Gap Junction Modulation in Rat Uterus. I. Effects of Estrogens on Myometrial and Serosal Cells

ROBERT C. BURGHARDT, REBECCA L. MATHESON and DANA GADDY

Department of Biology
Texas A&M University
College Station, Texas 77843

ABSTRACT

The ability of estradiol benzoate (E$_2$B) and diethylstilbestrol (DES) to affect the formation and internalization of gap junctions was examined in several uterine cell types from hypophysectomized rats. Both myometrial and serosal cells respond to daily administration of E$_2$B or DES by increasing gap junction membrane in a dose-dependent fashion. The myometrial cell response arises from a zero base with gap junctions detected within 24 h of a single injection of 500 µg E$_2$B, while five daily injections of 5 µg E$_2$B were required to induce their formation at the lower dosage. Uterine serosal cells exhibit small macular gap junctions 60 days posthypophysectomy with E$_2$B stimulation leading to an increase in the number of macular gap junctions and the induction of annular gap junctions. Myometrial cell gap junctions were modulated by L-thyroxine and progesterone when administered in combination with E$_2$B but were without effect when administered alone. Combination of indomethacin with E$_2$B injections antagonized E$_2$B-stimulated junction growth in both myometrial and serosal cells, however, only serosal cells responded to exogenous prostaglandin (PG) injections, with PGE$_1$, increasing and PGF$_{2\alpha}$ decreasing the number of serosal cell gap junctions. These studies support the assumption that the induction of gap junctions in uterine myometrium is hormone dependent.

INTRODUCTION

The temporal relationship between the development of coordinated electrical syncytium properties in uterine myometrium during labor and parturition (Krishnamurti et al., 1982) and the appearance of gap junctions between myometrial cells (Garfield et al., 1977, 1978, 1980a, 1982), provides a biophysical basis for the establishment of uterine contractile activity during pregnancy and parturition. Since gap junctions provide low-resistance pathways between cells (reviewed by Loewenstein, 1981), it is thought that their appearance is an important step in the coordination of uterine activity at this time.

There is good experimental evidence, beginning with the pioneering studies of Bergmann (1968), that gap junctions between myometrial cells are hormonally controlled (Garfield et al., 1980a,b). In particular, the relationship between estrogenic stimulation and myometrial gap junctions is significant since these intercellular contacts in virgin, castrate and hypophysectomized rats could be induced between myometrial cells following administration of very large doses of exogenous estrogen (Bergmann, 1968; Dahl and Berger, 1978; Merk et al., 1980) but their spontaneous appearance in vitro was blocked or delayed by progesterone (P) administration or by inhibitors of prostaglandin (PG) and protein synthesis (Garfield et al., 1978, 1980a). Although gap junction contacts seem to be a nearly universal feature of cells organized into tissues (Loewenstein, 1981), junctions between myometrial cells appear to be unique since they are present only during functional periods, i.e., during labor and parturition (Garfield et al., 1977; Garfield and Hayashi, 1981) and since they arise from a zero base during hormone stimulation.

Since in vitro preparations of the uterus do not behave like the intact organ in vivo (Fuchs, 1978; Garfield et al., 1980a) and since the endocrine status of pregnant animals is complex, we have examined uterine responses to exogenous hormone stimulation in juvenile hypophysectomized rats. Particular attention has been directed towards evaluation of uterine luminal epithelial cell stimulation and the analysis of
junctional membrane in endometrial, myometrial and serosal cells. We report here that exogenous estrogen stimulation causes the induction and modulation of gap junction membrane in myometrial cells from hypophysectomized rats in a dose-dependent fashion. One other cell type, the uterine serosal cell, also exhibits a dose-dependent increase in gap junction membrane, although the response is superimposed upon basal levels of junctional membrane that persist in the absence of hormones from the pituitary-ovarian axis. These studies also provide baseline data for a companion paper in this issue which examines the actions of a variety of ligands that bind cytoplasmic estrogen receptors for their ability to induce and/or modulate gap junction membrane in uterine tissues.

MATERIALS AND METHODS

Reagents

Diethylstilbestrol (DES), β-estradiol-3-benzoate (E₂ B), progesterone (P), prostaglandin E₁ (PGE₁), L-thyroxine (T₄), indomethacin (IDM) and dexamethasone (DEX) were purchased from Sigma Chemical Co. (St. Louis, MO). Prostaglandin F₂α (PGF₂α) or dino-

Animal Treatments and Tissue Preparation

Immature, intact and hypophysectomized female CD rats were obtained from the Charles River Breeding Labs. (North Wilmington, MA). Hypophysectomized rather than ovariec-
tomized animals were used in these investigations to eliminate possible effects of adrenal steroid production, to monitor the involvement of T₄ in synergistic uterotrophic effects with estrogen-receptor binding ligands, and because we wished to maintain the ovarian-tubal-uterine relationships (Merk et al., 1980) during hormonal manipulations. Hypophysec-
tomy was performed at 21 days of age and animals were kept 3 per cage, with hypophysectomized rats receiving 5% glucose water ad libitum in addition to standard laboratory diet. Intact animals of the same age were used as controls for verification of the efficacy of hypophysectomy according to criteria de-
tailed previously (Burghardt and Matheson, 1982). Exogenous hormone treatments were initiated 60 days posthypophysectomy. Animals were divided into groups consisting of 6 animals per treatment group. Steroid hormones, DES, IDM and DEX were dissolved by stirring in warm (50°C) sesame oil containing not more than 5% absolute ethanol. T₄ and PGE₁ were dissolved in Dulbecco’s PBS and PGF₂α was used as the dinoprostone tromethamine stock solution (5 mg/ml) diluted in PBS to desired concentrations. All animal injections were delivered intraperitoneally (i.p.) in 200-μl volumes of hormone carrier (sesame oil or PBS).

Following hormone injections, rats were sacrificed by cervical dislocation following mild ether anesthesia. Uteri were excised and immediately immersed in 2% paraformaldehyde, 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). The middle one-third segment of each uterine horn was dissected out after 5 min in fixative. This initial fixation was necessary to provide for a relative measure of uterine fluid accumulation and resulting stretch of the uterine wall. Tissues were subsequently processed further for light and electron microscopy. In several experiments the serosal cell covering of intestine and liver were ex-
inamined following identical processing of liver and intestine segments. Following the initial fixation for 2 h, tissue was washed several times in 0.1 M cacodylate buffer, postfixated in 1% OsO₄ in 0.1 M cacodylate and washed in the same buffer. Subsequent to several rinses in distilled water, tissues were en bloc stained in 1% aqueous uranyl acetate and washed overnight in distilled water. Tissues were then dehydrated in ethanol series and embedded in Epon-Araldite. For light microscopy, 0.25- to 0.5-μm sections were stained with 0.1% toluidine blue in 1% sodium borate and examined with a Zeiss IM-35 light microscope. Thin sections were mounted on 300-mesh copper grids and examined with a Philips 400T electron microscope. The morphological parameters of hormonal stimulation examined include the extent of hypertrophy of uterine luminal epithelium and a relative measure of myometrial and serosal cell gap junctions.

Measurements

All observations of uterine cells were made from sections of the entire uterine wall in cross section. Light microscopy was used to evaluate the extent of hypertrophy of uterine luminal epithelium following hormone treatments. Light microscopy was also used to evaluate the extent of fluid accumulation by measuring inner and outer diameters of the uterine wall. A relative measure of gap junction numbers as a function of hormone treatments was obtained in a manner similar to the method used by Garfield and Daniel (1974) and Merk et al. (1980). The number of gap junctions in myometrium was counted from grid squares (approx. 2500 μm² open area) uniformly covered by smooth muscle cells, with equal numbers of measurements made from longitudinal and circular muscle bands. Endometrial and serosal cells were evaluated if continuous sheets of these epithelia traversed the width of each grid square (at least 50 μm width). The number of gap junctions in eligible grid squares were counted, with 50 grid squares examined for each cell type and treatment group. Analysis of sample means was carried out with a one-tailed Student's t test at a significance level of P<0.05. An inherent weakness of this procedure is that hormone treatments causing increases in cell size (only estrogens in these studies) would reduce the fraction of open grid square occupied by cell membrane while increasing that fraction occupied by cytoplasm. Since estrogens also led to dose-dependent increases in gap junction numbers, these values therefore only underestimate the junctional response and do not alter the interpre-
tation of these data.
GAP JUNCTION MODULATION

RESULTS

Gap Junction Formation in Uterine Myometrium

Estradiol benzoate or DES administration initiated a dose-related series of events in the uterus of hypophysectomized rats (Fig. 1) which included uterine fluid accumulation leading to stretch of the uterine wall in addition to modification of the gap junction composition of two uterine cell types, the myometrial and serosal cells. Preliminary studies of the junctional content of uterine luminal epithelial cells revealed only minor differences following hormone treatments and were therefore not examined further. Figure 2 illustrates that the induction of macular (or cell surface) gap junctions in uterine myometrium and the number of junctions detected were dependent upon the dosage of E₂B administered. Gap junctions were observed both within the longitudinal and circular muscle bundles of myometrium, although junctions in the circular layers were detected approximately three times as frequently as in the longitudinal layer (data not shown). Higher doses of E₂B also led to the appearance of annular (or internalized) profiles of junctional membrane in the cytoplasm of these cells.

Modulation of this estrogenic response was obtained by combining T₄ or P with E₂B while T₄ or P alone were without direct effect on junctional membrane. Combination of T₄ with E₂B amplified the number of gap junctions while a P:E ratio of 50:1 but not 1:1 or 10:1 was found to depress this estrogenic response (Fig. 3). The time course for the appearance of myometrial gap junctions also varied with E₂B dose (Fig. 4). Macular gap junctions could be detected 24 h following a single injection of 500 µg E₂B, whereas no junctions were detected on Days 1–4 following the daily 5-µg injections. Five separate daily injections were required before detection of gap junctions in thin-sectioned uteri was possible using the latter dosage of E₂B.

The possible involvement of estrogen-directed PG synthesis on myometrial gap junctions was also examined in vivo using the PG synthesis
inhibitors DEX and IDM (Fig. 5). Pretreatment of animals with up to 2.5 mg DEX/24 h prior to 5 daily injections combining 2.5 mg DEX and 50 µg E₂B failed to antagonize the induction of myometrial gap junctions, whereas a similar regimen using IDM at a dosage of 1.0 mg effectively antagonized but did not completely abolish the response. However, exogenous injections of 1.0 mg PGE₁ or PGF₂α given every 8 h over 48 h (6 injections totaling 6 mg of PG) failed to promote induction of myometrial gap junctions in vivo.

**Gap Junction Modulation in Uterine Serosa**

One other cell type which responded dramatically to exogenous E₂B stimulation is illustrated in Fig. 6. The serosal cell covering of the uterus, in contrast to myometrium, retained basal numbers of macular gap junction contacts for as long as 60 days following withdrawal of hormones of the pituitary-ovarian axis. The effect of exogenous E₂B was to increase the number of macular gap junctions in a dose-dependent fashion, with the minimum dosage of 0.5 µg/day for 5 days capable of causing a significant increase in junctional membrane compared to control treatments from hypophysectomized animals (Fig. 7). Comparison of the gap junction composition of the serosal cell covering (peritoneal mesothelium) of small intestine and liver following 5 daily injections of 500 µg E₂B with uterine serosa from hypophysectomized animals revealed no significant increase in the number of junctions in the serosa of small intestine and liver, indicating the specificity of the estrogenic effect on uterine serosal cells.

Characteristic of estrogenic stimulation in hypophysectomized animals was the induction of annular gap junctions in the cytoplasm of serosal cells (Fig. 6). The presence of internalized junctions in these cells proved to be diagnostic of exogenous estrogen stimulation and were detected with doses as low as 50 ng E₂B/day for 5 days.

![Graph](image-url)
to a phase of rhythmic and coordinated activity (Krishnamurti et al., 1982), coupled with the temporal appearance of gap junctions in myometrium just before birth (Garfield et al., 1977, 1978, 1979), provides a biophysical basis for the behavior of myometrium as a single unit of electrical synecytium during parturition. In studies reported here and in a companion paper in this issue, we have examined the dose-response relationship between levels of sex steroids and the induction of morphological coupling by gap junctions in uterine myometrium. In long-term (60-day) hypophysectomized rats, daily i.p. injection of 5 μg E₂B in 0.2 ml oil carrier over 5 days caused the appearance of gap junctions in thin sections of myometrium, while doses of 500 μg E₂B resulted in their detection as early as 24 h later. In the absence of freeze-fracture data, however, we cannot exclude the possibility that very small gap junctions escaped detection in tissues subjected to the 5-μg doses over Days 1–4. Similar results were obtained following injections of the nonsteroidal estrogen DES. Exogenous T₄ or P alone do not elicit any junctional response in the uterus, yet both hormones modulate the effect of E₂B on myometrial cell gap junctions. The effect of T₄ is to agonize while P antagonizes the action of E₂B on the number of gap junctions present in myometrium.

Thyroid hormones have previously been found to modulate several delayed (24 h) uterine responses to estrogens while not significantly affecting early responses (Gardner et al., 1978; Stancel et al., 1979), presumably by modulating the concentration of estradiol binding sites (Cidlowski and Muldoon, 1975). The induction of gap junctions in uterine myometrium appears to be a late response that requires thyroid hormones to permit the full expression of gap junctions in response to E₂B. The antagonistic effect of P is probably the more physiologically relevant effector of the junctional response to E₂B. However, in the present study a P:E₂B ratio of 50:1 but not 1:1 or 10:1 effectively diminished the response but did not abolish it. The increase in estradiol combined with the reduction in placental production of P just before labor begins is well established (Thorburn and Challis, 1979; Fuchs, 1978). The high plasma levels of P that exist until this period may prevent the induction of gap junctions (Garfield et al., 1977, 1978) and may be partially responsible for the well-known

In contrast to myometrial cells, treatment combining T₄ or P with E₂B did not significantly alter the number of serosal cell gap junctions compared to E₂B treatments alone (Fig. 8). Analysis of PG synthesis inhibitor and exogenous PG treatments were also conducted on serosal cells. As was observed in myometrium, IDM (1.0 mg) but not DEX (2.5 mg) pretreatment followed by 5 daily injections combining inhibitor with 50 μg E₂B reduced the number of serosal cell gap junctions. Furthermore, injections of PGE₁ and PGF₂α resulted in small but significant alterations in serosal cell junctional membrane. PGE₁ treatment led to a slight increase, while PGF₂α decreased the number of macular gap junctions (Fig. 9). However, annular gap junctions were not encountered following either treatment.

DISCUSSION

Gap Juncions Formation
in Uterine Myometrium

The sudden transition from asynchronous electrical behavior of uterine myometrial cells...
FIG. 6. Electron micrographs of uterine serosal cells subjected to 5 daily injections of 50 μg E₂B/day. a) E₂B causes the induction of annular gap junctions (arrowheads) and increases both the number and size of macular junctions (arrow) compared to hypophysectomized control treatments. A macular gap junction (arrow) is also shown connecting two myometrial cells. ×14,000. b) Concentric annular gap junction profiles are also characteristic of sustained E₂B stimulation of uterine serosa. ×25,000.

quiescent effect of P known as the "progestosterone block" (Csapo, 1956) on uterine contractility.

Hormonal Control of Myometrial Gap Junctions

High levels of estrogen are clearly involved in the hormonal control of the birth process and also play a role in the development of gap junctions in vivo (Bergmann, 1968; Dahl and Berger, 1978; Merk et al., 1980). Whether estrogen stimulation results in the transcription of a specific mRNA coding for the connexon protein has not yet been determined. The report of Dahl et al. (1980) indicating that a mRNA preparation isolated from estrogen-dominated rat myometria codes for protein involved in the expression of junctional communication, included a suggestion of this possibility. On the other hand, a number of investigations indicate an indirect effect of estrogen on junctional membrane. Recent studies by Wathes and Porter (1982) have demonstrated that stretch of the uterine wall following insertion of uterine balloons was sufficient to cause the appearance of a few myometrial junctions in ovariectomized animals but that estradiol stimulation greatly amplified this response. They suggest that both E₂B and uterine wall distension may act on gap junction formation via their effects on PG synthesis.

Estradiol has been shown to stimulate the production of PGs in the uterus (Ham et al., 1975) possibly by modulating the levels of phospholipase A₂, an enzyme which provides free arachidonic acid for the synthesis of PGs (Dey et al., 1982). Furthermore, Csapo (1977) has shown that uterine distention doubled the synthesis of PGF without affecting PGE levels. These data suggest that stretching strips of uterine tissue incubated in vitro without steroids may be responsible in part for the development of spontaneous gap junctions seen
FIG. 7. Effects of E₂B and DES on the number of uterine serosal cell gap junctions in hypophysectomized (HX) rats. A dose-dependent increase in the number of gap junctions results from E₂B or DES treatment. The increase is superimposed upon basal numbers of gap junctions that persist 60 days following hypophysectomy. The induction of annular gap junctions (batched bars) results from 5 daily injections of 0.05 μg E₂B, while significant increases (P<0.05) in the number of macular junctions (open bars) over HX controls results from 5 daily injections of 0.5 μg E₂B. Hormone dose is expressed in micrograms of compound injected per day in 200 μl oil carrier over 5 days.

FIG. 8. Effects of T₄ and P on serosal cell gap junctions. T₄ or P administered alone or in combination with E₂B did not significantly alter the number of serosal cell gap junctions. Hormone dose is expressed in micrograms of compound injected per day in 200 μl carrier.

FIG. 9. Effects of prostaglandin (PG) synthesis inhibitors, DEX and IDM and the PGs, PGF₂α and PGE₁, on serosal cell gap junctions. IDM but not DEX significantly (P<0.05) antagonized E₂B stimulation of serosal cell gap junctions. Exogenous PG injections led to small but significant (P<0.05) modulation of the number of macular gap junctions (open bars). PGF₂α decreased and PGE₁ increased the number of macular junctions compared to HX controls. PGE₁, however, did not cause the induction of annular gap junctions (bathed bars) at doses given. Hormone dose is expressed in micrograms of compound injected in 200 μl carrier. E₂B and inhibitors were administered daily over 5 days. PGs were administered every 8 h over 2 days.

after 2 h in culture by Garfield and co-workers (1978, 1980a,b). Nevertheless, either numbers or size of myometrial gap junctions in this culture system were increased by estrogen, endoperoxides, thromboxane A₂, and cAMP, while their numbers were decreased by inhibitors of PG and protein synthesis (Garfield et al., 1980a,b).

The studies of Garfield et al. (1978, 1980a,b), and the IDM antagonism of E₂B-stimulated myometrial gap junction growth in hypophysectomized animals reported here, both support the possible role of PGs and other prostanoids of the cyclooxygenase pathway in regulating myometrial gap junctions. Since PGs modulate intracellular cAMP levels (Kuehl and Egan, 1980), it remains a possibility that the presence and amount of gap junction membrane in myometrial cells may be regulated by cAMP as has been suggested for other cell types (Loewenstein, 1981).
In support of an early suggestion that cAMP could regulate the size of gap junctions between cells (Johnson et al., 1974), a number of investigations have correlated cAMP stimulation and cAMP-mediated hormone action to the "up-regulation" of junctional membrane permeability and gap junction size in several mammalian cell lines (Flagg-Newton, 1980; Flagg-Newton et al., 1981; Flagg-Newton and Loewenstein, 1981; Radu et al., 1982). Further, the induction of gap junctions and promotion of membrane permeability in a gap junction-deficient mouse cancer cell type by administration of exogenous cAMP or by treatments elevating endogenous cAMP levels is significant since the response, like the formation of myometrial cell gap junctions, also arises from a zero base (Azarnia et al., 1981).

In this context, the recent report of the spontaneous assembly of gap junctions in cultured rat prostatic epithelial cells (another steroid hormone-responsive cell type) following treatments that involve cytoskeletal perturbations (Tadvalkar and Pinto da Silva, 1983) are significant. These investigators proposed that junctional assembly resulted from a convergent migration of preexisting junctional precursor molecules whose "positional control" in the membrane is released by perturbation of cytoskeletal elements. The involvement of the adenylate cyclase-cAMP system as the principal modulator of the cytoskeleton, and the close association of cytoskeleton with the plasma membrane (reviewed by Zor, 1983) may be an important link between cAMP stimulation and the reported increases in gap junction size and membrane permeability cited above. We are therefore currently testing the hypothesis that the role of estrogen in myometrial cell junction formation may involve increased synthesis and/or insertion of connexons into the plasma membrane. The assembly of connexons into junctional plaques could subsequently proceed following the acquisition of membrane-saturating levels of connexons, or more likely, following the elevation of intracellular levels of cAMP. The stimulatory effects of β-agonists, endoperoxide and exogenous cAMP on myometrial gap junctions (Garfield et al., 1980a,b) are compatible with the hypothesis that elevated cAMP levels could cause the assembly of connexons into junctional plaques if estrogen-stimulated increases in connexons were available in the plane of the membrane. Although no data has yet been reported to indicate such a role for estrogen in myometrial cells, this possibility is currently under investigation.

**Gap Junction Modulation in Uterine Serosa**

In addition to its effect on myometrial junctions, exogenous E₂B or DES stimulation also resulted in modification of gap junction membrane within the serosal cell covering of the uterus. However, the effect of the exogenous estrogens is to increase the number of junctions from the basal level of junctional membrane seen 60 days following the withdrawal of hormones from the pituitary-ovarian axis. Accompanying the significant increases in numbers of gap junctions detected with increased E₂B dose is a consistently observed increase in the number of annular gap junctions within the cytoplasm of uterine serosal cells. These structures probably provide for the bulk removal of junctional membrane from the surface of cells (Merk et al., 1973; Albertini et al., 1975). The appearance of annular gap junctions and the increases in the number of macular gap junctions in serosal cells following estrogenic stimulation were surprising since we are not aware of reports indicating the presence of cytosolic estrogen receptors in uterine serosa. Examination of the serosal covering of liver and intestine following exogenous E₂B treatments failed to reveal increases in the junctional content of these cells, indicating the specificity of estrogenic action on uterine serosa.

These observations are compatible with information cited above that estrogens may exert their effects on junctional membrane in the serosal layer, as in myometrium, through effects on PG synthesis induced by estrogens (Ham et al., 1975; Csapo, 1977; Dey et al., 1982; Wathes and Porter, 1982). Support for this suggestion is also provided from the IDM antagonism of E₂B-induced increase in serosal junctions and from the effects of exogenous PG injections. In the present studies, PGE₁ treatment resulted in a small but significant increase, while PGF₂α treatment caused a decrease in the number of serosal gap junctions compared to controls. PGE₁ is characterized by the ability to stimulate cAMP (Kuehl and Egan, 1980) while PGF₂α lowers the production of cAMP (Hammersstrom, 1982). The present observations are compatible with the hypothesis of Loewenstein (1981) that cAMP increases the number of cell-to-cell channels, and the concentration of cAMP in the cell would determine the steady-
state number of channels. Based on the present studies we believe that investigations examining the reestablishment of gap junction contacts as a function of exogenous hormonal stimulation using isolated myometrial and serosal cells will allow us to distinguish the direct contribution of estrogen versus cAMP in regulating junctional membrane in uterine tissues. Studies are currently underway to resolve this question.

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REFERENCES


