working rat hearts

Hearts were excised from pentobarbital anesthetized, female, Sprague–Dawley rats (225–275 g). Following cannulation of the aorta retrograde perfusion of the hearts was initiated (aortic pressure = 60 mm Hg). Without delay, the left atrium was cannulated and working perfusion established as previously described (Taegtmeyer *et al.*, 1980). Hearts were perfused at moderate workload (preload = 7.4 mm Hg, afterload = 74 mm Hg). The perfusion medium was Krebs–Henseleit (K–H) bicarbonate buffer containing 5 mM glucose and gassed with 95% O_2/5% CO_2. Bovine serum albumin (0.5 g/100 mL buffer) was added to the perfusate to provide a binding capacity for the lipophilic radiotracers. The perfusion apparatus utilized two parallel perfusion circuits. One circuit was utilized for administration of radiotracer to the hearts (wash-in). The other circuit did not contain radiotracer and was used for perfusion before and after the wash-in period. Input to the heart was switched between the two circuits by means of a three-way valve just preceding the left atrial cannula. The apparatus utilized water-jacketed vessels and a heater/circulator to deliver the medium to the heart at a temperature of 37°C. The perfusion medium was filtered using 0.45 μm in-line filters (Millipore Corp., Bedford, Mass., U.S.A.). Aortic pressure was monitored by a pressure transducer and allowed measurement of heart rate, diastolic and systolic pressures. Coronary and aortic flows were measured manually. Neither coronary nor aortic outputs were recirculated to the hearts. Hearts were not externally paced.

The perfusion protocol consisted of a 20 min stabilization period, a 4 min radiotracer wash-in period and a 20 min wash-out period in succession. Before each perfusion, the radiotracer perfusion circuit was filled with ~500 mL of perfusion medium containing 4–7 kBq/mL (+)- or (−)FEOBV. The radioactivity concentration of the wash-in medium, C_p (cpm/mL), was measured in a NaI well-counter. Coronary and aortic outflows were discarded at all times. At the end of perfusion, ventricles were opened and gently blotted on paper toweling. Hearts were then separated into three fractions: atria, ventricles and the remainder. The last fraction comprised the aorta, connective tissues that were not removed at cannulation and the surgical thread used to secure the cannulae. The fractions were placed in pre-weighed tubes, weighed and counted in the NaI well-counter. To account for all of the radioactivity that was monitored by the coincidence probes (described below), the paper toweling was also counted. This radioactivity was <2% of that in heart. As a consequence of the prolonged wash-out period, no radioactivity remained in the left heart chambers or perfusion cannulae at the end of perfusion. Accounting for ^{18}F decay, the retention index for each fraction of the heart (RI_i) was calculated from the radioactivity in each fraction (A_i), the mass of the fraction of tissue (M_i) and C_p:

$$RI_i\left(\frac{\text{cpm/g heart}}{\text{cpm/mL perfusate}}\right)=\frac{A_i}{M_i C_p} \quad (1)$$

The retention index of radioactivity in the whole heart (RI_h) was calculated from the sum of radioactivity in all three fractions of the myocardium and the paper toweling (A_h), the entire mass of the heart (M_h) and C_p:

$$RI_h\left(\frac{\text{cpm/g heart}}{\text{cpm/mL perfusate}}\right)=\frac{A_h}{M_h C_p} \quad (2)$$

Hearts were divided into three groups: Group I ($n = 6$), (−)FEOBV under control conditions; Group II ($n = 5$), (−)FEOBV and 10 μM (−)vesamicol; Group III ($n = 4$), (+)FEOBV under control conditions.

Acquisition and normalization of time–activity curves

^{18}F radioactivity was externally monitored by γ,γ-coincidence counting of the whole heart using two lead-collimated 2.54 × 2.54 cm bismuth germanate scintillation probes as previously described (DeGrado *et al.*, 1993). The heart was suspended between the detectors by the aortic and left atrial cannulae. The apertures of the lead collimators were 2.54 cm in diameter, allowing monitoring of radioactivity over the whole heart and including a small portion of the aortic and left atrial cannulae. Single, coincidence and random coincidence events were sampled every second and stored on an IBM PC/AT computer. The true coincidence count rate [Y_i (counts/s)] was calculated as measured coincidence minus random coincidence count rates. The entire time–course of the true coincidence count rate was referred to as the time–activity curve.

Each time–activity curve was normalized to the concentration of radioactivity in the wash-in perfusate and the mass of the heart. This was accomplished by multiplication of the time–activity curve by a calibration factor, f_c:

$$Y_i'\,(\text{mL/g}) = Y_i \cdot f_c \quad (3)$$

where Y_i' is the normalized true coincidence count rate at the ith point and f_c is given by the equation:

$$f_c\left(\frac{\text{mL/g}}{\text{counts/s}}\right)=\frac{RI_h}{Y_e} \quad (4)$$

RI_h is obtained from equation (2) and Y_e is the average true coincidence count rate over the last 10 s of data.

Kinetic analysis of time–activity curves

Indices of radiotracer uptake and clearance were derived from each of the myocardial time–activity curves. The uptake rate, k_i (mL/min/g), was estimated from the linear portion of the curve over the 10–70 s interval. The net extraction, E_{net}, was calculated as the quotient of K_i and coronary flow.

The clearance kinetics over the wash-out interval were described by the biexponential model: $y = A_1 \cdot \exp(-K_1 \cdot t) + A_2 \cdot \exp(-K_2 \cdot t)$. Estimates of the constants A_1, A_2, K_1 and K_2 were obtained by a weighted non-linear least-squares fitting procedure (Bevington, 1969).

Statistical analysis

Data are expressed as means ± SE. The *t*-test (two-tailed) for unpaired samples was used to compare means.

Results

Hemodynamic parameters

All hemodynamic parameters of the isolated working rat heart preparation were stable over the tracer kinetic study period. Data are shown in Table 1 for working rat hearts in control conditions and with 10 μM (−)vesamicol. Vesamicol did not influence any hemodynamic parameter.

Myocardial kinetics of (−)FEOBV and (+)FEOBV

Figure 1 shows the normalized time–activity curves of (−)FEOBV in a control isolated rat heart (Group I). Table 2 shows indices of tracer uptake and clearance derived from the fitting of time–activity curves. Although the rapid uptake of the tracer is nearly linear within the first 1–2 min, saturation of uptake rate is clearly seen by 4 min. The uptake rate, as measured over the interval of 10–70 s, did not significantly differ among the three experimental groups. The uptake rate correlated with coronary flow in all groups ($r > 0.7$). The net extraction of (−)FEOBV by control hearts was 37%. This index also showed no significant difference among the three experimental groups.

Visual inspection (Fig. 1) and biexponential analysis (Table 2) of the wash-out kinetics showed a large fraction (>90%) of the accumulated radioactivity to clear the hearts of all groups rapidly within 5 min of wash-out. The slower clearance phase presumably indicates the slower release of tracer from binding sites within the myocardium. The clearance rate constants of the rapid and slow clearance phases were similar among the three experimental groups.

The sensitivity of (−)FEOBV kinetics to pharmacologic blockade of the vesamicol binding site was investigated. Figure 2 shows representative time–activity curves from control and (−)vesamicol (10 μM) perfused hearts (Group II). The relative magnitude of the rapid phase $[A_1/(A_1 + A_2)]$ was significantly higher in hearts perfused with (−)vesamicol (Group II) versus control conditions (Group I). The fraction of ^{18}F-activity remaining in the heart at the end of perfusion relative to the value of the time–activity curve at 4 min (tail:peak ratio) was 3.5% for Group I and was significantly decreased in Group II hearts (Table 2).

The stereospecificity of FEOBV binding in rat heart was also investigated. No consistent differences were observed by visual or quantitative analysis of the time–activity curves (Table 2) of (+)FEOBV and (−)FEOBV.

Measurement of specific binding of (−)FEOBV

The distribution of radioactivity in the hearts at the end of perfusion is shown in Table 3. The retention of radioactivity in atria with (−)FEOBV was 3–4 times that in ventricles. Perfusion of hearts with (−)vesamicol caused 70–80% reduction in retention of (−)FEOBV in both atria and ventricles. The remaining tissues of the heart (composed primarily of connective tissue) showed no decrease of (−)FEOBV binding with (−)vesamicol. Retention of (+)FEOBV in ventricles was 50% that of (−)FEOBV (Table 3). The lesser differences observed in the retention of the two stereoisomers in atria and whole heart were not statistically significant.

Discussion

It was the purpose of the present work to evaluate (−)FEOBV as a probe of cholinergic neurons in the heart with PET. The externally monitored, perfused rat heart provides a stable and well-defined experimental model for tracer kinetic measurements of PET tracers (Ng *et al.*, 1991). Due to its lipophilic character (−)FEOBV is highly extracted by the myocardium (Fig. 1) and likely distributes initially in membranes and other lipid-rich sites. The early myocardial accumulation rate of ^{18}F in relation to the coronary flow indicates a net extraction of about 40% for FEOBV in the isolated rat heart. Presuming FEOBV is transported across the capillary endothelium by passive diffusion, higher extractions of tracer would be expected *in vivo* considering the supraphysiologic coronary flow rates of the buffer-perfused rat heart.

The extensive clearance of ^{18}F in the wash-out phase indicates a high fraction (>90%) of the initial (−)FEOBV accumulation to be freely diffusible. The low retention of radiotracer by the heart is consistent

Table 1. Hemodynamic parameters of isolated working rat hearts

Group	*n*	Coronary flow (mL/min/g)	Cardiac output (mL/min/g)	Heart rate (min^{-1})	Diastolic/systolic pressure (mm Hg)
Group I: control	6	12.9 ± 2.7	29.9 ± 5.5	243 ± 54	57 ± 2/93 ± 3
Group II: 10 μM (−)vesamicol	5	13.5 ± 2.9	33.7 ± 6.4	198 ± 30	57 ± 2/93 ± 2

Measurements were averaged for each heart over the duration of the tracer kinetic study.

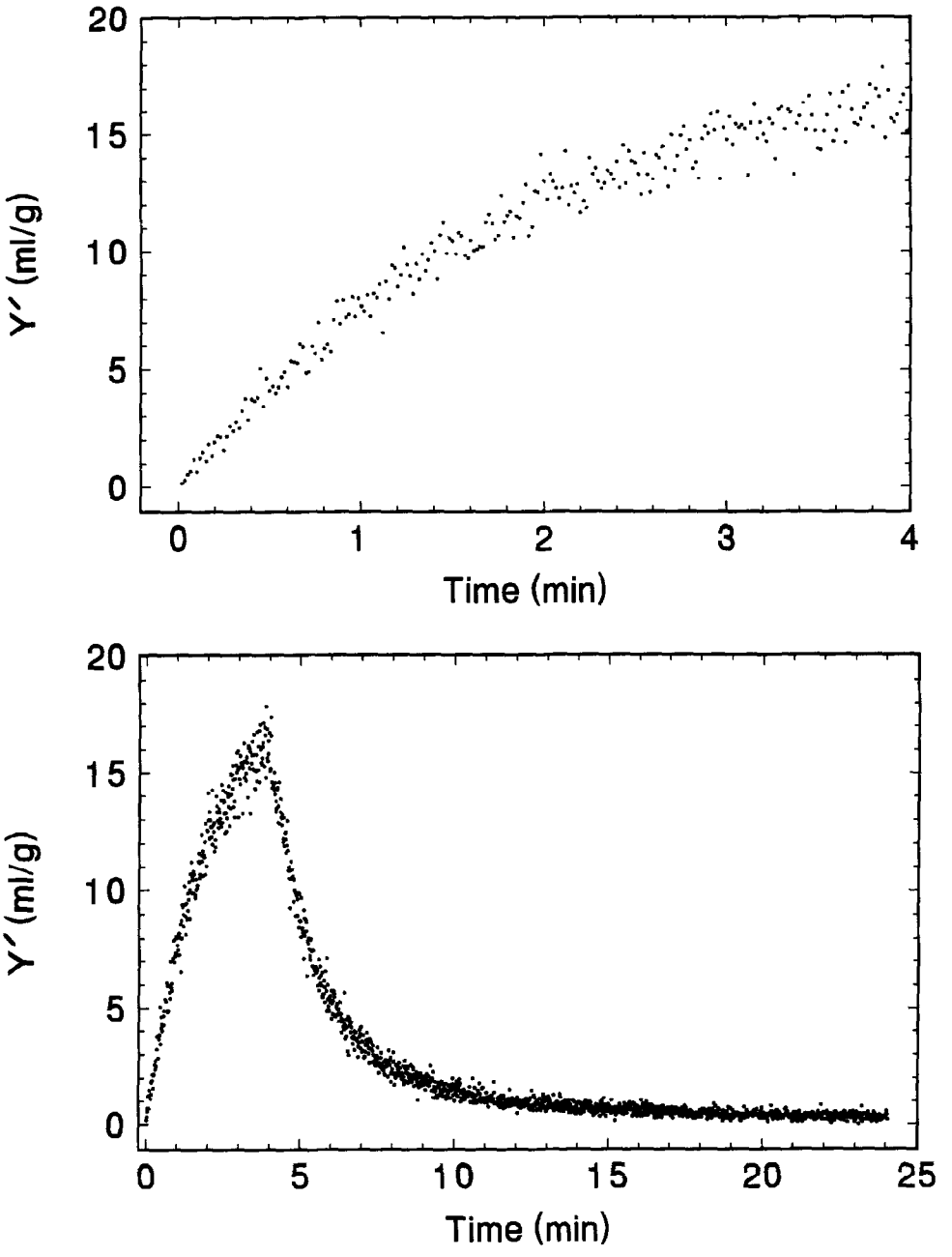

Fig. 1. Kinetics of (−)FEOBV in a representative isolated rat heart perfused under control conditions. Radiotracer is present in the perfusate at constant concentration (accounting for radioactive decay) from 0 to 4 min. The uptake kinetic is non-linear (upper graph) and the clearance kinetic is multiphasic (lower graph).

with a low density of cholinergic neurons (Lund *et al.*, 1978; Slavikova and Tucek, 1982) possessing specific vesamicol binding sites. The possibility of competitive inhibition of (−)FEOBV binding at the vesicular acetylcholine transporter by non-radioactive material in the radiopharmaceutical preparations is unlikely with the HPLC purified, no-carrier-added ^{18}F-labeled radiotracers utilized in this work. By comparison, there is very little (<2%) non-specific accumulation of the sympathetic neuronal probe [^{11}C]hydroxyephedrine in the isolated rat heart (DeGrado *et al.*, 1993). In the latter case, the specificity of retention is enhanced by the hydrophilicity of the radiotracer, the dense innervation of the myocardium by sympathetic neurons and the avidity of the neuronal catecholamine uptake process.

The specificity of the binding of (−)FEOBV to the vesicular acetylcholine transporter was investigated by (a) blocking the specific binding of (−)FEOBV with (−)vesamicol (Group II) and (b) direct

Table 2. Indices of myocardial kinetics of (−)FEOBV and (+)FEOBV in isolated rat heart

Group/tracer	Perfusate composition	Uptake rate K_1 (mL/min/g)	Net extraction (%)	Tail:peak ratio (%)	Clearance kinetics: fitted biexponential constants		
					$A_1/(A_1+A_2)$	K_1 min^{-1}	K_2 min^{-1}
Group I/(−)FEOBV ($n=6$)	Control	4.9 ± 1.8	37 ± 9	3.6 ± 0.8	0.84 ± 0.06	0.66 ± 0.21	0.062 ± 0.022
Group II/(−)FEOBV ($n=5$)	+10 μM (−)vesamicol	3.5 + 1.9	27 ± 14	2.5 + 0.7*	0.94 ± 0.02**	0.64 ± 0.09	0.053 ± 0.022
Group III/(+)FEOBV ($n=4$)	Control	4.5 ± 2.4	35 ± 11	3.0 ± 1.8	0.88 ± 0.03	0.61 ± 0.14	0.070 ± 0.048

*$P < 0.05$; **$P < 0.01$ versus Group I hearts.

comparison of (−)FEOBV with its inactive stereoisomer (+)FEOBV (Group III). The retention of radiotracer was markedly reduced relative to the control group (Group I) in both cases (Table 3), indicating the non-specific binding of (−)FEOBV to be <30% in both atria and ventricles. Higher retention of radiotracer by the atria relative to ventricles may reflect denser cholinergic innervation in agreement with higher levels of choline acetyltransferase (Slavikova and Tucek, 1982).

Measurements of the distribution of FEOBV by heart dissection at the end of perfusion (RIs, Table 3) were generally more sensitive to the effects of pharmacologic blockade and stereochemistry than were estimates of tracer uptake and retention derived from external monitoring of radioactivity in the whole rat heart (Table 2). In particular, the lower binding of (+)FEOBV relative to (−)FEOBV found in ventricles was more weakly evidenced in the time–activity curves. Several factors may be responsible for this discrepancy. Since the coincidence probes monitor radioactivity in the whole organ, the time–activity curve represents the sum of radioactivity contained in a heterogenous mixture of tissue. On the basis of radiotracer retention as measured at the end of each study, the ventricles (representing >90% of the mass of the heart) contribute predominantly to the time–activity curves. Yet the kinetics of (−)FEOBV in the atria and overlying tissues, for example, may be different from that in the ventricles. Also, the influence of connective tissues, vessels and surgical thread that are present during perfusion of the rat heart but dissected free following the perfusion should be considered. Although representing a small fraction of the mass of the heart, the retention (expressed on a per mass basis) of either stereoisomers of FEOBV by these fractions was greater than that of atria or ventricles and was independent of

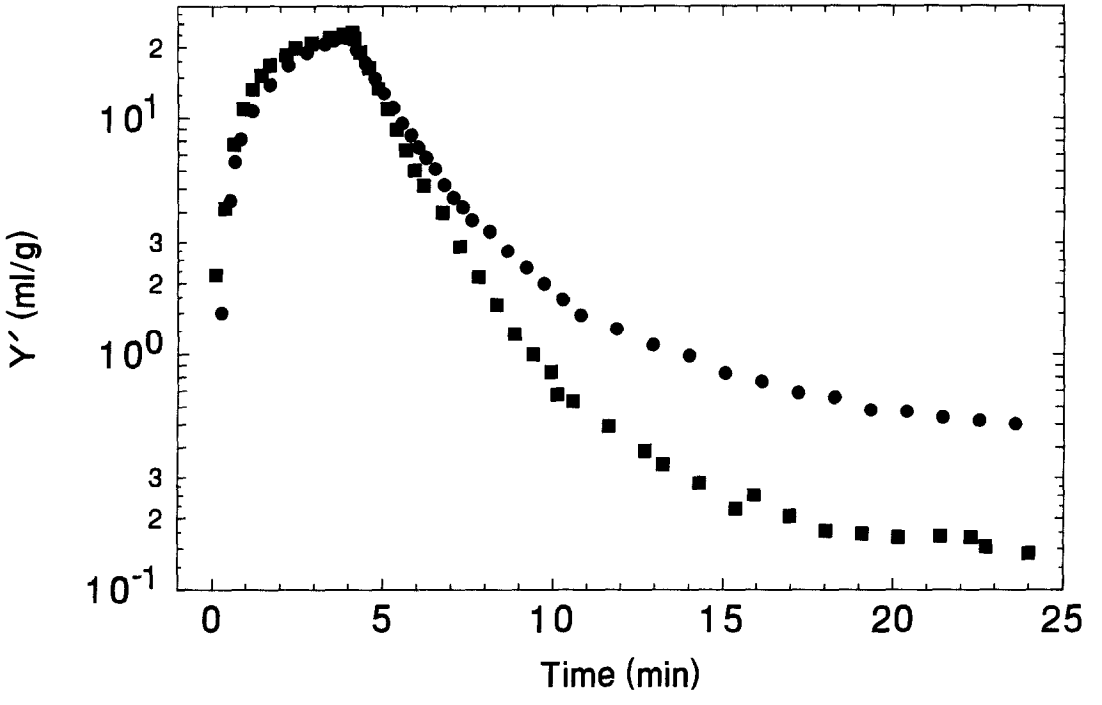

Fig. 2. Effects of pretreatment with (−)vesamicol on representative normalized time–activity curves of (−)FEOBV in isolated rat hearts. Hearts were perfused with 0 (●) and 10 μM (−)vesamicol (■). Although initial uptake into the myocardium is similar for both conditions, more extensive clearance of tracer is shown in (−)vesamical treated hearts.

Table 3. Retention of ^{18}F-labeled (−)FEOBV and (+)FEOBV in isolated rat heart

Group	Retention index (RI) of ^{18}F at end of perfusion (mL/g)				
	Total	Atria (A)	Vent. (V)	Other†	A:V ratio
Group I: (−)FEOBV; control ($n = 6$)	0.39 ± 0.09	0.87 ± 0.21	0.26 ± 0.06	1.25 ± 0.49	3.9 ± 0.4
Group II: (−)FEOBV; (−)vesamicol ($n = 5$)	0.18 ± 0.02**	0.25 ± 0.04**	0.063 ± 0.025**	0.92 ± 0.15	4.3 ± 0.9
Group III: (+)FEOVB ($n = 4$)	0.26 ± 0.01	0.66 ± 0.22	0.13 ± 0.11*	1.16 ± 0.30	6.2 ± 1.9

*$P < 0.05$; **$P < 0.005$ versus Group I hearts.
†Vessels, connective tissues and surgical thread.

perfusion condition (Table 3). Unlike the atria and ventricles, this fraction is very poorly perfused and probably exhibits incomplete wash-out of unbound tracer.

The very low binding of (−)FEOBV in ventricles in comparison to the uptake and retention of other cardiac PET tracers will likely impede its application *in vivo*. In light of *in vitro* studies showing high affinity of benzovesamicol derivatives to the vesicular acetylcholine transporter (Rogers *et al.*, 1989), the present study suggests that the slow rate of binding of the tracer within tissue is primarily due to a very low concentration of the transporter in the tissue. This is consistent with a low density of cholinergic innervation of the heart (Lund *et al.*, 1978; Slavikova and Tucek, 1982). Thus, the uptake and clearance patterns of (−)FEOBV in the myocardium determined from external monitoring are largely dependent on uptake and turnover of tracer from non-specific sites.

Although specific binding could be evidenced in the isolated rat heart, caution should be employed in extrapolating this result to the *in vivo* situation. Lower specific:non-specific ratios of (−)FEOBV retention in the myocardium would be expected for *in vivo* experiments using PET for several reasons. Most notably, radiotracer was not present in the perfusate during the wash-out period of isolated hearts, allowing extensive clearance of the unbound fraction in the myocardium. When administered *in vivo*, however, (−)FEOBV may remain in significant quantities in arterial blood for the duration of a PET study. The equilibration of the tracer between diffusible pools within the myocardium and the blood would likely obscure the small bound fraction of radioactivity. Other differences in experimental conditions in PET studies to those in the present study should be considered, including the supraphysiologic coronary flow of the buffer-perfused heart which promotes clearance of the unbound fraction of radiotracer; the different nature of volume-averaging effects; and the potential effects of systemic metabolites of (−)FEOBV are avoided in the isolated heart but encountered *in vivo*. It is concluded that the accurate prediction of specifically bound fractions of (−)FEOBV through means of tracer kinetic analysis is likely to be limited by poor image quality (low tracer retention), inadequate contrast of cholinergic innervated tissues from non-innervated tissues and close dependence of uptake and non-specific wash-out rates on myocardial perfusion.

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