Relaxin Increases Heart Rate by Modulating Calcium Current in Cardiac Pacemaker Cells

X. Han, Y. Habuchi, W.R. Giles

Relaxin (RLX), a reproductive hormone of the insulin family, increases heart rate in experimental animals. The cellular and ionic mechanisms responsible for this positive chronotropic effect remain unknown. We have investigated the actions of RLX on the action potential and underlying transmembrane ionic currents in single sinoatrial node cells of the rabbit heart under whole-cell voltage-clamp conditions, using both nystatin-perforated-patch and membrane-ruptured techniques. In this preparation RLX (0.8 to 80 nmol/L) caused reversible increases in the rate of spontaneous action potentials and a dose-dependent increase in the L-type calcium current, I_{CaL}. The best-fit Langmuir relation for the augmentation of I_{CaL} yielded a threshold concentration of 1 nmol/L and a K_{D} of 14 nmol/L. These effects of RLX appear to be mediated by increases in intracellular cyclic AMP (cAMP), since RLX was without effect after application of (1) the β-adrenergic agonist isoproterenol (1 μmol/L) or (2) superfusion of the intracellular second messenger cAMP (100 μmol/L) or 8-Br-cAMP (100 to 200 μmol/L). Internal dialysis with an inhibitor of cAMP-dependent protein kinase (PKI, 7 μmol/L) abolished the effects of RLX. These results provide the first electrophysiological evidence that RLX modulates heart rate and contractility by increasing I_{CaL}, and suggest that the biochemical mechanisms involve the formation of cAMP and activation of cAMP-dependent protein kinase. (Circ Res. 1994;74:537-541.)

Key Words • relaxin • sinoatrial node cell • calcium current • heart rate • cAMP

Materials and Methods

Cell Isolation

Single sinoatrial node cells were isolated from rabbit hearts as described previously.11,12 In brief, hepatized rabbits weighing 1.5 to 2 kg were anesthesiazed with pentobarbital and killed by a blow on the neck. Hearts were quickly excised, then Langendorff-perfused at 37°C with HCO3-buffered Tyrode's solution for 1 to 3 minutes to wash out the blood. Each heart was then perfused with Ca^{2+}-free Tyrode's solution for 5 minutes before switching to an enzyme-containing (12.5 U/mL collagenase, Yakult, Tokyo), low-Ca^{2+} (5 μmol/L) Tyrode's solution for a further 10 minutes. Small segments of the sinoatrial node were excised from the intact hearts, and the surrounding tissues were removed under a dissecting microscope. The final preparations (about 4×4 mm) were cut into five pieces and stirred at 37°C in Ca^{2+}-free, HEPES-buffered Tyrode's solution containing 500 U/mL collagenase (type I Sigma), 73 U/mL elastase (Sigma), and 0.1% bovine serum albumin (fraction V, Sigma). At selected time intervals, cells were collected, transferred into modified Tyrode's KB solution, and centrifuged at 140g for 3 to 5 minutes. After the supernatant was removed, cells were stored at 4°C in the same KB solution.

Solutions

The HCO3-buffered Tyrode's solution contained (mmol/L): NaCl 121, KCl 5.0, sodium acetate 2.8, MgCl2 1.0, Na2HPO4 1.0, NaHCO3 24, glucose 5.5, and CaCl2 0.5. This solution was equilibrated with 95% O2 and 5% CO2 (pH 7.4). The KB solution used for storing the cells contained (mmol/L): K+ -glutamate 90, K+ -oxalate 10, KCl 25, KH2PO4 10, NaOH 6, MgSO4_2H2O 1.0, taurine 20, HEPES 5, and glucose 10; pH was adjusted to 7.2 with KOH. The HEPES-buffered Tyrode's solution in which all experiments were carried out contained (mmol/L): NaCl 145, KCl 5.4, MgCl2 1.0, Na2HPO4 1.0, HEPES 5.0, CaCl2 1.8, and glucose 10. pH was adjusted to 7.4 with NaOH.

Recordings and Data Acquisition

In most experiments the nystatin-perforated-patch technique was used to current- and voltage-clamp these cells.

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The pipette solution contained (mmol/L): KCl 140, NaCl 6, MgCl₂ 1, and HEPES 5; pH was adjusted to 7.2 with KOH, and DC resistance was 2 to 3 MΩ. Nystatin was first dissolved in dimethyl sulfoxide and then added to the pipette solution at a final concentration of 0.4 mg/mL. The conventional membrane-ruptured patch-clamp technique in the whole-cell configuration was used for internal dialysis of adenosine 3':5'- cyclic AMP (cAMP) and a peptide inhibitor of cAMP-dependent protein kinase (PKI). In these experiments the pipette solution contained (mmol/L): K⁺-aspartate 90, KCl 30, HEPES 5, EGTA 10, K⁺-ATP 3, Na⁺-phosphocreatine 3, MgCl₂ 1, and CaCl₂ 1. pCa was ≈ 7.4 to 7.5. pH was adjusted to 7.2 with KOH. A liquid junction potential of 10 mV (pipette negative) was corrected electrically. All recordings were made at 32°C to 33°C. Membrane currents were digitized at 2.5 kHz. The recording chamber volume was 0.5 mL, and a constant flow rate of 1.5 mL/min was used to superfuse the cells. cAMP (200 μmol/L) and PKI (7 μmol/L) were dissolved in the pipette solution.

**Drugs**

Synthetic human RLX (MW approximately 6000) was kindly supplied by Genentech Ltd (San Francisco, Calif). PKI was purchased from Peninsula Lab Inc (Belmont, Calif). All other drugs were purchased from Sigma Chemical Co (St Louis, Mo).

**Statistical Analyses**

Statistical significance was evaluated with Student’s unpaired t test, where appropriate. Differences with values of P < .05 were considered to be significant.

**Results**

The single cells from rabbit sinoatrial node used in this study were similar to those described by Irisawa et al. Stable spontaneous action potentials could be recorded routinely when the nystatin-perforated-patch technique was used. Fig 1A shows that addition of RLX (80 nmol/L) to the superfusate caused both the rate of the diastolic depolarization and the amplitude of action potential to increase. The average increase in firing rate, calculated from four cells, was 42 ± 20% (mean ± SEM); this effect was almost completely reversible on removal of RLX. The observed increase in automaticity in sinoatrial node cells is consistent with recent reports that RLX can increase heart rate both in vivo and in vitro.

The ionic currents that underlie the pacemaker depolarization in sinoatrial node cells include T- and L-type calcium currents (ICa(T), ICa(L)); a hyperpolarization-activated inward current, IC₆; and the delayed rectifier K⁺ current, IC₈. In our initial experiments, RLX-induced changes in IC₈ were studied because contractility was known to be increased by this hormone. RLX (80 nmol/L) significantly increased IC₈ (85 ± 29%, n = 10, mean ± SEM), and this effect was reversible on removal of the agonist (Fig 1B and 1C). The current-voltage relation (Fig 1D) shows that this increase in IC₈ developed without a significant change in voltage dependence. Little effect was seen on the background inward current activated at potentials negative to −50 mV, indicating a selective effect of RLX on IC₈.

No information regarding RLX binding in rabbit heart is available at present. However, in rat atrium, the KD for RLX is approximately 1.4 nmol/L, and 5 nmol/L RLX causes a 36% increase in heart rate. We have studied the effects of five concentrations of RLX: 0.8, 8, 16, 80, and 200 nmol/L, using the nystatin-perforated-patch recording technique. The resulting RLX-induced increase in peak IC₈ is plotted as a dose-response relation in Fig 2. The threshold RLX concentration was approximately 1 nmol/L, and the maximum increase in IC₈ was obtained at 80 nmol/L. The best-fit Langmuir relation of these data yielded a KD of 14 nmol/L. These concentration-effect data are comparable to other peptide compounds that have electrophysiological effects on mammalian cells.

Unlike other peptide hormones of the insulin family, RLX has been reported to cause an increase in intracellular cAMP in a number of different tissues. The possibility that the observed RLX-induced increase in IC₈ was mediated by a cAMP-dependent mechanism...
was therefore investigated. We first compared the effects of β-adrenergic agonist isoprenaline (ISO) and RLX on I\textsubscript{Ca(L)}. ISO is known to stimulate I\textsubscript{Ca(L)} by increasing cAMP and activating a cAMP-dependent protein kinase, PKA, which phosphorylates L-type calcium channels.\textsuperscript{23} As shown in Fig 3A, after these pacemaker cells had been stimulated by ISO, subsequent addition of RLX caused no further increase in I\textsubscript{Ca(L)} (n=6). In contrast, a significant enhancement in I\textsubscript{Ca(L)} was observed when ISO was applied after this current had been increased significantly by a maximum dose of RLX (Fig 3B). Similar observations were made in four other cells. These results (Fig 3) indicate that (1) the RLX-induced increase in I\textsubscript{Ca(L)} may be modulated by intracellular cAMP, but (2) even at a maximum concentration, RLX cannot increase intracellular cAMP sufficiently to saturate the cAMP-dependent biochemical pathway.

If the RLX-induced increase in I\textsubscript{Ca(L)} is cAMP-dependent, no additive effect should be observed after I\textsubscript{Ca(L)} is increased maximally by raising intracellular cAMP. This hypothesis was tested directly by (1) applying the membrane-permeable cAMP derivative 8-Br-cAMP and (2) internally dialyzing cells with cAMP. As shown in Fig 4A, after I\textsubscript{Ca(L)} was increased by superfusing the cell with 100 μmol/L 8-Br-cAMP, application of RLX failed to cause any significant change in I\textsubscript{Ca(L)}. Results similar to those shown in Fig 4A were obtained in three other cells. The data in Fig 4B show that when a sinoatrial node cell was dialyzed with cAMP (200 μmol/L), an increase in I\textsubscript{Ca(L)} was seen soon after the membrane was ruptured and dialysis had begun. Note that after the cAMP-dependent increase in I\textsubscript{Ca(L)} reached a steady-state level, addition of RLX failed to cause any further increase (n=4). Moreover, in two of these cells, after I\textsubscript{Ca(L)} had been increased maximally by intracellular dialysis with cAMP, switching from RLX- to ISO-containing solution also failed to cause any significant change in I\textsubscript{Ca(L)} (Fig 4B). In combination, these results strongly suggest that generation of cAMP is essential for the effects of both RLX and ISO.

Our final experiments tested whether PKA was involved in the RLX-induced increase in I\textsubscript{Ca(L)}. In these experiments, the conventional whole-cell voltage-clamp technique was used to allow intracellular dialysis with a relatively large peptide, the PKA inhibitor PKI.\textsuperscript{24} The results in Fig 5A demonstrate that repeated application of RLX (200 nmol/L) did not cause an appreciable desensitization (n=4), because both the time course and the magnitude of increase in I\textsubscript{Ca(L)} after the second exposure were similar to those changes caused by the

![Fig 2](http://circres.ahajournals.org/doi/abs/10.1161/01.ATP.95.2.539)

**Fig 2.** Graph showing dose-response relation for the relaxin (RLX)-induced increase in I\textsubscript{Ca(L)} in rabbit sinoatrial node cells. Each point represents mean±SEM of three to eight measurements in separate cells. To activated I\textsubscript{Ca(L)}, 200-millisecond pulses to 0 mV from a holding potential of -40 mV were delivered at 0.067 Hz. Selected concentrations of RLX were added to the superfusing solution at three-minute intervals. Because of the "rundown" of I\textsubscript{Ca(L)} (even with the nystatin-perforated-patch technique), no more than three RLX doses were tested on each cell. The solid line shows the best-fit Langmuir relation, yielding a K\textsubscript{D} of 14 nmol/L.

![Fig 3](http://circres.ahajournals.org/doi/abs/10.1161/01.ATP.95.2.539)

**Fig 3.** Graphs showing effect of previous application of a β-adrenergic agonist, isoprenaline (ISO), on the relaxin (RLX)-induced increase in I\textsubscript{Ca(L)} in rabbit sinoatrial node cells. A, Changes in peak I\textsubscript{Ca(L)} when a sinoatrial node cell was first exposed to ISO (2 μmol/L) and then was exposed to RLX (80 nmol/L) in the presence of ISO. B, Time-course of change in peak I\textsubscript{Ca(L)} when a sinoatrial node cell was first exposed to RLX (80 nmol/L) and then was exposed to ISO (2 μmol/L) in the presence of RLX. The horizontal bars indicate the time during which each drug was applied. Superimposed current traces corresponding to the time points denoted a, b, and c in each panel are shown at the bottom. To elicit I\textsubscript{Ca(L)}, 200-millisecond depolarizations to 0 mV from a holding potential of -40 mV were delivered at 0.067 Hz.
initial challenge. Results obtained after internal dialysis of PKI (7 μmol/L) and subsequent application of RLX and then ISO are shown in Fig 5B. Three minutes after the membrane was broken, RLX (80 nmol/L) still caused a significant increase in ICa(L), indicating that the dialysis procedure was not yet complete. However, only a small increase in ICa(L) was seen in response to 2 μmol/L ISO 8 minutes after the dialysis with PKI had begun. After 11 minutes of dialysis, even a maximal concentration of RLX (80 nmol/L) caused very little change in peak ICa(L). Results similar to those shown in Fig 5B were obtained from three other cells in which RLX was tested directly after 10 minutes of internal dialysis with 7 μmol/L PKI. Since ISO increases ICa(L) in the presence of RLX (Fig 3B) and there is no significant desensitization to repeated applications of RLX (Fig 5A), these results suggest that activation of PKA is involved in the RLX-induced increase in ICa(L).

Discussion

Our findings provide the first direct evidence that RLX can significantly increase ICa(L) in primary pacemaker cells of the mammalian heart. Since ICa(L) is essential for generating spontaneous pacemaker activity in these cells,18,25 this increase in ICa(L) could explain the observed positive chronotropic and inotropic effects of RLX.9,10 Specific RLX binding having a KD similar to that found in uterus8 has been demonstrated in rat atrium. It therefore seems likely that the RLX-induced increase in ICa(L) is a receptor-mediated response. The
increase in $I_{Ca(L)}$ in response to RLX is dependent on both cAMP accumulation and PKA activation (Figs 4 and 5). In this regard, it is similar to the augmentation of $I_{Ca(L)}$ by β-adrenergic agonists. However, significant differences exist between the effects of ISO and RLX; for example, RLX cannot saturate the cAMP pathway (Fig 3).

During the first 3 weeks of human pregnancy, circulating RLX levels can be elevated to >800 pg/mL (=0.13 nmol/L).26 In the present single-cell experiments, RLX effects on $I_{Ca(L)}$ were observed at concentrations approximately 10-fold higher than these circulating levels. At least two factors may be responsible for this apparent discrepancy: species differences and/or alteration of RLX receptors during cell isolation. Concentrations of RLX (0.03 to 3 nmol/L) similar to the circulating RLX levels have been shown to increase the spontaneous beating rate of the isolated rat hearts by 10% to 75%.9,10 The lowest RLX concentration that significantly increased $I_{Ca(L)}$ in our experiments was approximately 1 nmol/L (Fig 2).

RLX-induced increases in $I_{Ca(L)}$ may have important implications in the pathogenesis of certain cardiac disease states. For example, during pregnancy, elevated circulating RLX levels increase both the heart rate and the stroke work of the myocardium. Under some circumstances, this extra energy requirement may cause deterioration of cardiac function.

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