Neuronal Sodium Homoeostasis and Axoplasmic Amine Concentration Determine Calcium-Independent Noradrenaline Release in Normoxic and Ischemic Rat Heart

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Calcium-independent noradrenaline release was studied in the isolated perfused rat heart under conditions of normoxia, cyanide intoxication, and ischemia. The release of endogenous noradrenaline and dihydroxyphenylglycol were determined by high-performance liquid chromatography. The release of dihydroxyphenylglycol, the main neuronal noradrenaline metabolite, was used as an indicator of the free axoplasmic amine concentration. When storage function of neuronal vesicles was disturbed by Ro 4-1284 or trimethyltin, high dihydroxyphenylglycol release was observed without concomitant overflow of noradrenaline. If, however, these agents were combined with inhibition of Na\(^+\),K\(^+\)-ATPase or with veratridine-induced entry of sodium into the neuron, both dihydroxyphenylglycol and noradrenaline were released. Noradrenaline release was independent of extracellular calcium and was suppressed by blockade of neuronal catecholamine uptake (uptake\(_{\text{Na}}\)), indicating nonexocytotic noradrenaline liberation from the sympathetic nerve ending. This release critically depended on two conditions: 1) increased cytoplasmic concentrations of noradrenaline within the sympathetic neuron and 2) intraneuronal sodium accumulation. Both conditions together were required to induce noradrenaline efflux across the plasma membrane using the uptake\(_{\text{Na}}\) carrier in reverse of its normal transport direction. A disturbed energy status of the sympathetic neuron, induced by cyanide intoxication or ischemia, likewise caused calcium-independent noradrenaline release by interfering with both vesicular storage function and neuronal sodium homoeostasis. Again, release was sensitive to uptake, blockade. Since neuronal sodium accumulation was the rate-limiting step, release was further accelerated when residual Na\(^+\),K\(^+\)-ATPase activity was inhibited. Na\(^+\)-H\(^+\) exchange was identified as the predominant pathway of sodium entry into the sympathetic nerve ending in ischemia, and its inhibition by amiloride and ethylisopropylamiloride markedly suppressed ischemia-induced noradrenaline release. (Circulation Research 1988;63:214–226)

Myocardial ischemia is associated with the accumulation of high catecholamine concentrations within the extracellular space of the underperfused area.\(^1\)–\(^4\) Deleterious consequences, such as the occurrence of fatal arrhythmias\(^5\)–\(^6\) and the acceleration of cell damage,\(^7\)–\(^8\) have been attributed to an adrenergic excess stimulation of the ischemic myocardium. The ischemia-induced catecholamine accumulation is predominantly due to noradrenaline release from local sympathetic nerve endings that is not influenced by central sympathetic activity.\(^3\)–\(^9\) Such local metabolically mediated release of noradrenaline has been shown to be independent of extracellular calcium and has been characterized as nonexocytotic release in myocardial ischemia,\(^3\)–\(^9\)–\(^11\) anoxia,\(^9\)–\(^11\) and cyanide intoxication.\(^11\) The release has been proposed to be a two-step process induced by energy deficiency in the sympathetic nerve ending.\(^11\) First, noradrenaline is lost from the storage vesicles, resulting in increased axoplasmic concentrations. Second, the amine is transported across the plasma membrane into the extracellular space using the neuronal catecholamine uptake (uptake\(_{\text{Na}}\)) carrier in reverse of its normal transport direction.

The first purpose of this study was to identify conditions that induce outward transport of noradrenaline across the cell membrane of the sympa-
thetastic neuron. The inward transport of catecholamines into the neuron via uptake, depends on the extracellular presence of sodium. Therefore, we focused our interest on the role of intraneuronal sodium in causing a reversal of the carrier's net transport direction. The second aim was to establish the significance of free axoplasmic amine concentration and disturbed neuronal sodium homeostasis for nonexocytotic noradrenaline release in ischemia and cardiac energy deficiency caused by cyanide intoxication.

The experiments were performed in isolated rat hearts using a modified Langendorff preparation, and the overflow of endogenous noradrenaline and 3,4-dihydroxyphenylglycol (DOPEG) were measured. DOPEG was chosen because it is the main product of intraneuronal oxidative deamination of noradrenaline by monoamine oxidase, and it has been demonstrated to reflect axoplasmic noradrenaline concentrations that cannot be detected directly. To avoid exocytosis, experiments were done in the absence of calcium in the perfusion buffer.

In the experiments with undisturbed energy metabolism, vesicular storage function was impaired pharmacologically, and the release of endogenous noradrenaline and DOPEG were measured. In these experiments, we investigated the effects on noradrenaline release of increased sodium influx into the sympathetic neuron, sodium pump inhibition, and depolarization of the plasma membrane.

In the two experimental models of energy depletion, neuronal sodium balance was modulated by interfering with various pathways of transmembrane sodium movement. The energy metabolism of the sympathetic nerve ending was disturbed by either inhibition of oxidative phosphorylation with cyanide in combination with glucose-free perfusion or by global ischemia of the heart.

Materials and Methods

Male Wistar rats (180–250 g; Ivanovas, Kislegg, FRG) were anesthetized with thiobutabarbital (50 mg/kg i.p.). After injection of 500 IU heparin, the thorax was opened, the heart was rapidly removed and weighed, and the ascending aorta was cannulated for retrograde coronary perfusion. Hearts were perfused at a constant flow of 5 ml/min/g heart wt using a multichannel peristaltic pump. Initial perfusion was done with a modified Krebs-Henseleit solution (KHS; composition in mmol/l: NaCl 125, NaHCO3 16.9, Na2HPO4 0.2, KCl 4.0, CaCl2 1.85, MgCl2 1.0, glucose 11, and EDTA 0.027). The buffer was gassed with 95% O2 and 5% CO2 and the pH adjusted to 7.4. The temperature of the chamber and of the perfusate at the point of entry 20.027). The buffer was gassed with 95% O2 and the hearts were subjected to the experiments.

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Experimental series with blockade of energy metabolism were done at constant flow (5 ml/min/g heart wt). The perfusion buffer was deficient of glucose, and oxidative phosphorylation was inhibited by 1 mM sodium cyanide. Samples for high-performance liquid chromatography (HPLC) determination of noradrenaline and DOPEG were taken cumulatively for 2-minute periods.

For ischemia experiments, the hearts were subjected to global ischemia by stopping perfusion flow, and they were subsequently reperfused at the initial flow rate. If drugs were used, they were added 10 minutes prior to ischemia and were continued to the end of the experiment. Samples for noradrenaline estimation were taken throughout the last minute before ischemia and during the first and second minute of reperfusion.

Samples for the estimation of endogenous noradrenaline and DOPEG were taken from the coronary venous effluent, put on ice, and stabilized by the addition of sodium EDTA to a final concentration of 10 mM. The samples were stored at -80°C until assayed by an HPLC method.

Agents used in this study were amiloride (Sigma, Munich, FRO), desipramine (CIBA-Geigy, Basle, Switzerland), monensin (Sigma), ouabain (Merck, Darmstadt, FRO), reserpine (Sigma), Ro 4-1284 (2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-1,2,3, 4,6,7-hexahydro-11b-H-benzo(a)-quinolizine; Hoffmann-La Roche, Basle, Switzerland), sodium cyanide (Merck), and trimethyltin (Merck). Ethylisopropylamiloride was synthesized according to Cragoe et al. The agents not soluble in water were dissolved in ethanol, with final concentrations of ethanol being less than 0.05%. For control experiments, identical solvent and ion concentrations were used.

Assay of Noradrenaline and DOPEG

Endogenous noradrenaline and DOPEG were measured using an HPLC method as described by Schomig et al. Briefly, the pH of the sample was adjusted to 8.5 by the addition of NH4Cl-NH4OH buffer (2 M, pH 8.5) containing 0.5% sodium EDTA and 0.2% diphenylborate. Catecholamines and DOPEG were extracted into a 5-m有机 phase consisting of 99% n-heptane and 1% octanol in the presence of 0.25% tetraoctylammonium bromide. Back-extraction of catecholamines into an aqueous phase was performed by shaking the organic phase with 0.15 ml 0.2 M phosphoric acid. To increase the recovery of DOPEG, the same step was repeated after the addition of 1 ml octanol. One hundred microliters of the aqueous phase was injected into the HPLC system (Latek, Heidelberg, FRG). Separation was performed using a 5 μm C18 reversed phase column (Latek) with a flow of 0.8 ml/min. The solvent was 0.2 M phosphate buffer (pH 3.0) containing sodium EDTA (40 μM) and octylsulphate (25 μM). Quantitative analysis was performed by electrochemical detection (LC 4B, Bioanalytic Systems, West Lafayette, Indiana) at 0.6 V. Retention times for noradrenaline and DOPEG were 4.5 and 6.2 minutes. Recovery was 98% for noradrenaline and
92% for DOPEG, the limits of detection were 0.1 and 0.2 nmol/l, and the coefficients of variation were 5.9% and 5.8%, respectively. None of the drugs used in the experiments interfered with extraction, separation, or detection of noradrenaline and DOPEG.

Statistical methods
In text and figures, results are expressed as mean±SEM. The significance of differences was assessed by Student’s t test or analysis of variance.

Results
Increased Cytoplasmic Noradrenaline Concentration in Sympathetic Neuron Induced by Pharmacological Agents
In this study, DOPEG release was used to reflect free axoplasmic noradrenaline concentrations because previous studies have shown that DOPEG formation is related to the concentration of noradrenaline within the cytoplasm of the sympathetic neuron.

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Inhibition of the vesicular catecholamine transporter by reserpine or Ro 4-1284 resulted in a concentration-dependent overflow of DOPEG and no significant noradrenaline release (Figure 1, upper panels). At 1 μM, the effect of Ro 4-1284 was maximal, and this concentration was utilized in further experiments. Likewise inhibition of vesicular H⁺-ATPase by trimethyltin resulted in a concentration-dependent overflow of DOPEG, and only high concentrations, which were supramaximal concerning DOPEG release, resulted in a measurable overflow of noradrenaline (Figure 1, lower left panel). In the concentration of 30 μM, which was used in further experiments, trimethyltin induced maximal DOPEG overflow without major noradrenaline release. Addition of the ionophore monensin, which directly interferes with the vesicular proton gradient, resulted in the overflow of high amounts of DOPEG. The release reached approximately one third of total cardiac noradrenaline content within 10 minutes at a concentration of 1 μM monensin. DOPEG release was accompanied by noradrenaline release only when monensin concentrations higher than 1 μM were applied. This effect may be related to a sodium influx into the neuron induced by high concentrations of monensin.

Effect of Increased Neuronal Sodium Influx on Noradrenaline Release in Presence of High Axoplasmic Noradrenaline Concentrations
The effect of 1 μM Ro 4-1284 on the time course of DOPEG and noradrenaline overflow is demonstrated in the left panel of Figure 2A. Despite loss of noradrenaline from storage vesicles and increased cytoplasmic noradrenaline concentrations within the neuron, reflected by high DOPEG overflow, no significant amounts of noradrenaline were released. In the absence of extracellular calcium, veratridine (30 μM) resulted only in a minor noradrenaline release from the heart (Figure 2A, middle panel). After enhancement of cytoplasmic noradrenaline levels by Ro 4-1284, however, stimulation of sodium influx by veratridine caused major noradrenaline release at the expense of DOPEG release (Figure 2A, right panel). In the presence of 1 μM tetrodotoxin, veratridine following Ro 4-1284 did not induce any noradrenaline release (Figure 2B, left panel). The data indicate that sodium influx via tetrodotoxin-sensitive channels is necessary for veratridine-induced noradrenaline release.

Noradrenaline release induced by the combined action of Ro 4-1284 and veratridine could be attenuated by 80% when uptake, was specifically inhibited by 0.1 μM desipramine (Figure 2B, middle panel). In
contrast, blockade of plasmalemmal Na\(^+\)-H\(^+\) exchange by 10 \(\mu\)M ethylisopropylamiloride did not influence noradrenaline release induced by Ro 4-1284 and veratridine (Figure 2B, right panel), indicating that ethylisopropylamiloride has neither tetrodotoxin-like nor desipramine-like properties.

**Release of Noradrenaline and DOPEG Induced by Inhibition of Vesicular H\(^+\)-ATPase and Plasma Membrane Na\(^+\),K\(^+\)-ATPase**

Inhibition of vesicular H\(^+\)-ATPase by 1 \(\mu\)M trimethyltin\(^{19}\) resulted in a marked release of DOPEG, the deaminated metabolite of noradrenaline, that was not accompanied by any release of noradrenaline itself. However, if the hearts had been preperfused with 1 mM ouabain for 30 minutes to suppress the outward transport of sodium by Na\(^+\),K\(^+\)-ATPase, identical concentrations of trimethyltin induced the simultaneous release of noradrenaline and DOPEG in a proportion of 1 to 2 (Figure 3A). The combined overflow of the catecholamine and its metabolite did not exceed the DOPEG overflow found in the absence of ouabain. Blockade of the uptake carrier by 0.1 \(\mu\)M desipramine prior to the addition of trimethyltin almost completely suppressed the noradrenaline release that was found after ouabain and trimethyltin. DOPEG overflow was not reduced by uptake blockade (Figure 3A, lower panel).

Extracellular potassium depletion, used instead of ouabain to reduce Na\(^+\),K\(^+\)-ATPase activity, resulted in a similar effect on noradrenaline release (Figure 3B). Following 30 minutes of potassium-free perfusion, the noradrenaline release induced by trimethyltin amounted to one quarter of the concomitant DOPEG overflow.

**Effect of Membrane Depolarization by Increased Potassium Concentrations on the Overflow of Noradrenaline and DOPEG**

In the absence of extracellular calcium, stepwise rise of potassium concentrations in the perfusion buffer had no effect on the release of noradrenaline and DOPEG (Figure 4). If the axoplasmic noradrena-
Circulation Research
Vol 63, No 1, July 1988

overflow of noradrenaline

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FIGURE 3. Effects of combined inhibition of vesicular H^+-ATPase and plasmalemmal Na^+,K^+-ATPase on noradrenaline (upper panels) and DOPEG release (lower panels). Left panels (A): Rat hearts perfused with calcium free Krebs-Henseleit buffer were exposed to 1) the inhibitor of vesicular H^+-ATPase trimethyltin (30 μM; n=7), 2) 30 μM trimethyltin and 1 mM ouabain (n=7), and 3) 30 μM trimethyltin, 1 mM ouabain, and 0.1 μM desipramine (n=7). Right panels (B): Rat hearts were perfused with calcium free Krebs-Henseleit buffer containing either 4 mM potassium (n=7) or no potassium (n=7). In both series hearts were exposed to 30 μM trimethyltin. The time course of electrolyte variation and drug exposure are indicated in the figure. Mean±SEM.

aline level was increased by Ro 4-1284, indicated by a high DOPEG overflow, potassium induced a concentration-dependent release of noradrenaline. In comparison with the noradrenaline release obtained by Na^+,K^+-ATPase inhibition or sodium-channel opening, the release caused by high extracellular potassium was small. Blockade of sodium channels by 1 μM tetrodotoxin further reduced significantly the potassium-induced noradrenaline release without interfering with the concomitant DOPEG overflow (Figure 4). Comparable effects were observed when the potassium concentration was increased from 4 to 40 mM in one step (without figure).

Effect of Cyanide Intoxication on Noradrenaline and DOPEG Overflow

The time course of noradrenaline and DOPEG overflow induced by cyanide intoxication and glucose deprivation is shown in Figures 5-7. Starting 10 minutes after addition of cyanide and withdrawal of glucose, noradrenaline was progressively released. Peak values were reached after 30 minutes. With progressing noradrenaline depletion of the hearts the overflow continuously decreased (Figure 5A, upper panel). Addition of calcium to the perfusate attenuated and retarded the noradrenaline overflow (Figure 5B, upper panel). Irrespective of calcium content of the perfusion buffer, the release of DOPEG consistently preceded that of noradrenaline by about 5 minutes (Figures 5, 6, and 7A).

Blockade of sodium channels with tetrodotoxin resulted in a concentration-dependent inhibition of noradrenaline release that was more pronounced in the absence (Figure 5A, upper panel) than in the presence (Figure 5B, upper panel) of extracellular calcium. The overflow components insensitive to tetrodotoxin were not influenced by the presence or absence of calcium. Tetrodotoxin had no effect on the overflow of DOPEG irrespective of the presence of calcium in the perfusion buffer (Figure 5, lower panels).

Inhibition of transmembrane Na^+-H^+ exchange with 10 μM ethylisopropylamiloride^25 did not influ-
Calcium-independent noradrenaline release has been demonstrated to critically depend on two conditions: 1) increased cytoplasmic concentrations of noradrenaline within the sympathetic neuron and 2) enhanced intraneuronal concentrations of sodium ions (scheme in Figure 9).

Increased Cytoplasmic Noradrenaline Concentration in the Sympathetic Nerve Ending

By accumulating protons within the neuronal catecholamine storage vesicles the H⁺-ATPase located in the vesicular membrane generates a transmembrane H⁺ electrochemical potential of close to 200 mV that is composed of the vesicular membrane potential (inside positive) and a proton gradient (inside pH 5.5). The proton potential is the driving force of vesicular noradrenaline uptake, and amine inward transport is coupled with proton outward transport by a specific carrier located within the vesicular membrane.

In this study, the capability of neuronal storage vesicles to retain catecholamines was experimentally disturbed by agents interfering either with the
overflow of noradrenaline

FIGURE 5. Role of sodium influx via tetrodotoxin (TTX) sensitive channels for noradrenaline release induced by energy deficiency. Blockade of energy metabolism was induced by glucose-free perfusion and the addition of 1 mM cyanide (10–50 minutes). Left panels (A): Overflow of endogenous noradrenaline (upper panel) and DOPEG (lower panel) from rat hearts perfused with calcium free Krebs-Henseleit buffer. The time course of overflow was studied in the absence of tetrodotoxin (TTX; n=14), in the presence of 10 nM TTX (n=7), and 1 μM TTX (n=7). Right panels (B): Overflow of endogenous noradrenaline (upper panel) and DOPEG (lower panel) from rat hearts perfused with buffer containing 1.8 mM calcium. The time course of overflow was studied in the absence of TTX (n=7) and in the presence of 1 μM TTX (n=7). Mean±SEM.

vesicular amine transport or with vesicular proton potential. Inhibition of the H⁺-ATPase by trimethyltin results in a progressive collapse of the H⁺ potential at first affecting the electrical component. Consequently, the capability of maintaining the high intravesicular amine concentrations is severely hampered, and catecholamines are lost from the storage vesicles into the axoplasm. The trimethyltin concentrations necessary to induce vesicular noradrenaline loss in the rat heart are without effect on Na⁺,K⁺-ATPase and are in the same range as those used to inhibit H⁺-ATPase in isolated vesicular membranes. A similar release could be achieved by the monovalent ionophore monensin, which causes a proton and sodium ion leak and thus directly interferes with vesicular H⁺ potential. At high concentrations, this agent induces swelling and damage of vesicles.

Inhibition of the vesicular amine carrier by reserpine or Ro 4-1284 likewise results in a net loss of noradrenaline from the vesicles and a less pronounced axoplasmic accumulation of the amine.

Axoplastic noradrenaline concentrations cannot be assessed directly. The release of DOPEG had to serve as an indirect measure of cytoplasmic noradrenaline. Outside the storage vesicles, neuronal catecholamines are substrates of monoamine oxidase located at the outer mitochondrial membrane. In the presence of oxygen, noradrenaline is deaminated and DOPEG is the predominant metabolite formed in the sympathetic neuron. In contrast to the hydrophilic amine, the more lipophilic DOPEG easily diffuses through the plasma membrane with an efflux/content ratio of 0.35/min. Hence, DOPEG release reflects axoplastic noradrenaline concentrations as long as deamination is not saturated or inhibited. The $K_m$ oxygen value of the oxidative deamination of noradrenaline is 50 mm Hg oxygen pressure, and therefore, oxygen limits the reaction only under hypoxic conditions such as ischemia.
Increased axoplasmic noradrenaline concentrations by themselves were not sufficient to cause a release from the nerve ending into the extracellular space where the transmitter can interact with its receptors. In contrast to its lipophilic metabolite DOPEG, \textsuperscript{32} unfacilitated transmembrane diffusion is negligible in the case of the hydrophilic amine, which is ionized at physiological pH. Thus, catecholamine transport across intact membranes is not conceivable without a specific carrier system.

**Carrier-Mediated Neuronal Noradrenaline Transport and Role of Intraneuronal Sodium**

The inward transport of noradrenaline from the extracellular space into the sympathetic neuron via uptake, has been extensively studied, and has been demonstrated to be a cotransport with sodium ions energetically depending on the physiological electrochemical sodium gradient. Furthermore, the transport depends on the extracellular presence of chloride, \textsuperscript{33,34} is independent from extracellular calcium, and is specifically inhibited by various antidepressants such as desipramine and cocaine.\textsuperscript{12,23}

A reversed transport direction of uptake, had first been proposed by Paton to potentially act as a release mechanism under conditions of extracellular sodium depletion or inhibition of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase in the rabbit atrium.\textsuperscript{35} These observations were confirmed by other investigators, who measured the release of tritium\textsuperscript{36} or \textsuperscript{3}H-noradrenaline\textsuperscript{13,37} from rat vas deferens labeled with \textsuperscript{3}H-noradrenaline. Either extracellular sodium depletion\textsuperscript{13,37} or veratridine\textsuperscript{38} induced a calcium-independent release, which was suppressed by inhibitors of uptake, such as cocaine or desipramine. In all of these studies, the tissues were pretreated with reserpine to inhibit vesicular function and pargyline to block monoamine oxidase activity. In cultured sympathetic nerve cells\textsuperscript{39} and PC-12 cells\textsuperscript{40} prelabeled with \textsuperscript{3}H-noradrenaline, a comparable efflux of tritium was observed when the transmembrane sodium gradient was altered. Using rat vas deferens with active monoamine oxidase, Stute and Trendelenburg measured the release of \textsuperscript{3}H-noradrenaline and \textsuperscript{3}H-DOPEG.\textsuperscript{16} In the presence of reserpine, inhibition of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase or stimulation of sodium influx led to release of \textsuperscript{3}H-noradrenaline at the expense of \textsuperscript{3}H-DOPEG. From these results it was concluded that a reduced transmembrane sodium gradient is a precondition for reversed amine transport by uptake.

In the present study, the release of endogenous noradrenaline and endogenous DOPEG, its deaminated metabolite, were measured. Thus, we were able to avoid problems potentially associated with the use of radiolabeled catecholamines, such as isotope effects and inhomogenous distribution of the labeled amine and its metabolites in various compartments. In studies using labeled amines, therefore, the quantification of the liberated amine is hampered because of undefined and variable ratios between the labeled and the endogenous compound during release.

Three interventions have been utilized to increase cytoplasmic sodium concentrations: stimulation of sodium influx by veratridine and inhibition of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase by ouabain or potassium-free perfusion. With methods currently available, sodium concentrations within the sympathetic neuron cannot be quantified. Our considerations, therefore, have to rely on microelectrode studies of papillary muscle\textsuperscript{40} and Purkinje fibers\textsuperscript{40,41} and on nuclear magnetic resonance studies of perfused rat hearts.\textsuperscript{42} In Purkinje fibers, inhibition of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase using high concentrations of digitalis glycosides or potassium-free perfusion resulted in a twofold to threefold rise in intracellular sodium activity from basal levels in the range of 5 to 8 mM.\textsuperscript{40,41} Similar effects were achieved using micromolar concentrations of veratridine.\textsuperscript{42}
In our experiments, neither ouabain nor potassium depletion caused a significant noradrenaline release when the axoplasmic concentrations of the amine were not increased. Veratridine, however, induced a measurable noradrenaline overflow sensitive to tetrodotoxin. The mechanism of this sodium-mediated release remains unclear, and since intracellular liberation of calcium cannot be excluded, residual exocytosis may be the cause.

When the rise in neuronal sodium concentration was combined with high axoplasmic noradrenaline levels, a brisk noradrenaline overflow was observed, whereas DOPEG release was reduced. Two processes are thought to compete for noradrenaline in the axoplasmic pool: deamination of noradrenaline by monoamine oxidase resulting in DOPEG formation and DOPEG release on the one side, and the outward transport of unchanged noradrenaline on the other side. The ratio NA/DOPEG overflow reflects the ratio between the rate constants of both processes. Assuming that the rate constants for DOPEG formation and diffusion remain unaltered, the relative increase of the rate constant of noradrenaline outward transport is quantified by

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\frac{\text{NA/DOPEG}_{\text{high sodium}}}{\text{NA/DOPEG}_{\text{low sodium}}}
\]

where NA is noradrenaline. The rate constants of noradrenaline efflux were increased by 30 \( \mu \)M veratridine by a factor of 72, by 1 mM ouabain by a factor of 80, and by extracellular potassium deficiency by a factor of 26. Thus, a presumably threefold increase of intracellular sodium activity gives rise to a drastic efflux of noradrenaline from sympathetic nerve cells mediated by the uptake, carrier.

In comparison with the transmembrane sodium gradient, the membrane potential has been demonstrated to be of minor significance for noradrenaline efflux. High potassium concentrations resulted only in a moderate noradrenaline release and tetrodotoxin blockade of the sodium channel further reduced this release.

The presented data are compatible with the uptake model by Graefe who proposed a complex interaction of sodium with the amine transport. In agreement
with this hypothesis, sodium ions not only increase the affinity of noradrenaline to the carrier but also enhance the availability of the carrier at that side of the plasma membrane where the sodium concentration is high by forming an immobile sodium-carrier complex (transinhibition). In the presence of a physiological sodium gradient, number and affinity of transport sites are low at the cytoplasmic side and high at the outside of the plasma membrane, thus facilitating inward and preventing outward amine transport. With increasing axoplasmic sodium concentrations, the barrier of transinhibition is lost, and driven by high axoplasmic noradrenaline concentrations, an outward amine flux is found, even against the residual inward-directed electrochemical sodium gradient.

**Neuronal Sodium Balance and Nonexocytotic Noradrenaline Release Induced by Energy Deficiency**

Inhibition of two ion pumps in the sympathetic nerve cell has been shown for the first time to be a sufficient cause for nonexocytotic noradrenaline release. Blockade of H⁺-ATPase led to noradrenaline loss from storage vesicles and cytoplasmic amine accumulation. Inhibition of Na⁺,K⁺-ATPase, by increasing neuronal sodium levels, induced carrier-mediated efflux of the amine. It is not surprising, therefore, that energy deficiency, which ultimately reduces ATPase activities, caused amine release from sympathetic neurons with the same characteristics as those found after pharmacological inhibition of both ATPases: independence from extracellular calcium and inhibition by uptake, blockade, both being incompatible with the assumption of exocytosis.

The rate-limiting step of metabolically induced noradrenaline release is the carrier-mediated amine transport across the plasma membrane. Following cyanide intoxication and glucose depletion, the rise in axoplasmic noradrenaline, reflected by DOPEG overflow, preceded the noradrenaline release from the nerve ending by 5 minutes. This delay of noradrenaline release disappeared when the Na⁺-K⁺ pump had been inhibited prior to cyanide intoxication. Similarly, blockade of Na⁺,K⁺-ATPase prior to ischemia resulted in a marked acceleration of noradrenaline release. These data demonstrate a high sensitivity of storage vesicles to energy deficiency that resulted in fast axoplasmic noradrenaline accumulation. However, ongoing activity of the Na⁺-K⁺ pump prevented a major rise of intraneuronal sodium concentration and thereby prevented noradrenaline release for the first 10 minutes of energy deficiency.

Virtually no direct information is available concerning intraneuronal sodium accumulation during myocardial ischemia, anoxia, or cyanide intoxication, and we are aware of the reluctance to apply knowledge about ischemic processes in the myocyte to the sympathetic nerve cell. In principle, however, the alterations of transmembrane ion gradients that are caused by energy deficiency are likely to be comparable in both cell types. In rat myocardium, intracellular sodium has been shown to increase fivefold within 10 to 15 minutes of anoxia and more than threefold within 12 minutes of total ischemia. Intracellular sodium accumulation is determined not only by decreasing activity of the Na⁺,K⁺-ATPase but also by changes of passive sodium influx, which may involve various pathways of entry.

The pathways of neuronal sodium entry have been demonstrated to differ in ischemia and cyanide intoxication. Blockade of sodium channels by tetrodotoxin effectively reduced noradrenaline overflow during cyanide intoxication in the absence of calcium. When calcium was present in the perfusion buffer, noradrenaline release was reduced in comparison with calcium-free perfusion, and its further inhibition by tetrodotoxin was less pronounced. In ischemia, blockade of sodium channels did not reduce noradrenaline release at all. These observations may

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**FIGURE 8.** Cumulative overflow of endogenous noradrenaline from perfused rat hearts during 2-minute reperfusion following 5-, 10-, and 20-minute periods of total ischemia in the absence of calcium in the perfusion buffer. Left panel: Noradrenaline overflow induced by 5 and 10 minute ischemia in the absence and presence of 1 mM ouabain 30 minutes prior to ischemia. Right panel: Noradrenaline overflow induced by 20 minutes of ischemia in the absence of drugs and in the presence of 0.1 μM desipramine, 1 μM tetrodotoxin, 10 μM ethylisopropylamiloride (EIPA), and 1 mM amiloride. Addition of the agents began 10 minutes prior to ischemia and lasted to the end of the experiments. Each column n=7; mean±SEM.
Hypothetical scheme of nonexocytotic, calcium-independent noradrenaline (NA) release (left part) and its relation to neuronal sodium homeostasis (right part). Various interventions used in the experiments and their specific sites of action are indicated. Four different agents have been used to interfere with vesicular catecholamine storage. The inhibitor of vesicular H\(^+\)-ATPase trimethyltin (1) and the ionophore monensin (2) disturb intravesicular pH and transmembrane potential, which are essential for vesicular catecholamine transport and storage. Reserpine (3) and Ro 4-1284 (4) have been used to directly block vesicular noradrenaline transport. Application of any of the four agents resulted in a loss of noradrenaline from storage vesicles that could be detected by markedly increased DOPEG release. Increased axoplasmic noradrenaline concentrations did not cause relevant noradrenaline release from the nerve ending as long as neuronal sodium homeostasis was undisturbed. Only the combination of increased noradrenaline and sodium within the axoplasm resulted in a major noradrenaline release via uptake, which reversed its normal transport direction under those conditions. Blockade of uptake, by desipramine (5) inhibited nonexocytotic noradrenaline release, but not DOPEG overflow. Several interventions have been used to interfere with neuronal sodium homeostasis (right part) and to modify nonexocytotic noradrenaline release thereby. Inhibition of Na\(^+\),K\(^+\)-ATPase by ouabain (6) or potassium-free perfusion (7) on the one hand and increased sodium influx induced by veratridine (8) on the other hand caused noradrenaline release if high cytoplasmic noradrenaline concentrations were present. A disturbed energy status, induced by ischemia or cyanide intoxication, of the sympathetic nerve ending interfered with both vesicular storage function and sodium homeostasis and therefore resulted in nonexocytotic noradrenaline release. Blockade of sodium channels by tetrodotoxin (9) and inhibition of Na\(^+\)-H\(^+\) exchange by amiloride (10) and ethylisopropylamiloride (11) have been used to identify Na\(^+\)-H\(^+\) exchange to be the major route of neuronal sodium influx in early ischemia.

Acidosis activates another pathway for sodium entry into the nerve ending: the Na\(^+\)-H\(^+\) exchange. This carrier-mediated transport system has been identified in plasma membranes of various cell types, including nerve cells. The exchange plays a critical role in the regulation of intracellular pH and is maximally activated by intracellular acidosis. The extrusion of protons is coupled with a sodium entry, which leads to intracellular sodium accumulation, especially when Na\(^+\),K\(^+\)-ATPase activity is suppressed. The Na\(^+\)-H\(^+\) exchange is inhibited by amiloride and analogues. One of the most potent derivatives is ethylisopropylamiloride, which is selective for Na\(^+\)-H\(^+\) exchange in comparison with other sodium coupled transport systems such as Na\(^+\)-Ca\(^{2+}\) exchange. Furthermore, it has been demonstrated not to interfere with tetrodotoxin-sensitive sodium channels or with uptake, (Figure 2) in concentrations that were applied in this study.

In ischemia, but not during cyanide intoxication, noradrenaline release was markedly inhibited by amiloride and ethylisopropylamiloride, indicating that in ischemia Na\(^+\)-H\(^+\) exchange is the predomi-
nant pathway for sodium entry into the sympathetic nerve ending. This activated, neuronal sodium influx (in combination with reduced activity of plasmalemmal Na\(^+\)-K\(^+\) pump) and disturbed vesicular storage function are necessary and sufficient conditions to cause calcium-independent noradrenaline release in myocardial ischemia.

Acknowledgment

We would like to thank Anette Brückner, Michaela Oestriinger, and Peter Stefan for excellent technical assistance.

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**Key Words**: calcium-independent noradrenaline release • uptake • vesicular H+-ATPase • Na⁺,K⁺-ATPase • Na⁺-H⁺ exchange • amiloride • ischemia
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doi: 10.1161/01.RES.63.1.214

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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World Wide Web at:
http://circres.ahajournals.org/content/63/1/214

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