N-Type Calcium Channels Control Sympathetic Neurotransmission in Human Heart Atrium

G.J. Molderings, MD; J. Likungu, MD; M. Göthert, MD

Background—Because knowledge about the type of calcium channels involved in action potential–induced norepinephrine release from the human peripheral sympathetic nervous system is sparse, we investigated which types of calcium channels are functionally important in the sympathetic nerves of human cardiac tissue.

Methods and Results—In superfused segments of human right atrial appendages, the type of calcium channels that control 

\[ ^{3} \text{H} \] norepinephrine release evoked by transmural electrical stimulation was determined. \[ ^{3} \text{H} \] norepinephrine release was almost abolished by 0.2 \( \mu \text{mol/L} \) \( \omega \)-conotoxin GVIA (a selective blocker of N-type channels) but was not modified by 0.1 \( \mu \text{mol/L} \) \( \omega \)-agatoxin IVA (a selective blocker of P- and Q-type channels). Mibebradil (a T-type and N-type calcium channel blocker) at concentrations of 0.3 to 3 \( \mu \text{mol/L} \) reduced the evoked tritium overflow in a frequency- and calcium-dependent manner, whereas 0.1 to 10 \( \mu \text{mol/L} \) amlodipine, diltiazem, and verapamil (selective blockers of L-type channels) were ineffective.

Conclusions—Norepinephrine release from cardiac sympathetic nerves is triggered by \( \text{Ca}^{2+} \) influx via N-type but not L- and P/Q-type calcium channels. The inhibitory effect of mibebradil on norepinephrine release at clinically relevant concentrations is probably due to its blocking action on N-type \( \text{Ca}^{2+} \) channels. This property of mibebradil is unique among the calcium channel blockers that have been or still are therapeutically applied and may considerably contribute to its slight negative chronotropic effect in vivo. (Circulation. 2000;101:403-407.)

Key Words: atrium ■ calcium channels ■ norepinephrine ■ mibebradil

The depolarization-induced norepinephrine release from central and peripheral norepinephrine neurons occurs by exocytosis triggered by an influx of extracellular calcium via voltage-dependent calcium channels. The latter are activated when the surface membrane is depolarized by an action potential. At present, 6 classes of voltage-dependent transmembrane calcium channels have been identified on the basis of functional properties such as voltage- and time-dependent kinetics, single-channel conductance, pharmacology, and cellular distribution (for review, see Reference 1): long-lasting conductance or L-type channels (inhibited by verapamil, diltiazem, and dihydropyridines); neural or N-type channels (blocked by \( \omega \)-conotoxin GVIA); transient or T-type channels (blocked by mibebradil with moderate selectivity over N-type channels); P-type or Q-type channels (blocked by \( \omega \)-agatoxin IVA); and R-type channels (blocked by Ni\(^{2+}\)). L-, N-, P-, Q-, and R-type calcium channels have been identified in central neurons. L- and T-type calcium channels are abundant in the periphery, particularly in the cardiovascular system.

In the peripheral sympathetic nervous system of humans, little is known about the type of calcium channels involved in depolarization-induced norepinephrine release. Therefore, the aim of the present study was to examine which types of calcium channels are functionally important in the sympathetic nerve terminals of human cardiac tissue. This question was also addressed in the context of the possibility that the favorable bradycardic influence of the novel calcium channel blocker mibebradil might be due to an inhibition of norepinephrine release by inhibiting calcium influx into the cardiac sympathetic nerve terminals. The purpose of the present experiments with mibebradil was to provide evidence for the suggestion that mibebradil inhibits norepinephrine release by blocking calcium channels in the postganglionic sympathetic axon terminals.

Preliminary accounts on the present data have been given at the 9th International Symposium on Vascular Neuroeffector Mechanisms.

Methods

Segments of macroscopically normal human right atrial appendages were obtained from normotensive 30- to 75-year-old male or female patients undergoing open-heart surgery. After the atrial appendages were removed (via a routine procedure for cannulation of the right atria), they were immediately transferred into fresh, cold physiological salt solution (for composition, see below) and stored at 4°C until the beginning of the experiment. The patients were not treated with adrenoreceptor agonists or antagonists or with drugs that influence the...
Storage or release of norepinephrine. After premedication with pethidine and promethazine, the patients were anesthetized (both induction and maintenance) with flunitrazepam and fentanyl. During maintenance of anesthesia, they were ventilated with mixtures of oxygen and air. Pancuronium was administered for neuromuscular blockade. The study was approved by the local ethics committee.

The experiments on strips (≈3 × 15 mm) of the atrial appendages began, as a rule, within 20 hours (or sooner, in a few cases) after removal; preliminary experiments, started 2 or 20 hours after removal, had revealed that norepinephrine release and its drug-induced modification did not differ. The strips were incubated for 60 minutes in 1.5 mL of physiological salt solution (37°C) containing

- 0.03 (aerated with 95% O2 and 5% CO2).
- Throughout superfusion, removal, had revealed that norepinephrine release and its drug-induced modification did not differ.


determined.

Drugs used were (-)-(ring-2,5,6-3H)-norepinephrine (specific activity 42 Ci/mmol; New England Nuclear); desipramine hydrochloride (Ciba-Geigy); corticosterone, ω-conotoxin GVIA, ω-agatoxin IVA, diltiazem, verapamil, and amiodarone (Sigma); and mibefradil dihydrochloride (a gift of Hoffmann-La Roche, Grenzach-Wyhlen, Germany). Drugs were dissolved in saline or water with 1 exception: corticosterone was dissolved in 1.2-propanol, and the stock solution was further diluted with saline. All experiments with verapamil and amiodarone were performed under exclusion of light.

<table>
<thead>
<tr>
<th>Control Values for Stimulation-Evoked Tritium Overflow</th>
<th>Experimental Condition</th>
<th>S1, nCi</th>
<th>S2, % of Tissue Tritium</th>
<th>S1/S2</th>
<th>S3/S2</th>
<th>S4/S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.66 Hz, 1.6 mmol/L Ca2+</td>
<td>41.8±8.0</td>
<td>1.38±0.22</td>
<td>0.80±0.04</td>
<td>0.78±0.06</td>
<td>0.82±0.14</td>
<td></td>
</tr>
<tr>
<td>2 Hz, 1.6 mmol/L Ca2+</td>
<td>30.3±6.1</td>
<td>1.00±0.16</td>
<td>0.80±0.05</td>
<td>0.81±0.04</td>
<td>0.67±0.13</td>
<td></td>
</tr>
<tr>
<td>2 Hz, 3.2 mmol/L Ca2+</td>
<td>53.3±12.1</td>
<td>2.20±0.43</td>
<td>0.82±0.05</td>
<td>0.77±0.06</td>
<td>0.67±0.04</td>
<td></td>
</tr>
<tr>
<td>2 Hz, 4.8 mmol/L Ca2+</td>
<td>30.8±5.7</td>
<td>1.03±0.19</td>
<td>0.80±0.05</td>
<td>0.74±0.03</td>
<td>0.65±0.05</td>
<td></td>
</tr>
<tr>
<td>6 Hz, 1.6 mmol/L Ca2+</td>
<td>18.3±6.1</td>
<td>0.90±0.33</td>
<td>0.93±0.09</td>
<td>0.83±0.09</td>
<td>0.62±0.04</td>
<td></td>
</tr>
<tr>
<td>10 Hz, 1.6 mmol/L Ca2+</td>
<td>17.8±3.0</td>
<td>0.66±0.13</td>
<td>0.76±0.04</td>
<td>0.71±0.10</td>
<td>0.55±0.08</td>
<td></td>
</tr>
</tbody>
</table>

Strips of human atrial appendages were preincubated with [3H]norepinephrine and superfused with [3H]norepinephrine-free solution containing 0.6 μmol/L desipramine plus 40 μmol/L corticosterone. Five 9-minute (0.66 Hz), 3-minute (2 Hz), 1-minute (6 Hz), or 36-second (10 Hz) periods of transmural electrical stimulation (S1–S5) were applied to each preparation after 94 (S1), 126 (S2), 158 (S3), 190 (S4), and 222 (S5) minutes of superfusion. Results from representative control experiments are given, corresponding to those in Figures 3 and 4. Mean±SEM of 5 to 8 experiments.

Results

Basal Efflux and Electrically Evoked Tritium Overflow in Control Experiments

When no test drug was administered before, during, and after S1 through S4, basal tritium efflux during t1 from segments of atrial appendages preincubated with [3H]norepinephrine was 4.55±0.54 nCi/min (in a representative series of control experiments; n=8; 2 Hz, 1.6 mmol/L calcium), which corresponds to a fractional rate of efflux of 0.00054±0.00002 min⁻¹. Basal efflux decreased with time, as reflected by t1/t2 ratios that declined from t2/t1 (0.94±0.02) to t3/t1 (0.87±0.02). Neither changes in Ca2⁺ concentration nor application of the test drugs in the concentration range tested modified basal tritium efflux.

In control experiments in the absence of the test drugs, transmural electrical stimulation elicited an increase in tritium overflow above basal efflux (Table). At all frequencies of stimulation and calcium concentrations, the evoked overflow decreased from S1 to S3, as reflected by S3/S2 ratios that declined from S3/S2 to S3/S3 (Table).

Effects of Calcium Channel Antagonists

ω-Conotoxin GVIA 0.2 μmol/L nearly abolished the electrically evoked (2 Hz, 3 minutes, 360 impulses) [3H] overflow (Figure 1), whereas ω-agatoxin IVA 0.1 μmol/L was without effect (Figure 1). The L-type calcium channel blockers amiodarone, diltiazem (Figure 2), and verapamil (Figure 3A) failed to modify the evoked [3H] overflow. Verapamil also did not change the electrically evoked [3H] overflow at the increased stimulation frequencies of 6 and 10 Hz (360 impulses; Figure 3A).
Under the standard experimental conditions (2 Hz, 360 impulses; 1.6 mmol/L Ca^{2+}), the T- and N-type calcium channel blocker mibefradil concentration-dependently inhibited the electrically evoked [3H] overflow with a pIC_{25} value of 6.64 (Figure 4, open circles). The concentration-response curve of mibefradil was shifted right when the Ca^{2+} concentration was increased to 3.2 mmol/L (Figure 4, closed circles; pIC_{25} 5.95). An additional increase in Ca^{2+} concentration to 4.8 mmol/L did not induce a further rightward shift of the concentration-response curve compared with that at 3.2 mmol/L (Figure 4, closed squares; pIC_{25} 6.09). A rightward shift of the concentration-response curve for mibefradil was also observed when the preparations were stimulated electrically for 1 minute with an increased frequency of 6 Hz (360 impulses; Figure 3B, closed squares; pIC_{25} 6.00). In contrast, lowering of the stimulation frequency to 0.66 Hz (9 minutes, 360 impulses) tended to shift the concentration-response curve for mibefradil to the left (Figure 3B, closed circles; pIC_{25} 6.71).

**Discussion**

The aim of the present study was to characterize the calcium channels that are involved in the control of sympathetic transmission in human heart atrium and to extend our findings previously obtained in the same preparation with mibefradil.6 The electrically evoked tritium overflow reflects quasi-physiological, Ca^{2+}-dependent release of labeled and unlabeled norepinephrine from the postganglionic sympathetic axon terminals.9 This view is supported by our previous findings10 in human atrial appendages that the electrically evoked tritium overflow is abolished by tetrodotoxin, as well as in the absence of Ca^{2+} in the superfusion fluid. On the basis of these considerations, the evoked tritium overflow may be denoted as norepinephrine release.

**Figure 1.** Effects of ω-conotoxin GVIA and ω-agatoxin IVA on electrically evoked (2 Hz) tritium overflow from segments of human atrial appendages. Ordinate: ratios S_{3}/S_{2} expressed as percentages of those in the corresponding drug-free control experiments. Drugs were present from 12 minutes before until 20 minutes after onset of S_{3}. Mean±SEM of 3 to 6 experiments. ***P<0.001 (compared with corresponding control experiments).

**Figure 2.** Effect of amiodipine and diltiazem on electrically evoked tritium overflow from superfused segments of human atrial appendages preincubated with [3H]norepinephrine. Five periods of transmural electrical stimulation (2 Hz, 360 impulses; S_{1}-S_{5}) were applied. Amlodipine (▼) and diltiazem (□) were present at increasing concentrations from 12 minutes before until 20 minutes after onset of S_{3}, S_{4}, and S_{5}. Ordinate: ratios S_{n}/S_{2} expressed as percentages of those in corresponding drug-free control experiments. Mean±SEM of 7 to 9 experiments.

**Figure 3.** Effect of verapamil (A) and mibefradil (B) on electrically evoked tritium overflow from superfused segments of human atrial appendages preincubated with [3H]norepinephrine at different frequencies of stimulation. Five periods of transmural electrical stimulation (S_{1} through S_{5}, 360 impulses) were applied. Verapamil and mibefradil were present at increasing concentrations from 12 minutes before until 20 minutes after onset of S_{3}, S_{4}, and S_{5}. 0.66 Hz; O, 2 Hz; ▼, 6 Hz; and ▼, 10 Hz. Ordinates: ratios S_{n}/S_{2} expressed as percentages of those in corresponding drug-free control experiments. Mean±SEM of 5 to 10 experiments. *P<0.05, **P<0.02, ***P<0.001 (compared with corresponding control experiments); +P<0.05, ++P<0.01 (compared with corresponding drug concentration in experiments performed at 2 Hz). In the case of experiments with individual verapamil concentrations, data obtained at various stimulation frequencies did not significantly differ from each other.
the electrically evoked norepinephrine release. Hence, the ability of ω-conotoxin GVIA to almost abolish the evoked norepinephrine release, whereas ω-agatoxin IVA was without effect, indicates that Ca\(^{2+}\) influx into the human cardiac sympathetic axon terminals occurs via N-type Ca\(^{2+}\) channels, whereas P- and Q-type channels are not involved.16

Mibefradil mimicked the inhibitory effect of ω-conotoxin GVIA on norepinephrine release. This inhibitory effect of mibefradil was dependent on the extracellular Ca\(^{2+}\) concentration. An increase in extracellular Ca\(^{2+}\) concentration from 1.8 to 3.6 mmol/L, leading to an elevated intraneuronal Ca\(^{2+}\) availability for stimulus-release coupling by an increased influx,17 reduced the potency of mibefradil in inhibiting evoked norepinephrine release. This finding is compatible with a quasi-competitive type of inhibition, i.e., at a certain state of blockade of a given number of calcium channels, more Ca\(^{2+}\) ions can permeate the channel if the extracellular Ca\(^{2+}\) concentration is increased; conversely, a higher concentration of a calcium channel blocker is necessary at increased extracellular Ca\(^{2+}\) concentration to produce a certain degree of inhibition of Ca\(^{2+}\) influx. This interpretation is not ruled out by the finding that a further increase in Ca\(^{2+}\) concentration above 3.2 mmol/L to 4.8 mmol/L did not produce an additional reduction in the inhibitory potency of mibefradil: beyond a saturation level of extracellular Ca\(^{2+}\) concentration at which the maximum amount of Ca\(^{2+}\) already penetrates the channel at a given level of blockade (in the present experiments, this level appeared to be \(\approx\)3.2 mmol/L Ca\(^{2+}\)), an increase in extracellular Ca\(^{2+}\) concentration will not lead to an additional increase in intraneuronal Ca\(^{2+}\) availability for stimulus-release coupling. Moreover, to maintain the level of channel blockade, it will not be necessary to increase the amount of the calcium channel blocker. Similarly, an increase in stimulation frequency, which also leads to an increase in intraneuronal Ca\(^{2+}\) concentration (see References 9 and 18), also reduced the potency of mibefradil to inhibit evoked norepinephrine release, whereas a decrease in stimulation frequency tended to increase its potency. Because mibefradil can potently block neuronal N-type calcium channels in addition to T-type channels,2,3 the ability of mibefradil to mimic the effect of ω-conotoxin GVIA in human atrial appendages strongly suggests that the inhibitory effect of mibefradil on norepinephrine release from the cardiac sympathetic nerves is due to blockade of N-type Ca\(^{2+}\) channels.

Verapamil, diltiazem, and the dihydropyridine derivatives amlodipine and, as shown previously, nifedipine,4 which block the L-type calcium channel, did not inhibit the evoked norepinephrine release in human atrial appendages at concentrations up to 1 and 10 μmol/L, respectively. These results obtained at the standard stimulation frequency of 2 Hz suggest that L-type channels are not involved in Ca\(^{2+}\) influx into human cardiac sympathetic nerves during depolarization. Whether or not verapamil, diltiazem, and the dihydropyridines are capable of interacting with presynaptic calcium channels at clinically relevant concentrations is still a matter of debate. High-affinity binding of dihydropyridines to neuronal tissue has been demonstrated, and it has been proposed that these binding sites are L-type calcium channels.39 However, in functional studies in which an inhibition of evoked norepinephrine release was observed by dihydropyridines and verapamil, it only occurred either at high stimulation frequencies (≥10 Hz) or at drug concentrations ≥1 μmol/L.19–23 In the present study, even 1 μmol/L verapamil failed to inhibit evoked norepinephrine release at stimulation frequencies of up to 10 Hz. Because the upper limit of the clinically relevant plasma concentration of the L-type calcium channel blockers is 0.1 μmol/L,24 it is rather unlikely that an inhibition of norepinephrine release will occur with these drugs in vivo at therapeutic doses. However, combined L- and N-type calcium channel blockers would be suitable to inhibit norepinephrine release. Such drugs may be assumed to be clinically beneficial, because they would counteract the reflex tachycardia in response to blood pressure reduction.

Taken together, in human heart, norepinephrine release from sympathetic nerves is triggered by Ca\(^{2+}\) influx via N-type but not L-type calcium channels. The inhibitory effect of clinically relevant concentrations of mibefradil (which has been applied therapeutically until recently) on norepinephrine release is probably due to its blocking effect on N-type calcium channels. This unique property of mibefradil may contribute to the slight negative chronotropic effect of the drug in vivo. In the Mortality Assessment in Congestive Heart Failure (MACH)-1 trial, mibefradil did not result in clinical benefits in patients with cardiac failure and showed a trend toward an increased mortality; this may be assumed to be due to an interaction with T-type calcium channels in the conduction system and/or to drug interactions.25 In the meantime, mibefradil has been withdrawn from the market because of its complex pharmacokinetic interactions. Nevertheless, it might serve as a leading compound to design future
calcium channel antagonists devoid of its unfavorable pharmacokinetic interactions but sharing its beneficial properties. In particular, drugs that block both the vascular L-type and N-type calcium channels and that do not substantially pass the blood/brain barrier should lead to vasodilatation without direct negative inotropism, and they should not induce sympathetic reflex activation but may cause mild bradycardia. Such drugs may represent progress in the treatment of cardiovascular diseases such as hypertension and coronary heart disease.

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**References**

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