Use of microdialysis for monitoring sympathetic and parasympathetic innervation of heart in conscious rats

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Cremers, T. I. F. H., A. C. H. Teisman, W. H. van Gilst, and B. H. C. Westerink. Use of microdialysis for monitoring sympathetic and parasympathetic innervation of heart in conscious rats. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2850–H2856, 1997.—A microdialysis method was developed to sample norepinephrine and acetylcholine from the heart of freely moving rats. A flexible dialysis fiber (length 14 mm), with a copper wire inserted inside, was implanted into the heart. Extracellular norepinephrine was detectable for at least 72 h after implantation. Basal output levels 24 h after surgery were 140 pg/ml when corrected for in vitro recovery. Evidence was provided that the major part of norepinephrine in dialysates is derived from local neurotransmission. Acetylcholine was only detectable in cardiac dialysates when an esterase inhibitor was infused. Corrected basal output levels 24 h after surgery were 223 pg/ml when neostigmine was infused in a concentration of 100 µmol/l. In addition, the presence of local muscarinic autoreceptors on cholinergic neurons in the heart was shown. It is concluded that microdialysis is a reliable method that can be used to study the innervation of the heart in subchronic preparations in freely moving rats.

Keywords: muscarinic receptors; neurotransmission

DURING THE LAST DECADE microdialysis has been used frequently to sample the extracellular compartment of the central nervous system (3, 8, 17, 21, 22). The method has contributed significantly to our understanding of the neurochemistry and pharmacology of neurotransmitter release in the brain of conscious animals.

The use of microdialysis in peripheral organs is a relatively new application, and until now only a few studies appeared that monitored peripheral neurotransmission by this method. Microdialysis of myocardial norepinephrine has been described in acute experiments using anesthetized cats and rats (1, 3, 13, 14). In addition, acetylcholine was studied in the heart of anesthetized cats (2). We are aware of two studies in conscious animals in which peripheral neurotransmission was monitored in the heart (13) and pineal gland (9), respectively.

In the present study, we have investigated the suitability of microdialysis to monitor the release of norepinephrine and acetylcholine from peripheral nerves in the heart of conscious rats. For that purpose a microdialysis probe was designed that is compatible with the movements of the beating heart. To prevent the probe from collapsing by the movements of the beating heart, a copper wire was inserted inside the microdialysis membrane. This probe was loosely fixed in the heart muscle and connected subcutaneously with polyethylene tubing to a stainless steel inlet and outlet that was fixed on top of the skull.

Extracellular norepinephrine in microdialysates was detectable for at least 72 h after implantation. The effect of infusion via the dialysis probe of norepinephrine uptake inhibitors and tetrodotoxin (TTX) was studied. In addition, the response to water immersion was investigated. Acetylcholine was detectable in the dialysates in the presence of an acetylcholine esterase inhibitor; the effect of local application of a muscarinic blocker was established.

MATERIALS AND METHODS

Animals, Drug Treatment, and Chemicals

Male albino rats of a Wistar-derived strain (275–320 g) (Harlan, Zeist, The Netherlands) were used for the experiments. The rats were housed individually in plastic cages (35 × 35 × 40 cm). During the experiments the animals had free access to food and water.

The following drugs were used: TTX and methylatropine (Sigma, St. Louis, MO), cocaine hydrochloride, desipramine hydrochloride (RBI, Natick, MA), and neostigmine bromide (Centrachemie, Etten Leur, The Netherlands).

The experiments were approved by the Animal Care Committee of the Faculty of Mathematics and Natural Science of the University of Groningen.

Preparation of Probe

The probe designed for microdialysis of the heart is depicted in Fig. 1A. A dialysis fiber was taken from an artificial kidney (AN 69, Hospal, Bologna, Italy). The fiber consisted of polyacrylonitrile-sodium methalyl sulfonate copolymer (ID, 0.22 mm; OD, 0.31 mm, molecular-mass cutoff 4,000 Da). The probe was prepared in a U shape with a copper wire (length 20 cm, diameter 0.1 mm) inserted inside. The copper wire strengthens the probe and prevents the fiber from collapsing within the beating heart. The exposed length of the membrane was 14 mm. The inlet and outlet of the cannula were made of polyethylene flexible tubing (PE-10) (ID, 0.28 mm; OD, 0.60 mm). At the top of the U-shaped probe, a piece of suture (with a needle already attached) was glued to the probe. All connections in the probe were made with Superglue (Patex).

Surgery

Subchronic experiments. Before surgery 5 mg amoxicillin dissolved in 1 ml of saline were administered intramuscularly in the hindlimb of the animal. Probes were implanted during general chloral hydrate anesthesia (400 mg/kg ip) followed by halothane. Rats were kept at a temperature of 38°C with a heating pad.

The rat was intubated and artificially respirated (400 ml/min; 70 times/min; Infant Respirator, Losco, Amsterdam, The Netherlands). An incision was made between the sixth and the seventh rib.
The intercostal muscles were cut over a distance of ~3 cm. The ribs were spread with the help of a wound clamp. The expiratory end pressure was increased to minimize the oscillating movements of the heart and to facilitate probe insertion. Next, the pericardium was removed. With the help of small forceps, we inserted the probe caudal to rostral, attempting to position it from the apex into the wall of the right ventricle and septal area of the left ventricle for the norepinephrine measurements. For the measurement of acetylcholine, the probe was inserted in the upper side of the right ventricle toward the base of the heart approaching the aorta. During all insertions, we carefully verified that the probes were positioned in the myocardium. To fix the cannula, a stitch was made near the area where the cannula left the heart. The implanted cannula is depicted in Fig. 1B.

After the lungs were reinflated by increasing the expiratory end pressure, the sixth and seventh ribs were stitched together with three firm stitches, while the PE-10 tubings of the probe were allowed to exit the thoracic cavity in the middle of the incision. After the intubation was removed, the PE-10 tubing was transferred subcutaneously to the skull of the rat. A hole was drilled in the skull, and a stainless steel screw was placed into the skull. The PE tubing was connected to the inlet and outlet (stainless steel) tubes with Superglue and fixed to the screw with dental cement.

Acute dual-probe experiments. During constant isoflurane anesthesia (2%) and controlled body temperature, probes were implanted both in the right ventricular wall/septal area and in the lumen of the left ventricle as described above. After the probes were implanted, the thorax was closed and the left jugular vein was cannulated. Animals were allowed to breathe spontaneously throughout the experiments.

In vitro experiments. To determine the adequate perfusion speed and optimal recovery of the heart probe, we bathed cannulas in a 38°C Ringer solution, with a fixed concentration of norepinephrine (0.49 × 10^{-8} M) and acetylcholine (10^{-7} M). Samples were collected at different perfusion speeds (1–7 µl/min). Relative recovery [(perfusate concentration/standard concentration) × 100%] and absolute recovery (concentration in fmol/min) were calculated.

To evaluate the effect of the copper wire on the recovery capacities of the probe, the recovery of norepinephrine was also determined for probes without copper wire.

Microdialysis

Microdialysis experiments were carried out 6–72 h after implantation of the probe. Infusion of drugs and induction of stress were only performed 24 and 48 h after surgery. The dual-probe experiments were started immediately after surgery. The probes were perfused with a Ringer solution at a flow rate of 2.0 µl/min (Perfusor VI, B. Braun, Melsungen, Germany). The composition of the Ringer solution was (in mmol/l) 140.0 NaCl, 4.0 KCl, 3.4 CaCl_{2}, and 1.0 MgCl_{2}. Fifteen-minute fractions were collected either in 300-µl glass vials (Chromacol, Trembull) with the help of a CMA/142 microfraction collector (CMA/Microdialysis, Stockholm, Sweden) for the norepinephrine experiments or directly into a 50-µl high-performance liquid chromatography (HPLC) loop for the online quantification of acetylcholine.

Evaluation of heart function. Blood pressure was monitored by means of a standard tail-cuff registration (Life Science) under mild isoflurane anesthesia. In short, rats were anesthetized for 5 min with O_{2} and 2% isoflurane. Thereafter, the mean blood pressure and heart rate were registered by a computerized system.

Chemical Assays

Norepinephrine was derivatized with diphenylethlyenediamine (18) and quantified by HPLC with fluorimetric detection as described earlier (9). Samples were automatically derivatized, injected, and analyzed with the help of the CMA/200 refrigerated microsampler (CMA/Microdialysis). The chromatographic system of the norepinephrine assay consisted of a Pharmacia 2248 pump (Pharmacia, Uppsala, Sweden) in conjunction with a Waters M470 fluorescence
detector (excitation, 350 nm; emission 480 nm). Samples were separated on a reversed-phase C18 column (Supelco, 250 × 4.6 mm). The mobile phase consisted of 50 mmol/l sodium acetate (adjusted to pH 6.5 with concentrated acetic acid) (62%), acetonitrile (30%), and methanol (8%). The detection limit of the assay was 1.8 fmol/injection (on column).

Acetylcholine in dialysates was assayed online using HPLC after enzymatic conversion and electrochemical detection as previously described (6). Briefly, the samples were injected onto a reversed-phase C18 column preloaded with sodium lauryl sulfate. Acetylcholine was converted into hydrogen peroxide and betaine in a postcolumn enzyme reactor containing immobilized acetylcholine esterase and choline oxidase (Sigma). Subsequently, hydrogen peroxide was detected using a platinum electrode (Antec, The Netherlands) set at +500 mV. The mobile phase consisted of a 0.1 mol/l potassium phosphate buffer (pH 8.0) containing 0.5 mmol/l tetramethylammonium chloride and delivered at a rate of 0.5 ml/min. The detection limit of the assay was 10 fmol (on column).

Expression of Results and Statistics

The lag time to sudden concentration changes, caused mainly by the passage of the sample through the tubing, was ~5 min. Figures 3 and 5–8 are corrected for this lag time. Absolute values are given as femtomoles per minute, not corrected for recovery. Dialysate contents of norepinephrine and acetylcholine are expressed as percentages of controls. The average concentration of three to four stable samples (deviation <20%) was considered as the control and defined as 100%. Data were analyzed using a statistical program (SigmaStat). Nonparametric one-way repeated measurements analysis of variance on ranks analysis was followed by the Dunnett’s multiple comparisons test when appropriate. The level of significance was set at P < 0.05. Differences in basal output (Fig. 4) were determined by the Mann-Whitney rank sum test.

RESULTS

Cannula

Several probe designs were tested. Transversal fibers did not have enough solidity in freely moving animals. I-shaped cannulas containing fused silica were frequently ejected from the heart. Finally, a U-shaped fiber strengthened by a copper wire was found to be reliable for subchronic implantation. Figure 2, A and B, shows relative and absolute recoveries as a function of the perfusion rate. The relative and absolute recoveries at 2 µl/min were 62.1 ± 1.7% and 6.0 ± 0.2 fmol/min, respectively, for norepinephrine and 65.8 ± 1.9% and 130.0 ± 3.3 fmol/min, respectively, for acetylcholine. The recoveries excluded the recovery in the chemical assay.

Although the norepinephrine recoveries of the copper wire-containing heart probe were slightly lower than the empty probe (67.7 ± 2.1%, 6.6 ± 0.2 fmol/min at 2.0 µl/min), these differences were not statistically significant.

Surgery and Telemetric Observations

The probe implantations reached a success rate of virtually 100%. Animals recovered faster when compared with rats that underwent intracerebral microdialysis. Food intake and alertness were normalized the day after surgery.

### Table 1. Effect of probe implantation on development of body weight, tail-cuff blood pressure, and heart rate parameters in rats compared with values after sham operation

<table>
<thead>
<tr>
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<th>Before Operation</th>
<th>After Operation</th>
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<tr>
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<td>1 day</td>
<td>0 days</td>
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<tr>
<td>Body wt, g</td>
<td>356 ± 12</td>
<td>356 ± 11</td>
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<tr>
<td>Probe</td>
<td>352 ± 13</td>
<td>359 ± 12</td>
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<tr>
<td>Sham</td>
<td>350 ± 13</td>
<td>350 ± 12</td>
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<tr>
<td>Mean blood pressure, mmHg</td>
<td>101 ± 5</td>
<td>111 ± 5</td>
</tr>
<tr>
<td>Probe</td>
<td>112 ± 6</td>
<td>116 ± 8</td>
</tr>
<tr>
<td>Sham</td>
<td>356 ± 6</td>
<td>355 ± 13</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>354 ± 10</td>
<td>353 ± 11</td>
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Values are means ± SE; n = 5 rats. No significant differences were observed in probe vs. sham data.
Acute Dual-Probe Experiments

Figure 3 shows that the norepinephrine concentration of the blood significantly increased to 125% of control after infusion of a bolus of 0.2 ml of a 10 ng/ml solution of norepinephrine via the jugular vein. Norepinephrine levels in the heart muscle, however, remained unchanged. Bolus injections of saline had no effect on the norepinephrine content sampled from both probes.

Extracellular Norepinephrine in Heart

Average levels of extracellular norepinephrine are shown in Fig. 4. Six hours after implantation, the norepinephrine output was 1.1 fmol/min. These levels gradually declined after implantation but were still detectable 72 h after implantation when the levels had dropped to 0.34 fmol/min.

Effect of reuptake inhibition on extracellular levels of norepinephrine. When the reuptake inhibitor desipramine (in a concentration of 100 µmol/l) was infused via the microdialysis probe, extracellular norepinephrine in the heart increased to 150% of controls (Fig. 5). When TTX (10 µmol/l) was coinfused, the levels of norepinephrine decreased to 30% of controls. Cocaine (100 µmol/l) induced a similar increase in extracellular norepinephrine (Fig. 6). TTX, here followed for only 15 min, again suppressed the output of norepinephrine.

Effect of Stress and Behavioral Activation on Extracellular Norepinephrine in Heart

Rats were immersed in 6 cm of water (37°C) for a period of 15 min. The rats had never been immersed in the water before. Extracellular norepinephrine levels rose to 200–300% of controls. The rise in norepinephrine lasted for at least 2.5 h. A second 15-min forced water immersion, applied 105 min after the first period, did not further increase the levels of norepinephrine (Fig. 7). When TTX (10 µmol/l) was infused after the immersion period, norepinephrine levels dropped to 100% of controls. During TTX infusion, the second

![Fig. 3](image-url) Change of norepinephrine levels measured simultaneously in the ventricular muscle (squares) and heart chambers (circles) upon an infusion, via the jugular vein, of saline (open symbols) and 10 ng/kg norepinephrine (solid symbols). Data are means ± SE; n = 5 rats. *P < 0.05, heart chamber vs. ventricular muscle.

![Fig. 4](image-url) Extracellular levels of norepinephrine as a function of time after implantation. Values are given as means ± SE in fmol/min. *Extracellular levels were decreased 72 h after implantation (P < 0.05) compared with acute implantations (day 1, n = 4 rats; day 2, n = 14 rats; day 3, n = 8 rats; day 4, n = 3).

![Fig. 5](image-url) Effect of local infusion of desipramine (100 µmol/l) and tetrodotoxin (TTX, 10 µmol/l) on extracellular levels of norepinephrine in heart. Bars indicate time period of infusion. Levels are given as percentage of controls ± SE; n = 5 rats. *P < 0.05 vs. controls.

![Fig. 6](image-url) Effect of local infusion of cocaine (100 µmol/l) and TTX (10 µmol/l) on extracellular levels of norepinephrine in heart. Bars indicate time period of infusion. Levels are given as percentage of controls ± SE; n = 5 rats. *P < 0.05 vs. controls.
immersion had only a slight stimulatory effect on the extracellular content of the transmitter.

**Extracellular Levels of Acetylcholine**

Acetylcholine was detectable in dialysates of the right ventricle 24 h after surgery when neostigmine (100 µmol/l) was included in the perfusion fluid (Fig. 8). The detected values (2.68 ± 1.15 fmol/min; n = 4) were close to the detection limit of the method. When atropine was included in the perfusion fluid (in a concentration of 100 µmol/l), the output of acetylcholine was clearly stimulated to >250% of controls. The levels of acetylcholine dropped to <50% of controls when atropine and neostigmine were removed from the perfusion fluid.

**DISCUSSION**

**Probe Design and Implantation**

To measure the local neuronal innervation of the heart, a microdialysis probe was designed that was suitable for subchronic implantation in the beating heart. The loose fixation of the U-shaped dialysis fiber allowed synchronization of the movements of the heart and the probe. As the solidity of the probe was increased by insertion of a copper wire, the probe could be more easily fixed to the myocardium. Although the absolute recoveries increased on increasing the dialysis flow, a flow rate of 2 µl/min was chosen in order to avoid increased pressure in the probe. The relatively long length of the exposed membrane (14 mm) resulted in a high in vitro recovery rate (62.1% for norepinephrine and 65.8% for acetylcholine). However, it should be emphasized that the in vitro recoveries often do not match in vivo recoveries.

We experienced that rats recovered faster from the cardiac surgery than after implantation of a microdialysis probe into the brain. Twenty four hours after implantation of the probe, the behavior of the animal was fully normalized, blood pressure and heart rate were not affected, and no loss of weight was noticed. It is concluded that the copper wire-containing flexible probe and its subcutaneous connections to an inlet and outlet mounted on the skull of the animal represent a robust cardiac microdialysis model that can be subchronically applied to freely moving rats for several days.

**Norepinephrine**

Histochemical studies (11) indicated that the right ventricle is more densely innervated by the sympathetic nervous system than the left ventricle. From these results, we observed that basal norepinephrine levels were lower when the probe was inserted in the left ventricle than in the right ventricle/septal area of the left ventricle (data not shown). With the present approach, norepinephrine was detectable for at least 72 h after implantation of the probe.

Until now only a few studies have appeared that used cardiac microdialysis to study neurotransmitter function. Akiyama et al. (1) and Shindo et al. (16) implanted microdialysis probes in the heart of anesthetized cats and determined extracellular norepinephrine after coronary occlusion. Mertes et al. (13) recorded norepinephrine in the heart of pigs after brain death. Cardiac microdialysis of conscious rats was described by Obata et al. (14). These authors have used the model to study norepinephrine and •OH generation in anesthetized rats after myocardial ischemic injury.

When the basal value of norepinephrine (detected 24 h after surgery) is corrected for the in vitro recovery, a value of ~140 pg/ml was obtained. This value is close to the values found in two comparable studies using cats, 131 (1) and 256 pg/ml (16), respectively. The decrease over time of the norepinephrine output may have several causes. First, as the probe is loosely fixed, neuronal tissue damage could occur due to the constant...
beating of the heart. Second, de Lange et al. (7) showed that repeated perfusion of a microdialysis probe in the brain gives rise to hypercellularity and infiltration of granulocytes, which, in turn, could have an effect on the norepinephrine output. Taking surgical stress and decline in norepinephrine output into account, we conclude that experiments could best be conducted 24–48 h after surgery.

Next, we investigated whether the sampled norepinephrine was directly derived from local neurotransmission. For that purpose we included the reuptake inhibitors cocaine or desipramine in the perfusion fluid. A moderate increase in extracellular norepinephrine was detected (to −100–150% of controls) during infusion of the uptake inhibitors; this increase was less pronounced than the response of norepinephrine to cocaine or desipramine in the perfusion fluid. A large increase in extracellular norepinephrine was directly derived from local neurotransmission in microdialysates of the pineal gland, in which addition of 10 µmol/l cocaine to the perfusion fluid resulted in a rise of norepinephrine content to −200% of controls (9). We interpret the increase in extracellular norepinephrine as an indication that a part of the recorded norepinephrine is locally produced.

Another method to investigate the origin of neurotransmitters in dialysates is infusion of the sodium channel blocker, TTX. Several studies (19, 21, 22) have demonstrated that extracellular norepinephrine and acetylcholine recorded by microdialysis from brain areas are virtually TTX dependent, indicating that the sampled transmitters are directly derived from synaptic processes. Figure 5 indicates that 70% of the basal norepinephrine is TTX dependent. A similar TTX response on extracellular norepinephrine has been noticed in the spleen, in which norepinephrine levels decreased to 40% of controls (15). We therefore conclude that the sampled norepinephrine is derived from two sources: the major part of the basal norepinephrine originates from local neurotransmission, and a minor part is not directly related to local neuronal release. A possible source of nonneuronal norepinephrine is the blood compartment. A rapid exchange between norepinephrine present in the blood and the extracellular fluid seems likely. It is of significance in this respect that the corrected extracellular norepinephrine values are close to reported blood values (e.g., 12). The results from the acute dual-probe experiments, however, did not show any elevation of the norepinephrine output due to a systemic infusion of norepinephrine. These data indicate the norepinephrine measured in the heart muscle is at least in majority not derived from the blood. As mentioned above, microdialysis of norepinephrine in the pineal gland was fully TTX dependent (9) and only partly in the spleen (15) and in the heart (present study). Microdialysis of peripheral organs could therefore be characterized by a nonneuronal pool, which may well be derived from neuronal tissue damage, because the probe is not as firmly fixed as in intracerebral microdialysis.

In addition, we studied a condition in which local norepinephrine release was stimulated by stress and behavioral activation. We induced stress in rats naive to water by immersing them in water. This immersion increased norepinephrine output severalfold. A large part of this stimulated norepinephrine release was proven to be TTX dependently. The biphasic increase of norepinephrine after immersion was also observed by de Boer et al. (6), who measured norepinephrine and epinephrine in blood under the same conditions. In this study, epinephrine rapidly returned to baseline levels after the 15 min of immersion, indicating the initial increase of norepinephrine and epinephrine to be due to an increased activity of the sympathetic and adrenomedullary systems caused by stress. The prolonged elevation of norepinephrine, however, is related to a period of increased sympathetic activity due to vigorous grooming and wet-shaking behavior, which was also observed in our experiments.

Because the water immersion-induced stimulation of norepinephrine release is to a great extent TTX dependent, this model could serve as a physiological model of stimulated norepinephrine release. With receptor-specific compounds infused, the regulation of local norepinephrine release can be studied in the myocardium of conscious animals.

Acetylcholine

As far as we know only one study has described microdialysis of acetylcholine from peripheral organs. In that study acetylcholine was determined in the heart of anesthetized cats (1). In the present study, acetylcholine was detectable in microdialysates of the upper side of the right ventricle of the conscious rat. The transmitter was only detectable when an esterase inhibitor was added to the perfusion fluid. It is emphasized that in brain microdialysis studies, addition of an esterase inhibitor is routine when acetylcholine is recorded (4). The pronounced enzymatic activity of extracellular acetylcholine esterase is an explanation for the need to block the degradation of the transmitter. In contrast to norepinephrine the blood compartment is not likely to contribute to the extracellular levels because acetylcholine is not detectable in blood by sensitive electrochemical methods (5). We therefore conclude that the acetylcholine recorded by cardiac microdialysis is directly derived from local parasympathetic innervation. That the recorded acetylcholine is indeed directly related to receptor-regulated synaptic processes was demonstrated by infusion of the anticholinergic agent atropine via the microdialysis probe. The observed increase in acetylcholine during atropine infusion is explained by blockade of cholinergic muscarinic autoreceptors. Similar effects have been noticed in microdialysis studies in brain tissue (4).

In conclusion, the present study describes the use of microdialysis to monitor sympathetic and parasympathetic innervation of the heart of the conscious rat. It is concluded that cardiac microdialysis is a promising method to study the innervation of the heart in subchronic preparations.

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