Failure of the Cholinergic Modulation of Norepinephrine Release During Acute Myocardial Ischemia in the Rat

Xiao-Jun Du, Anthony M. Dart, Rudolph A. Riemersma, and Michael F. Oliver

The effect of ischemia on cholinergic presynaptic inhibition of exocytotic norepinephrine release was studied in the innervated perfused rat heart. In normoxic hearts, vagal nerve stimulation significantly reduced exocytotic norepinephrine overflow to 75% of control values. This inhibitory effect was not affected by 3 minutes of low-flow ischemia (67% of control overlap values), but was attenuated or abolished by 10-minute low-flow ischemia or by 1-, 3-, and 5-minute stop-flow ischemia (107%, 85%, 101%, and 120% of control overlap values, respectively). The α-adrenergic antagonist phentolamine could completely or partly restore the failure of vagally induced inhibition of norepinephrine overflow in hearts with 1-, 3-, and 5-minute stop-flow ischemia (72%, 73%, and 85% of control overlap values, respectively). The muscarinic agonist methacholine substantially inhibited norepinephrine overflow to 18% of control overlap values in normoxic hearts. This effect was also significantly attenuated by 10-minute low-flow ischemia or by 1-, 3-, and 5-minute stop-flow ischemia (46%, 38%, 53%, and 55% of control overlap values, respectively). The cholinesterase inhibitor physostigmine did not restore the methacholine-induced inhibition of norepinephrine overflow after 3-minute stop-flow ischemia to normoxic level (55% vs. 17%). These results indicate that myocardial ischemia interferes with endogenous and exogenous cholinergic presynaptic inhibition of norepinephrine overflow in the rat heart. The extent of this attenuation depends on the severity and duration of ischemia. Reduced exocytotic acetylcholine release, which is at least in part due to an enhanced adrenergic presynaptic modulation, and dysfunction of presynaptic muscarinic receptors are suggested as two possible mechanisms. (Circulation Research 1990;66:950–956)

The autonomic nervous system plays an important role in the pathogenesis of malignant arrhythmias during acute myocardial ischemia.1–6 The arrhythmogenic effects of enhanced sympathetic stimulation, whether produced by electrical, pharmacological, or psychological means, are well known,1–5 and surgical or chemical sympathetic denervation with depletion of cardiac norepinephrine content is protective against subsequent ischemic arrhythmias.4,5 The influence of parasympathetic activation is less clearly defined, and some studies have shown an inhibitory effect on early ischemic arrhythmias,4–6 which is believed to be due largely to antiadrenergic mechanisms, including the presynaptic inhibition of exocytotic norepinephrine release.4–8 However, opposite results have also been reported.5,9,10 Recent clinical and experimental studies have demonstrated that an autonomic imbalance may be critical for the occurrence of lethal ischemic arrhythmias.4,11–13

Exocytotic norepinephrine release within the heart is greatly affected by myocardial ischemia.14–17 Although presynaptic sympathetic-parasympathetic interactions, mediated by α-adrenergic and muscarinic cholinergic receptors, have been well recognized in normoxic preparations,7,8,18 little is known about how this aspect of sympathetic nerve function may be affected by ischemia. As coexistence of efferent sympathetic and parasympathetic overactivity may be a frequent pathophysiological event during early myocardial ischemia,3–5,19,20 we have extended our studies on the effects of myocardial ischemia on sympathetic nerve function to examination of the influence of ischemia on the cholinergic presynaptic modulation of exocytotic norepinephrine release in the rat heart.

From the Cardiovascular Research Unit, Department of Medicine, University of Edinburgh, Edinburgh, Scotland, UK.

Supported by a grant from the British Heart Foundation. X.J.D. is a visiting Research Fellow sponsored by the State Education Commission of the People's Republic of China.

Address for correspondence: Dr. X.-J. Du, Cardiovascular Research Unit, Hugh Robson Building, George Square, University of Edinburgh, Edinburgh EH8 9XF, Scotland, UK.

Received May 25, 1989; accepted October 25, 1989.
Materials and Methods

Preparation

Male Wistar rats (200–250 g) were anesthetized with pentobarbitone (60 mg/kg i.p.). The thorax was opened and a cannula inserted and tied into the ascending aorta for retrograde coronary perfusion by use of a multichannel peristaltic pump.14 Each heart (mean weight 0.82±0.01 g) was perfused at a constant flow rate of 4.5 ml/min (5.1±0.1 ml/min/g) with a modified Krebs-Henseleit solution of the following composition (mM): Na+ 148, K+ 3.98, Ca2+ 1.84, Mg2+ 1.05, HCO3– 25, PO43– 0.50, glucose 11, pyruvate 1.8, and EDTA 0.027. The perfusate was gassed continuously with 95% O2–5% CO2, giving a pH of 7.42±0.01 and a Po2 of 580±6 mm Hg. The temperature of the perfusate at the point of entry into the aorta was 37º C. All hearts underwent an initial 30-minute stabilization period before the experiments. Hearts were made globally ischemic by either reduction of perfusion flow rate (0.26±0.03 ml/min/g, low-flow ischemia) or cessation of the pump (0 ml/min/g, stop-flow ischemia). During the ischemic period, hearts were covered with a thermostatic chamber and myocardial temperature was kept between 35.3º and 36.5º C.

A cannula was inserted into the right atrium for collection of samples of coronary venous effluent. The timing of collection of coronary effluent samples for norepinephrine assay varied depending on the protocol used. In normoxic heart (5 ml/min/g), effluent was collected for 1 minute after the start of the nerve stimulation. In ischemic (0 or 0.26 ml/min/g) experiments, coronary effluent was collected during the first 1.5 minutes of reperfusion, as there was little effluent overflow in the first 30 seconds. All samples were cooled on ice, stabilized by the addition of perchloric acid (0.3 N), and stored at -40º C until radioenzymatic assay in duplicate or triplicate.21 The coefficient of variation of samples with a norepinephrine concentration of 2 pmol/ml is 7%. A polyethylene cannula (1 mm o.d.) connected to a pressure transducer (model EM 751, Elecomatic, Glasgow, UK) was introduced into the left ventricular cavity through the apex. Left ventricular pressure and the first derivative of ventricular pressure (dP/dt) were measured with a polygraph. Heart rate was counted with a chronometer triggered by electrocardiographic signals and continuously displayed on the polygraph. All signals were recorded with an ultraviolet recorder (model 2106, Visicorder, Honeywell, Hemel-Hempstead, UK).

The left cervicothoracic ganglion (with intact cardiac nerves attached)44 and both vagal nerves were dissected free and mounted on two pairs of bipolar platinum electrodes by use of micromanipulators. The nerves were constantly superfused with warmed and oxygenated buffer except when stimulated. Nerve stimulation was performed by means of a Model S88 Stimulator (Grass Instruments, Quincy, Massachusetts) with stimulus isolation units (SIU 7).

Electrical stimuli had a pulse width of 2 msec, a current of 0.8 mA, and a frequency of 5 pulses/sec for sympathetic ganglion stimulation and 15 pulses/sec for vagal nerve stimulation.

Drugs and Model Characteristics

All experiments were performed in the presence of 0.1 μM desipramine HCl (Sigma Chemical, St. Louis) for inhibition of neuronal reuptake of norepinephrine.14 Choline chloride (Sigma Chemical), physostigmine (Sigma Chemical), phentolamine mesylate (CIBA Laboratories, Horsham, UK), and methacholine chloride (Sigma Chemical) were also used as specified. Desipramine, physostigmine, and phentolamine were initially dissolved in ethanol; the final concentration of ethanol was <0.05%. All additions of drugs to the perfusate started at least 12 minutes before nerve stimulation.

To define the concentration of methacholine to be used, we examined the relation between methacholine concentration and exocytotic norepinephrine overflow in seven normoxic hearts. After an initial sympathetic nerve stimulation (S1, 30-second duration), methacholine was infused into the hearts to give final concentrations of 0.1, 1, 5, and 10 μM, respectively. Four more consecutive sympathetic nerve stimulations (S2–S5, 30-second duration with 15-minute intervals) were then performed at each concentration of methacholine.15,16 Norepinephrine overflow by S1 was 16.5±1.3 pmol/g/min, and S2–S5–evoked norepinephrine overflow was inhibited by methacholine, in a dose-dependent manner, to 79±7% (p<0.05), 39±6% (p<0.001), 21±6% (p<0.001), and 16±4% (p<0.001), respectively. On the basis of these results, methacholine was used for all studies at a dose of 10 μM.

In 35 normoxic hearts, sympathetic stimulation resulted in a significant increase in heart rate (209±7 to 257±8 beats/min), peak +dP/dt (1,587±28 to 2,196±35 mm Hg/sec), and peak -dP/dt (769±22 to 1,175±27 mm Hg/sec). A profound reduction in heart rate and peak dP/dt was recorded during vagal nerve stimulation (heart rate, 235±5 to 90±7 beats/min; +dP/dt, 1,640±35 to 857±32 mm Hg/sec; -dP/dt, 863±39 to 456±18 mm Hg/sec). When combined sympathetic and vagal nerve stimulation was performed, all the functional parameters showed marked reductions from prestimulation levels (heart rate, 229±16 to 77±14 beats/min; +dP/dt, 1,589±28 to 904±42 mm Hg/sec; -dP/dt, 812±26 to 437±15 mm Hg/sec). Of 185 preparations, 33 were rejected because of either a weak cardiac response to nerve stimulation (<25% increase in peak +dP/dt for sympathetic stimulation and <30% decrease in heart rate for vagal stimulation) or a low effluent recovery (<85% of perfusion flow).

In eight normoxic hearts, it was found that four consecutive sympathetic nerve stimulations (S1–S4, 1-minute duration separated by 15-minute recovery periods) produced a reproducible amount of norepinephrine overflow (32.6±6.4, 34.3±5.5, 32.1±3.7,
and 33.4±7.0 pmol/g/min, respectively, p=NS). The norepinephrine overflow percentage of S2–S4 over S1 were 115±13%, 110±11%, and 103±7%, respectively. Therefore, a recovery period of 15 minutes was always allowed between nerve stimulations.

Spontaneous norepinephrine overflow (i.e., absence of nerve stimulation) was always found to be very low (about 1 pmol/g/min) and was not affected by the drugs used.

To check if vagal nerve stimulation alone could contribute to norepinephrine overflow, we collected coronary effluent in six normoxic hearts immediately before and during vagal nerve stimulation (15 pulses/sec for 1 minute). Spontaneous norepinephrine overflow was 1.41±0.13 pmol/g/min, and vagal nerve stimulation did not affect this value (1.43±0.19 pmol/g/min).

**Protocols**

**Vagal nerve stimulation and norepinephrine overflow.** The effect of vagal nerve stimulation on stimulation-evoked norepinephrine overflow was examined during normoxia and during either low-flow or stop-flow ischemia of varying duration.

In 14 normoxic hearts, sympathetic and combined sympathetic and vagal nerve stimulation were each performed, in random order, for 1 minute. In ischemic experiments, each heart was subjected to two equal periods of low-flow or stop-flow ischemia separated by a 15-minute recovery period of normoxic perfusion. Sympathetic nerve stimulation or combined sympathetic and vagal stimulation was applied during the final 1 minute of each ischemic period. The order of stimulation was random. The duration of low-flow ischemia was 3 (n=8) or 10 minutes (n=8) and of stop-flow ischemia was 1 (n=12), 3 (n=8), or 5 minutes (n=9). In all experiments, physostigmine (1 μM) was added to the perfusate to minimize acetylcholine (ACh) hydrolysis by cholinesterase, and choline chloride (10 μM) was added as a substrate for ACh resynthesis. Physostigmine at this dose potentiated the heart rate–lowering effect of vagal nerve stimulation (15 Hz for 1 minute) from −96±7 (n=16) to −170±8 beats/min (n=13, p<0.01).

**Effect of α-adrenergic antagonist on vagally induced inhibition of norepinephrine overflow.** In eight normoxic hearts, the effect of the α-adrenoceptor antagonist phentolamine on vagally induced inhibition of norepinephrine release was assessed. After an initial 1-minute sympathetic nerve stimulation, phentolamine was continuously infused into the heart (final concentration 1 μM). Sympathetic and combined sympathetic and vagal nerve stimulation were then each performed, in random order, for 1 minute. The same protocol was also followed during stop-flow ischemia of 1 (n=8), 3 (n=8), or 5 minutes (n=9). Physostigmine (1 μM) and choline chloride (10 μM) were present throughout these experiments.

**Muscarinic agonist and norepinephrine overflow.** The effect of the exogenous muscarinic agonist methacholine (10 μM) was examined during normoxia (n=6), low-flow ischemia of 3 (n=6) and 10 minutes (n=5), and stop-flow ischemia of 1 (n=6), 3 (n=9), and 5 minutes (n=8). Sympathetic nerve was stimulated twice (1-minute duration) in the presence or absence of methacholine.

Methacholine can also be hydrolyzed by cholinesterase; therefore, to exclude this possibility, we subjected nine hearts to two periods of 3-minute stop-flow ischemia, and sympathetic nerve stimulation was performed in the presence, in random order, of physostigmine (1 μM) or physostigmine (1 μM) and methacholine (10 μM).

**Statistical Analysis**

Results are presented as mean±SEM. Analysis of variance and paired or unpaired Student’s t test were used for comparison of the data. Statistical significance was set at p<0.05.

**Results**

**Effects of Ischemia on Vagally Induced Inhibition of Norepinephrine Overflow**

Vagal nerve stimulation reduced exocytotic norepinephrine overflow to approximately 75% of control in normoxic hearts (p<0.01, Table 1 and Figure 1). Norepinephrine overflow was similarly reduced by vagal stimulation during 2–3 minutes of low-flow ischemia (p<0.01), but not if vagal nerves were stimulated during 9–10 minutes of low-flow ischemia even though sympathetic stimulation–induced increase in heart rate (12±12 to 74±8 beats/min, p<0.01) was prevented (11±11 to 7±7 beats/min, p=NS). During 1-minute stop-flow ischemia, vagal stimulation tended to reduce norepinephrine overflow, although this reduction was no longer statistically significant (p=0.06). If stop-flow ischemia was extended to 3 or 5 minutes, vagal stimulation had no effect on stimulation-evoked norepinephrine overflow (Table 1 and Figure 1), yet a suppression of the chronotropic effect of sympathetic stimulation was achieved in the 3-minute ischemic group (sympathetic stimulation, 73±18 to 99±8 beats/min, p<0.01; combined nerve stimulation, 65±9 to 4±4 beats/min, p<0.001).

| TABLE 1. Effects of Vagal Nerve Stimulation on Left Sympathetic Ganglion Stimulation-Evoked Norepinephrine Overflow During Normoxia, Low-Flow Ischemia, and Stop-Flow Ischemia |
| --- | --- | --- |
| n | GS | GS+VS |
| Normoxia | 14 | 30.9±4.1 | 22.3±3.0* |
| Low-flow ischemia | 3 minutes | 8 | 29.8±2.3 | 19.4±1.9* |
| | 10 minutes | 8 | 32.8±5.0 | 31.0±4.3 |
| Stop-flow ischemia | 1 minute | 12 | 36.6±7.2 | 27.8±6.0 |
| | 3 minutes | 8 | 31.3±5.1 | 29.2±5.1 |
| | 5 minutes | 9 | 24.7±2.5 | 27.9±4.0 |

Values are expressed in pmol/g/min. n, Number of rat hearts; GS, ganglion stimulation; VS, vagal nerve stimulation.

*p<0.01 vs. GS value in same group.
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Prevention of flow ischemia. Neuronal uptake of norepinephrine was reduced by 0.1 μM desipramine, and 1 μM physostigmine was used for prevention of hydrolysis of acetylcholine. Norepinephrine overflow produced by combined nerve stimulation (GS+VS) is expressed as percent of overflow produced by GS alone. Group numbers are shown in Table 1; all values are mean±SEM. **p<0.01 for GS+VS vs. GS. #p<0.05 for percent of norepinephrine overflow vs. 0-minute group. NA, norepinephrine.

**Effect of Phentolamine on Vagal-Sympathetic Interactions**

During normoxia, phentolamine resulted in a 136% increase in norepinephrine overflow (p<0.001, Table 2), corresponding to a higher level of peak +dP/dt during sympathetic stimulation (1,850±253 vs. 2,145±354 mm Hg/sec, p<0.05). Vagal stimulation in the presence of phentolamine reduced norepinephrine overflow to 74% of control (p<0.02), a value similar to that observed in the absence of phentolamine (75%). In 1-, 3-, and 5-minute stop-flow ischemic groups, phentolamine resulted in a 139% (p<0.001), 55% (p<0.01), and 45% (p=0.07) increase, respectively, in norepinephrine overflow. The values were reduced by vagal stimulation to 71% (1-minute ischemia, p<0.02), 72% (3-minute ischemia, p<0.01), and 88% (5-minute ischemia, p=0.16), respectively, of control overflow values (Table 2). In the presence of phentolamine, sympathetic nerve stimulation increased heart rate from 58±18 to 111±13 beats/min in the 3-minute ischemic group (p<0.001) and from 12±9 to 71±12 beats/min in the 5-minute ischemic group (p<0.001); this chronotropic effect was prevented by concomitant vagal stimulation (3-minute ischemia, 96±12 to 5±0 beats/min, p<0.001; 5-minute ischemia, 30±14 to 9±8 beats/min, p=NS).

**Effect of Ischemia on Methacholine-Induced Inhibition of Norepinephrine Overflow**

In normoxic hearts, over 80% of norepinephrine overflow was inhibited by 10 μM methacholine (p<0.001) (Table 3 and Figure 2). In all the ischemic experiments, norepinephrine overflow was also significantly reduced by 10 μM methacholine but with less effectiveness (p<0.05), depending on the degree of flow reduction and duration of ischemia (Table 3 and Figure 2). Addition of the cholinesterase inhibitor

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<th>Table 3. Effects of Muscarinic Agonist Methacholine (10 μM) on Sympathetic Ganglion Stimulation–Evoked Norepinephrine Overflow During Normoxia, Low-Flow Ischemia, and Stop-Flow Ischemia</th>
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Values are expressed in pmol/g/min. n, Number of rat hearts; GS, ganglion stimulation; MCh, methacholine. *p<0.01, †p<0.05 vs. GS in same group. ‡With physostigmine (1 μM).
itor physostigmine to the perfusate did not restore the methacholine-induced inhibition of norepinephrine overflow after 3-minute stop-flow ischemia to normoxic value (55% vs. 17%, p<0.01, Figure 2). The net increases in the heart rate-to-nerve stimulation were similar between the two groups of 3-minute stop-flow ischemia without and with physostigmine (77±14 vs. 66±10 beats/min, p=NS), although the heart rate immediately before nerve stimulation was significantly lower in the group with physostigmine (51±12 vs. 10±10 beats/min, p<0.02).

**Discussion**

These experiments document, for the first time, that short periods of ischemia attenuate vagally induced inhibition of exocytotic norepinephrine overflow in the rat heart. The results show 1) that the longer the period of ischemia, the less effective is vagal stimulation in inhibition of norepinephrine overflow, and 2) that vagally induced inhibition of norepinephrine overflow, though still detectable after 3-minute low-flow ischemia, is abolished by 3-minute stop-flow ischemia. Thus, an essential feature of this ischemia-induced attenuation is a dependency on the duration and severity of ischemia. Two important mechanisms for the clearance of released ACh, namely washout from synaptic clefts and hydrolysis by cholinesterase,7,22 are no longer effective in our preparation due to a very low or zero coronary flow rate and to the administration of cholinesterase inhibitor. Therefore, a pronounced presynaptic inhibition of exocytotic norepinephrine release by vagal nerve stimulation might have been expected; however, the reverse was observed.

Several mechanisms could account for this ischemia-induced failure of vagally mediated presynaptic inhibition. Hydrolysis of ACh in the ischemic heart appears not to be responsible because enzyme inhibitor (physostigmine) was present throughout vagal nerve stimulation experiments. In addition, physostigmine did not reverse the diminished inhibitory effect of methacholine on norepinephrine overflow observed during 2–3 minutes of stop-flow ischemia. It has been found that cholinesterase activity in normoxic mammalian hearts is low,7 but nothing is known of ischemic effects on this hydrolyzing enzyme.

A reduction in ACh release from vagal nerve terminals during ischemia could be a possible mechanism. In principle, at least three factors may cause reduced ACh release in the present model: 1) Ischemia may directly interfere with exocytotic release of ACh according to studies that used rat brain slices or synaptosomes,23,24 in which it was found that 10-minute hypoxia or anoxia reversibly inhibited K+-evoked ACh release and that with a concomitant acidosis (pH=6.2), this impairment became irreversible.24 Lack of ATP may be particularly important in the heart because efferent vagal neurotransmission is dependent on two synapses (i.e., between preganglionic and postganglionic neurones and between postganglionic neurones and effectors), and the main biochemical events in the vagal nerve terminals, including choline uptake, ACh synthesis, transport, and exocytotic release, depend on energy supply and ionic homeostasis.7 Indeed, reduced ATP supply has been suggested as a major cause for the failure of sympathetic neurotransmission during ischemia,14,15 although a concomitant acidosis seems not to contribute to this failure.16 2) Electrically evoked ACh release from brain tissue or peripheral vagal terminals can be partly inhibited by micromolar levels of adenosine or the adenosine analogue phenylisopropyladenosine.25,26 Adenosine may well play a similar role in the present heart model since Schöning and coworkers17 have recently demonstrated micromolar adenosine levels in the coronary venous effluent drained from rat heart within a few minutes of stop-flow ischemia. 3) ACh release from cholinergic nerve terminals in the heart can be reduced by exogenous norepinephrine via activation of the presynaptic a-adrenoceptors.7,18 However, there has been no study to show that exocytotic norepinephrine release inhibits exocytotic ACh release. During early ischemia, a high concentration of norepinephrine may accumulate in the synaptic clefts due to the combined effects of sympathetic nerve stimulation, inhibition of neuronal reuptake of norepinephrine, and ineffectiveness of washout from the synaptic clefts; therefore, this inhibitory modulation on ACh release is expected to be more pronounced than during normoxia. This possibility is supported by the present results that during 1- and 3-minute stop-flow ischemia, phentolamine restored the inhibitory efficacy of vagal stimulation on norepinephrine overflow to the control level, probably by removal of this enhanced transaxonal inhibitory modulation. However, this effect of phentolamine was no longer significant after 5-minute stop-flow ischemia.

Another mechanism contributing to the reduced vagal effect may be ischemia-induced dysfunction of the presynaptic muscarinic receptors, as suggested by the fact that the modulatory effect of methacholine is similarly attenuated during ischemia. In various cardiac preparations, both the number and affinity state of postsynaptic muscarinic receptors can be modulated by multiple factors, including muscarinic agonists or antagonists, ionic activity, guanine nucleotides, and binding state of adjacent nonmuscarinic receptors.7,27–30 We are not aware of any study on the modulation of presynaptic muscarinic receptors in either physiological or pathological conditions. The present study suggests an ischemia-induced interference with the function of presynaptic muscarinic receptors. No effort was made in the present study to examine the mechanisms, but an increase in extracellular potassium concentration may be involved as it has been found that potassium is capable of reducing the affinity state of muscarinic receptors to agonist,7,27–30 and that the extracellular K+ concentration will double or even triple within the first few minutes of myocardial ischemia.31,32
The results from vagal stimulation and methacholine, although qualitatively similar, were not identical. This could be due to a lower prevailing concentration of ACh than of methacholine in the synaptic clefts. However, until ACh levels are measured, the mechanisms whereby ischemia interferes with vagal inhibition of norepinephrine overflow remain speculative. An interesting finding in the current studies is that when vagal stimulation no longer reduces norepinephrine overflow, its negative chronotropic effect is still maintained. This may be explained by a denser vagal innervation in atria and conducting system than in ventricles, whereas the latter are probably the source of most norepinephrine overflow. In addition, it is not known if there is any difference between presynaptic and postsynaptic muscarinic receptors in their agonist affinity or their sensitivity to ischemia.

Clinical and experimental observations suggest a simultaneous sympathetic and vagal activation during the very early phase of myocardial ischemia.3–5, 19, 20 If our findings from the perfused rat heart can be extrapolated to the in vivo situation, then the presynaptic inhibition of vagal activity on norepinephrine release will vary within the ischemic heart, depending on the severity and duration of ischemia. This condition may lead to a local imbalance of autonomic activity with an increased exocytotic norepinephrine release evoked by efferent sympathetic impulse within the ischemic region. Thus, electrical instability and arrhythmias may occur as a result of heterogeneous adrenergic stimulation. These events may happen within the first few minutes of myocardial ischemia, a time when sudden cardiac death frequently occurs. Indeed, combined stimulation of cardiac sympathetic and vagal nerves during ischemia produced a higher incidence of serious arrhythmias than either alone in an in vivo study.10 Clinical and experimental studies have also suggested that withdrawal of vagal activity could predispose to the onset of ischemia-induced ventricular fibrillation and sudden cardiac death.4,11–13

In conclusion, these results demonstrate that cholinergic presynaptic inhibition of exocytotic norepinephrine release in the heart is impaired during acute ischemia, and suggest that reduced ACh release and/or dysfunction of the presynaptic muscarinic cholinoreceptors are two of the responsible mechanisms.

Acknowledgment

The authors thank Miss M. Millar for excellent technical assistance.

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**KEY WORDS** • acute myocardial ischemia • sympathetic and parasympathetic interactions • norepinephrine release • presynaptic inhibition • nerve stimulation • rat
Failure of the cholinergic modulation of norepinephrine release during acute myocardial ischemia in the rat.

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Circ Res. 1990;66:950-956
doi: 10.1161/01.RES.66.4.950

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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