Endothelin Activation of an Inwardly Rectifying K⁺ Current in Atrial Cells

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Various tissues including heart express specific binding sites for endothelin. Endothelins have been reported to increase the force of contraction of cardiac muscle, presumably via specific receptors. Specific binding of endothelin to atrial tissue is particularly high. In spontaneously contracting rat atrial cells used in this study, all three isoforms of endothelin (endothelin-1, endothelin-2, and endothelin-3) decreased the rate of beating and caused an increase in inwardly rectifying K⁺ current in voltage-clamped whole cells. Endothelin-3 was the most potent isoform, and its effects on beating rate and K⁺ current were present at a concentration as low as 100 pM (Kₐ ~ 1 nM). The atrial cells did not have the hyperpolarization-activated current (the pacemaker current), Iₛ. In excised inside-out patches, all three isoforms of endothelin activated a population of K⁺ channels with kinetic properties identical to those of acetylcholine (muscarinic)-activated K⁺ channels, and this was GTP dependent. Endothelin failed to decrease the beating rate or to elicit the K⁺ current in pertussis toxin–treated cells. These results indicate that endothelin has a potent negatively chronotropic effect by activation of the inwardly rectifying, muscarinic K⁺ channel and therefore could be an important regulator of heart function. (Circulation Research 1991;69:250–255)

Endothelin is a potent 21 amino acid vasoconstrictor peptide originally isolated from porcine aortic endothelial cells.⁴⁻⁶ Three isoforms of endothelin, endothelin-1, endothelin-2, and endothelin-3, have been identified and shown to bind to various types of tissues via specific receptors to elicit different physiological responses such as release of atrial natriuretic factor, inhibition of renin release, and proliferation of smooth muscle and fibroblasts.⁴⁻⁶ In rat, guinea pig, and human hearts, endothelin was reported to produce a positive inotropic effect,⁷⁻¹¹ suggesting that intracellular Ca²⁺ or the Ca²⁺ sensitivity of myofilament is increased.

Studies to measure changes in intracellular Ca²⁺ concentrations as well as calcium entry via Ca²⁺ channels in response to endothelin have so far yielded conflicting results.¹⁰⁻¹⁴ Involvement of phosphatidylinositol hydrolysis in the inotropic effect of endothelin-1 has also been reported,⁹ suggesting that Ca²⁺ release from intracellular stores may be important. The mechanism of action of endothelin may thus be similar to that of phenylephrine, which produces an increase in force of contraction via α₂-receptor–mediated hydrolysis of phosphatidylinositol.¹⁵⁻¹⁷ The qualitative differences in the development of force of contraction and in chronotropic effects by endothelin and other cardiac hormones indicate, however, that endothelin’s actions may involve other mechanisms.

In initial experiments to examine the mechanism of action of endothelin, it was found unexpectedly that endothelin caused cessation of beating in spontaneously contracting neonatal rat atrial cells. Therefore, the mechanisms by which endothelin inhibited the atrial pacemaker activity in these cells were investigated, with particular emphasis on membrane K⁺ conductance.

Materials and Methods

Cell Preparation

Hearts from 1–2-day-old newborn rats were dissociated with collagenase and trypsin. Right and left atrial tissues from whole hearts were cut out and placed in calcium-free Hanks’ medium (Sigma Chemical Co., St. Louis). The tissues were then cut into small pieces with a sharp blade and placed in Hanks’ balanced salt medium containing 0.05% collagenase type II and 0.03% trypsin (Worthington Biochemical Corp., Freehold, N.J.). Tissues were incubated at 37°C for 10 minutes. Suspended cells were then removed and added to the same volume of 50% fetal calf serum to inhibit enzyme activity. Remaining tissues were
incubated in new enzyme solution and allowed to dissociate for another 10 minutes. This procedure was done five times. Dissociated cells were collected, centrifuged, and placed into culture medium consisting of Dulbecco's modified Eagle medium, 10% fetal calf serum, and 0.1% penicillin-streptomycin. Cells were plated on glass coverslips and incubated at 37°C in 5% CO₂. The atrial cells began to beat spontaneously after 2 days in culture.

Contractility Measurements
Changes in beating rate of individual cells were assessed using an optical video apparatus. A glass coverslip with attached beating cells was placed on an inverted phase-contrast microscope and superfused with bicarbonate-buffered physiological solution containing (mM) KCl 5, NaCl 140, MgCl₂ 1.2, CaCl₂ 2.0, and NaHCO₃ 25 (pH 7.3, 37°C) and 1% fetal calf serum at a rate of 4 ml/min. Cells were magnified with a ×32 objective, and the image was monitored by a low light level video camera connected to a video motion detector that monitored a selected raster line segment and provided position data every 16 msec for an image point moving along the raster line. The analog output voltage from the motion detector was calibrated to indicate actual micrometers of cell wall motion. After 5–10 minutes of equilibration, cells were perfused with a solution containing a specific test hormone or drug.

Electrophysiology
Gigaseals were formed with pipettes having 1–2-MΩ (for whole-cell configuration) or 4–6-MΩ (excised patches) resistances as described by Hamill et al. Whole cells were formed by suction, and the capacitive transients were cancelled at the beginning of each experiment. Cells were perfused with solutions containing (mM) KCl 10, NaCl 130, EGTA 5, Mg²⁺ 2, and HEPES 10 (pH 7.2) unless noted otherwise. The pipette solution contained (mM) KCl 140, EGTA 5, MgCl₂ 2, and HEPES 10 (pH 7.2). For single-channel studies, cell-attached and inside-out patches were formed as described by Hamill et al. and perfused with bath solution identical to the pipette solution. Currents were recorded with a patch-clamp amplifier (Axopatch 1C, Axon Instruments, Foster City, Calif.), low-pass filtered at 5 kHz (eight-pole Bessel filter, Frequency Devices Inc., Haverhill, Mass.), and stored on magnetic tape via a digital data recorder (Instrutech, Elmont, N.Y.). Later, digitized data were entered into a computer (Atari) and analyzed to obtain duration and amplitude histograms and channel activity (channel activity is measured as averaged Np, where N is the number of channels in a patch and p, is the probability of channel opening). Single-channel dwell times were plotted on a logarthmic time scale using binned maximal likelihood fitting with constant logarithmic bin width (10 bins/decade). The 50% threshold method was used to determine the times of channel openings, and minimum duration was set at 100 µsec. Experiments were generally done at room temperature (24–26°C).

Miscellaneous
Endothelins were purchased from Peninsula Laboratories, Inc., Belmont, Calif., and Calbiochem Corp., La Jolla, Calif. Acetylcholine, atropine, and theophylline were purchased from Sigma. ATP, GTP, GTPγS, and GDP were purchased from Boehringer Mannheim Corp., Indianapolis, Ind. Pertussis toxin was purchased from List Biological Laboratories, Inc., Campbell, Calif.

Results
Studies of the Beating Rate
To examine the effect of endothelin on the beating rate, spontaneously contracting atrial cells were allowed to equilibrate in the perfusion solution and then were perfused with a solution containing endothelin. Beating rates in control cells varied among coverslips and cultures. In general, rates ranged from 60 to 150 beats/min. The addition of endothelin-3 (100 pM, human and rat) to the perfusion solution resulted in an immediate, marked reduction of beating rate (Figure 1). Wash-out of endothelin-3 resulted in complete recovery of
the beating rate. In some cells, 1–2 minutes of washout was necessary to cause full recovery. In many cells, the beating rate after the washout of endothelin was greater than the rate before treatment with the hormone. At 500 pM, endothelin-3 caused cessation of beating, which recovered completely when the peptide was washed off. Endothelin-2 (10 nM, human) and endothelin-1 (20 nM, porcine) also caused cessation of beating but at higher concentrations. The order of potency was endothelin-3 > endothelin-2 > endothelin-1. Acetylcholine (ACh), a muscarinic agonist, produced no change in beating rate at 500 pM (Figure 1C) and caused slowing of the beating rate at 10 nM and above. These results show that endothelins are inhibitors of atrial pacemaking activity that are more potent than ACh.

Whole-Cell Current Studies

The above effects of endothelin on the beating rate suggest that membrane K⁺ permeability could be altered. Therefore, K⁺ currents were studied using the whole-cell voltage-clamp technique. Cell membrane potential was held at −60 mV, and currents were recorded during 200-second voltage steps from −140 to +60 mV in 20-mV increments. The pipette solution, which equilibrates with the cytoplasmic content of the cell, contained 100 μM GTP and 2 mM ATP. Figure 2A shows typical inwardly rectifying whole-cell K⁺ currents (resting K⁺ current) in a bathing solution containing 10 mM K⁺. The addition of 1 nM endothelin-3 to the bath increased the inward currents by approximately twofold without an effect on the outward current (Figure 2B). Current–voltage relations showed that endothelin-induced currents were inwardly rectifying.

Current changes were studied in more detail using whole cells whose membrane potentials were held constant at −60 mV in solution containing 140 mM K⁺. Extracellular application of endothelin-3 (1 nM) elicited an inward current that decayed slowly to a steady-state level approximately half of the initial peak current (Figure 2D). Endothelin-1 (1 nM) and endothelin-2 (1 nM) also evoked inward currents, but
of lesser magnitudes (Figures 2E and 2F). Concentration–effect relations for endothelin and ACh are shown in Figure 2G. Maximal $K^+$ current was evoked at $\sim 100$ nM for all three endothelins; no further increases in current were observed above 100 nM. Although not shown in the figure, ACh produced a maximal effect at 10 $\mu$M and the magnitude of ACh-induced current was approximately threefold greater (9.5 nA, $n=3$) than that produced by 10 nM endothelin-3. These results show that endothelin-3 is more potent than ACh in the activation of the muscarinic $K^+$ current at nanomolar concentrations, but ACh has a greater efficacy than endothelins.

Replacement of GTP in the pipette solution with a high concentration of GDP could effectively block GTP-dependent effects such as the activation of muscarinic $K^+$ channel by ACh. When GTP in the pipette was replaced with 5 mM GDP, the resting $K^+$ current was not changed but endothelin-3 failed to elicit the typical response (Figure 2H). In the same patch of membrane, ACh had very little effect on the $K^+$ current, as expected. This strongly suggested that endothelin-evoked current changes are mediated by a GTP-binding protein (G protein). The involvement of an inhibitory G protein was further confirmed by the observation that endothelin-3 failed to activate the $K^+$ current in cells treated with 100 ng/ml pertussis toxin for 12 hours (Figure 2I). Thus, the G protein that couples the endothelin receptor is of the inhibitory G protein ($G_i$) type similar to that which couples the muscarinic and adenosine receptors.20–22 Atropine (10 $\mu$M) or theophylline (100 $\mu$M) did not block the increase in current produced by endothelin, indicating that endothelin probably acts on its own receptor, and not on the muscarinic or adenosine receptors, to activate the $K^+$ current via G protein. When ACh maximally stimulated the $K^+$ current, none of the endothelins elicited any additional current at concentrations up to 50 nM, indicating that endothelin and ACh probably activate the same population of $K^+$ channels.

**Single-Channel Studies**

The GTP dependency of the endothelin-evoked $K^+$ current indicated that either the muscarinic-gated or the ATP-sensitive $K^+$ channel is involved. The role of the resting $K^+$ current was ruled out since this channel is known not to depend on GTP. The results described above in whole-cell studies also show clearly that the resting $K^+$ channel current is not coupled to G proteins. The current activated by endothelin was studied at the single-channel level by using cell-attached and inside-out patches with endothelin in the pipette in symmetrical 140 mM $K^+$ solution. Endothelin-3 (1 nM) activated single-channel currents in 31 of 38 cell-attached patches. After formation of inside-out patches, the channel activity decreased rapidly to basal levels (one opening every few seconds). Subsequent application of GTP (100 $\mu$M) to the bath resulted in reactivation of the channels having a conductance of $36\pm2$ pS and a mean open time of 0.9±0.1 msec, similar to those activated by ACh.20,21 The GTP-dependent channel current exhibited inward rectification (Figure 3A). The reversal potential shifted 59 mV to the right when [K+] was changed from 140 to 14 mM (126 mM choline), indicating that the channel was $K^+$ selective. When added outside the pipette in cell-attached patches, endothelin-3 failed to activate any channel current. When $K^+$ channels were activated with 50 nM endothelin-3, application of GTP$\gamma$S to the cytosolic surface of inside-out patches did not cause a further increase in channel activity (channel activity represented as $N_p$, changed from 0.26 to 0.27, mean of three determinations). Endothelin-1 (20 nM) and endothelin-2 (10 nM) also activated the single-channel currents with identical kinetics under similar conditions but with less frequency of success (12/30 for endothelin-1 and 11/27 for endothelin-2).

In cells preincubated with 100 ng/ml pertussis toxin for 12 hours, endothelin-3 failed to activate the $K^+$ channel in cell-attached patches or in inside-out patches with GTP in the bath ($n=16$). Endothelin-3 (5 nM) also failed to inhibit spontaneous contractions in pertussis toxin–treated cells. To examine whether the effect of endothelin on the beating rate could be due to inhibition of the pacemaker current ($I_p$) that is reported to be present in sinoatrial nodal cells, the existence of $I_p$ in rat atrial cells used in this study was first studied. In all 12 atrial cells tested, no hyperpolarizing-activated current ($I_n$) was present when the membrane potential of whole cells was pulsed for 1 second from $-20$ to $-120$ mV. Thus, the activation of the inwardly rectifying $K^+$ current via G protein very likely underlies the inhibition of the atrial spontaneous contractile activity produced by endothelin.

**Discussion**

The major finding of the present study is that endothelin, an endothelium-derived contracting factor, produces a potent ACh-like activity in atrial cells by activation of an inwardly rectifying $K^+$ current. Initial studies of endothelins on heart function revealed that these peptides have potent positively inotropic as well as chronotropic actions.7–11 Although the underlying cellular mechanisms are not clearly known, the results implied that endothelins must be increasing [Ca$^{2+}$], or the sensitivity of myofibrils to Ca$^{2+}$. Such effects would be expected to be associated with an increase in beating rate in spontaneously contracting cells, since elevation of [Ca$^{2+}$] by different procedures generally increases the beating rate. The potent negative chronotropic action of endothelins was therefore surprising. However, the subsequent finding that endothelins can activate the muscarinic-gated $K^+$ channels could explain the strong ACh-like activity of endothelins that more than likely overshadowed any existing stimulatory effects.

As a test of the role of the muscarinic $K^+$ channels in the control of beating rate in atrial cells, the chronotropic effect of endothelin was also examined.
in spontaneously beating neonatal rat ventricular cells in which there is a relative lack of coupling between muscarinic receptors and the K⁺ channel. In preliminary studies, all three isoforms of endothelin at concentrations up to 100 nM failed to cause cessation of beating in ventricular cells. Although the beating rate itself depends on several cellular processes, comparison of the results in the atrial and ventricular cells suggests that the negative chronotropic action of endothelin in atrial cells is likely to be due to opening of muscarinic K⁺ channels.

Separate receptors for the three isoforms of endothelin have been found by radioligand studies,⁷⁻¹⁰ and at least two distinct types of endothelin receptors with differing affinities have been described for heart tissues.⁷⁻¹⁰ Different contractile effects of endothelin in atrial and ventricular cells could well be due to the presence of distinct types of endothelin receptors with differing affinities for endothelin isoforms, as well as to coupling of the receptor to different intracellular effectors. This could also account for the different contractile responses to endothelin in neonatal atrial cells in this study and in adult heart tissues by others⁷⁻⁹ in which no effect of the peptide on the beating rate was noted.

In neonatal rat atrial cells, the lack of additional effect of endothelin on the K⁺ current maximally activated by 10 μM ACh indicates that endothelin and ACh open the same population of K⁺ channels. The failure of endothelin to activate the K⁺ current in pertussis toxin–treated cells indicates that endothelin receptors are coupled to the inhibitory G protein, similar to that of muscarinic receptors. Thus, there are at present at least five different hormone receptors that are coupled to GTP-binding protein in atrial cells. They include muscarinic (M₁), adenosine (A₁), somatostatin,⁹ endothelin, and calcitonin gene–related peptide receptors.⁹

The K⁺ channel current activated by endothelins in membrane patches could not be increased further by GTP½ (10 μM), which produces a maximal activation of the channel. Therefore, the coupling of endothelin receptor to the K⁺ channel via G protein is probably as efficient as those of muscarinic receptors. If the coupling of endothelin receptor to G protein is one to one, then the comparison of whole-cell currents evoked by endothelin and ACh shows that the number of receptors for endothelin-3 is approximately one third of that for ACh. This is supported by the finding that in a continuous whole-cell recording, ACh (10 μM) plus endothelin-1 (100 nM) elicited 5.2 nA of current at steady state (average of two values); washout of ACh alone resulted in a decrease of the current to 1.4 nA (27%). If a value of 90 fmol/mg protein for muscarinic receptors as judged by specific binding of [³H]quinuclidinyl benzylate binding is assumed,¹⁰ binding sites for endothelin-3 in atrial cells is ~30 fmol/mg protein. The number of receptors for endothelin-1 and endothelin-2 is probably lower than that for endothelin-3, because the two endothelin isoforms evoked less K⁺ current than did endothelin-3.

**Figure 3.** Single-channel currents activated by endothelin. Panel A: Cell-attached patch with 1 nM endothelin-3 (ET-3) in the pipette shows single K⁺ channel currents with kinetics identical to that of acetylcholine-activated (muscarinic) K⁺ current. After formation of an inside-out patch, channel activity subsided to basal levels (almost no opening in this patch). Addition of 100 μM GTP to the cytosolic side of the membrane rapidly reactivated the channels. Holding potential, −80 mV. At the end, membrane potential was changed from −80 to +80 in 20-mV increments to obtain single-channel current–voltage relations. Panel B: Open time histogram (mean open time, −1 ms). Note the logarithmic and square root scales for representing the open time histogram.¹⁹ Panel C: Plot of the current–voltage relation showing clear inward rectification. Panel D: Single-channel openings at a higher time resolution.
Coronary endothelium may, via release of endothelin, exert vagal-like activity on the heart. Endothelium-derived contracting factors have been reported to be released in response to stretch and anoxia. Perhaps when released locally during humoral, neural, or mechanical stimuli, endothelin opposes the potential increase in heart rate that would accompany the stimulus or helps to reduce the heart rate during local vasoconstriction. Although at present there is no direct evidence that locally released endothelin does act on its receptors in cardiac muscle cells in vivo, data from this and other studies show that endothelin and endothelium-derived contracting factors could be very important in the regulation of heart function.

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Key Words: endothelin • atrial cells • muscarinic K+ channel • G protein
Endothelin activation of an inwardly rectifying K+ current in atrial cells.

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Circ Res. 1991;69:250-255
doi: 10.1161/01.RES.69.1.250

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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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