Relaxin binding in the rat heart atrium

(insulin family polypeptide/relaxin receptor/steroid hormone regulation/cardiovascular function)

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ABSTRACT   Relaxin is a member of the insulin family of polypeptides that is best known as a reproductive hormone. In an effort to elucidate the mechanism of action of relaxin we previously localized the specific binding sites of a ^{32}P-labeled relaxin in the rat uterus and brain. These studies suggested that, in addition to its classical role in pregnancy, relaxin might have other physiological functions. In the present paper we describe the specific and high-affinity binding of relaxin to the cardiac atrium of both male and female rats. The relaxin binding could not be displaced by peptides belonging to the same family [insulin, insulin-like growth factor I (IGF-I)] or by peptides that were identified in the atrium or were known to have cardiovascular functions (atrial natriuretic peptide, angiotensin II). The dissociation constant for relaxin in the atrium was estimated to be 1.4 nM, which was similar to that found in the uterus (1.3 nM) and the brain (1.4 nM). In view of the close association of relaxin with reproduction, an experiment was also performed to compare the relaxin binding in the uterus and heart after gonadectomy and sex steroid treatment. It was found that the relaxin binding in the rat uterus was diminished by 53% overall following ovariectomy but was restored to 90% of normal levels when treated with estrogen (but not with testosterone). In contrast, the relaxin binding in the rat heart was not affected by castration or sex steroid treatment. We conclude that specific and high-affinity relaxin receptors exist in the atrium of both the male and female rat heart and that these are regulated differently than the relaxin receptors in the uterus.

Relaxin is structurally related to insulin and insulin-like growth factors and is best known as a pregnancy-associated hormone (1, 2). In addition to its classical actions on cervical ripening, uterine contraction, and pubic symphysis elongation (1, 2), relaxin has been suggested to participate in the regulation of blood pressure (3–8) and neuropeptide release, notably oxytocin and vasopressin (9–13). However, the results from these studies were conflicting, and the site of action of relaxin was undetermined. Biochemical support for these hypotheses was provided when it was shown that, in addition to the uterus and cervix (14), relaxin bound with specificity and high affinity ($K_d \approx 1$ nM) to several sites in the male and female rat brain (15). Some of these sites, in particular two of the circumventricular organs (subfornical organ and organum vasculosum of the lamina terminalis) and the magnocellular hypothalamic nuclei (paraventricular and supraoptic nucleus) are known to be involved in the control of blood pressure, fluid balance, and the release of neuropeptides. To further investigate the mechanism of action of relaxin, we continued the search for relaxin target tissues and were surprised to observe the specific and high-affinity binding of relaxin to the atrium of the rat heart. We also show that the atrial binding is regulated differently than the uterus.

MATERIALS AND METHODS

Materials. Synthetic human relaxin H2 was supplied by E. Rinderknecht (Genentech). It was active in the mouse pubic symphysis assay (16) and a cAMP bioassay (14, 17). The concentration of relaxin was determined by amino acid analysis. Human insulin was obtained from Eli Lilly. Insulin-like growth factor I (IGF-I) was from Genentech. Synthetic human angiotensin II and atrial natriuretic peptide were obtained from Sigma.

Phosphorylation of Relaxin. Phosphorylation of relaxin with the catalytic subunit of cAMP-dependent protein kinase (from bovine heart muscle, Sigma) and [γ-^{32}P]ATP (specific activity, >5000 Ci/mmol, Amersham; 1 Ci = 37 GBq), and subsequent purification of the ^{32}P-labeled relaxin (^{32}P-relaxin) on Sep-Pak C$_{18}$ cartridge (Waters), and cation-exchange HPLC (Poly CAT A, Poly LC, Columbia, MD) were as described (14).

Binding of ^{32}P-Relaxin to Rat Uterus and Heart Tissue Sections. Ten- to 11-week-old normal male and female Sprague–Dawley rats (Charles River Breeding Laboratories) were used for the binding of ^{32}P-relaxin to various tissues. The animals were given standard food and water ad libitum and housed under a 12/12-hr light/dark schedule at 21 ± 2°C. For experiments testing the effects of steroid hormones, female rats were ovariectomized and then injected subcutaneously 2 weeks later with 10 μg of estradiol cyclopentylproprionate in a 0.2% peanut oil vehicle or with vehicle alone. Likewise, male rats were castrated and were injected 2 weeks later with either 10 μg of estradiol cyclopentylproprionate, 10 μg of testosterone, or vehicle alone. The animals were sacrificed 7 days later by asphyxiation with CO$_2$, and the relevant tissues were removed rapidly and frozen immediately in powdered dry ice. Tissue cryosectioning and binding of ^{32}P-relaxin followed by autoradiography were performed as described (14). Regions of binding were determined by overlaying the autoradiographs with adjacent sections counterstained with hematoxylin/eosin. Binding was performed on sections from several different regions covering the entire tissue. For relaxin-binding displacement experiments, consecutive 16-μm sections were incubated with 100 pM of ^{32}P-relaxin in the absence and presence of increasing concentrations (serial 3-fold increases) of unlabeled relaxin in the range of 0.1–100 nM. Pseudocolor reconstruction and quantitative analysis of ^{32}P-relaxin binding autoradiographs were performed with a RAS-3000 image analysis system (Amersham). The optical density (OD) or radioactivities (cpm) of bound ^{32}P-relaxin (maximum binding within a particular region) were obtained by computerized densitometry based on parallel sets of ^{32}P-relaxin standards (ranging from 50–2000 cpm) blotted onto nitrocellulose membranes (Trans-Blot SF, Bio-Rad). The binding displacement data were fit to a four-parameter equation to obtain the ED$_{50}$ (the concentration of unlabeled relaxin yielding 50% displacement of the binding of ^{32}P-relaxin). The dissociation constant (K_d) for

Abbreviation: IGF-I, insulin-like growth factor I.
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relaxin was calculated by the method of Cheng and Prusoff (18).

RESULTS

Relaxin Binding Sites in the Rat Heart. Nine different rat tissues, including the liver, spleen, thymus, kidney, adrenal gland, heart, lung, skin, and testis were examined in the present studies for the binding of ^{32}P-relaxin. Of all of these tissues, specific binding was seen clearly in the heart atria (Fig. 1*A*). The ventricles did not show detectable binding under the present experimental conditions. The specificity of binding was demonstrated by the binding displacement of 100 pM ^{32}P-relaxin by 100 nM unlabeled relaxin (Fig. 1*B*) but not by 100 nM IGF-I (Fig. 1*C*), insulin, angiotensin II, and atrial natriuretic peptide (data not shown). Major cell types in the atrial sections included the cardiac muscle, elastic fibers, and fibroblasts, as seen in adjacent sections counterstained with hematoxylin/eosin (data not shown). To which of these cell types the observed binding was attributed was not known from the autoradiographs. However, recent evidence has shown that relaxin increases the rate and force of contraction in isolated rat heart atria, which suggests that the cardiac muscle is likely the site of relaxin action (39). Similar binding seen in the atria of the normal male rat heart (Fig. 1*D*) could also be fully displaced by 100 nM unlabeled relaxin (data not shown). Binding was also seen in the aorta (marked by an arrow in Fig. 1*B*) and other arteries (see Fig. 2*D*) but was not readily displaceable by up to 100 nM unlabeled relaxin. The skin was another tissue where binding was above background

yet could not be fully displaced by up to 100 nM unlabeled relaxin (data not shown).

Affinity of Relaxin Binding to Rat Heart and Rat Uterus. The densities of specific relaxin binding sites in the atrium and uterus were similar, showing maximum binding per unit area (9 mm^2 on the RAS-3000 screen) of 1026 ± 16 cpm and 921 ± 85 cpm, respectively (see the legend to Fig. 2 for quantification of binding intensity), calculated as the mean ± SEM of two separate determinations, each using tissues from different animals. To determine the dissociation constant of relaxin binding, the inhibition of ^{32}P-relaxin binding in the presence of increasing concentrations of unlabeled relaxin was measured. The data yielded a displacement curve consistent with a single class of relaxin binding sites in the rat uterus (Fig. 2*A*) and heart (Fig. 2*B*). While total displacement was achieved in the heart (Fig. 2*B*), partial displacement of 75% was observed in the uterus (Fig. 2*A*). The residual 25% nondisplaceable binding could be due to nonspecific interactions. Fig. 2*D* shows the pseudocolor representation of the binding autoradiographs corresponding to points 1–5 in Fig. 2*B*. The arrow in autoradiograph 5 in Fig. 2*D* points to nondisplaceable binding in the pulmonary artery. The dissociation constant (K_d) for relaxin estimated from two separate regions on consecutive tissue sections from one female rat was 1.3 ± 0.22 nM in the uterus and 1.37 ± 0.13 nM in the heart. The results were repeated in a separate experiment in which the dissociation constant for relaxin in the male rat heart was determined in the same fashion with one male animal, and a similar value (1.53 ± 0.03 nM) was obtained (data not shown).
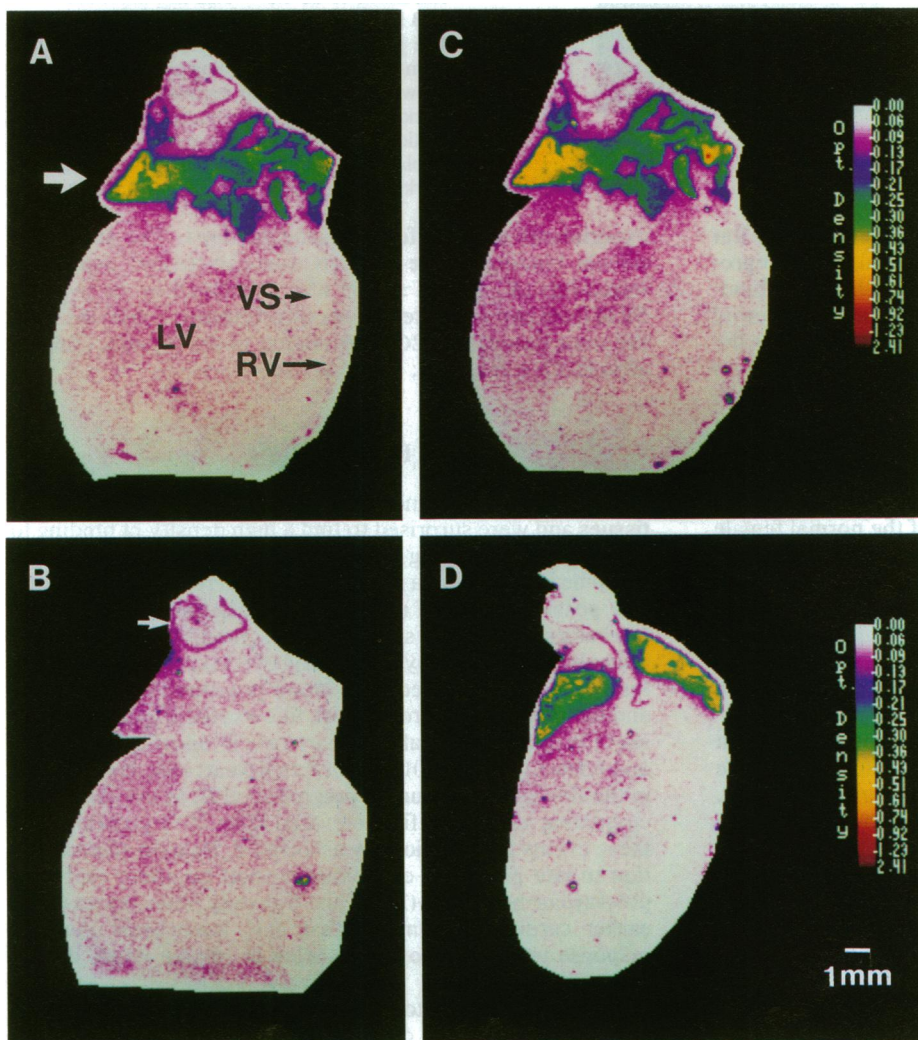

FIG. 1. Pseudocolor representation of the binding of 100 pM ^{32}P-relaxin to female rat heart in the absence (*A*) and presence of 100 nM unlabeled relaxin (*B*) and 100 nM IGF-I (*C*). Binding of 100 pM ^{32}P-relaxin to male rat heart is shown in *D*. Tissue sections shown represent cross sections of the heart. The left ventricles (LV) and right ventricles (RV) are separated by the ventricular septum (VS). Binding to tissue sections and computer-assisted image analysis of the binding autoradiographs were performed as described in text. Low- to high-binding intensities are shown in OD units and represented by a color spectrum from magenta to red. The arrow in *A* points to the regions of atria, and the arrow in *B* points to the aorta, where there appeared to be nonspecific binding of labeled relaxin. See Fig. 4*E* for histology. The same magnification was applied to images *A–D*. (Bar = 1 mm.)
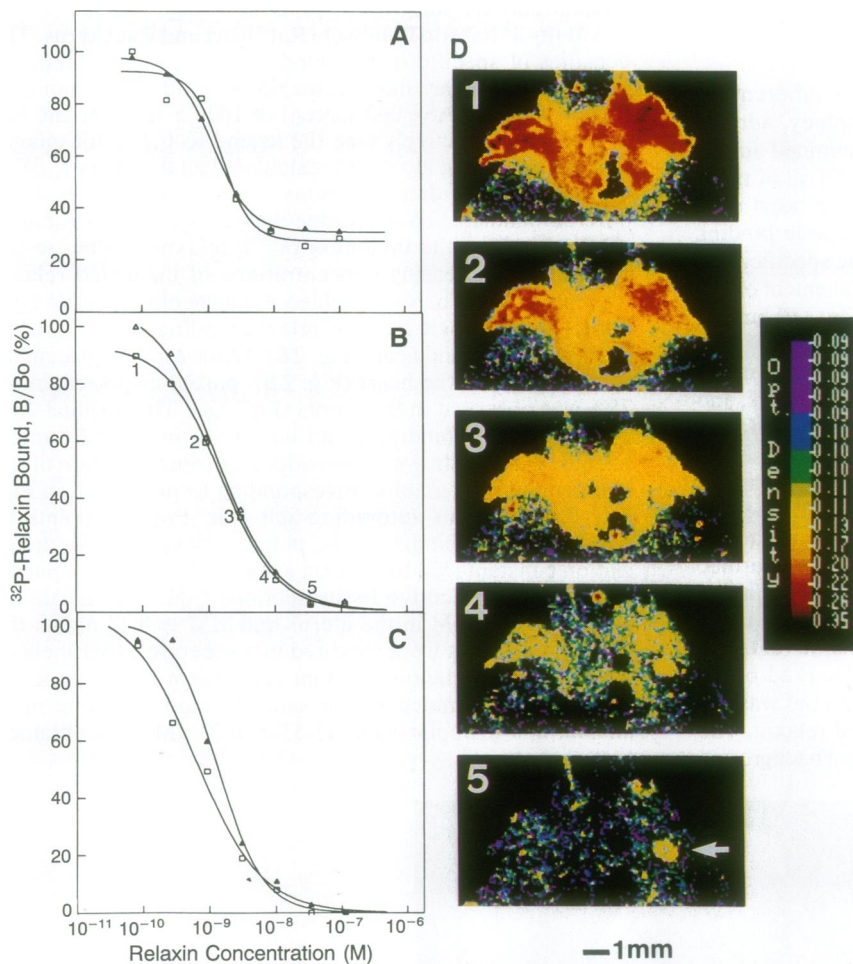
FIG. 2. (*A–C*) Displacement of the binding of ^{32}P-relaxin by unlabeled relaxin in the rat uterus (*A*), the atria of female rat heart (*B*), and the atria of estrogen-treated ovariectomized rat (*C*). Consecutive tissue sections were incubated with 100 pM ^{32}P-relaxin in the absence of unlabeled relaxin (Bo) and the presence of increasing concentrations of unlabeled relaxin (B). Binding intensities were quantified by computerized densitometry of the binding autoradiographs and transformed into optical density (OD) units or radioactivity (cpm). In detail, a square of uniform predetermined size (9 mm^2 on the RAS-3000 screen) was placed on the areas of maximum binding in each particular region. The OD or cpm represents the integrated intensity within that particular square, based on parallel ^{32}P-relaxin standards ranging from 50 to 2000 cpm. Background binding was subtracted from all readings. Binding intensities corresponding to an OD of >0.05 were readily detected and resolved from background binding. Each point represents the mean of the five highest binding areas within each region. The two curves in each panel represent determinations on two separate regions on each tissue. (*A*) ▲, Uterine horn; □, cervix. (*B* and *C*) △, ▲, Right atrium; □, left atrium. The displacement data (expressed as B/Bo % as a function of B) were fit to a four-parameter equation, and the dissociation constant (K_d) for relaxin was calculated to be 1.3 ± 0.22 nM in *A*, 1.37 ± 0.13 nM in *B*, and 0.86 ± 0.35 nM in *C*. (*D*) The points marked 1–5 in *B* are quantified from corresponding binding autoradiographs shown as pseudocolor reconstructions (also marked 1–5) in *D*. Orientation of the heart section in *D* is the same as in Fig. 1. The nondisplaceable binding to the pulmonary artery is marked by an arrow in 5. (Bar = 1 mm.)

Effect of Steroid Hormones on Relaxin Receptors in the Rat Uterus. In view of the close association of relaxin to reproduction, an experiment was performed to evaluate the binding of relaxin under different ovarian status and sex steroid treatment. Fig. 3 shows that specific ^{32}P-relaxin binding in the rat uterus was diminished by 53% overall (quantified over three to five areas of highest binding, each within the regions of uterine cervix and uterine horns) after ovariectomy. However, when the ovariectomized female was treated with estrogen, the ^{32}P-relaxin binding was increased to 90% of the intact level (Fig. 3*E*), while treatment with testosterone had no effect (Fig. 3*F*). Estrogen treatment of the normal female did not affect the ^{32}P-relaxin binding (data not shown) as compared with vehicle control animals. The above data were obtained by using one animal for each condition, but the results were repeated in two other independent experiments, each time using one animal for each condition, except for the testosterone treatment of the ovariectomized female, which was repeated once.

Effect of Steroid Hormones on Relaxin Receptors in the Rat Heart. Castration did not change the ^{32}P-relaxin binding in the female (Fig. 4 *A* and *B*) or male (data not shown) rat heart. Furthermore, estrogen treatment of intact or ovariectomized female rats had no effect on relaxin binding in the heart (Fig. 4 *C* and *D*). These results were obtained from the same groups of animals that showed significant changes in uterine relaxin binding in response to ovariectomy and estrogen treatment (see Fig. 3). Likewise, testosterone treatment of intact or castrated male rats, or estrogen priming of castrated male rats, did not change the atrial binding of relaxin (data not shown). These latter data were repeated in a separate set of

experiments using different tissue sections. The affinity of relaxin binding to the atria in estrogen-treated ovariectomized rats (K_d = 0.86 ± 0.35 nM, calculated as the mean ± SEM from two separate regions on consecutive sections from the same tissue; Fig. 2*C*) was similar to that in intact female atria (1.37 ± 0.13 nM; Fig. 2*B*).

DISCUSSION

In this study we examined the binding of relaxin to various rat tissues and were surprised to find a high density of binding to the heart. The binding was seen clearly in the atria, which might be indicative of a novel function for relaxin in the heart. The possible effect of relaxin on the cardiovascular system has been suggested by several studies, mostly in anesthetized animals (3–8, §), and by the binding of relaxin to brain regions known to be associated with blood pressure, fluid balance, and the release of neuropeptides like oxytocin and vasopressin (15). Relaxin can affect heart rate in anesthetized (7, 8) and conscious rats (40). As maternal heart rate is increased in the first weeks of human pregnancy (19), at the same time circulating relaxin is first elevated (20), it is possible that these events are functionally associated. Relaxin administration to late pregnant, conscious rat had no effect on blood pressure or heart rate (21). This, however, does not exclude earlier cardiac adjustments that may have been made in response to the rat's own elevation in relaxin levels during pregnancy.

Relaxin was shown to modulate the synthesis and secretion of procollagenase and collagen by human dermal fibroblasts
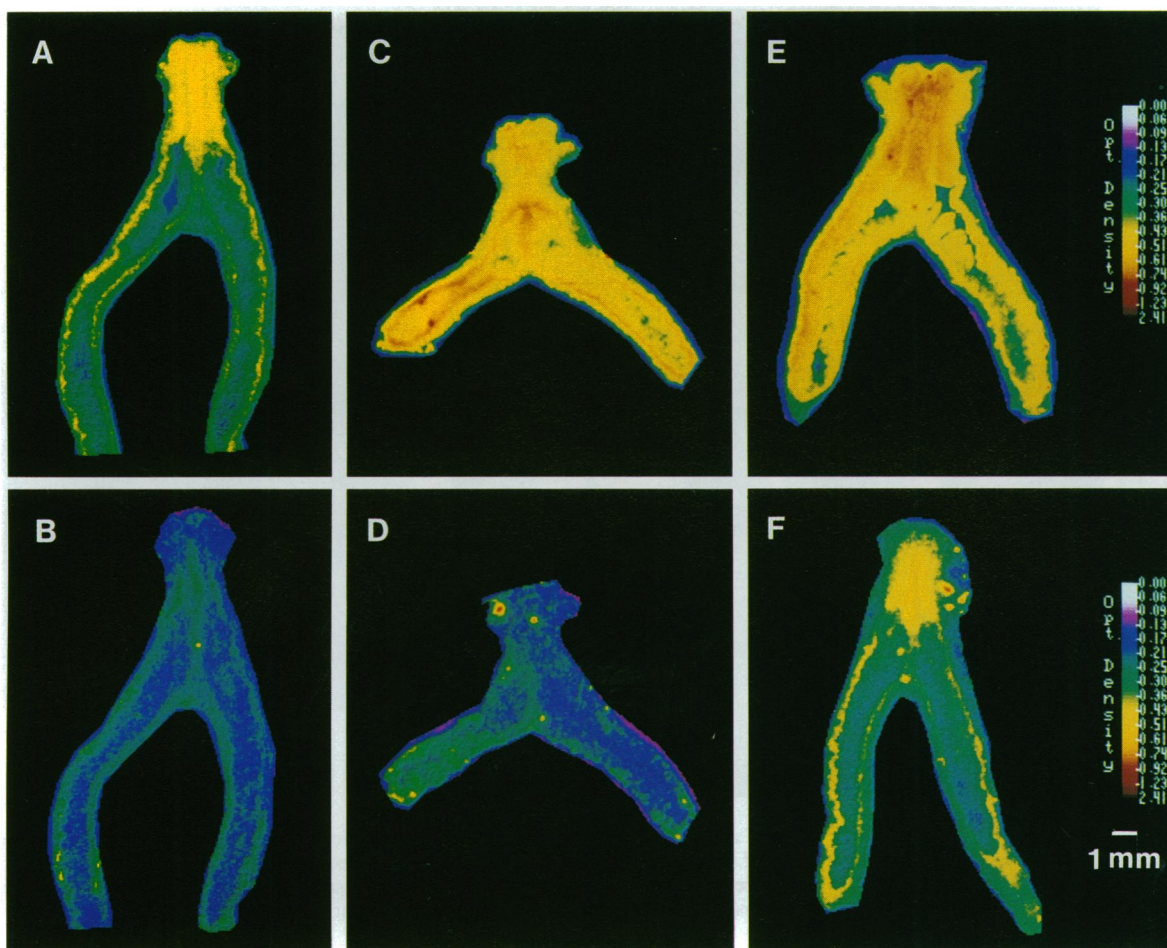
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FIG. 3.   Binding of 100 pM ^{32}P-relaxin in the uterus of an ovariectomized rat (*A*), an ovariectomized rat but in the presence of 100 nM unlabeled relaxin (*B*), a normal intact rat (*C*), a normal intact rat but in the presence of 100 nM unlabeled relaxin (*D*), an ovariectomized rat treated with estrogen (*E*), and an ovariectomized rat treated with testosterone (*F*). The same magnification was applied to images in *A–F*. (Bar = 1 mm.)

(22). In the present study the binding of ^{32}P-relaxin to the rat skin was higher than background, but could not be fully displaced by unlabeled relaxin. Similar nondisplaceable binding was seen in the aorta and other arteries leading into and out of the heart. The reason for this nondisplaceable binding is not clear.

The source of relaxin for the atrial binding sites is unknown at present. Although the highest circulating levels of relaxin are found during pregnancy, detectable levels (30–150 pg/ml) have been reported in the peripheral blood during the non-conceptive luteal phase in humans (20). Circulating levels of relaxin have not been established in the male. However, immunoreactive and biologically active relaxin has been reported in human seminal plasma (average 45 ng/ml) with the prostate as the likely source (23–25). The expression of H1 as well as H2 human relaxin genes (1) in the heart also cannot be ruled out.

The specificity and affinity of relaxin binding to the atrium, uterus, and brain (15) were similar to one another. A significant decrease in uterine relaxin binding after ovariectomy and restoration of such binding upon estrogen treatment suggested that uterine relaxin binding was estrogen dependent. Other examples of steroid hormone modulation of polypeptide growth factor receptors are the epidermal growth factor receptors and IGF-I receptors (26). A different picture existed in the heart. The affinity of relaxin binding in the male was indistinguishable from that in the female atrium and similar to the picture in the rat brain (15). Furthermore, the relaxin binding in the atrium was independent of sex steroids.

These data demonstrated that the regulation of relaxin receptors was tissue specific. Whether this represents different receptor subtypes or the presence of steroid response element (27) or other steroid-dependent regulatory elements (28) remains to be elucidated. It is also known that certain polypeptide hormones and growth factors have the ability to bind to the same family of receptors which share regions of amino acid sequence identity. Among these are the insulin receptor/IGF-I receptor family (29, 30) and the growth hormone/prolactin receptor family (31–33). We also have found that a synthetic human H1 relaxin analog (deletion of the C-terminal Lys-Arg-Ser-Leu of B chain) could displace the binding of ^{32}P-relaxin with a dissociation constant very similar to that of H2 relaxin (unpublished results of PLO). The crossreactive binding of these peptide hormones to other members of the receptor family raises the possibility that relaxin may display tissue-specific binding to additional receptor(s), possibly of the insulin receptor family (34–38), with either higher or lower affinities. Thus, the discovery of these relaxin binding sites in the brain and heart may uncover a complex biology in several organ systems. Isolation or expression cloning (e.g., by ^{32}P-relaxin binding) of the receptors from uterus, heart, or brain will help to elucidate the observed differences in relaxin receptor regulation.
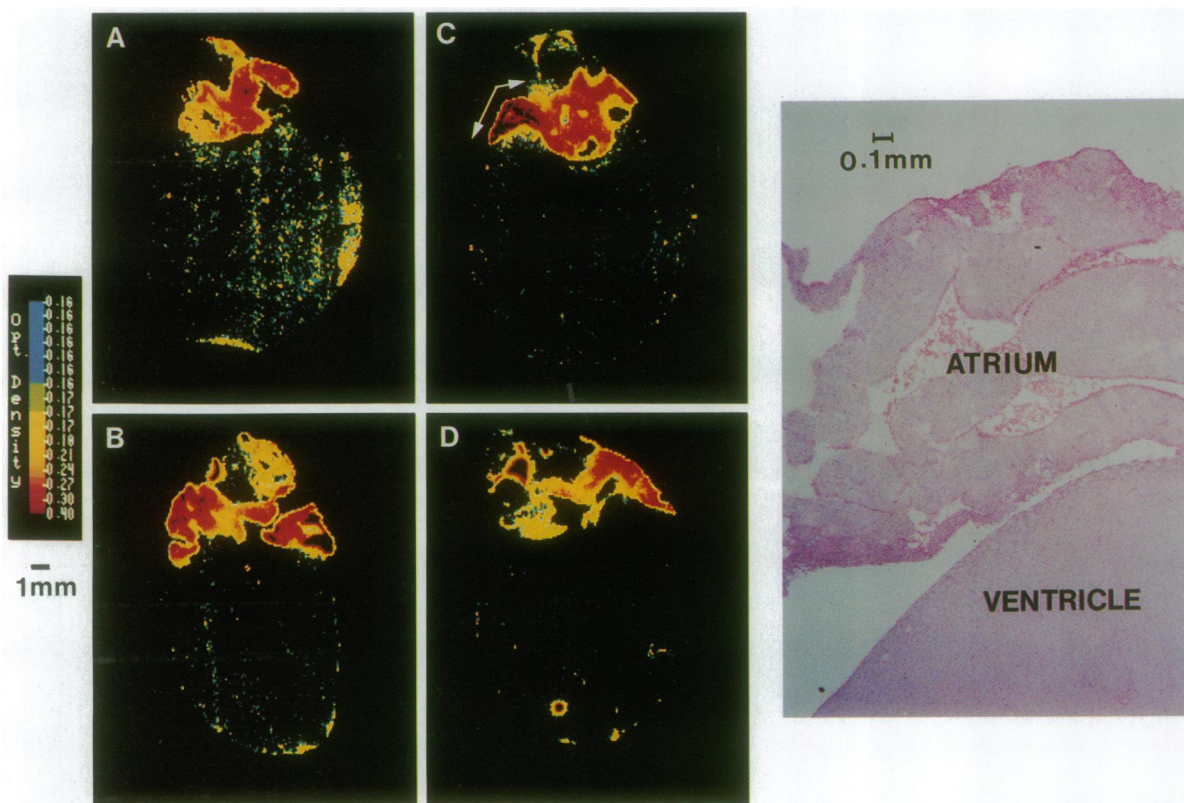
FIG. 4.   Binding of 100 pM ^{32}P-relaxin in the atria of an ovariectomized female rat (*A*), an ovariectomized female treated with estrogen (*B*), a normal intact female (*C*), and a normal female treated with estrogen (*D*). The enhanced color spectrum is shown in *D*. The orientation of the heart section is the same as in Fig. 1 except in *B*, where the right atrium and left atrium are reversed. The same magnification was applied to *A–D*. (Bar = 1 mm.) (*E*) Portion of an hematoxylin/eosin-stained female rat heart section that corresponds to the region marked by ↔ in *C*. (Bar = 100 μm.)

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