Regulation of Lipoprotein Synthesis

Studies on the Molecular Mechanisms of Lipoprotein Synthesis and Their Regulation by Estrogen in the Cockerel

LAWRENCE CHAN, RICHARD L. JACKSON, AND ANTHONY R. MEANS

SUMMARY We used the estrogen-treated cockerel as a model to study the regulation of very low density lipoproteins (VLDL) at the molecular level. A single injection of estrogen induced marked elevation of plasma VLDL in the cockerel. Messenger RNA (mRNA) activity for a major VLDL apoprotein (apoVLDLα) in hepatic polyribosomes was assayed in vitro, and increased at the same rate as plasma VLDL levels. Simultaneous determinations of mRNA activities for albumin and apoA-I (a major HDL apoprotein) showed that these were unchanged. Specific estrogen-binding sites were measured in the liver cell nuclei and a single class of sites with a Kd of 2 x 10^-9M was observed. The number of such sites increased after estrogen treatment prior to any detectable increase in plasma VLDL. Simultaneously, RNA polymerase I and II activities were markedly stimulated by the hormone. ApoVLDLα mRNA was purified to apparent homogeneity by (1) total nucleic acid extraction, (2) zonal ultracentrifugation, (3) Sepharose 4B chromatography in EDTA, (4) Sepharose 6B chromatography, (5) Sepharose 4B chromatography in MgCl₂, and (6) sucrose gradient centrifugation. The in vitro translation product of apoVLDLα mRNA was about 12 amino acids larger than apoVLDLα isolated from the blood. The identification of such a product (designated pre-apoVLDLα) is compatible with Blobel’s hypothesis that, like other secretory proteins, VLDLα is synthesized initially as a larger protein and the N-terminal sequence is cleaved prior to completion of synthesis and secretion of the VLDL particle.

ALTHOUGH a large number of studies have characterized the plasma lipoproteins physically and chemically, little has been reported on the molecular mechanisms involved in lipoprotein synthesis. We have studied the cellular events in the synthesis of very low density lipoproteins (VLDL). The system developed to study these processes is the estrogen-treated cockerel. Non-laying hens or cockerels have only small amounts of VLDL. However, at the onset of egg production or after administration of estrogen, there is a dramatic increase in plasma VLDL followed by the development of atherosclerosis in the animal.²³ Thus, estrogen is a well-defined stimulus to the development of hyperlipoproteinemia and a useful tool in our studies of the regulation of VLDL synthesis in this animal. Furthermore, it is now well known that estrogen-containing contraceptives, VLDLα is synthesized initially as a larger protein and the N-terminal sequence is cleaved prior to completion of synthesis and secretion of the VLDL particle.

Methods

Animals

Three-week-old white Leghorn cockerels (200 g) were treated with a single injection of estrogen (estradiol-17β in propylene glycol or diethylstilbestrol in sesame oil) at the dose described in the figure legends. Estradiol was injected intramuscularly in the thigh, and diethylstilbestrol, subcutaneously under the left wing.

Purification of Plasma Apolipoproteins

Plasma apoVLDLα was purified from hen plasma as previously described.⁷ Briefly, the procedure consists of ultracentrifugal flotation at plasma density. The VLDL fraction was recentrifuged twice at a density of 1.006 g/ml. After lyophilization, the lipoprotein was delipidated by extracting twice with diethyl ether-ethanol (3:1) at 4°C followed by two washes with diethyl ether. Apo-VLDL were solubilized in 0.1 M Tris-HCl, pH 8.2, containing 0.1 M sodium decyl sulfate (SDS) and were fractionated on Sephadex G-150 and DEAE-c as previously described.⁷ Plasma apoA-I was isolated from hen plasma as previously described.⁴ Total high density lipoproteins (HDL) were isolated from plasma by ultracentrifugal flotation between densities 1.063 and 1.210 g/ml. After delipidation, the lipid-free proteins were fractionated by chromatography on Sephadex G-150 in urea; one major apo-
lipoprotein was isolated which had characteristics quite similar to the major apoprotein of human HDL, apoA-I, and the avian protein was similarly designated apoA-I.  

Plasma vitellogenin was purified from hen plasma as previously described. Hen plasma was collected and the VLDLs were removed by ultracentrifugation. The infranatant fraction then was subjected to DEAE-cellulose chromatography. Hen egg phosvitin was purified by an aqueous extraction of delipidated egg yolk granules, followed by DEAE-cellulose chromatography, as previously described.

Chick plasma albumin was purchased from Sigma Company and was found to be homogeneous and similar to cockerel plasma albumin by SDS gel electrophoresis.

Preparation of Antisera against Plasma Proteins

Antisera against apoVLDL₄₄, apoA-I, phosvitin, and chick albumin were prepared by injection of the pure protein into goats (for apoVLDL₄₄, apoA-I, and phosvitin) and rabbits (for albumin), respectively. The antisera were purified by ammonium sulfate precipitation (0-50%) twice. Antiserum specificity in all cases was determined by immunodiffusion on Ouchterlony plates against total plasma, as well as against the purified proteins. Antisera against apoVLDL₄₄, apoA-I, and chick albumin were monospecific for these proteins, whereas that against phosvitin cross-reacted well against vitellogenin, as we have previously reported.

Immunoprecipitation and Rocket Immunoelectrophoresis

Specific immunoprecipitation of the various plasma proteins was accomplished by incubation at room temperature for 30 minutes followed by incubation at 4°C overnight. The antigen-antibody complex was routinely purified from contaminating proteins by low-speed centrifugation through a cushion of 1 M sucrose, as previously described. Protein content in the immunoprecipitates was determined by the method of Lowry et al., and in the case of vitellogenin, phosphorus content was determined by the method of Bartlett. In instances in which counting of the immunoprecipitate was done, the pellet was dissolved in NCS (Amersham-Searle Co.); ScintiVerse (Fisher Scientific Co.) was added and samples counted by liquid scintillation spectrometry. Rocket immunoelectrophoresis was performed by the method of Laurell.

Preparation and Analysis of Hepatic Polyribosomes and Polyribosomal RNA

Cockerel liver polyribosomes were prepared by the method of Jost and Pehling. For analysis of polysomal profiles, the polyribosomes were layered on 0.5-1.2 M linear sucrose gradient in 25 mM NaCl, 50 mM Tris-HCl, pH 7.7; 5 mM MgCl₂, heparin Na, 0.5 mg/ml; and the gradients were centrifuged at 40,000 rpm at 4° for 100 minutes in a Beckman SW40 rotor. Gradients were unloaded from top to bottom by a pump, and UV absorbance at 254 nm was monitored by means of a UA-5 UV monitor (Instrument Specialties Co.). Polysomal RNA was extracted from the polysome pellets by a phenol-SDS method, as previously described.

Purification of ApoVLDL₄₄ mRNA

The following procedures were carried out in sequence in the purification of apoVLDL₄₄ mRNA: (1) Total nucleic acid extraction was performed on estrogen-primed cockerel livers, as previously described. Zonal ultracentrifugation was performed in a Beckman Ti50 zonal rotor in a 1-20% sucrose gradient in 40 mM Tris acetate, pH 7.2, 20 mM Na acetate, 2 mM EDTA, 1% SDS. Centrifugation was at 30°C at 32,000 rpm for 18 hours. (2) Sepharose 4B chromatography was performed in 100 mM Na acetate, 1 mM EDTA, pH 5.0, as previously described. (3) Sepharose 6B chromatography was performed under the same buffer conditions. (4) Sepharose 4B chromatography was repeated by the method of Konecki et al., in 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, pH 7.5. Sucrose gradient centrifugation was performed on 5-10% sucrose gradients in 1% SDS in 40 mM Tris-HCl, pH 7.0, 2 mM Na acetate, 5 mM EDTA. The centrifugation was run in an SW40 rotor at 20°C for 16 hours at 39,000 rpm.

Each step of purification was monitored by: (1) agarose gel electrophoresis in 7 M urea, as described by Rosen and Monahan. (2) in vitro protein synthesis in a wheat germ system and analysis of product, as described by Chan et al. The mRNA was assumed to be pure when it sedimented as a single peak on sucrose gradient, ran as a single band on agarose gel as well as acrylamide gel in 99% formamide, and when 100% of the in vitro translation product was identified as apoVLDL₄₄.

Slab Gel Electrophoresis and Fluorography

Slab gel electrophoresis was performed in 12% polyacrylamide gel in 0.5 M urea and 0.4% SDS in 1.5 mM Tris-HCl, pH 8.8. Electrophoresis was performed for 4 hours at 20°C. Fluorography of methionine[^35S] labeled in vitro translation product was performed by exposure to x-ray film, as described previously.

Specific Nuclear Binding for Estrogen

Liver cell nuclei were prepared from cockerels at various times after estrogen treatment by the method of Schibler and Weber. Specific binding sites for estradiol[^3H] were determined by the estradiol[^3H] exchange assay of Anderson et al. Incubations were carried out at 30°C for 30 minutes in the presence of 0.4-12 nM estradiol[^3H] with or without a 100-fold excess of unlabeled diethylstilbestrol (DES). Binding data were analyzed by the method of Scatchard.

Sucrose Gradient sedimentation profile of estrogen receptor

Nuclei were isolated from DES-injected animals (16 hours) and incubated with 12 nM estradiol[^3H] as described in the last section. They were extracted with 0.4 M KCl at 4°C for 30 minutes then with charcoal to remove unbound radioactivity. Samples were layered on 5-20% linear sucrose gradients in 0.01 M Tris-HCl, 15 mM EDTA,
Effects of Estrogen on Plasma Proteins

To determine the specificity of estrogen action, four plasma proteins were measured before and after hormone treatment. These included the plasma lipoproteins VLDL and HDL, the phosphoprotein vitellogenin, and albumin.

Chicken VLDL apoproteins have been fractionated and shown to contain a high molecular weight fraction which is similar to human apoLDL (apoB), and a low molecular weight protein. The primary amino acid sequence of the low molecular weight protein has been determined and has been designated apoVLDL\textsubscript{a1}. It consists of two polypeptides of identical sequence which are linked by a disulfide bond at cystine-76. Chicken HDL is very similar to human HDL in that it contains one major protein, apoA-1. The protein has a weight of 28,000, and preliminary amino acid sequence studies show that it is similar to apoA-1.\textsuperscript{a} Vitellogenin is a phosphoprotein consisting of 3% phosphorus and has a molecular weight of 240,000.\textsuperscript{b, c}

The concentrations of apoA-1 and albumin were determined on delipidated plasma by rocket immunoelectrophoresis. As shown in Figure 1, A and B, after a 5 mg injection of estradiol-17\textbeta, there was a marked increase in the plasma levels of vitellogenin and apoVLDL\textsubscript{a1}. Vitellogenin concentration was increased within 5 hours of hormone treatment from undetectable levels to 0.3 mg/ml at 5 hours, and to 9.2 mg/ml at 30 hours. As shown in Figure 1B, plasma apoVLDL\textsubscript{a1} also increased rapidly following estrogen administration. The increase in apoVLDL concentration was followed by a marked increase in plasma triglyceride levels (Table 1). The concentrations of albumin and apoA-1, in contrast, were relatively unaltered (Fig. 1B). Thus, a single injection of estrogen appeared to elicit a specific response, i.e., the selective augmentation of apoVLDL\textsubscript{a1} and vitellogenin levels without significant effects on the levels of two other major plasma proteins. Since the plasma concentration of a protein is a balance between the rate of its synthesis and that of its removal, we have undertaken studies on the rate of synthesis of vitellogenin and apoVLDL\textsubscript{a1} in the intact animal.

Effects of Estrogen on Vitellogenin and apoVLDL\textsubscript{a1} Synthesis

With the specific antiserum prepared against vitellogenin and apoVLDL\textsubscript{a1}, it was possible, by immunochromatographic techniques, to determine the effects of estrogen on the synthesis of the two proteins in the intact animal. As shown in Figure 2, estrogen treatment resulted in a rapid increase of radioactivity incorporated into vitellogenin from undetectable levels to \(10^7\) counts/min per ml plasma. Similarly, the radioactivity incorporated into apoVLDL\textsubscript{a1} was increased 10-fold in 30 hours. Because the trichloro-

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**Table 1** Plasma Triglyceride in the Cockerel after Intramuscular Estradiol-17\textbeta (5 mg)

<table>
<thead>
<tr>
<th>Time (hrs)*</th>
<th>Triglyceride (mg/100 ml plasma)</th>
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<tbody>
<tr>
<td>0</td>
<td>162</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
</tr>
<tr>
<td>18</td>
<td>594</td>
</tr>
<tr>
<td>48</td>
<td>2780</td>
</tr>
</tbody>
</table>

*The early depletion of triglyceride is highly reproducible and varies with the dose of estradiol administered. Whereas the triglyceride level shows an initial decrease, apoVLDL actually started increasing during this time (see Fig. 1B). Plasma triglyceride was measured by autoanalyzer methods. Each value represents results from plasma pooled from four different experimental animals.

* After estradiol.
EFFECTS OF ESTRADIOL ON PLASMA VITELLOGENIN

**Figure 1**
A: Plasma vitellogenin concentration after estradiol. Each point represents results on plasma pooled from four different experimental animals. Plasma vitellogenin concentration was determined by specific immunoprecipitation with a specific antibody against phosvitin at different times after a single injection of estradiol-17β (5 mg). Protein content was determined by the method of Lowry et al., and the phosphorus content by the method of Bartlett.

B: Plasma apoVLDL, albumin, and apoA-I after estradiol. Each point represents results on plasma pooled from four experimental animals. Plasma apoVLDL level was determined by specific immunoprecipitation. Plasma albumin and apoA-I levels were determined by rocket immunoelectrophoresis.

**Figure 2**
Effects of estrogen on vitellogenin and apoVLDL synthesis. Four 3-week-old cockerels were treated with a single intramuscular injection of estradiol-17β (5 mg). Three hours before being killed, each animal received an intraperitoneal injection of 1 mCi of L-leucine (4,5-²H) (specific activity, 46 Ci/mmol). At the times indicated, the cockerels were killed by decapitation and blood was collected and pooled. Immunoprecipitable radioactivity was determined by precipitation of samples of delipidated plasma with specific antisera against phosvitin or apoVLDL. The technique for immunoprecipitation was as previously described.

Acetic acid-soluble counts were comparable, changes in pool-size or permeability were unlikely to be the cause of the estrogen effects on these proteins. Hence, estrogen appears to stimulate the synthesis of vitellogenin and apoVLDL.

This observation in vivo is a direct confirmation of our previous studies of the effects of estrogen on VLDL synthesis in liver slices in vitro. In the latter situation, we have shown that actinomycin D, an RNA synthesis inhibitor, completely inhibited the effects of estrogen on VLDL biosynthetic rate. This observation suggested that the hormone regulates VLDL synthesis by increasing the transcription of the specific VLDL mRNAs. To obtain a direct demonstration of the selectivity of the effect of estrogen on apoVLDL mRNA activity, we have purified liver polyribosomes at various times after hormone treatment. RNA was extracted from the isolated polysomes and tested for specific mRNA activities for apoVLDL, albumin and apoA-I (Fig. 3 and Table 2). Estrogen markedly stimulated apoVLDL mRNA activity without significantly affecting apoA-I mRNA activity (Table 2). Albumin mRNA activity appears to show a slight de-
crease in its specific activity or no noteworthy change in total activity (Fig. 3).

**Purification of apovLDL<sub>41</sub> mRNA**

The specific mRNA for apovLDL<sub>41</sub> was purified by the following steps: (1) total nucleic acid extraction of estrogen-treated cockerel liver, (2) zonal ultracentrifugation, (3) Sepharose 4B chromatography, (4) Sepharose 6B chromatography, (5) Sepharose 4B chromatography under a different salt condition as in (3) and, finally, (6) sucrose gradient ultracentrifugation. The gel electrophoretic pattern of the RNA for each of the above steps is shown in Figure 4. As shown in gel pattern F, the purified mRNA showed a single band on electrophoresis in acid urea. The RNA sediments at 6-7S on sucrose gradients and had an electrophoretic mobility compatible with an apparent molecular weight of about 150,000. It thus contains approximately 400 nucleotides.

The various steps of RNA purification were monitored by translation of the various fractions in a heterologous protein synthesis system prepared from wheat germ. When the purified mRNA was translated in this system, using methionine<sup>135S</sup>, the product analyzed on SDS-urea slab gel by fluorography, a single band of radioactivity was detected. As shown in Figure 5, the in vitro translation product, designated pre-apoVLDL<sub>41</sub>, migrated on the slab gel as a single band with a molecular weight of approximately 11,000. The purified apoVLDL<sub>41</sub>, which has a molecular weight of 9,400, migrated ahead of the in vitro product. The difference in molecular weights of the two species was also observed when the immunoprecipitated in vitro translation product from a less pure apoVLDL<sub>41</sub> mRNA fraction was similarly analyzed. This observation indicates that the mRNA codes for a protein which is about 10-12 residues longer than the protein isolated from plasma. If the pre-apoVLDL<sub>41</sub> is truly analogous to precursors of other secretory peptides, the extra amino acid sequence is located at the amino-terminal. 25

We are currently carrying out primary amino acid sequence analysis, starting from the N-terminal of the pre-apoVLDL<sub>41</sub>.
Effects of Estrogen on Gene Transcription

Thus far, we have presented evidence that estrogen administration leads to a selective increase in VLDL synthesis. The hormone appears to act at the transcriptional level, stimulating the accumulation of the specific mRNA for the apoprotein. To fully understand the mechanism by which such stimulation occurs, we have examined other biochemical events which take place in the liver nucleus following the administration of estrogen.

First, we examined the number of specific nuclear binding sites for the hormone. Liver cell nuclei isolated from cockerels treated with 2.5 mg of DES 16 hours prior to sacrifice demonstrated a 7-fold increase in specific estrogen-binding sites compared to nuclei from untreated controls (Fig. 6). These sites were specific and were competed with by estrogenic hormones (100% with estradiol-17β and DES, and 67% with estrone and estriol) but not by nonestrogenic hormones like progesterone, corticosterone, or testosterone. When the binding data were analyzed according to the method of Scatchard, the nuclei from the treated cockerels contained a single class of high-affinity binding sites with an apparent dissociation constant of $2 \times 10^{-9}$ M (Snow et al., manuscript in preparation). To characterize the nuclear estrogen receptor, the estradiol[$^3$H] receptor complexes were extracted from liver nuclei of DES-treated cockerels with 0.4 M KCl. Sucrose gradient sedimentation analysis of the KCl-extractable complex in a 5-20% linear sucrose gradient showed a peak of radioactivity sedimenting at the 6S region (Snow et al., manuscript in preparation). The presence of 100-fold excess of unlabeled DES completely abolished this peak. These observations suggest that, in the liver cell nuclei, estrogen is bound specifically to sites with high affinity but relatively low capacity. The extractable estrogen receptor complex behaves like a macromolecule with sedimentation characteristics similar to those from other estrogen target cell nuclei.

We next examined the effect of estrogen on endogenous RNA polymerase I and II activities in the cockerel liver. These enzymatic activities were measured in purified liver nuclei at various times after hormone treatment. As shown in Figure 6, estrogen treatment results in marked stimulation of both polymerase I and II activities, which remained at a plateau for at least 24 hours.

To examine whether estrogen treatment "opens up" additional initiation sites for RNA synthesis in liver cell chromatin, we next studied the effect of the hormone on the number of RNA synthesis initiation sites. As shown in Figure 6, the number of RNA synthesis initiation sites...
is increased by 88% within an hour of estrogen treatment. It stays at a stimulated level over control for at least 24 hours after hormone administration.

Discussion

Studies on the regulation of lipoprotein metabolism have been hampered by the lack of suitable experimental models. We believe that the estrogen-treated cockerel is a valuable model for such studies. Estrogen has been used as an experimental tool because the hormone induces hyperlipoproteinemia due predominantly to VLDL in both man and the cockerel. We have studied the mechanism by which estrogen "turns on" the transcription of the apoVLDL1 gene: the hormone appears to bind first to specific receptors in the liver cell nuclei, resulting in (1) enhanced RNA polymerase I and II activities, (2) increase in number of RNA synthesis initiation sites, and (3) accumulation of apoVLDL mRNA. This is followed by an increased rate of VLDL synthesis and, finally, by hyperlipoproteinemia. In addition to estrogen, a number of other factors result in hyperlipoproteinemia. For example, cholesterol feeding leads to hypercholesterolemia and an elevation of a number of apoproteins, especially arginine-rich lipoprotein, in the pig.\textsuperscript{29} Studies similar to those we have performed for the estrogen-induced hyperlipoproteinemia will help dissect out the specific site(s) of regulation of such factors on lipoprotein metabolism.

The isolation and translation in vitro of eucaryotic lipoprotein mRNA was initially performed in our laboratory a year ago.\textsuperscript{7} In the meantime, we have completed the purification of the apoVLDL1 mRNA. It is the first lipoprotein purified by mRNA to apparent homogeneity. There are a few interesting features of this RNA. It is about 400 nucleotides long. Assuming that the length of the structural gene for pre-apoVLDL1 (approximately 94 amino acids) is 282 nucleotides, the specific mRNA appears to contain stretches of nontranslated sequence totaling over 100 nucleotides. Nontranslated sequences have been reported essentially in all eucaryotic mRNAs.\textsuperscript{30-33} These sequences are located at both the 5' and 3' ends of the coding sequence. Another potentially significant observation we have made during the purification of the apoVLDL1 mRNA is the identification of a precursor molecule to the apoprotein. On the basis of observations on a number of secretory proteins, Blobel and coworkers\textsuperscript{35, 36} postulated that there are intracellular precursors to most, if not all, of these proteins. According to their "signal" hypothesis, segregation of specific secretory proteins in the rough endoplasmic reticulum is accomplished by a metabolically short-lived "signal" sequence in the nascent polypeptide chain. This unique sequence would result from the translation of codons located in immediate proximity to the 3' end of the initiation codon. Once the signal sequence "leads" the nascent polypeptide chain across the endoplasmic membrane, it is removed by a specific peptidase and no longer present in the finally secreted molecule. Since lipoproteins are secretory proteins, it is quite possible that they contain the signal sequence. The size of the in vitro apoVLDL1 mRNA translation product is compatible with such a hypothesis.

On the basis of our studies in the cockerel, we can now construct a model for the regulation of lipoprotein synthesis in the liver (Fig. 7). Estrogen and, possibly, other lipoprotein-regulating agents appear to bind to specific receptors in the liver. It is likely, but not proven, in the cockerel liver that such receptors exist in the cytoplasm. The receptors are then translocated into the cell nucleus where they associate with the chromatin. This association results in the enhancement of RNA polymerase I and II activities and the stimulation of specific gene transcription. As a consequence, the synthesis of VLDL mRNA and probably of some other specific mRNAs is stimulated. The VLDL mRNA is exported into cytoplasm where its translation is initiated on free ribosomes. Translation of the initial "signal" codons results in a unique sequence of amino acid residues on the amino terminal of the nascent VLDL chain. Emergence of this signal sequence is thought to result in attachment of the ribosomes to the rough endoplasmic membrane. Translation then continues on the bound ribosomes. The signal sequence, still attached to the nascent protein, is thought to be cleaved by a specific peptidase.

On completion of translation of the mRNA, the apoVLDL is released and vectorially discharged.\textsuperscript{35, 36} The apoVLDL then binds triglycerides which are synthesized mainly in the smooth endoplasmic reticulum.\textsuperscript{36, 37} The VLDL are transported to the Golgi apparatus and are concentrated in secretory vesicles. They are finally secreted by fusion of the vesicular membrane with the plasma membrane of the hepatocyte.

Different parts of the proposed model are entirely speculative. However, with the availability of the pure apoVLDL1 mRNA, the pure apoVLDL and preapoVLDL, reconstitution experiments can be performed and many of the hypothetical steps in lipoprotein synthesis and assembly can be directly tested. Furthermore, the
mechanism of other lipid-modifying agents can be studied using experimental designs which we have developed for estrogen.

Acknowledgments

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References

Phase-Related Sensitivity of the Sinoatrial Node to Vagal Stimuli in the Isolated Rat Atrium

SHERRY L. STUESSE, MATTHEW N. LEVY, AND HARRISON ZIESKE

SUMMARY In isolated rat atria, endogenous neurotransmitters were released by electrical pulses that were below threshold for activation of the myocardial cells. A brief train of pulses was delivered at a specified time after each atrial activation to determine the relationship between the time of stimulus delivery during an atrial cycle (P-St interval) and the subsequent cardiac cycle length (P-P interval). These stimuli caused a change in P-P interval from a mean control level of 592 msec to a mean maximum P-P interval of 527 msec. This change was attributed to the release of neurotransmitters from sympathetic and parasympathetic fibers, but the effects from the parasympathetic nerve terminals predominated. Increasing the number of pulses per burst increased the atrial slowing. The extent of slowing was dependent on the time of stimulus delivery in an atrial cycle. The region of minimal effectiveness (mean P-P interval of 412 msec during stimulation) was obtained with P-St intervals that were only slightly greater (about 10 msec) than those that were maximally effective. Stimuli delivered at times in the cardiac cycle that fell between the P-St intervals that elicited maximum and minimum changes in heart rate occasionally caused profound irregularities in heart rate. These changes in cycle length do not appear to be due to pacemaker shifts, but are probably the result of the interaction between acetylcholine and the sinoatrial nodal cell membrane. The responsiveness of this membrane to acetylcholine changes with time during the cardiac cycle.

SEVERAL laboratories have demonstrated that heart rate varies with the time of vagal stimulus delivery during a cardiac cycle.1-7 Over a certain range of stimulation frequencies, an increase in frequency caused an increase in heart rate instead of the expected decrease (the "paradoxic" effect of vagal stimulation). Previous experiments have used whole animals, and the vago stimuli were applied to the SA nodal region, resulting in a considerable distance from the heart. The underlying basis for this phase dependency of neural stimulation is still unresolved.

Endogenous neural transmitters can be released from the heart by direct electrical stimulation at voltages that do not excite the myocardial cells.8 We have applied this technique of neurotransmitter release to investigate the phase-dependent sensitivity of the sinoatrial node to acetylcholine in the isolated right atrium of the rat. With this preparation the effect of selective stimulation of the nerve fibers in the sinoatrial (SA) nodal region itself may be examined, and the factor of variable neural conduction times encountered during stimulation of the vagal trunks in the neck is avoided. Very brief bursts of subthreshold stimuli were applied to the SA nodal region, resulting in the release of small amounts of endogenous neurotransmitter during a cardiac cycle. The increase in cycle length was related to the time of stimulus delivery. This phase dependency is characterized in this paper, and it is compared with the phase dependency elicited by cervical vagal stimulation9 in intact dogs.

Methods

Atrial Preparation

White male rats (300-500 g) were injected, ip, with sodium pentobarbital (3 mg/100 g). The heart was excised.
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