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Changes in Hormone Levels and Gap Junctions in the Rat Uterus During Pregnancy and Parturition

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ABSTRACT

To study the temporal relationship between changes in hormone levels with the development of myometrial gap junctions, rats were sacrificed to collect uterine vein blood and uterine tissues daily from Day 15 to Day 20 of pregnancy, every 3 h on Day 21, during delivery, and 3 h after parturition. Levels of progesterone, estradiol and prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$) were measured by specific radioimmunoassay methods, and myometrial gap junctions were measured by quantitative electron microscopy. Levels of progesterone declined markedly on Day 19 of pregnancy, at least 60 to 70 h before the expected time of parturition and the decrease was more rapid in uterine tissue than in plasma. Levels of estradiol started to increase after Day 19, showed a marked rise on Day 21, and peaked at the time of parturition. Levels of $PGF_{2\alpha}$ also increased rapidly after Day 20, when progesterone levels had already dropped by 67% and 84% of Day 15 levels in plasma and tissue samples, respectively. Gap junctions, identified as 5- or 7-lined structures with a gap of about 2 nm at 100,000X magnification were present in the pregnant uterus. However, their number, size and area were increased significantly on Day 21 and during parturition. Increased numbers of gap junctions were still present in rats killed 3 h after parturition. These studies demonstrate that a decrease in progesterone levels followed by increases in estradiol and $PGF_{2\alpha}$ are coincident with the formation of gap junctions which in turn may coordinate the increased uterine activity required for parturition.

INTRODUCTION

The development of labor in pregnant animals is associated with a series of hormonal changes that proceed sequentially to achieve normal parturition (Nathanielsz, 1978; Thorburn and Challis, 1979; Challis and Mitchell, 1981). The decrease in progesterone levels followed by increases in estrogens and prostaglandins have been suggested as the hormonal events responsible for upsetting the regulatory balance which maintain pregnancy (Csapo, 1981; Challis and Mitchell, 1981). These changes in the hormonal balance between uterine suppressants and stimulants prior to term are thought to convert the relaxed and refractory uterus to an active and reactive organ which expels the products of conception (Csapo, 1981). However, little is known of the molecular changes which occur in the uterine muscle cells to result in synchronized and coordinated contractions which facilitate parturition.

Recently several electron microscopic studies (Garfield et al., 1977, 1978, 1979a,b; Garfield and Hayashi, 1981) have shown that gap junctions between myometrial cells are greatly increased in size and number in tissues taken from animals and humans during labor than present earlier in pregnancy. Other studies have shown that progesterone inhibits, whereas estrogens and prostaglandins may be necessary for, gap junction formation in the myometrium during labor (Garfield et al., 1980a, 1982). In sheep there is good correlation between changes in the steroid hormones prior to term and the

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appearance of increased gap junctional area (Garfield et al., 1979b). It is likely that the hormonal changes that occur prior to parturition precipitate the formation of the gap junctions, and the development of increased gap junctional area during labor is believed to be the structural basis for the conversion of uterine muscle from the inactive to active state. However, the exact sequence of changes in steroid hormones, prostaglandins, and gap junctions is not known.

In this quantitative study, we have determined by electron microscopy the number, size and area of gap junctions in pregnant, parturient and postpartum rat uterus. Levels of progesterone, estradiol and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were also measured in the uterine tissue and uterine vein plasma samples to correlate the endocrine changes with the development of gap junctions. A significant increase in the number, size and area of the gap junctions was observed during parturition after the levels of progesterone had declined and the levels of estradiol and $PGF_{2\alpha}$ were elevated.

MATERIALS AND METHODS

Rats

Fifty-two pregnant Sprague-Dawley (Holtzman) rats were used in this study. Pregnancy was assessed by "sperm positivity" at Day 0 and was confirmed at the time of autopsy. Rats were individually caged in rooms maintained at a constant temperature (22°C) and photoperiod, 14L:10D, and were fed a Purina diet.

Collection of Samples

On Days 15 through 20 of pregnancy, 3 or 4 rats were killed between 0900–1000 h. On Day 21, 3 or 4 rats with intact pregnancies were killed every 3 h until the morning of Day 22. Five animals were killed during delivery and 2 were killed 3 h after they had delivered all the fetuses. Samples of uterine vein blood and uterine tissue were immediately taken from all the animals. Plasma and tissue samples for hormone analysis were stored below −20°C. A part of the uterine tissue from the midportion of either the right or left uterine horns was fixed in glutaraldehyde and sodium cacodylate buffer for the electron microscopy studies.

Estimation of Hormones

Levels of progesterone, estradiol and $PGF_{2\alpha}$ were measured by specific radioimmunoassay (RIA) methods described previously for human (Puri et al., 1975, 1976) and rat tissues and plasma (Garfield et al., 1982). In brief, to assay steroid hormones, uterine tissue was homogenized in water, extracted with petroleum ether and then purified through a dual chromatographic system (Runnebaum et al., 1978) to eliminate chromogens and lipids. Progesterone was measured by using antiserum raised against progesterone-11, α-hemisuccinate-bovine serum albumin (BSA) and 1,2,6,7-[^{3}H]progesterone (sp. act. 98 Ci/mmol) as a tracer. Estradiol was assayed by using an antiserum raised against estradiol-6-(0-carboxymethyl)-oxime-BSA and 2,4,6,7-[^{3}H]estradiol (110 Ci/mmol) as a tracer. To measure the levels of $PGF_{2\alpha}$, the samples were acidified to pH 4.5, homogenized and extracted with ethyl acetate. $PGF_{2\alpha}$ was separated from other prostaglandins on silicic acid columns using benzene:ethyl acetate:methanol as developing solvents (Caldwell et al., 1971). Antisera raised against $PGF_{2\alpha}$-BSA and [^{3}H]$PGF_{2\alpha}$ (10 Ci/mmol) were used to assay $PGF_{2\alpha}$ levels. Free and bound hormone were separated by using dextrane-coated charcoal. The characteristics of the assay procedures are summarized in Table 1.

Electron Microscopy

The detailed procedure to process the uterine tissue for electron microscopy has been described previously (Garfield et al., 1980b). A longitudinal strip of uterine muscle (approx. 1 × 3 cm), dissected from the portion of the uterine horn directly above a fetus (nonplacental), was stretched to approximately the in vivo length and pinned on dental wax. It was immediately fixed in 2% glutaraldehyde containing sodium cacodylate buffer (75 mM) for 2 h at room temperature (22°C). After initial fixation, the tissue

TABLE 1. Characteristics of the assay methods.

Hormone	Recovery mean % + SD	Precision (% Coefficient of variation) Intraassay Pool a[a]	Intraassay Pool b[b]	Interassay Pool a	Interassay Pool b	Sensitivity pg/tube
Progesterone (P)	78 ± 3	7.5	6.7	9.8	10.3	15
Estradiol (E)	80 ± 4	5.3	6.9	8.7	9.5	10
PGF	86 ± 2	6.2	7.4	11.2	8.9	25

[a] *Pool a* contained 15.2 ng/ml P, 0.40 ng/ml E and 0.51 ng/ml PGF.

[b] *Pool b* contained 73.0 ng/ml P, 1.34 ng/ml E and 2.10 ng/ml PGF.

was washed in 100 mM cacodylate buffer and then post-fixed in 2% osmium tetroxide, in 50 mM cacodylate buffer, for 90 min. All tissues were stained en bloc with saturated uranyl acetate for 40 min, dehydrated and embedded in Spurr-resin. The tissues were then oriented in molds to cut the longitudinal muscle layers in transverse section. Sections were cut using a diamond knife (Dupont) on a Porter-Blum MT2-B ultramicrotome, mounted on 200 mesh grids, stained for 1 min with lead citrate and examined in a Philips 301 transmission electron microscope.

Quantitative Measurements

To determine the length of myometrial cell membranes surveyed for gap junctions, 20 to 22 nonoverlapping photographs were taken from one section of each tissue. Each negative was then enlarged and printed at 34,000X magnification of 8" X 11" photographic paper. The cell membrane length was estimated using a transparent grid superimposed over the assembled photographs. Each suspected gap junction in the photograph was further enlarged to 100,000X magnification for identification and length measurement. A gap junction was identified if it showed either a 5- or 7-lined structure at 100,000X magnification.

From all the photographs taken for each tissue, we tabulated the number of gap junctions observed in all photographs, the total length of the nongap junctional membranes, the mean number (frequency) of gap junctions per 1000 μm of nongap junctional membrane length, and the mean length of the gap junctions. The methods for calculation of gap junction area (twice the total length of gap junction membranes divided by the total length of nongap junctional membrane plus total length of gap junction membrane) was described in detail earlier (Garfield and Hayashi, 1981).

Statistical Analysis

Student's *t* test was used for statistical comparisons of hormone levels, fetal and placental weights and gap junction values on different days of pregnancy. Analysis of variance was applied to indicate differences for multiple comparisons between tissue samples.

RESULTS

The mean time (± SEM) of parturition in the animals was 06.36 ± 1.1 h on the morning of Day 22 of pregnancy. Nine rats delivered between 0320 and 1150 h.

The mean (± SEM) fetal and placental weights during pregnancy are shown in Fig. 1. A gradual and significant increase in the weight of the fetuses and placentas was observed after Day 15 of pregnancy. The placental weights increased from 0.34 ± 0.03 g on Day 15 to 0.59 ± 0.02 on Day 20 ($P<0.01$) and thereafter were almost plateaued. On the other hand, the increase in the fetal weight was more marked and it continued to increase until around 1930 h on Day 21 of pregnancy.

Levels of progesterone, estradiol and $PGF_{2\alpha}$, and the area of gap junctions during pregnancy and parturition are shown in Fig. 2. The levels of progesterone in both uterine vein plasma and uterine tissue started to decline on Day 19 of pregnancy, at least 60 to 70 h before the anticipated time of parturition in the rats. The decrease in the progesterone levels to basal concentrations in uterine tissues appeared to be more rapid as compared to that in plasma. After Day 20, no further significant drop in progesterone levels was observed in the tissues. The mean levels in five animals killed during delivery were 13.2 ng/ml in the plasma and 8.6 ng/g in the tissue.

The levels of estradiol in the uterine tissue began to rise after Day 20 of pregnancy. The increase in the levels was more marked on Day 21 and it increased further as the time of parturition approached. During delivery mean estradiol levels were 1485 ± 505 pg/g, about 3 to 4 times the levels found in tissues between Days 15 to 19 of pregnancy. The quantity of uterine vein plasma samples left after analysis of the other hormones was insufficient to assay for estradiol levels.

Levels of $PGF_{2\alpha}$, which were always higher

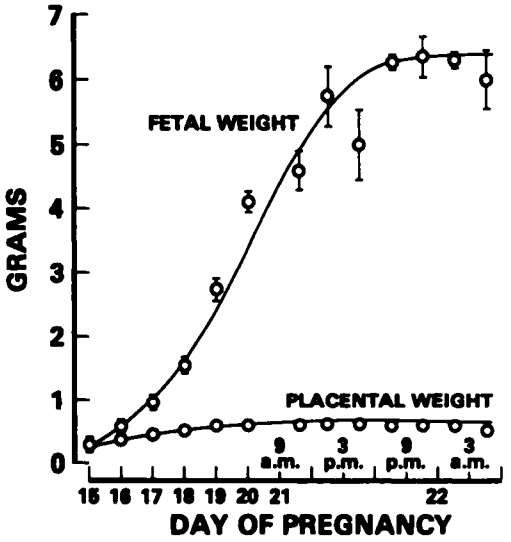


FIG. 1. Changes in fetal and placental weights in rats during pregnancy. Rats (n=3 or 4) were sacrificed daily from Day 15 to 20 of pregnancy, then every 3 h on Day 21 until delivery on Day 22. Shown are mean values ± SEM for all fetuses at each time. The SEM for all placental weights fell within the range of values indicated by the open circles. The time scale is expanded between Day 21 to delivery.

in the uterine tissue (37 ± 3 ng/g, Day 15) than in the uterine vein plasma (185 ± 10 pg/ml, Day 15) showed a gradual increase after Day 16 of pregnancy. However, the increase was more marked after Day 20, when progesterone levels had dropped by about 67% and 84% of Day 15 levels in the plasma and uterine tissues, respectively. At the time of parturition the mean $PGF_{2\alpha}$ levels had risen to 2.48 ng/ml in plasma and 435 ng/g in tissue.

The gap junction area was low through Day 20 of pregnancy. From Day 21 to parturition a gradual increase in the gap junction area was observed.

Figure 3 shows the results of the quantitative analyses of the frequency (number of gap junctions/1000 μm of nonjunctional membrane), size (length), and area (% of area gap junctional membrane/area total plasma membrane) of gap junctions in the longitudinal

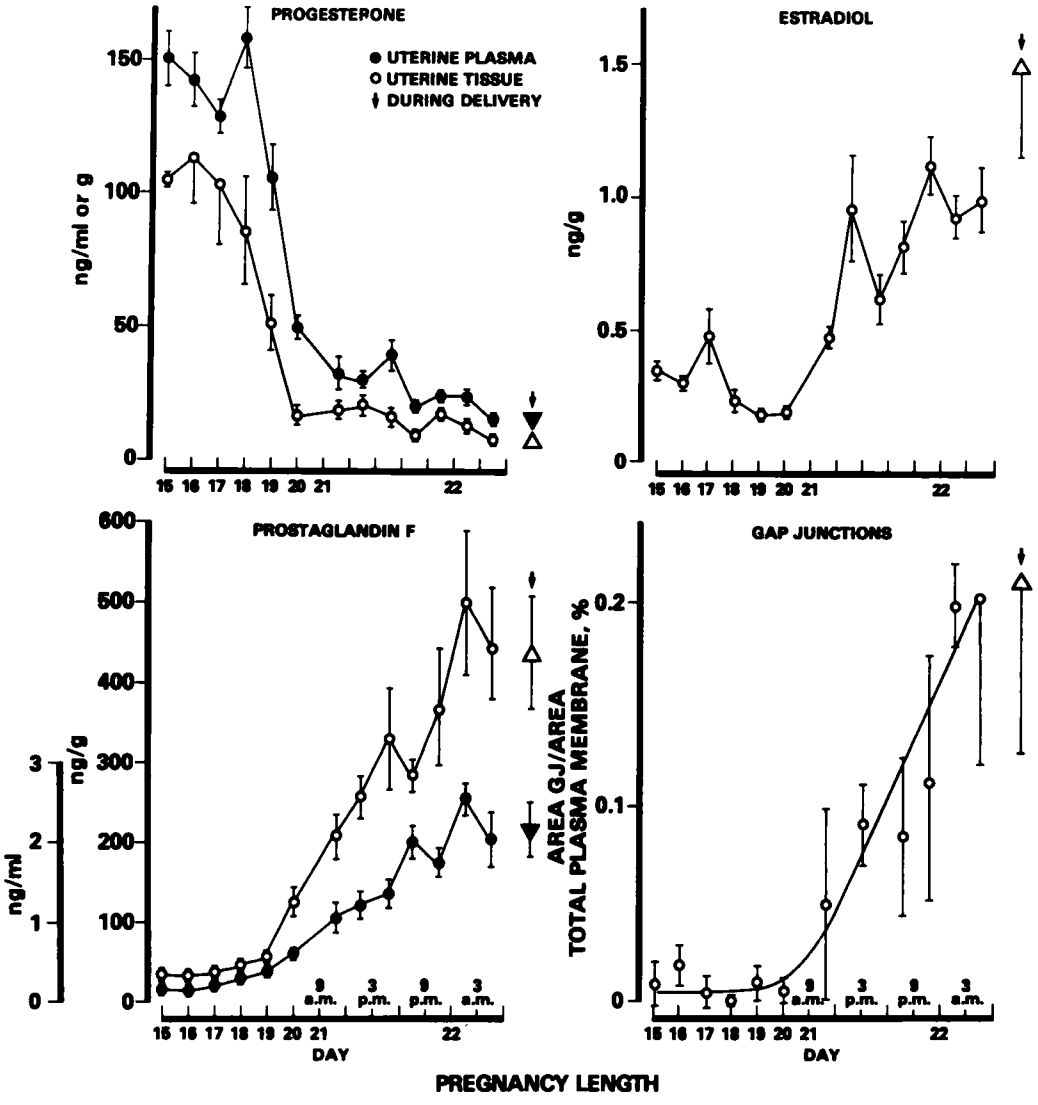


FIG. 2. Changes (mean ± SEM) in the levels of progesterone, estradiol and PGF in uterine vein plasma (*closed circles*) and uterine tissues (*open circles*) and the myometrial gap junctional area during the latter days of pregnancy and during delivery (*triangles*). Note that the axis of the time scale is expanded from Day 21 onwards to delivery. Number of samples at each point=3 or 4.

muscle of rat uteri from Day 15 to Day 21, during delivery and postpartum. It is interesting to note that gap junctions were not observed in all the tissue samples fixed prior to Day 21. A mean frequency of about 0.5 gap junctions/1000 μm of nonjunctional membrane was observed in 18 rats examined between Days 15 to 21 of pregnancy. However, the frequency of gap junctions was significantly ($P<0.01$) higher (mean 1.7/1000 μm) in rats killed during Day 21 of pregnancy. The number of gap junctions was also significantly higher in tissues from animals during delivery as compared to tissues at ($P<0.05$) or prior to Day 21 ($P<0.01$). Gap junctions were found in all tissues from the 5 rats killed during delivery, with a mean frequency of 4.0 junctions/1000 μm membrane. The increased number of gap junctions was still present in rats killed 3 h after delivery.

The size of the gap junctions was also significantly larger ($P<0.01$) in the tissues from animals on Day 21, delivering and postpartum, as compared to animals pregnant between Days 15 to 20 (Fig. 3). The increase in frequency and size resulted in a significant increase ($P<0.001$) in area of gap junctions in tissues at Day 21, during delivery and postpartum compared to tissues taken prior to these times.

Figures 4 and 5 show electron micrographs of gap junctions between uterine smooth muscle cells in tissues from delivering animals. At high magnification the 7-lined (4 dark and 3 light lines) appearance of the gap junction is apparent (Fig. 4). Figure 5 shows an unusually large number of gap junctions present within one field.

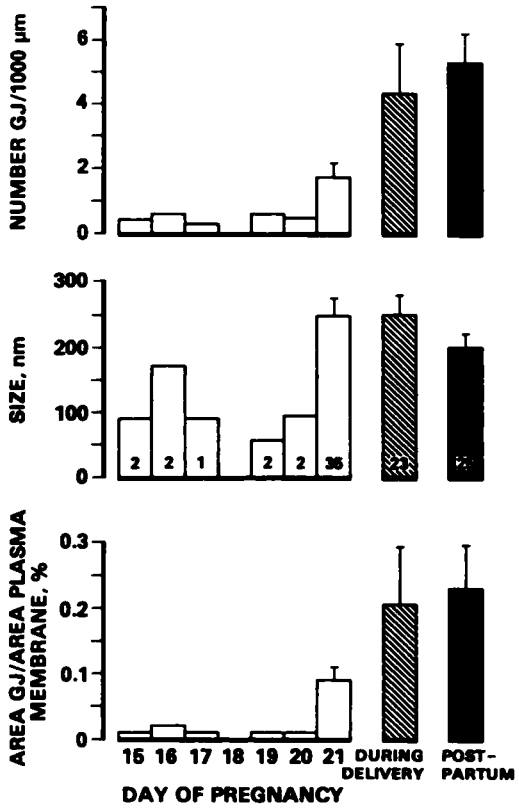
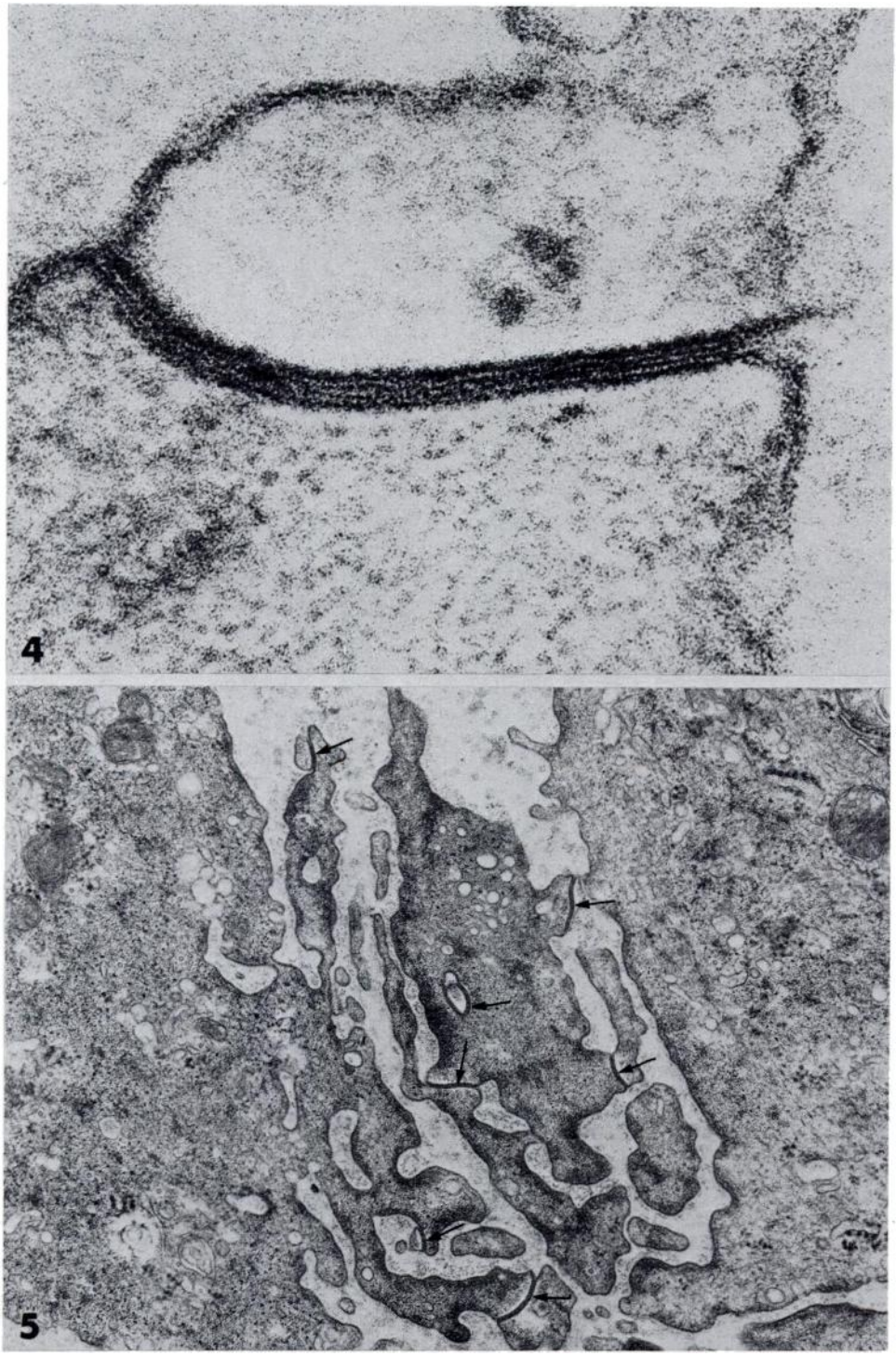


FIG. 3. Histograms of quantitative measurements of gap junctions in rat myometria showing the frequency (number of gap junctions/1000 μm nongap junctional membrane) size (length of gap junction profile, nm) and area (% of area gap junction membrane/area total membrane) before delivery (Day 15 to 21 of pregnancy, *clear bars*) during delivery (*crosshatched*) and postpartum (*darkened*). Height of each histogram represents mean ± SEM. The number within the bars on the "size" histogram indicates the total number of gap junctions present in tissues examined at the specified time. The number ($P<0.01$) and area ($P<0.001$) of gap junctions determined on Day 21, during delivery and in postpartum tissues were significantly different when compared with all values prior to Day 21.

DISCUSSION

This study describes the changes in steroid hormones, prostaglandin F and gap junctions that occur in plasma and uterine tissues during the last few days of pregnancy and during parturition in the rat. The observed changes indicate that the sequence of events is a decline in progesterone levels starting on Day 19 of pregnancy followed by simultaneous increases in the levels of estradiol and prostaglandin F after Day 20, and an increase in myometrial gap junction area commencing at Day 21. These events culminate in labor and delivery on Day 22 of pregnancy. These results are consistent with the hypothesis (Garfield et al., 1978, 1980) that changes in the levels of hormones which precede (progesterone) or accompany (estrogen and prostaglandins) the development of gap junctions are responsible for regulation of their appearance.

The development of gap junctions is thought to provide the low resistance electrical pathways between cells which establish the synchronized and coordinated muscle activity of labor (Garfield et al., 1977, 1980b). The contention that gap junctions are essential for parturition is supported by observations which show that they occur in large numbers in rats and other animals during normal spon-



FIGS. 4 and 5. Electron micrographs of uterine smooth muscle at high (Fig. 4, ×337,000) and low magnifications (Fig. 5, ×36,000) showing gap junctions between muscle cells from tissues of delivering animals. At high magnification the 7-lined structure of a gap junction is apparent (Fig. 4). Figure 5 shows an unusually large number of gap junctions (*arrows*) within one field.

taneous delivery or whenever premature labor is initiated, and they are absent or reduced in number in similar animals where premature or normal labor is prevented experimentally (Garfield et al., 1977, 1978, 1979a,b, 1980, 1982; Garfield and Hayashi, 1981). However, previous investigations have not clearly established the time of appearance of the increased gap junctional area in relationship to hormone changes.

Our analyses are based on the premise that the changes we observed occurred more or less equally in all animals. We have not sequentially sampled from the same animals throughout pregnancy and parturition. Sequential sampling of blood and tissues in the manner required for this study is not possible in the rat. All animals which delivered did so when expected, on the morning of Day 22 of pregnancy and within a short period of time (approx. 8 h). We assume that animals which were sacrificed earlier would also have delivered at the same time. It is unlikely that the changes we observed in animals prior to delivery reflect differences in animals or that any of these animals were delayed or advanced in their progress to significantly affect our results. Also, our hormone values for tissues reflect concentrations within the whole uterine wall (myometrium + endometrium) and the hormone levels in the myometrium are expected to be more relevant in relation to the development of gap junctions.

A decline in plasma or tissue progesterone levels (progesterone withdrawal) prior to parturition has been described by others for rats (Weist, 1970) and other animals (Nathanielsz, 1978; Thorburn and Challis, 1979). The suppressant effects of progesterone on uterine contractility and labor in animals has been demonstrated convincingly (Csapo, 1981). That the absence of gap junctions may be the structural basis for the suppression is supported by this and other studies. Progesterone treatment prevents the formation of gap junctions and labor in rats treated at Day 19 of pregnancy (Garfield et al., 1978). Progesterone also inhibits premature delivery and the development of gap junctions in rats ovariectomized at Day 16 of pregnancy (Garfield et al., 1982). In sheep there is also a pronounced progesterone withdrawal preceding gap junction development (Garfield et al., 1979b), and in this species progesterone is thought to control gap junction development (Garfield et al., 1979b). In addition, evidence from studies of rat myometrial tissues in vitro indicate that progesterone suppresses gap junction formation (Garfield et al., 1980a). However, in vitro, the delay between total progesterone withdrawal and gap junction development appears to be less (Garfield et al., 1980a), than that found in this study.

If progesterone is responsible for the inhibition of formation of gap junctions, it is not evident how this is accomplished. This process may occur through several steps, including inhibition of protein synthesis, a direct effect on the plasma membrane or an effect on prostaglandin synthesis. Protein synthesis is thought to be necessary for gap junction formation in the myometrium (Garfield et al., 1980b) and direct effects of progesterone on membrane enzymes possibly associated with gap junctions (Garfield et al., 1980b) have been demonstrated in oocytes (Finidori-Lepicard et al., 1981). Also, progesterone withdrawal has been shown to stimulate prostaglandin synthesis (Nathanielsz, 1978; Thorburn and Challis, 1979).

Changes in prostaglandin synthesis and metabolism resulting in increases in these compounds during labor have been previously demonstrated (Nathanielsz, 1978; Thorburn and Challis, 1979; Csapo, 1981). Prostaglandins stimulate uterine muscle to contract (Csapo, 1981) and indirectly effect steroid production (luteolysis) (Horton and Poyser, 1976; Fuchs et al., 1976; Fuchs, 1978). Prostaglandin synthesis in the uterus is stimulated by estrogens and inhibited by progesterone (Nathanielsz, 1978; Thorburn and Challis, 1979). Our study suggests that progesterone withdrawal leads to increases in prostaglandins as progesterone declines prior to increases in $PGF_{2\alpha}$ (Fig. 2).

Prostaglandins may be essential for gap junction development in the myometrium because indomethacin, a prostaglandin synthesis inhibitor, prevents gap junction formation in vitro (Garfield et al., 1980a,b). There have been no previous studies of gap junction development and their relationship to prostaglandins in vivo. The changes in $PGF_{2\alpha}$ measured in this study may reflect changes in the synthesis of several or all the prostaglandins, as PGF may not be the prostanoid responsible for gap junction formation (Garfield et al., 1980b). $PGF_{2\alpha}$ does produce uterine contractility (Thorburn and Challis, 1979; Csapo, 1981) and thus gap junctions may provide the setting for synchronous contractility stimulated by $PGF_{2\alpha}$

and other stimulants such as oxytocin (Soloff et al., 1979).

The appearance of gap junctions is accompanied by a 3- to 4-fold increase in tissue estradiol levels (Fig. 2). These results are in agreement with observations which show an increase in plasma levels of estradiol in rats (Fuchs, 1978). Whether the increase in estradiol is essential for the development of the junctions is questionable. Estrogens are known to stimulate gap junction formation in the myometrium as well as other tissues (Garfield et al., 1980a). Estrogens are not essential for gap junction development in myometrial tissues in vitro but myometrial tissues exposed to estrogens develop higher numbers (Garfield et al., 1980a). In addition, estrogens seem to be essential for normal or premature labor (Csapo et al., 1973). These data indicate that an elevation in estradiol levels above those normally found before progesterone withdrawal may not be necessary for gap junction formation or labor but may reinforce these events to effectively terminate pregnancy.

The effects of distention of the uterine wall have been suggested as one possible mechanism responsible for events leading to gap junction formation (Garfield et al., 1978) and labor (Csapo, 1981). As seen in this study, the appearance of large numbers of gap junctions occurs during the later stages of fetal development when there is little change in fetal weight (Figs. 1 and 2). Therefore, stretch of the uterine cells is likely to have little direct influence on gap junction development. Furthermore, distention or stretch has little influence on gap junction formation in vitro (Garfield et al., 1980a), or in vivo after unilateral ovariectomy where distention does not occur in one horn during pregnancy (Garfield et al., 1978).

In summary, our study is descriptive and shows the changes in steroid hormones, prostaglandin F and gap junctions that occur in plasma and uterine tissues during the last week of pregnancy and during parturition. The observed changes indicate that the possible sequence of events is: *1*) a decline in progesterone levels on Day 19, *2*) increases in PGF (on Day 20), estradiol and gap junctions (on Day 21), and *3*) labor and delivery of fetuses. The results of this study are consistent with previous studies and support the hypothesis (see Garfield et al., 1980b) that gap junction development between myometrial cells may be one of the final necessary steps in a sequence of events that occur to terminate pregnancy in the rat. However, the development of gap junctions may be only one of many changes which occur in the muscle cells in preparation for labor to promote excitability, cell coupling and contractility.

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