THE ROLE AND REGULATION OF GAP JUNCTIONS IN UTERINE SMOOTH MUSCLE

BY

© WILLIAM CRAWFORD COLE

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

August, 1985

GAP JUNCTIONS IN
UTERINE SMOOTH MUSCLE

DOCTOR OF PHILOSOPHY (1985) McMASTER UNIVERSITY

(Medical Sciences) Hamilton, Ontario

TITLE: The Role and Regulation of Gap Junctions in Uterine Smooth Muscle

AUTHOR: William Crawford Cole, BSc. (University of Toronto)

MSc. (University of Toronto)

SUPERVISOR: Professor Robert E. Garfield

NUMBER OF PAGES: xvi, 224

ABSTRACT

Regular, coordinated contractions of uterine smooth muscle are thought to facilitate the delivery of the fetus(es) at parturition. The development of synchronous activity at term follows the development of many, large gap junctions between uterine smooth muscle cells. The objectives of this thesis were to test the hypothesis that the formation of gap junctions improves direct intercellular communication between the muscle fibers and to determine whether the functional properties of the junctions are regulated. Increased intercellular communication in uterine tissues may facilitate synchronous activity during labor and the regulation of such cell-cell interactions by modulating the function of the gap junctions may participate in the control of uterine contractility during pregnancy and parturition.

A technique was developed to study the diffusion of a small radiolabelled glucose analog, 2-deoxyglucose, through small strips of myometrium. Tissues with many gap junctions from rats in labor demonstrated a significantly greater redistribution of tracer compared to muscle removed from days 17-20 pregnant and days 2-3 post partum animals not in labor which had few junctions. This movement of tracer was shown to be the result of intracellular and direct, cell-to-cell diffusion. Thus, there is evidence for improved intercellular communication in the myometrium during parturition when gap junctions are present.

The extent of intercellular communication in the parturient myometrium was reduced by elevating the intracellular concentrations of Ca^{++} and cyclic AMP in the absence of a change in the extent of gap junctions. More significantly, however, reduced communication was shown in tissues exposed to specific agents which are thought to play a role in the regulation of pregnancy and parturition. Thus, in addition to providing for the increase in gap junctions in the myometrium at term, the hormonal alterations which precede and accompany labor may also regulate the functional properties of these cell-to-cell membrane channels.

Acknowledgements

I would like to express my gratitude to my supervisor, Dr. Bob Garfield for his support and encouragement during the course of this study. Many opportunities were provided for my growth and for these I am deeply grateful.

I cannot adequately return the friendship which Dr. M.S. Kannan has extended to me - his advice, criticsm and encouragement will long be remembered as will his support during my oral defense.

I am indebted to my supervisory committee consisting of Dr. Denis Crankshaw, Dr. Jack Diamond, and Dr. Jack Kirkaldy for their interest in me and this project. Their many helpful suggestions and criticisms were very appreciated.

Many other individuals at McMaster University made my sojourn an enjoyable experience. The stimulating companionship and advice of Dr. P.K. Rangachari was a highlight. I want to thank Jean Crankshaw for the many cups of tea that I stole. Colin Ikeson gave his time and technical expertise without reservation and I shall always be indebted to him for the manufacture of the apparatus used in this study. Thanks also to Debbie Merrett, Elizabeth Welsh, and Peter Bowker who helped out with some of the drudgery in this project.

I am very thankful for the Research Traineeship award and continued support granted to me by the Canadian Heart Foundation.

The ever present support and love given to me by my parents made this work worthwhile.

Mr. & Mrs. T.T. Cole provided the distractions necessary to put life into context and I thank them for this and for their patience when I wasn't around and when I was to busy to run.

Finally, my wife, Catherine, for her contributions to my studies, thesis, and life - without the balloons I couldn't have made it.

TABLE OF CONTENTS

page

CHAPTER 1.0: INTRODUCTION 1

1.1 GENERAL INTRODUCTION AND OBJECTIVES 2

1.2 GAP JUNCTION STRUCTURE............................ 4

1.2.1 Thin Section Electron Microscopy of GJs.......... 5

1.2.2 Freeze-fracture Microscopy of GJs................ 5

1.2.3 Diffraction Analysis of GJs...................... 6

1.3 DIRECT INTERCELLULAR COMMUNICATION...................10

1.3.1 Evidence for Cell-to-Cell Coupling...............12

1.3.2 Characteristics of the Cell-to-Cell Pathway......14

1.3.3 Regulation of Cell-to-Cell Coupling..............16

1.3.4 Roles of Cell-to-Cell Coupling...................20

1.4 UTERINE SMOOTH MUSCLE...............................21

1.4.1 Structure of the Uterus and Myometrium...........21

1.4.2 Alterations in Myometrial GJs at Term............22

1.4.3 Control of Myometrial GJs........................25

1.4.4 Electrical Properties of Uterine Smooth Muscle...30

1.5 MECHANISM OF PARTURITION.............................34

1.6 STRATEGY OF THE PRESENT STUDY........................39

CHAPTER 2.0: METHODS AND MATERIALS 42

2.1 TISSUES AND PREPARATION OF MUSCLE STRIPS FOR

THE DIFFUSION EXPERIMENTS.......................... 43
2.2 DIFFUSION APPARATUS AND TECHNIQUE.................... 46
2.3 ANALYSIS OF DIFFUSION DATA.......................... 47
2.4 UPTAKE AND EFFLUX EXPERIMENTS........................ 50
2.5 ELECTRON MICROSCOPY AND MORPHOMETRIC ANALYSIS........ 51

CHAPTER 3.0: ALTERATIONS IN FUNCTIONAL COUPLING AND GAP JUNCTIONS IN UTERINE SMOOTH MUSCLE 55

3.1 INTRODUCTION.. 56
3.2 METHODS AND MATERIALS............................... 57
3.3 RESULTS... 58
3.3.1 Diffusion Experiments............................. 58
3.3.1.1 2-DG diffusion in longitudinal myometrium.... 58
3.3.1.2 Extracellular tracer diffusion in longitudinal myometrium.................. 65
3.3.1.3 2-DG and sucrose diffusion in hepatic portal vein longitudinal smooth muscle.............. 66
3.3.2 Structural Analysis............................... 67
3.3.2.1 GJ area and muscle fiber size in longitudinal myometrium...................... 67
3.3.2.2 GJ area in hepatic portal vein.............. 73
3.3.3 2-DG, Sucrose and Mannitol Uptake and Efflux..... 77
3.4 DISCUSSION.. 82
3.4.1 GJs Between Uterine Smooth Muscle Cells.......... 82
3.4.2 Route of 2-DG Diffusion.......................... 84

3.4.3 Role of GJs and Intercellular Communication in Uterine Smooth Muscle........................ 88
3.4.4 Calculation of a Theoretical Value of Da for 2-DG Diffusion in Longitudinal Myometrium.... 90
3.5 SUMMARY... 98

CHAPTER 4.0: REGULATION OF MYOMETRIAL GAP JUNCTION PERMEABILITY BY [Ca++]i AND CALMODULIN 99

4.1 INTRODUCTION... 100
4.2 METHODS AND MATERIALS.............................. 101
4.3 RESULTS... 102
4.3.1 Diffusion Experiments............................ 102
4.3.1.1 Calcium ionophore reduces 2-DG diffusivity....102
4.3.1.2 Calmodulin antagonists block the effects of ionophore........................ 104
4.3.2 Lack of an Effect of Ionophore and Calmodulin Antagonist on Structural Coupling..... 106
4.3.3 Lack of an Effect of Ionophore and Calmodulin Antagonist on 2-DG Efflux............. 106
4.4 DISCUSSION... 118
4.4.1 Mechanism for Altered 2-DG Diffusivity.......... 118
4.4.2 Uncoupling Induced by Elevated $[Ca^{++}]_i$........... 121
4.4.3 Roles of Calmodulin in the Control of GJ Permeability................................ 124
4.5 SUMMARY.. 132

CHAPTER 5.0: REGULATION OF MYOMETRIAL GAP JUNCTION PERMEABILITY BY [cAMP]i 133

5.1 INTRODUCTION 134
5.2 METHODS AND MATERIALS 135
5.3 RESULTS 135
5.3.1 Diffusion Experiments 135
5.3.1.1 Db-cAMP and 8-Bromo cAMP reduce 2-DG diffusivity 135
5.3.1.2 Forskolin and theophylline reduce 2-DG diffusivity 139
5.3.2 Structural Analysis 144
5.3.2.1 Lack of an effect of elevated $[cAMP]_i$ on GJ area 144
5.3.2.2 Forskolin induces an alteration in GJ morphology 144
5.3.3 Lack of an Effect of Elevated $[cAMP]_i$ on 2-DG Efflux 146
5.4 DISCUSSION 151
5.4.1 Modulation of Functional Coupling by $[cAMP]_i$ 151
5.4.2 Possible Association between GJs and Adenylate Cyclase 154
5.5 SUMMARY 157

CHAPTER 6.0: EVIDENCE FOR PHYSIOLOGICAL REGULATION OF

MYOMETRIAL GAP JUNCTION PERMEABILITY 159

6.1 INTRODUCTION... 160

6.2 METHODS AND MATERIALS.................................. 161

6.3 RESULTS.. 162

6.3.1 Diffusion Experiments................................ 162

6.3.1.1 Agonists which elevate $[cAMP]_i$ reduce 2-DG diffusivity........................ 162

6.3.1.2 Effect of oxytocin, PG E_2, and PG $F_{2\alpha}$ on 2-DG diffusivity........................ 162

6.3.1.3 Effect of indomethacin and ETYA on 2-DG diffusivity........................ 166

6.3.2 Structural Analysis.................................. 172

6.3.2.1 Lack of any effect on GJ area.................... 172

6.3.2.2 Alterations in GJ morphology..................... 172

6.4 DISCUSSION... 172

6.4.1 Control of functional Coupling by Physiologically Relevant Agonists.............. 172

6.4.2 Possible Mechanisms for Enhancing Myometrial GJ Permeability..................... 176

6.4.3 Two Different Mechanisms Control Functional Coupling in the Myometrium................... 178

6.5 SUMMARY.. 183

CHAPTER 7.0: SUMMARY AND CONCLUSIONS 184

7.1 GENERAL CONCLUSIONS AND FURTHER QUESTIONS............ 185
7.2 SUMMARY... 188

REFERENCES.. 191

List of Tables

page

1. TABLE 3.1: The apparent diffusion efficient (Da) for 2-DG in myometrial tissues from rats on various days of pregnancy....................................62

2. TABLE 3.2: Gap junctions in the myometrium and hepatic portal vein *in vivo* and after the diffusion experiments *in vitro*..................................74

3. TABLE 4.1: The apparent diffusion coefficient (Da) for 2-DG in parturient myometrial tissues with ELEVATED $[Ca^{++}]_i$..105

4. TABLE 4.2: Gap junctions in parturient myometrium after treatment with ionophore (Iono), chlorpromazine (Chlor.), or Ca^{++} free solutions, during the diffusion experiments.......................115

5. TABLE 5.1: The apparent diffusion coefficient (Da) for 2-DG in parturient myometrial tissues with elevated $[cAMP]_i$.......................................138

6. TABLE 5.2: Gap junctions in parturient myometrium after treatment with dibutyryl cAMP (DBcAMP) or forskolin (Forsk.) during the diffusion experiments..145

7. TABLE 6.1: The apparent diffusion coefficient (Da) for 2-DG in parturient myometrial tissues treated

with prostaglandins, oxytocin, isoproterenol, relaxin, indomethacin and 5,8,11,14 eicosatetraynoic acid (ETYA)........................... 145

8. TABLE 6.2: Gap junctions in parturient myometrium after treatment with isoproterenol (Isopr.), relaxin (Relax.), carbacyclin (Carba.), or 5,8,11,14 eicosatetraynoic acid (ETYA)................. 171

List of Figures

page

1.1 Diagram of a gap junction............................9

1.2 Changes in gap junctions in several species at term.....24

2.1 Diagram of the two-compartment bathing chamber..........45

3.1 Distribution of 2-DG in myometrial tissues..............60

3.2 Gap junctions and Da for 2-DG in myometrium.............64

3.3 Distribution of ^{3}H-sucrose in myometrium...............68

3.4 Distribution of ^{3}H-mannitol in myometrium..............70

3.5 Distribution of 2-DG and sucrose in portal vein.........72

3.6 Electron micrographs of gap junctions in myometrium.....76

3.7 Uptake of tracers by myometrium.........................79

3.8 Efflux of tracers from myometrium.......................81

3.9 Diagram of the myometrium...............................93

4.1 Effect of ionophore on 2-DG diffusion...................104

4.2 Effect of calmodulin antagonists on uncoupling..........108

4.3 Dose-response curve for inhibition of uncoupling by calmodulin antagonists.........................110

4.4 Effect of chlorpromazine on 2-DG diffusion................112
4.5 Effect of calmidazolium on 2-DG diffusion................114
4.6 Tracer efflux after treatment to elevate Ca^{++}............117
4.7 Diagram of uncoupling by Ca^{++}..........................126

5.1 Effect of elevated cAMP on 2-DG diffusion................137
5.2 Effect of cAMP, 5'AMP and butyrate on 2-DG diffusion....141
5.3 Effect of forskolin and theophylline on 2-DG diffusion..143
5.4 Electron micrographs of gap junctions with deposits.....148
5.5 Tracer efflux after treatment to elevate cAMP...........150

6.1 Effect of relaxin, carbacyclin and isoproterenol on 2-DG diffusion.......................................164
6.2 Effect of oxytocin, PGs $F_{2\alpha}$ and E_2 on 2-DG diffusion....168
6.3 Effect of indomethacin and 5,8,11,14 eicosatetraynoic Acid on 2-DG diffusion.............................170
6.4 Model for control of myometrial gap junctions...........181

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION AND OBJECTIVES

The mechanisms which underlie and regulate the transformation of uterine muscular activity at the end of gestation and permit this organ to effectively expel the fetus(es) are incompletely understood. Uterine muscle is unique among smooth muscles in that it only displays very rare, brief episodes of highly active well-coordinated, synchronous contractility. Indeed, simultaneous contractions in different regions of the uterine wall are only associated with, and are believed to be necessary for, delivery of the young at parturition (Reynolds, 1949; Fuchs, 1969, 1978; Liggins, 1979; Thorburn & Challis, 1979; Csapo, 1981). Moreover, the absence of this activity and the predominance of a state of relative quiescence and asynchrony are thought to be required for implantation and the maturation of the fetus(es) during gestation (Reynolds, 1949; Liggins, 1979; Csapo, 1981).

In light of the multicellular nature of uterine and other visceral smooth muscles, Emil Bozler (1938) concluded that;

> "uncoordinated activity of the small muscle cells could never produce the regular movements which are observed;...in the absence of external stimuli, rhythmic contractions can only be understood by postulating some mechanism of conduction which coordinates the activity of the numerous elements".
>
> E. Bozler, Amer. J. Physiol., 211:614 (1938)

The innervation of uterus is not considered to directly stimulate activity in the myometrial muscle cells (Abe, 1970; Marshall, 1961, 1981), nor is a specialized conduction pathway similar to the Purkinje fiber system in the heart present in the myometrium. For these

reasons, it is thought that electrical activity must propagate from cell-to-cell to synchronize and coordinate mechanical events in the individual myometrial muscle fibers (Abe & Tomita, 1968; Abe, 1970). Thus, it is evident that the manner by which this activity propagates between the smooth muscle cells and the factor(s) which regulate this process during gestation and labor are of singular importance to an understanding of the mechanisms which maintain pregnancy and initiate parturition.

The recent observation that gap junctions (GJs) are only present in large numbers and sizes between uterine smooth muscle cells at parturition (Garfield et al., 1977, 1978) is thought to be significant with regards to propagation and the development of coordinated contractility during labor. These specialized sites of cell-to-cell contact are believed to provide pathways (ie. cell-to-cell channels) for direct intercellular communication and permit the synchronization of electrical and metabolic activities in other multicellular tissues (Peracchia, 1980; Loewenstein, 1981; Hooper & Subak-Sharpe, 1981; Spray et al., 1984). It was suggested, therefore, that the development of GJs at term might facilitate parturition by providing the structural basis for propagation of electrical activity from cell-to-cell throughout the uterine wall (Garfield et al., 1977). However, whether the junctions actually function in this capacity is unclear; previous studies provide conflicting evidence for a role for GJs in direct intercellular communication in smooth muscle (Daniel et al., 1976) and for a change in electrical cell-to-cell communication in the uterus at term (Daniel & Lodge, 1973; Kuriyama & Suzuki, 1976; Zelcer

& Daniel, 1979; Sims et al., 1982). In addition, it is not known whether the functional properties of GJs in the myometrium are regulated. The presence of the junctions appears to be controlled by the hormonal milieu of the uterus (Garfield et al., 1978, 1980a,b), but whether the GJs are rendered functional (open channels), or nonfunctional (closed channels), by similar factors is unknown. Control of the function of the junctions may provide an additional mechanism whereby the development of synchronous activity in the myometrium can be regulated physiologically, and prehaps, manipulated pharmacologically.

In light of these considerations, the general objectives of this thesis were to;

1) determine whether there is a change in direct intercellular communication in uterine muscle associated with the development of GJs at term by studying cell-to-cell diffusion of a low molecular weight radiolabelled metabolite in tissues from pregnant, parturient, and post-partum rats,

and 2) to determine whether there are any factors which may regulate the functional properties of GJs in uterine muscle.

1.2 GAP JUNCTION STRUCTURE

Gap junctions occur between almost all varieties of cells in vertebrate tissues with the exception of freely circulating blood cells and skeletal muscle fibers (Staehelin, 1974; Larsen, 1977b, 1983; Peracchia,1980). Their structure has been probed with thin section and freeze fracture electron microscopy as well as electron and X-ray diffraction analysis. The composite picture of GJs provided

by these techniques indicates that they are aggregates of intramembrane protein particles, or connexons (Caspar et al., 1977; Makowski et al., 1984), between closely apposed, plasma membranes of adjacent cells.

1.2.1 Thin Section Electron Microscopy of GJs

GJs are identified in thin sections of tissue samples as regions of close membrane-membrane apposition that possess a distinct multilamellar appearance. The junctional membranes are separated by a 2-3 nm extracellular space or 'gap', and have a characteristic 5- or 7-lined configuration in tissues stained en bloc with uranium salts (Staehelin, 1974; Peracchia, 1980). Opportune tangential sections reveal a pattern within the extracellular 'gap' composed of alternating electron-dense and -lucent bands aligned perpendicular to the junctional membranes. A similar but reverse pattern is observed if the extracellular space is delineated with small electron dense tracers, such as lanthanum or tannic acid (Staehelin, 1974). This periodicity is thought to reflect the presence of subunits within the 'gap' that bridge the extracellular space and provide the only actual physical contact between the adjacent membranes (Peracchia, 1980).

1.2.2 Freeze-fracture Microscopy of GJs

The macular appearance and individual subunits of GJs are readily apparent in replicas of freeze-fractured tissues. This technique provides an en face view of the internal surfaces of the membrane bilayers within the junction. Vertebrate GJs are discoid aggregates of either 8-10 nm in diameter intramembrane protein particles (the connexons) that protrude from the protoplasmic (P-) face of the lipid

bilayer or of corresponding depressions pits on the extracellular (E-) fracture face (Peracchia, 1980). Peracchia (1980) has suggested that an aggregate of intramembrane particles can only be positively identified as a GJ if the fracture plane passes through both bilayers and exposes both membrane faces within the junction or if there is a distinct association between the particles and a region of membrane-membrane contact. The particles are generally separated by 8-14 nm but this is quite variable and is dependent on the packing arrangement (Staehelin, 1974; Peracchia, 1980). Polygonal, irregular arrays are more random and display greater separation than hexagonal aggregates. Low angle rotary shadowed replicas reveal a shallow depression in the center of the particles which is interpreted to mark the site of the postulated cell-to-cell channel or pore within each connexon (Peracchia, 1980).

1.2.3 Diffraction Analysis of GJs

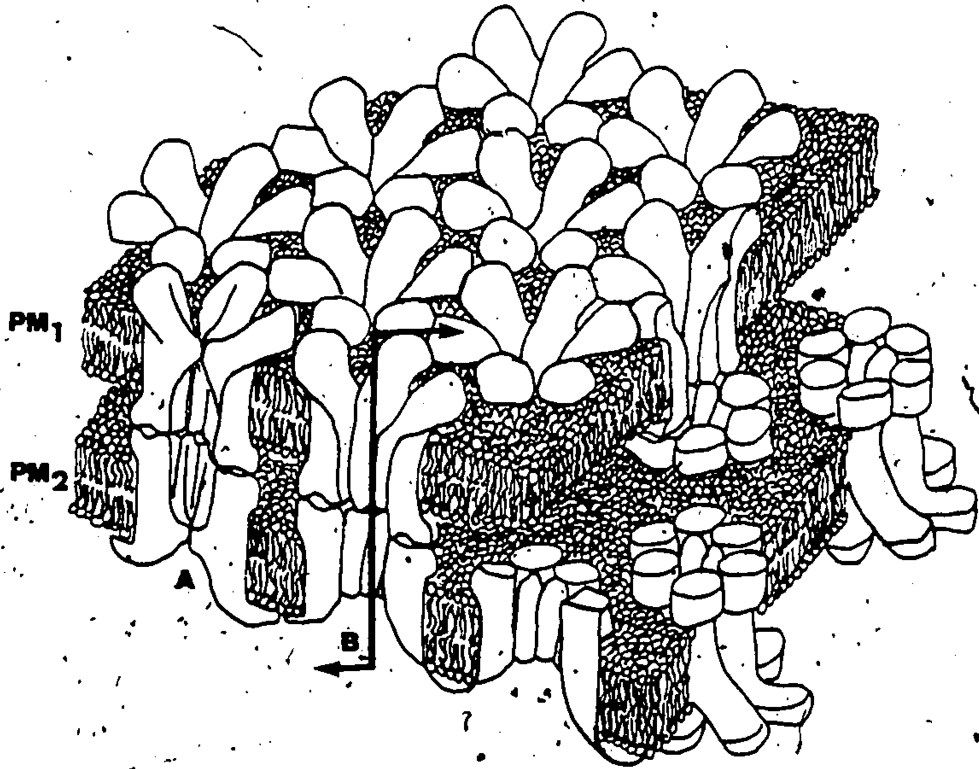
Structural studies of isolated junctional plaques from liver tissues by X-ray and electron diffraction demonstrate the connexons to consist of two identical subunits, called hemichannels (Spray et al., 1984) or protochannels (Loewenstein, 1981), that are symmetrically contributed to the junction by the apposing membranes. Each hemichannel comprises six rod-like, identical protein subunits, or connexins (Zampighi & Unwin, 1979; Unwin & Zampighi, 1980; Unwin & Innis, 1982; Makowski et al., 1984), which are 2.5 nm thick by 7.5 nm long and arranged in a ring around a central region of low density 1-2 nm in diameter (Makowski et al., 1984). This central region is thought to represent the postulated cell-to-cell channel that extends through the length of

each connexon. However, unequivocal evidence for the existence of a continuous channel is still lacking.

Interestingly, Unwin and co-workers (Zampighi & Unwin, 1979; Unwin & Zampighi, 1980; Unwin & Ennis, 1982) have observed two different connexin arrangements in dialyzed (in distilled water) liver junction samples and, in one conformational state, the central low density region was not observed. These authors speculated that these arrangements indicate that the connexons may exist in different conformations, permitting the channel to be either open or closed. Moreover, it was found that the so-called closed conformation could be induced by exposing the junctional plaques to solutions of relatively high Ca^{++} concentration (0.1 mM)(Unwin & Ennis, 1983). This change was interpreted to account for the inhibition of communication between cells with elevated levels of intracellular free calcium ion (see section 1.3.3). However, Makowski et al. (1984) have not observed similar variations in connexin arrangements in detergent isolated liver GJs. Furthermore, it was suggested that a region of high diffraction density at the cytoplasmic end of the postulated channel may represent a gating mechanism responsible for occluding the pore entrance and providing different states of channel permeability (Makowski, 1985). Whether either of these observations are valid for the intact, *in situ* junction remains to be determined.

The concept that GJs are aggregates of many individual, functional units, or connexons, that possess an aqueous channel linking the cytoplasmic compartments of adjacent cells is summarized in the diagramatic representation of a GJ shown in figure 1.1.

FIGURE 1.1: A diagrammatic representation of a gap junction. Several channels or connexons are shown to cross the extracellular space between adjacent lipid bilayers of opposing plasma membranes (PM_1 & PM_2). Each connexon is shown to be composed of a ring of six subunits which surround a central cell-to-cell channel. The arrow passes along the postulated position of the channel in connexon 'B'. The two postulated states of the cell-to-cell channel are shown in A and B; note that the gates are closed in A. (Based on a diagram in Makowski et al., 1984).


PM1
PM2
A
B

1.3 DIRECT INTERCELLULAR COMMUNICATION

It is postulated that the synchronization and coordination of cellular activities in communities of structurally independent cells is provided by a direct cell-to-cell exchange of low molecular weight ions, metabolites, second messengers and regulatory molecules, referred to as intercellular communication or cell coupling (Loewenstein, 1981; Hooper & Subak-Sharpe, 1981). This free exchange of small protoplasmic constituents couples the metabolic and electrical activities of cells while allowing for the maintenance of macromolecular individuality; it represents a compromise between cellular independence, as seen in many neurons and circulating blood elements, and cellular fusion into anatomical syncytia, as in skeletal muscle. In other words, diffusion of small molecules within a continuous, aqueous cellular and intercellular compartment permits the formation of a functional, albeit not anatomical, electrical and metabolic syncytium. The evidence that the 'private pathway' between cells is provided by GJs is compelling, however, it is indirect and circumstantial; the channel behavior of isolated connexons reconstituted into isolated membrane systems has not been adequately investigated.

The evidence that GJs provide the pathway for cell-to-cell coupling is summarized as follows;

1) GJs are the only variety of cell-to-cell contact between some communicating cell types in culture (Gilula et al., 1972; Johnson et al., 1974; Sheridan et al., 1978),

2) mutant cell lines incapable of intercellular communication do

not possess ultrastructurally identifiable GJs (Hooper & Subak-Sharpe, 1981),

3) the extent of electrical (Johnson et al., 1974; Flagg-Newton et al., 1981; Meyer et al., 1981; Sims et al., 1982) and metabolic (Flagg-Newton et al., 1981; Meyer et al., 1981; Cole et al., 1985) coupling increases and decreases in parallel with alterations in the area of GJs between communicating cell types in cultures and in intact tissue preparations (and see results in Chapter 3, section 3.3.1.1 below),

and 4) intracellular (but not extracellular) application of antibodies generated to isolated liver GJs (but not the affinity purified antibody or preimmune sera) will prevent electrical and fluorescent dye coupling between cells in cultures of hepatocytes, myocardial cells, and superior cervical ganglion neurones (Hertzberg et al., 1985).

Although it is generally accepted that GJs are sufficient for cell-to-cell communication, whether they are necessary for electrical coupling remains a point of considerable debate. Electrical coupling is observed in the apparent absence of ultrastructurally defined GJs in some tissues (Daniel et al., 1976; Williams & De Haan, 1981), including several smooth muscles (see section 1.4.2). For this reason it was suggested that there may be other low resistance pathways for current flow between cells (Daniel et al., 1976; Loewenstein, 1981; Williams & De Haan, 1981), or that cells may be functionally coupled by other processes, such as the electrical field mechanism postulated by Sperelakis and co-workers (see Sperelakis, 1979). Additionally, it

has been argued that coupling might be mediated by isolated, functional connexons scattered in the plasma membrane (Peracchia, 1980; Loewenstein, 1981; Williams & De Haan, 1981). Such pathways could not be identified as GJs using the ultrastructural criteria which are presently employed (Peracchia, 1980).

1.3.1 Evidence for Cell-to-Cell Coupling

Evidence for direct exchange of small ions and organic molecules between cells has been obtained by a variety of techniques. Measurements of electrical coupling between cells in systems with a simple structure, such as occur in epithelia or small clusters of cells, are generally made using a double microelectrode technique (Socolar & Loewenstein, 1982); one electrode is inserted into a cell and is used to pass current and record voltage changes (V_a) while a second electrode monitors the potential across the membrane of an adjacent cell (V_b). If the cells are coupled by low resistance pathways, then small hyperpolarizing and sub-threshold depolarizing potential changes in one cell will spread passively into the contiguous partner and result in a parallel shift in membrane voltage. The degree of coupling is indicated by the ratio of voltage changes in the two electrodes, V_b/V_a, which is referred to as the coupling coefficient. A coefficient of zero indicates electrical isolation. whereas unity suggests perfect coupling.

Optical and radioactive tracers have been used to probe for metabolite communication between adjacent cells. Small membrane-impermeant hydrophilic fluorescent dyes, such as 6-carboxyfluorescein and Lucifer yellow (see Socolar & Loewenstein, 1982), will diffuse

directly between coupled cells, with no evidence of dye in the intervening extracellular space, when iontophoretically injected into the intracellular compartment with microelectrodes. In contrast, large labelled molecules such as albumin and horseradish peroxidase are not transferred (Loewenstein, 1981). Donor cells containing diffusible, membrane impermeant radioactive tracers, such as phosphorylated nucleotides (including cyclic and high energy phosphate derivatives) or sugars (eg. glucose and 2-deoxyglucose), will impart radioactivity to nonlabelled cells in co-cultures (Hooper & Subak-Sharpe, 1981). This ability to exchange fluorescent dyes or radioactive molecules is dependent on cell-to-cell contact and on the presence of GJs (Hooper & Subak-Sharpe, 1981).

In general, however, quantitative studies of metabolite communication between cells are rare and primarily limited to systems with a simple geometry. Although cardiac tissues possess a complex 3-dimensional geometry, cells in small preparations of trabecular and Purkinje fibers are arranged as a series of parallel, linear cables. This simple geometry was exploited by Weidmann (1966) and others (Imanaga, 1974; Weingart, 1974; Tsien & Weingart, 1976) to study the longitudinal, cell-to-cell diffusion of radiolabelled (eg. K^+, tetraethylammonium ions (TEA^+), and cyclic adenosine-3'5'-monophosphate (cAMP)) and fluorescent (Procion yellow) tracers within small bundles of these synchronously contracting muscle tissues. A small portion of each muscle bundle was exposed to the tracer and its distribution within the remainder of the bundle determined subsequent to a period for longitudinal diffusion. The apparent diffusion

coefficient for tracer movement within the syncytium, which provides a quantitative evaluation of the extent of functional cell-to-cell coupling, was then calculated from the tracer distribution curves. A modification of this technique was used in the present study to evaluate the extent of metabolite communication between smooth muscle cells in the longitudinal uterine muscle of pregnant and parturient rats (see Chapter 3 below).

1.3.2 Characteristics of the Cell-to-Cell Channel

A wide variety of functional studies have shown the intercellular pathway or channel for cell-to-cell coupling to possess a number of important qualities. Firstly, Brink (1983; see also Brink et al., 1984) has shown that the pathway is an aqueous environment and that charged solute molecules diffuse through it surrounded by hydration shells. The fluorescent dye, dichlorofluorescein, was employed by Brink (1983) because it has a molecular diameter (1.0-1.1 nm) close to the suspected diameter of the channel (1.5-2.0 nm) across the junctional membrane between cells in the septate giant axon of the earthworm. The movement of the dye molecules through the junctions was found to be slower, and more sensitive to reduced temperature, in saline solutions composed of D_2O as compared to H_2O. These data were interpreted to indicate that the dye molecules and channel interior must be hydrated because the size of hydration shells would be expected to be fractionally larger in D_2O compared to H_2O solutions, and as well, the shell size would therefore increase slightly more rapidly in 'heavy' solutions with decreasing temperature giving rise to slightly different rates of movement (see Brink, 1983).

Secondly, the channel discriminates between permeant and nonpermeant molecules on the basis of size and charge. A number of synthetic and natural peptides were conjugated to fluorescent dyes to make a series of molecules of increasing size and charge (Simpson et al., 1977; Flagg-Newton et al., 1979). It was shown that molecules up to about 1200 daltons diffuse between mammalian cells and that more polar species are selectively impeded over those of similar size but bearing no charge (Simpson et al., 1977; Flagg-Newton et al., 1979; Brink & Dewey, 1978, 1980). Unfortunately, the fact that the diffusing molecules used by Loewenstein and co-workers (Simpson et al., 1977; Flagg-Newton et al., 1979) were anisotropic and possessed similar abaxial dimensions limits any speculation regarding the actual dimensions of the channel; what is lacking is a series of spherical probes of increasing diameter.

Thirdly, it seems that the pathway which permits electrical coupling is shared with small organic molecules moving between adjacent cells (Brink et al., 1985). The conductance of the cell-to-cell pathway was shown to decrease when large fluorescent dye molecules were simultaneously passing between cells. This was interpreted to indicate that the slowly diffusing dye molecules restricted the transport of the relatively much faster moving current carrying ions (Brink et al., 1985).

Finally, reversible drops in the conductance and permeability of the pathway are observed under a wide variety of experimental conditions (see section 1.3.3). This suggests that the channels can switch between states of low and high resistance (Loewenstein, 1981;

De Mello, 1982b; Spray et al., 1984). Whether this switch is all or none (channels are either open or closed) or iris-like, with states of partial conductance, remains to be determined. Evidence has been presented consistent with both mechanisms (see Loewenstein, 1980; Spray et al., 1984), but single channel recording techniques which could resolve this problem have not yet been employed.

1.3.3 Regulation of Cell-to-Cell Coupling

Alterations in the extent of coupling producing either increased or decreased cellular interactions are described for a variety of cell types (see Spray & Bennett, 1985). These changes are often, but not always, concomitant to variations in the extent of structural coupling by GJs (ie. the number and size of GJs). In theory, alterations in coupling could result from the addition or withdrawal of functional cell-to-cell channels from the plasma membrane, or conversely, from a change in the permeability of existing channels. That junctional resistance drops in uniform, step-like or quantal jumps during GJ formation (Ito et al., 1974; Loewenstein et al., 1978) is consistent with the idea that the extent of coupling can be altered by the insertion or withdrawal of individual, functional conducting units.

Concomitant alterations in the area of GJs and functional coupling between cells can be manipulated by several experimental procedures (Spray & Bennett, 1985). More importantly, however, there are several examples of tissues in which changes in structural coupling are associated with marked alterations in cellular activity and in which the altered coupling appears to be provoked by circulating or local hormones, neurotransmitters, secretagogues, and autacoids (Peracchia,

1980; Larsen, 1983; Spray & Bennett, 1985). Presumably, these alterations result from a modification in GJ synthesis, insertion into the plasma membrane, connexon formation and aggregation, or GJ degradation. The myometrium is an excellent example of a tissue demonstrating physiological regulation of structural coupling and a distinct temporal association between changes in GJs and intercellular coordination of activity (see sections 1.4.1.3 & 1.5).

Alterations in functional coupling in the absence of any change in the extent of GJs between cells have been reported for many cell types (Peracchia, 1980; Loewenstein, 1981; De Mello, 1982b). In this case, altered coupling is interpreted to result from changes in the conductance or permeability of the cell-to-cell pathway (Loewenstein ,1981; Spray et al., 1984). This mechanism is thought to provide a short term control of coupling (seconds to hours) whereas changes in structural coupling are long term (hours to days) (Spray & Bennett, 1985). The possible structural mechanism(s) which may account for altered GJ channel permeability were indicated previously (see section 1.2.4).

Studies on the factors which influence functional coupling in a variety of vertebrate tissues implicate intracellular Ca^{++}, H^{+}, and cAMP as important determinants of channel permeability (Loewenstein, 1981; Spray et al., 1984; Spray & Bennett, 1985). Transjunctional potential differences also seem to influence coupling in some vertebrate tissues (Spray et al., 1984), but this is most pronounced in embryonic cells and of rather less concern in adult tissues (with the exception of rectifying GJs in neuronal pathways) (eg. Auerbach &

Bennett, 1969). Elevated intracellular levels of Ca^{++} ($[Ca^{++}]_i$) (Loewenstein, 1981), or reduced cytosolic pH (Spray et al., 1984), appear to reduce coupling in a wide variety of cell tissues (and see results in Chapter 4, section 4.3.1 below), however, the effects of elevated $[cAMP]_i$ are tissue specific. Coupling between horizontal cells in the retina is decreased by cAMP (Laufer & Salas, 1981; Piccolino et al., 1982; Teranishi et al., 1982, and see the results in Chapter 5, section 5.3.1 below) but it is elevated in cardiac tissues (Estapé & De Mello, 1983; De Mello, 1982a,b, 1983, 1984). Evidence for control of channel permeability by physiologically relevant hormones and neurotransmitters is sparse, however, angiotensin II (Hermsmeyer, 1982), epinephrine (De Mello, 1982a,b), and norepinephrine (see Spray & Bennett, 1985) increase coupling in the heart, dopamine apparently inhibits communication between retinal horizontal cells (Piccolino et al., 1982; Teranishi et al., 1982), and rather high concentrations of acetylcholine, bombesin, and caerulein uncouple pancreatic acinar cells (Iwatsuki & Peterson, 1978a,b; Findlay & Peterson, 1982) (see also results in Chapter 6, section 6.3.1 below).

Loewenstein (see Loewenstein, 1981) originally advanced the hypothesis that elevated $[Ca^{++}]_i$ produces physiogically relevant alterations in GJ permeability and functional coupling. Subsequently, it was demonstrated that a variety of experimental manipulations which elevate $[Ca^{++}]_i$, such as membrane rupture, exposure to calcium ionophores, intracellular injection of Ca^{++} using microelectrodes, poisoning oxidative phosphorylation with dinitrophenol or cyanide, or inhibiting the sodium pump in cardiac muscle with ouabain, reduce

cell-to-cell coupling (Peracchia, 1980; Loewenstein, 1981; De Mello, 1982b). In some instances, the rise in $[Ca^{++}]_i$ was confirmed with Ca^{++}-sensitive dyes or electrodes (Rose & Loewenstein, 1976) and its effect dissociated from that of cytoplasmic acidification (Rose & Rick, 1978; Spray et al., 1984). However, Spray et al. (1984) have shown that the conductance of the cell-to-cell channels in amphibian embryonic cells is very much more sensitive (x10,000 fold) to altered cytoplasmic pH than elevated $[Ca^{++}]_i$. Furthermore, these authors contend that uncoupling is only produced at levels of $[Ca^{++}]_i$ (100-500 μM) which are well above the range observed in viable cells (0.1-10.0 μM). For this reason it was suggested that pH is the physiologically relevant factor and that the Ca^{++} phenomenon is only important in conditions of pathology or trauma (Spray et al., 1984). It must be noted, however, that it is very difficult to ascertain the precise level of $[Ca^{++}]_i$ in cells let alone within very small compartments such as that within the cytoplasm subjacent to the entrance of cell-to-cell channels. Although it is clear that $[Ca^{++}]_i$ is likely never raised to 100 μM in the general cytoplasm, localized elevations in Ca^{++} at the junctions to such a level might be possible, and for this reason, it seems premature to entirely dismiss a physiological role for Ca^{++}.

The mechanism by which the permeability of the cell-to-cell channels is modulated by Ca^{++}, H^{+}, and cAMP are not clear. These factors could influence the connexons or channel interior directly, or alternatively, their effect could be mediated by intermediate, junction-associated regulatory molecules. A halo of filamentous

electron dense material is almost always observed to be present along the cytoplasmic surface of GJs in thin section electron micrographs of most tissues (Larsen, 1977b, 1983). The significance of this material is unknown. One proteinaceous constituent of the material associated with cardiac junctions has a molecular weight of between 15 and 17K daltons (Manjunath et al., 1984). In this context, it is interesting that the calcium-dependent regulatory protein, calmodulin, has a molecular weight of 16.5K daltons (Cheung, 1980; Means et al., 1982). Furthermore, this protein binds to isolated liver GJs in a Ca^{++}-dependent fashion in vitro (Hertzberg & Gilula, 1981), and has been implicated to confer Ca^{++} and H^{+} sensitivity to GJs between cells in vertebrate cardiac (Wojtczak, 1984) and embryonic (Peracchia et al., 1983; Peracchia,1984) tissues.

1.3.4 Roles of Cell-to-Cell Coupling

The widespread occurrence of cell-cell coupling and the numerous possible candidates for exchangeable molecules has prompted speculation that direct intercellular communication plays a role in a variety of cellular functions (Loewenstein, 1981; De Mello, 1982b). In multicellular systems of excitable cells, coupling is postulated to facilitate synchronized electrical activity (Bennett, 1977; De Mello, 1982b). Cell-to-cell coupling, regardless of the mechanism, is thought to provide the basis for propagation of action and junction potentials between cells in smooth and cardiac muscles and facilitate coordinated, simultaneous contractile activity in these multicellular tissues (Barr et al, 1968a,b; Daniel et al., 1976; Holman & Neild, 1979; De Mello, 1982b; Sims et al., 1982). Burnstock (1970) suggested

that coupling may also provide for the neural modulation of electrical and mechanical activity in large numbers of muscle cells within smooth muscle tissues that demonstrate few nerve varicosities. Coupling was postulated to allow junction potentials and second messengers to spread from a limited number of directly innervated 'key' cells, into cells which are not directly innervated (Burnstock, 1970).

The specific role(s) of coupling in nonexcitable cells is not so readily apparent. Loewenstein (1981) postulates that it may permit cell-cell homeostasis by facilitating intracellular equilibration of permeant molecules. Coupling may also provide a pathway for regulatory molecules important in the control of growth, differentiation, development and pattern formation (Caveney, 1985; Sheridan & Atkinson, 1985).

1.4 UTERINE SMOOTH MUSCLE

1.4.1 Structure of the Uterus and Myometrium

The uterus of the rat is duplex, having two separate, hollow horns or tubes, which most often open into the vagina by way of independent cervical canals (Mossman, 1977). The horns are anchored to the posterior body wall by means of mesometrial peritoneal sheets, through which pass the vascular and neural supply to the organ. The wall of the uterus is composed of three distinct layers; an inner mucosal epithelium, the endometrium, and two layers of smooth muscle tissue, referred to as the myometrium. The outer, serosal muscle layer contains fibers that are oriented along the longitudinal axis of the uterine horns whereas the inner layer is circular muscle. These muscle layers are separated by an extensive vascular plexus. Cells in the

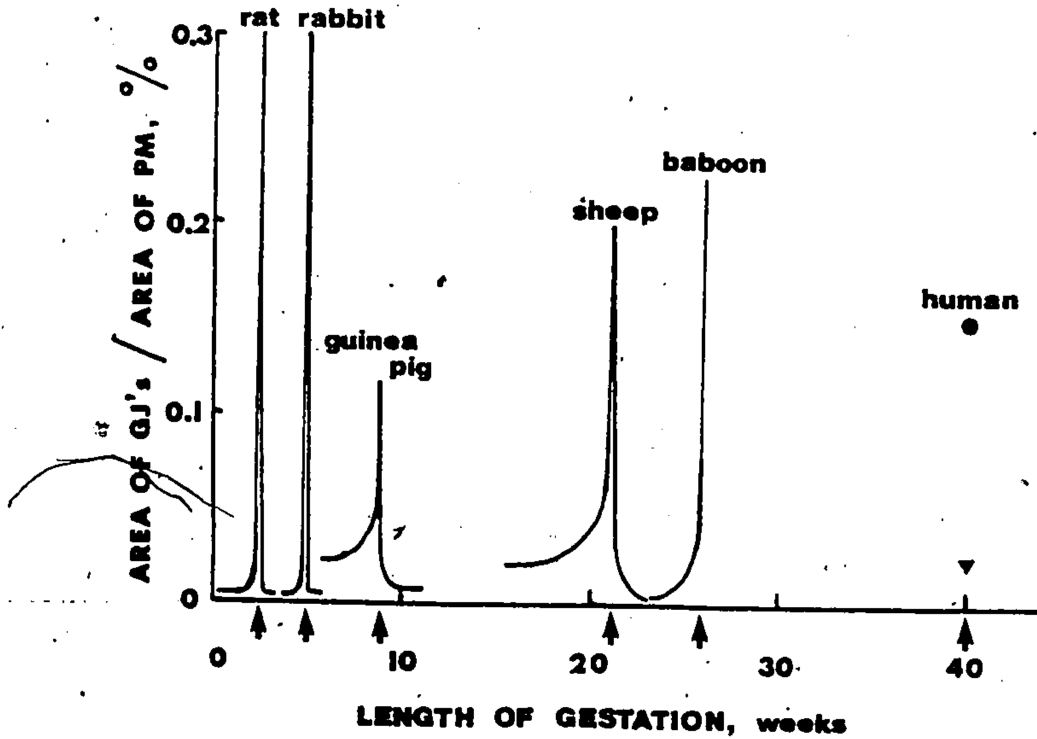
longitudinal layer are arranged into a distinct, anastomosing network of longitudinally oriented muscle bundles some 50-200 μm in cross-section. In contrast, the cells in the circular layer are not arranged into bundles, and appear to be more dispersed than the fibers in the longitudinal layer.

The muscle fibers in both layers display ultrastructural features typical of smooth muscle cells. Each fiber is distinctly bipolar and anisotropic, reaching maximal dimensions of 5-10 μm in diameter and 300-600 μm in length in tissues from late pregnant animals (Csapo, 1981, and see results in Chapter 3, section 3.3.2.1 below). They possess a central nucleus and typical complement of cytoplasmic organelles including mitochondria, RER, SER, contractile filaments and Golgi apparatus. A variety of cell-to-cell contacts have been described to be present between myometrial cells, including intermediate contacts, interdigitations, simple appositions, and GJs (Garfield & Daniel, 1974; Daniel et al., 1976).

1.4.2 Alterations in Myometrial GJs at Term

Gap junctions in the myometrium (see Fig. 3.6 Chap.3) of the rat and all other mammalian species studied to date display a very marked variation in size and number in tissues from pregnant, parturient, and post-partum animals (see Fig. 1.2) (Garfield et al., 1977, 1978, 1979a,b). This change in structural coupling appears to be dependent on the changing hormonal environment of the uterus just prior to and following parturition (see section 1.4.3 below). GJs only occupy a relatively large proportion (ca. 0.1-0.4 %) of the area of the plasma membrane just prior to, during, and following term or preterm labor

FIGURE 1.2: Diagrammatic representation of the dramatic changes in gap junctions in the myometrium of several species at term. The area of gap junction membrane is shown as a percentage of plasma membrane during late pregnancy and at parturition. Arrows indicate the date of parturition following conception. Note: human data are for tissues from women undergoing caesarean section either prior to labor (▼) or during labor (●). (From Cole & Garfield, 1985b).


MYOMETRIAL GAP JUNCTIONS IN
SEVERAL SPECIES AT PARTURITION
rat
rabbit
guinea
pig
sheep
baboon
human
AREA OF GJ's / AREA OF PM, %
0
0.1
0.2
0.3
0
10
20
30
40
LENGTH OF GESTATION, weeks

(Garfield et al., 1977, 1978). Conversely, they are reported to be absent (Garfield & Daniel, 1974; Garfield et al., 1977, 1978), or present in very low frequency and small size (Garfield el., 1980a,b; Merk et al., 1980; Sims et al., 1982), in tissues from nonpregnant, pregnant but not delivering, and post-partum animals. The junctions begin to form in pregnant rats about 24-36 hours prior to the onset of labor, they are present in abundant numbers (ca. 1000 per cell) and increased size (ca. 250 nm) during normal delivery of the fetuses, and disappear within 24-36 hrs subsequent to parturition (Garfield et al., 1977, 1978, 1980a,b). This pattern of change is particularly prominent and precipitous in rats and rabbits (Garfield et al, 1977, 1978, 1980a) (see Fig. 1.2). Guinea pigs, sheep and humans appear to differ slightly in that they demonstrate greater numbers of GJs prior to parturition, and in the former two species, the period of GJ development at term appears to be more prolonged (Garfield et al., 1979a,b; Garfield & Hayashi, 1981; R.E. Garfield, unpublished observations) (Fig. 1.2).

1.4.3 Control of Myometrial GJs

The role of several circulating and local hormones in the control of the presence of GJs in the myometrium has been studied _in vitro_ and _in vivo_. The evidence is consistent with the presence of an endogenous mechanism for regulating the presence of GJs between myometrial fibers. The extent of structural coupling appears to be dependent on the steroid hormones, estrogen and progesterone, as well as, specific prostaglandins (Garfield et al., 1980a,b)..

Ovariectomy of pregnant rats subsequent to day 15 following

conception leads to premature formation of GJs and labor within 24 to 48 hrs (Garfield et al., 1977). That this premature alteration in GJs results from an experimentally-induced progesterone withdrawal is suggested by a drop in circulating progesterone levels following surgery, and by the ability of progesterone therapy to prevent this drop, preclude the formation of GJs, and prolong pregnancy for as long as treatment is continued. Administration of progesterone to intact rats over the last few days of pregnancy (i.e. day 19 onward) will also prevent normal progesterone withdrawal, inhibit the appearance of GJs, and delay parturition (Garfield et al., 1977, 1978). Progesterone can inhibit GJ development in tissues exposed to estrogens _in vitro_ or _in vivo_, (Garfield et al., 1978). These observations prompted Garfield et al. (1977, 1978) to suggest that progesterone may inhibit labor and maintain pregnancy by repressing the formation of GJs and limiting electrical cell-to-cell coupling in the myometrium. Moreover, it was proposed that this inhibition of GJ development by this steriod may be the basis of the 'progesterone block' hypothesis (see section 1.5 below) for the control of pregnancy and parturition advanced by Csapo (see Csapo, 1981).

It is not entirely evident how the influence of progesterone on myometrial GJ formation is manifested. Puri and Garfield (1982) contend that this steroid could act at one or more of the following levels; inhibition of protein (connexon) synthesis either directly or indirectly through inhibition of the estrogen nuclear receptor-genome interaction, a direct effect on the plasma membrane, and/or an indirect effect through manipulation of the synthesis of prostaglandins.

Injection of relatively high doses of estrogen (either 17β-estradiol or diethylstibesterol, a synthetic estrogen) into immature (Merk et al., 1980; MacKenzie et al., 1984), mature ovariectomized nonpregnant (MacKenzie & Garfield, 1985) and intact, day 18-20 pregnant (L.W. MacKenzie & R.E. Garfield, unpublished observation) rats, will induce the formation of myometrial GJs and, in the latter animals, it will provoke premature labor. Similarly, estrogen will potentiate the formation of junctions in tissues incubated in vitro (Garfield et al., 1980a,b).

It is thought that estrogens give rise to the development of GJs by stimulating the synthesis of connexon proteins (Garfield et al., 1978, 1980a,b). The ability of these steroids to stimulate uterine protein synthesis is well recognized and is believed to result from a direct stimulation of transcription through an interaction between estrogen-nuclear receptor complexes and the genome (Gorski & Gannon, 1976). The ability of an anti-estrogen, tamoxifen (MacKenzie & Garfield, 1985), as well as, actinomyocin D and cycloheximide (Garfield et al., 1980b), to inhibit GJ development in response to estrogens is consistent with a nuclear control of connexon synthesis.

The ability of prostanoid metabolites of arachidonic acid liberated from the plasma membrane of smooth muscle cells, endometrial cells, or the fetal membranes to influence the development of GJs in the myometrium is suggested by several lines of experimentation. However, the role and mechanism by which they operate remain to be identified.

Garfield et al. (1977) demonstrated that GJs were less numerous in

the nondistended horns of unilaterally ovariectomized, parturient rats. Similarly, GJ frequency in the myometrium of post-partum rats following estrogen injections was the greatest, and more closely approached that observed in parturient animals, in uterine horns distended by intra-uterine balloons (Wathes & Porter, 1982). Distention of the myometrium is a well-documented stimulus of uterine prostaglandin synthesis (Thorburn & Challis, 1979) and on the basis of these observations it seems likely that prostanoids and stretch may be required to attain the extent of structural coupling observed in the distended horns of parturient animals.

In vitro experiments show that some prostaglandins may stimulate (thromboxanes & endoperoxides), and others inhibit (prostacyclin), the development of GJs (Garfield et al., 1980a,b). Similar evidence for both a stimulation and an inhibition of myometrial GJ development by prostaglandins has been obtained through the use of drugs (indomethacin and sodium meclofenamate) which inhibit the activity of the prostanoid forming enzyme, cyclooxygenase (or prostaglandin synthetase). When myometrial tissues from mid-term pregnant rats are incubated in the presence of indomethacin *in vitro*, the normal development of GJs is dramatically suppressed (Garfield et al., 1980a,b), suggesting a role for a stimulatory prostaglandin. Conversely, treatment of immature or ovariectomized mature rats (with nondistended uteri) with a combination of estrogen and either, indomethacin or meclofenamate, will potentiate the stimulatory effect of the steroid on the formation of GJs (MacKenzie et al, 1983; MacKenzie & Garfield, 1985). Neither inhibitor exerts an effect when

administered alone. These data exemplify the complex nature of the prostaglandin influence on myometrial GJs.

The *in vivo* actions of the cyclooxygenase inhibitors have been interpreted to indicate that some product of the cyclooxgenase pathway, perhaps prostacyclin, inhibits the stimulus provided by estrogen treatment or, alternatively, that an eicosanoid product of the lipoxygenase pathway of arachidonic acid metabolism may potentiate the estrogenic stimulation of GJs. (MacKenzie et al., 1984). In the presence of the cyclooxygenase inhibitors, the activity of lipoxygenase may be enhanced and the effect of estrogen potentiated (MacKenzie & Garfield, 1985).

The mechanism by which metabolites of arachidonic acid influence the presence of GJs in the myometrium remains to be clarified. They could regulate the synthesis of connexons directly, either at the level of the steroid receptors or translation (MacKenzie et al., 1984). Alternatively, they may influence the aggregation of the connexons by either altering membrane fluidity or influencing protein-protein cross-linking in the plasma membrane leading to a stabilization of developing GJs (Garfield et al., 1980).

Previous studies on myometrial GJs all stress the concept that the presence of the junctions is primarily regulated by processes which control their synthesis. Clearly, however, the destruction or degradation of the GJs may also play a significant role in this process. GJ degradation was previously suggested to involve an aggregation of small GJs into very large contacts, prior to the formation of annular junctions which are withdrawn and degraded in one

of the cell partners by an endocytotic mechanism (Garfield et al., 1980). It remains to be shown whether there are any agents which promote this process. It is fairly clear, however, that the presence of the ovaries and associated hormones are not required for the disappearance of the junctions after parturition (Berezin et al., 1982).

1.4.4 Electrical Properties of Uterine Smooth Muscle

The mechanical behavior of the uterus which is associated with delivery of the young is a consequence of the underlying electrical activity in the myometrial smooth muscle cells (Abe, 1970; Kao, 1977; Anderson et al., 1981). Ca^{++} ions required for contractile filament interaction enter, or are released from intracellular stores, in response to Ca^{++} influx during the action potential (Kuriyama, 1981; Vassort, 1981). Moreover, there is a relationship between the duration and frequency of the electrical events and the generation of tension (Kao, 1977). Contractions of high amplitude and long duration, which are thought to be necessary for the effective expulsion of the fetus(es) (Fuchs, 1969, 1978; Csapo, 1981), are only associated with high frequency bursts or trains of action potentials (spikes) that last for several seconds and propagate rapidly throughout the myometrium (Abe, 1970; Fuchs 1968, 1978; Kao, 1977; Csapo, 1981).

Electrical activity in the myometrium is generated by the muscle fibers in the absence of external stimuli and is referred to as being myogenic in origin (Marshall, 1962, Lodge & Sproat, 1981). This myogenic activity is attributed to periodic oscillations in the membrane potential of cells within small (2x4 mm), anatomically

indistinguishable pacemaker regions (Lodge & Sproat, 1981). As noted by Bozler (1938), in the absence of a specialized conduction pathway (eg. neural or modified muscle cell), the multicellular composition of the myometrium demands that this pacemaker activity spreads rapidly from cell-to-cell throughout the tissue in order that all the muscle cells are activated in a coordinated and synchronous fashion. It is generally believed that propagation between cells occurs by the same mechanism which conducts action potentials along the plasma membrane within a single cell. Low resistance pathways between contiguous cells are thought to permit local circuit currents which accompany action potentials in one cell to passively spread into, and activate, adjacent cells (Abe & Tomita, 1968; Tomita, 1970). In other words, the cells behave as if coupled into an electrical syncytium and for this reason propagation depends not only on the intrinsic properties of the smooth muscle (eg. fiber radius, excitability and passive membrane properties) but also on the extent of cell-to-cell spread of activity.

Indirect measurements of the extent of electrical coupling in the myometrium have been made, however, whether GJs represent the structural basis of this coupling and whether coupling in the myometrium improves concomitant to the appearance of large numbers of GJs at parturition is not entirely agreed upon (Daniel & Lodge, 1973; Kuriyama & Suzuki, 1976; Zelcer & Daniel, 1979; Sims et al., 1982). The measurement of electrical coupling in the myometrium is complicated by the complex 3-dimensional arrangement of the muscle cells. Previous attempts to determine the degree of coupling using the double microelectrode technique described above have been largely

unsuccessful. The density of injected current falls so rapidly with distance from the current electrode that it is difficult to record any voltage deflection in an adjacent electrode at more than a few tens of microns of separation (Holman & Neild, 1979). This difficulty prompted the development of techniques which are thought to evaluate the extent of electrical coupling indirectly. The so-called Abe-Tomita method employs extracellular polarization to reduce the complex geometry of the tissue to that of a one-dimensional cable (Abe & Tomita, 1968). The distance for the steady state amplitude of propagating, electrotonic potentials to fall by a factor of 1/e, referred to as the length or space constant (λ), is related to the membrane and internal resistances, with latter being a function of the resistance of the cytoplasm and of the junctional, cell-to-cell pathway (Abe & Tomita, 1968; Tomita, 1970).

The space constant in rat myometrial tissues, regardless of the gestational state of the animal, is always reported to be much greater than the length of a single smooth muscle cell when measured with the Abe-Tomita technique (Daniel & Lodge, 1973; Kuriyama & Suzuki, 1976; Zelcer & Daniel, 1979; Sims et al., 1982). This is generally interpreted to imply the presence of relatively good electrical coupling at all stages of pregnancy. However, as noted previously, few and frequently no GJs are observed in myometrial tissues from animals not in labor (see section 1.4.2). Furthermore, GJs are also absent in several other smooth muscles, including the longitudinal muscle of the guinea pig ileum and vas deferens, which are similarly reported to have good electrical coupling (Daniel et al., 1976). This led Daniel

and co-workers (1976) to question the role of GJs in electrical coupling in smooth muscle and to advance the hypothesis that low resistance pathways may be provided by another variety of cell-to-cell contact or that other mechanisms may be involved.

On the other hand, coupling in the nondelivering tissues might be provided by the small aggregates of intramembrane particles observed in freeze-fracture replicas of the myometrium (Merk et al., 1980) and other smooth muscles (Gabella & Blundell, 1981). These structures, or even isolated, functional connexons, might provide sites for communication and yet they could not be identified as GJs by the ultrastructural criteria in current use. Furthermore, GJs are known to develop in myometrial tissues in vitro (Garfield et al., 1980a,b; and see results in Chapter 3, section 3.3.2.1 below), so that a careful examination of the myometrial tissues used to evaluate coupling is necessary, but has not been routine in previous studies. Another possibility is that a washout of inhibitory agents (eg. relaxin, prostaglandins) occurs in vitro, allowing the permeability of the few GJs present to rise and contribute to the measured coupling in nondelivering tissues.

It should also be noted that the ability of techniques like the Abe-Tomita method to measure the extent of low resistance coupling has been questioned (Sperelakis, 1969). It was suggested that the extracellular polarization technique may elicit a small but preferential longitudinal flow of current within the restricted extracellular space around the muscle cells. This violates one of the fundamental assumptions of the mathematical model on which the method

is based; that there is equipotential polarization in the transverse direction across the muscle tissue. It is possible that this preferential flow of current may contribute to an overestimate of the extent of coupling (Sperelakis, 1969).

1.5 MECHANISM OF PARTURITION

Effective term or preterm labor in the rat and other species follows a precise series of hormonal events (Liggins, 1979; Thorburn & Challis, 1979). These changes are thought to be responsible for initiating alterations in the structural and functional properties of the myometrium, which include an increase in excitability, sensitivity to stimulants, and the development of GJs between myometrial smooth muscle cells (Garfield et al., 1980a; Puri & Garfield, 1982). The steroid hormones, estrogen and progesterone, appear to play a central role in this cascade, but two peptide hormones, relaxin and oxytocin, and several prostaglandins (PGs), PG $F_{2\alpha}$, E_2, E_1, and I_2 (prostacyclin) (Fuchs, 1978; Thorburn & Challis, 1979; Downing & Sherwood, 1985a,b,c) are also implicated. Although there are marked variations in the hormonal alterations which apparently provoke labor in different animals (Liggins, 1979; Thorburn & Challis, 1979), the development of GJs (Garfield et al., 1977, 1978, 1979a,b) and the appearance of synchronous electrical and contractile activity (Fuchs, 1978; Liggins, 1979; Csapo, 1981) in the myometrium at term is common to all species and is postulated to be necessary for effective delivery of the young (Garfield et al., 1977, 1978). Indeed, GJs are invariably present in tissues from animals undergoing term labor or premature delivery due to experimental manipulation or pathology

(Garfield et al., 1977, 1978, 1982). Moreover, if the appearance of GJs is delayed, then labor is delayed and pregnancy is prolonged (Garfield et al., 1978).

Regulation of structural coupling in the myometrium may not, however, represent the only mechanism by which coordinated myometrial contractility is influenced by GJs. A second level of control involving a modulation of GJ channel permeability may also affect the synchrony of electrical activity. Intracellular factors (perhaps H^+, Ca^{++} or cAMP) and specific circulating and/or local hormones may promote the opening or closure of the channels to promote or inhibit synchronization. Furthermore, there is the possibility that an imbalance or failure of these postulated control mechanisms might contribute to dysfunctional or premature labor by preventing communication at term or promoting coordinated contractility before term, respectively. This problem of the regulation of the functional properties of the myometrial GJs is addressed in Chapters 4,5, & 6 of this thesis.

The myometrium of the pregnant rat is reported to be relatively quiescent, refractory to stimulatory agonists, and to display asynchronous contractility before term (Fuchs, 1969, 1978; Csapo, 1981). Electrical activity is characteristically intermittent and relatively sparse and different regions of the uterine wall behave as if they were electrically independent (Csapo, 1981). It is believed that although tension is generated in the uterine wall at sites of electrical activity, significant changes in intrauterine pressure are not observed due to elastic lengthening of inactive regions (Csapo,

1981). Furthermore, discrete electrical stimulation at one end of large (8 cm) strips of pregnant myometrium was reported to produce mechanical activity which was restricted to the stimulated region (see Abe, 1970). This was interpreted to be evidence for the presence of a propagation block in the pregnant myometrium. Csapo (see Csapo, 1981) suggested that this block is responsible for the maintenance of pregnancy and that it can be attributed to progesterone released by the corpus luteum. More recently, the structural basis for the conduction block was suggested to be the inhibition of GJ development in the myometrium by progesterone (Garfield et al., 1978, 1980a). Other studies also implicate a role for progesterone in depressing the excitability and sensitivity of myometrial tissues to stimulants (Csapo, 1981).

The decline in progesterone levels over the final 3-4 days of gestation is thought to release the myometrium from this inhibition and provoke labor (Csapo, 1981). That premature development of GJs, synchronous activity, and labor can be provoked by an experimentally-induced progesterone decline (ie. ovariectomy prior to day 18 (Garfield et al., 1977, 1978, 1982)) is consistent with this hypothesis.

Recent data imply that relaxin may also participate in the maintenance of pregnancy (Porter & Downing, 1978; Downing & Sherwood, 1985a,b). The levels of this hormone in the rat are elevated during pregnancy, reach a peak during the 24-36 hours before parturition, but decline around the time of labor (Sherwood et al., 1980; Downing & Sherwood, 1985a). Relaxin inhibits spontaneous activity in the

myometrium of the rat (in vitro and in vivo) (Sanborn et al., 1980; Downing & Sherwood, 1985b) and it is postulated that this hormone may be responsible for the marked uterine quiescence and maintenance of pregnancy in the rat over the final 24-36 hours of pregnancy after the levels of progesterone have fallen considerably (Porter & Downing, 1978; Porter et al., 1979; Downing & Sherwood, 1985b). It is also evident that this hormone participates in the control of cervical maturation to permit the passage of the fetus(es) (Downing & Sherwood, 1985c).

During parturition, the electrical and mechanical activity of the smooth muscle cells is well-coordinated and synchronous at different sites in the uterine wall (Fuchs, 1969, 1978; Abe, 1970; Kao, 1977; Csapo, 1981). In addition, the myometrium is thought to be more reactive to stimulants (Fuchs, 1969; 1978; Abe, 1970; Kao, 1977; Csapo, 1981) and GJs are characteristically large and abundant (Garfield et al., 1977, 1978, 1979a,b, 1980a). In contrast to the result described above for pregnant tissues, discrete stimulation of strips of parturient myometrium in vitro, was reported to produce mechanical activity involving the entire strip (see Abe, 1970). It is postulated that the simultaneous contraction of large numbers of smooth muscle cells in the uterine wall is produced by bursts of spikes which propagate throughout the myometrium from pacemaker regions. This coordinated mechanical activity is thought to be responsible for the markedly increased development of intrauterine pressure during labor which provides the expulsive force to empty the uterine cavity (Abe, 1970; Kao, 1977; Csapo, 1981).

Although the decline in progesterone at term appears to be one factor in the cascade of events which terminate pregnancy, it is evident that other factors are involved as well. A variety of experimental evidence indicates that labor in the rat is provoked by a rise in the levels of estrogens and several PGs, as well as, a greater sensitivity to oxytocin at term (Fuchs, 1978). Administration of anti-estrogen (tamoxifen) or inhibitors of PG synthesis (eg. indomethacin and sodium meclofenamate) to intact rats will delay labor and prolong pregnancy (Thorburn & Challis, 1979; Anderson et al., 1981). Moreover, infusions of oxytocin will initiate labor in rats during the final 6-8 hrs prior to parturition (Thorburn & Challis, 1979). A role for these agents in the stimulation of labor is indicated by these observations but they do not permit any conclusions regarding mechanism of action. As noted above, estrogen and some PGs (thromboxanes and endoperoxides) are thought to stimulate the development of GJs in myometrial tissues and this may be one aspect of their actions. These agents may also influence the function of the GJs. Moreover, oxytocin, and the prostanoids $F_{2\alpha}$, E_2 and, E_1 are reported to increase myometrial activity and may alter Ca^{++} handling by the smooth muscle cells leading to more prolonged and forceful contractions (Kao, 1977; Fuchs, 1978; Thorburn & Challis, 1979; Csapo, 1981). The role of eicosanoid products of arachadonic acid breakdown by lipoxygenase remains to be explored.

On the basis of the functions ascribed to GJs in other tissues (see section 1.3.4 above), Garfield et al. (1977) postulated that the proliferation of junctions at term is responsible for the development

of synchronous activity in the myometrium. This hypothesis is supported by evidence which indicates that there is a good temporal correlation between these structural and functional events in the rabbit (Demianczuk et al., 1984). However, this indirect evidence is weak in that several different changes in the myometrial cells other than an alteration in GJs and coupling could theoretically account for increased synchrony in the uterus. For example, an increase in the length or diameter of the muscle cells or, alternatively, elevated membrane resistance or excitability, would increase the rate of spike propagation and enhance synchrony. For this reason it is necessary to measure the extent of intercellular communication between the myometrial cells to provide direct evidence of improved coupling in parturient tissues. Unequivocal evidence for a change in communication at term is lacking, however, in that of four published reports (Daniel & Lodge, 1973; Kuriyama & Suzuki, 1976; Zelcer & Daniel, 1979; Sims et al., 1982) which measured electrical coupling in small strips of myometrium from pregnant and parturient rats using the Abe-Tomita technique (see above), improved communication was only observed by Sims et al. (1982). The experimental technique employed in this thesis demonstrates improved intercellular communication in the myometrium at term by a method which does not involve the inherent difficulties associated with the measurement of electrical coupling in smooth muscle.

1.6 STRATEGY OF THE PRESENT STUDY

This thesis reports the results of studies on direct intercellular communication and the regulation of the functional properties of GJs

in rat uterine smooth muscle. A two-compartment bathing chamber similar to that of Weidmann (1966; and see section 1.3.1 above) was developed to study cell-to-cell diffusion of 2-[^{3}H]deoxy-D-glucose (2-DG) in small strips of longitudinal myometrium from rats at different stages of gestation, delivery, and post-partum. 2-DG is particularly suited to the study of metabolite communication because it enters the muscle cells by substituting for glucose on the facilitative diffusion carrier mechanism (Smith & Gorski, 1968; Smith & Stultz, 1971; Elbrink & Bihler, 1975). Furthermore, upon entering the muscle cells, 2-DG is immediately phosphorylated, and in this form, it is neither further metabolized (Smith & Gorski, 1968), nor does it recross the plasma membrane very quickly (Sokoloff,1981; and see Chapter 3, section 3.3.3 below). Thus, this low molecular weight tracer remains within the intracellular compartment and, provided that appropriate, functional cell-to-cell pathways are present between the smooth muscle cells, it may diffuse longitudinally through the muscle strip.

The experimental apparatus and technique, as well as, the method of analysis used to determine the apparent diffusion coefficient for 2-DG in the myometrial strips are described in Chapter 2. Chapter 3 documents the results of experiments in which the extent of metabolite communication was studied in tissues from days 17-20 pregnant (few GJs), day 22 parturient (many GJs), and days 2-3 post-partum (few GJs) rats. The results indicate that improved cell-cell coupling is associated with the presence of many large GJs at parturition. The subsequent chapters deal with the possible regulatory mechanisms which may control the permeability of GJs in the myometrium. The data show

coupling to be infuenced by Ca^{++}-calmodulin (Chapter 4), cAMP (Chapter 5), and physiologically relevant agonists, such as relaxin, β-adrenoceptor agonists, prostacyclin, and perhaps, eicosanoids (Chapter 6).

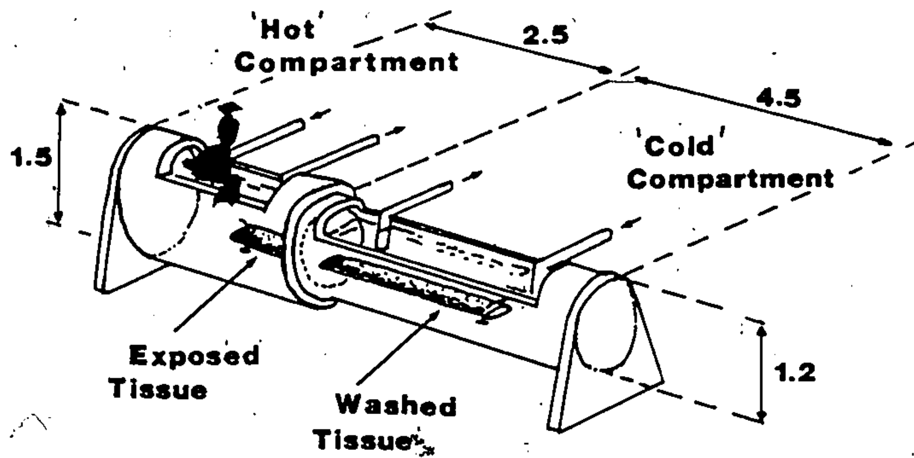
CHAPTER 2: METHODS AND MATERIALS

The tissues, equipment, methods and analysis of data used in the diffusion experiments reported in the subsequent chapters were identical and for this reason they are reported in this separate chapter. Any variations (eg. drug treatments and sources) in the individual experiments are supplied in a brief methods/materials section in each chapter.

2.1 PREPARATION OF MUSCLE STRIPS FOR THE DIFFUSION EXPERIMENTS

Pregnant rats (Charles River strain) were obtained from CanLab Breeders (Montreal, Canada) and maintained for at least three days prior to sacrifice. Animals were killed by cervical dislocation and their uteri excised and placed in well-aerated (95% O_2 & 5% CO_2) Krebs solution (composition in mM; 115.5 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 1.16 $MgSO_4$, 1.16 NaH_2PO_4, 21.9 $NaHCO_3$, 11.1 Glucose, and a pH of 7.4 at 37^oC). Small tissue samples were also removed for electron microscopic analysis (i.e., zero time tissues). The contents of each uterus were removed by opening the horns along their mesometrial attachment. Longitudinal strips (4.0 X 0.25 X 0.05 cm) of the uterine wall were cut from the ante-mesometrial region adjacent to the linea uteri of each horn using a block of razor blades fixed at 0.25 cm apart. The endometrium and circular muscle were removed under a dissecting microscope using iris scissors. Only those strips in which the longitudinal bundles of smooth muscle ran parallel to the long axis of the strip and which possessed only limited adherent connective tissue and circular muscle were used in the diffusion experiments (eg. Fig. 2.1). Longitudinal strips of hepatic portal vein vascular smooth muscle from female rabbits were prepared in a similar manner except that the adventitial layer was removed.

FIGURE 2.1: A diagrammatic representation of the bathing chamber and its components. The dimensions of the chambers are given in cm .


'Hot'
Compartment
2.5
4.5
1.5
'Cold'
Compartment
Exposed
Tissue
Washed
Tissue
1.2

2.2 DIFFUSION APPARATUS AND TECHNIQUE

Strips of smooth muscle were pulled through an interchangeable latex partition and pinned at their *in vivo* length in a two-compartment bathing chamber made of Perspex (Fig. 2.2). The partition fit snugly around the muscle but was not so tight as to crush the fibers. Both compartments of the bathing chamber were perfused with inactive Krebs for at least ½ hour prior to the addition of trace quantities of tritiated 2-[1,2-^{3}H(N)]-deoxy-D-glucose, (NEN; specific activity 37.3 Ci/mM), sucrose [fructose-1-^{3}H(N)]- (NEN; specific activity 10.8 Ci/mM), or mannitol [1-^{3}H(N)] (NEN; specific activity 10 Ci/mM), in low glucose (0.5 mM) Krebs to the 'hot' compartment. In all cases, the tritium label was bound to a carbon atom and was not part of an hydroxyl group. For this reason, tritium exchange was not considered to be a significant hazard in these experiments. The portion of the muscle strips in the 'hot' compartment (Fig. 2.2) was exposed to the tracer for 6 hours (1 hour for uptake of tracer to reach a stable plateau (see results in Chapter 3, section 3.3.3 below) and 5 hours for longitudinal diffusion). The inactive Krebs in the 'cold' compartment was exchanged continuously during the experiment whereas the radioactive solution in the 'hot' compartment was exchanged intermittently. All drugs used in the diffusion experiments reported in Chapters 4,5, and 6 were added to the perfusate circulated through the 'cold' compartment.

At the completion of 2-DG experiments the tissue in the 'hot' compartment was washed for 15 minutes with inactive Krebs to remove the extracellular content of the tracer. Strips exposed to sucrose and

mannitol were not washed in this manner. All strips were briefly exposed to toluidine blue dye to mark the position of the partition just before they were removed from the baths, pinned at their *in vivo* length on dental wax, and sliced into pieces of equal dimension using a block of 20 razor blades spaced 1.0 mm apart. A small portion of each strip from the 'cold' compartment was processed for electron microscopic analysis. The remaining pieces of tissue, 5-6 identical slices from the 'hot' side and 13-14 from the 'cold' compartment, were placed in minivials and dissolved with 0.5 ml of Protosol (NEN). Subsequently, 50.0 µl of glacial acetic acid, and 5.0 ml of Aquasol (NEN) was added to each vial and the content of radioactivity determined in a Beckman scintillation counter.

2.3 ANALYSIS OF DIFFUSION DATA

A strip of longitudinal myometrium longer than the length of a single smooth muscle cell and containing thin bundles of muscle fibers oriented in parallel to the long axis of the strip is analogous to a one-dimensional cable. When one portion of a strip is exposed to radiolabelled 2-DG, the tracer enters the cells, is phosphorylated and, at time t = 0, it begins to diffuse towards the nonexposed tissue with an apparent diffusion coefficient, Da, in cm^2sec^{-1}. If it is assumed that (i) the distribution of tracer within the exposed tissue is rectangular and homogeneous at t = 0, (ii) the tracer diffuses according to Fick's first law, (iii) there is no loss of tracer from the diffusing pool of solute due to efflux across the plasma membrane, binding, sequestration or chemical reaction, and (iv) the concentration of tracer in the nonexposed tissue is initially zero,

then the theoretical solution which describes the distribution of tracer after a period of time in a cable with these characteristics (i.e., diffusion in a one-dimensional, semi-infinite and homogeneous medium) is described in the following equation provided by Crank (1956).

$$C'(x,t) = C'_0(1-\mathrm{erf}(x/(2\sqrt{Da.t})) \qquad (2.1)$$

satisfying the following boundary conditions;

$$C'(x,t) = C'_0,\ x = 0,\ t > 0 \text{ and } C' \to 0 \text{ as } x \to \infty$$

and the initial condition;

$$C'(x,t) = 0,\ x > 0,\ t = 0$$

where $C(x,t)$ represents the concentration of tracer at any point, x (in cm), along the nonexposed tissue after a known time, t (in seconds), for diffusion to occur; C_0', the concentration of tracer at the source; erf, the error function; and Da, the apparent diffusion coefficient for solute movement.

Three different techniques have been used in previous studies to determine Da for diffusible solutes from longitudinal distribution data; (i) an equivalent electrical analogue model (Weidmann, 1966), (ii) curve fitting theoretical equations to the experimental data using trial values for Da (Weingart, 1974; Tsien & Weingart, 1976), or

(iii) graphical methods (Weingart, 1974). The latter technique was employed in the present study. The experimental conditions described above for 2-DG diffusion in the myometrium are equivalent to those outlined by Lauffer (1961) for solute diffusion in agar gels. For this reason the graphical method described in detail by Lauffer (1961) and in a subsequent paper by Schantz and Lauffer (1962) was used to obtain an estimate of Da for 2-DG from the distribution of tracer in myometrial strips.

The content of radioactivity in each slice in the 'cold' compartment (C') was compared to the average of several slices of equivalent size from the 'hot' compartment (C_0'). This gave a ratio for each slice which was referred to as the relative radioactivity (C'/C_0'). It was assumed that the radioactivity was concentrated at the mid-point of each slice. The value of C'/C_0' for each slice in the 'cold' compartment was then plotted as a function of distance (x) in centimeters from the partition to the mid-point of the slice on normal linear graph paper and on arithmetic probability graph paper. If the initial and boundary conditions are achieved and the diffusion of 2-DG is Fickian, then the distribution of the tracer will fall along a straight line when plotted on probability paper. A least-squares fit regression line was determined for each of these plots and only those with a correlation coefficient greater than 0.9 were accepted. An uncorrected value (Da^*) for Da may be calculated from the distribution of the tracer within the nonexposed tissue. Schantz and Lauffer (1962) show that Da is related to the slope of the regression lines in the following manner;

$$Da^* = 1/(\Delta y/\Delta x)^2\ 2t \quad (2.2)$$

where $(\Delta y/\Delta x)$ is the slope of the regression line and the other variables are as described above. Values of Da^* for each strip were then corrected to give Da and permit the assumption that the radioactivity was concentrated at the mid-point of each slice (from Lauffer, 1961).

$$Da = Da^* (1 - (P^2/48t.Da^*))^2 \quad (2.3)$$

where 'P' is the thickness of the slices and the other variables are as described above. Corrected values of Da were determined for several strips in each group and expressed as the mean ± SE. An unpaired Student's T test was used to compare for differences in the Da values between different groups at a significance level of 5%.

2.4 UPTAKE AND EFFLUX EXPERIMENTS

Small samples (ca. 10 mg) of longitudinal myometrium were equilibrated for at least ½ hour in low glucose Krebs solution prior to exposure to trace amounts of either tritiated 2-DG, mannitol or sucrose. To determine tracer uptake, the tissue samples were exposed to a well-stirred, low glucose Krebs solution (37°C) containing tracer for predetermined intervals of between 0 and 180 minutes. Each sample was removed, dipped quickly in inactive Krebs, and placed in a pre-weighed vial. The vials were weighed again, and the content of radioactivity in the samples determined as described for the diffusion

experiments. The uptake (%) of 2-DG, mannitol and sucrose in the tissues was determined from the ratio of the content in wet tissue (dpm/mg) to the content in the bathing medium (dpm/ml) multiplied by 100. Tracer uptake was then plotted as a function of time.

The tissue samples used the in efflux experiments were exposed to radioactive low glucose Krebs solution (37° C) (containing either trace amounts of tritiated 2-DG, mannitol or sucrose) for 15-20 minutes. Following a rapid pass through inactive Krebs the tissue samples were transferred at set intervals through a series of vials containing inactive low glucose Krebs. Each aliquot was evaporated to approximately 1 ml prior to the addition of cocktail and determination of the radioactivity in each vial. The residual content of radioactivity in each tissue which was not removed by washing was also determined. Plots were made of the radioactivity remaining in each tissue sample at the end of each collection period expressed as a percent of the content at time zero.

2.5 ELECTRON MICROSCOPY AND MORPHOMETRIC ANALYSIS

Samples of uterine and portal vein tissues (zero time or after the diffusion experiments) were sequentially fixed in 2% glutaraldehyde (2 hours) and 2% osmium tetroxide (1.5 hours) in 0.075 mM cacodylate buffer (with 4.5% sucrose and 1 mM $CaCl_2$). They were stained _en bloc_ with uranyl acetate (1 hour), dehydrated in a graded series of ethanol, and embedded in Spurr resin. Thin (0.5 µm) and ultrathin sections were obtained; the latter sec[illegible] were stained with lead citrate (2 minutes) and viewed in a P[illegible]00 or 301 electron microscope.

The technique used in this study to calculate the fractional area of the smooth muscle cell membrane occupied by GJs is based on the methods of Garfield et al. (1980a,b). This morphometric technique is based on stereological methods for the quantitation of cellular components developed by Weibel (1973). Measurements of the volume, surface area and frequency of cellular components can be made from micrographs because a mathematical relationship exists between the average dimensions of a given component in a cell or tissue and of the profile of the structure in thin section (Weibel et al., 1969; Weibel, 1973).

An estimation of the length of plasma membrane in a series of micrographs of myometrium was achieved using the multi-purpose test system of Weibel et al. (1969). Twenty-two, nonoverlapping photos of each tissue were obtained and printed at x33,000 magnification on 20 X 25 cm photographic paper. An 'all-purpose test grid' (Weibel et al., 1969; Weibel, 1973) was placed over each micrograph. The number of times the probe lines on the grid intercepted the plasma membrane of smooth muscle cells was determined. The length of membrane present in each photo is mathematically related to the number of these intercepts. Since the smooth muscle cells are often oriented in an anisotropic manner on the micrographs it was necessary to make two intercept counts at right angles to each other and use the average of these counts. The length of membrane in each micrograph is calculated according to the following formula from Garfield et al. (1980a,b).

$$B = (\pi/2)(I/L)(A)(Cf) \qquad (2.4)$$

where B is the length of plasma membrane, I, the average number of intercepts, L, the length of probe lines on the test grid, A, the test area on the grid, and Cf, which is a correction factor for the magnification. The total length of membrane scanned is the sum of the membrane lengths in each micrograph. Frequency is then expressed as the number of junctions per 1000 microns of plasma membrane.

The total length of gap junction membrane is the sum of the lengths of all the individual junctions in the micrographs for a given tissue. The proportional area of plasma membrane occupied by gap junctions as a percent of the plasma membrane may be determined by the following equation;

$$(A_{gj}/A_m)\% = (2L_j/(L_m + 2L_j))100 \qquad (2.5)$$

where A_{gj} and A_m are the area of gap junction and plasma membrane, respectively, L_j and L_m the total length of gap and nonjunction membrane measured, respectively. Note that the length of gap junction membrane must be multiplied by a factor of two because each junction comprises components of both membranes and in the determination of A_j both must be considered. In this study the following parameters were determined; (i) the length of smooth muscle cell plasma membrane, (ii) the number of GJs per 1000 microns of membrane, (iii) the average size of the GJs in each tissue, and (iv) the fractional area of the plasma membrane occupied by GJs. These values were determined for several strips in each group and expressed as the mean ± SE. An unpaired

Student's T test was used to compare for differences in the values between different groups at a significance level of 5%.

CHAPTER 3:

ALTERATIONS IN FUNCTIONAL COUPLING AND GAP JUNCTIONS IN UTERINE SMOOTH MUSCLE

3.1 INTRODUCTION

The problem considered in this chapter concerns the role of gap junctions (GJs) in providing sites for direct intercellular communication in the rat myometrium. Many large GJs appear precipitously between uterine smooth muscle cells at parturition (Garfield et al., 1977, 1978). The development of synchronized electrical and contractile activity in the uterus at term is thought to be dependent upon this morphological event (Garfield et al., 1977, 1978). That the GJs should facilitate synchronous activity follows from their well established role as low resistance pathways for current flow (electrical coupling) and metabolite diffusion (metabolic coupling), i.e., direct intercellular communication between cells in smooth muscle and other tissues (Peracchia, 1980; Hooper & Subak-Sharpe, 1980; Loewenstein, 1981; De Mello, 1982b; Sheridan & Atkinson, 1985). For example, the experiments of Barr et al. (1968a,b) were interpreted to indicate that a loss of functional coupling in smooth muscle tissues treated with hypertonic sucrose results from the disappearance of GJs. However, whether there is a change in intercellular communication in the myometrium at term remains unresolved. Only Sims et al. (1982) found evidence for improved coupling in parturient tissues.

In light of these conflicting data on electrical coupling, a technique was developed to measure cell-to-cell diffusion of a radiolabelled metabolite, 2-[^{3}H-] deoxy-D-glucose-6-phosphate (2DG) in small preparations of longitudinal myometrium (see Chapter 2). If the

presence of GJs represents a limiting condition for cell-to-cell coupling of uterine smooth muscle cells, and other factors which could influence the diffusion of a radiolabelled tracer through the intracellular compartment of the myometrium remain constant, then the diffusivity of the tracer should be greater in tissues with many GJs compared to those with few GJs. To test this hypothesis, one portion of strips of longitudinal myometrium from days 17-20 pregnant (few GJs), delivering (many large GJs), or days 2-3 post partum (few GJs) rats was exposed to 2-DG and the distribution of this tracer determined after a period for diffusion through the muscle. The experiments demonstrated that the diffusivity of 2-DG was considerably greater in tissues from delivering rats compared to samples from nondelivering animals and provide evidence that this elevated diffusivity was due to the greater area of GJs between the muscle fibers in the parturient tissues.

3.2 METHODS AND MATERIALS

Longitudinal myometrial tissues from ante-partum (days 17-20), parturient (Day 22-, following the birth of at least one pup), and post-partum (days 2-3) rats were excised, suspended in a two-compartment bathing chamber and utilized for diffusion experiments as described in Chapter 2. In other experiments, three different time periods (2.5, 5.0, & 7.5 hr) for 2-DG diffusion were used to determine whether the apparent diffusion coefficient (Da) for tracer movement in the delivering myometrium remained constant. Several additional diffusion experiments were conducted using smooth muscle tissues from hepatic portal veins in female rabbits. Longitudinal strips of portal

vein were prepared in a similar manner to that described for the myometrial strips except that only the adventitial layer was removed.

2-DG, sucrose, and mannitol uptake and efflux experiments were conducted as described in Chapter 2 using myometrial tissues from days 17-20 pregnant, parturient, and post partum rats.

Small tissue samples were obtained for electron microscopic analysis at the time of sacrifice (ie. zero time) and following the diffusion experiments and prepared according to the methods in Chapter 2. The length and cross-sectional area of myometrial smooth muscle cells in tissues fixed _in situ_ from day 19 pregnant and parturient rats were determined according to the methods reported by Gabella (1976).

3.3 RESULTS

3.3.1 Diffusion Experiments

3.3.1.1 2-DG diffusion in longitudinal myometrium. The longitudinal distribution of 2-DG in myometrial muscle strips from ante-partum, partum and post-partum rats is shown in Figure 3.1. Each data point represents the mean (± S.E.) of several preparations. The longitudinal distribution of 2-DG was consistently greater in parturient compared to nonparturient myometrial strips following five hours for diffusion (Fig. 3.1). 2-DG had diffused a greater distance from the partition, and the relative content of radioactivity in each slice within the first 0.55 centimeters of the tissue was always substantially greater, in parturient tissues compared to those from nondelivering animals. These data provide qualitative evidence that the longitudinal movement of 2-DG took place more rapidly in tissues with large numbers of GJs.

FIGURE 3.1: The longitudinal distribution of 2-DG in myometrial strips from day 17-20 pregnant, (n = 37 strips) parturient (n = 14 strips), and day 2-3 postpartum (n = 14 strips) rats following a 5 hour diffusion time plotted on arithmetic probability graph paper (3.1A) and normal graph paper (3.1B). Included in 3.1A is data for parturient tissues following 2.5 and 7.5 hour diffusion periods. 3.1B also includes the distribution of 2-DG after the 1 hr uptake period and a theoretical plot of 2-DG distribution calculated using equation 2.1 (see section 2.3) and a value for Da of 1.9×10^{-6} cm^2sec^{-1} is fitted to the data points. (data points in this figure, and in all subsequent distribution graphs, are the mean ± SE).

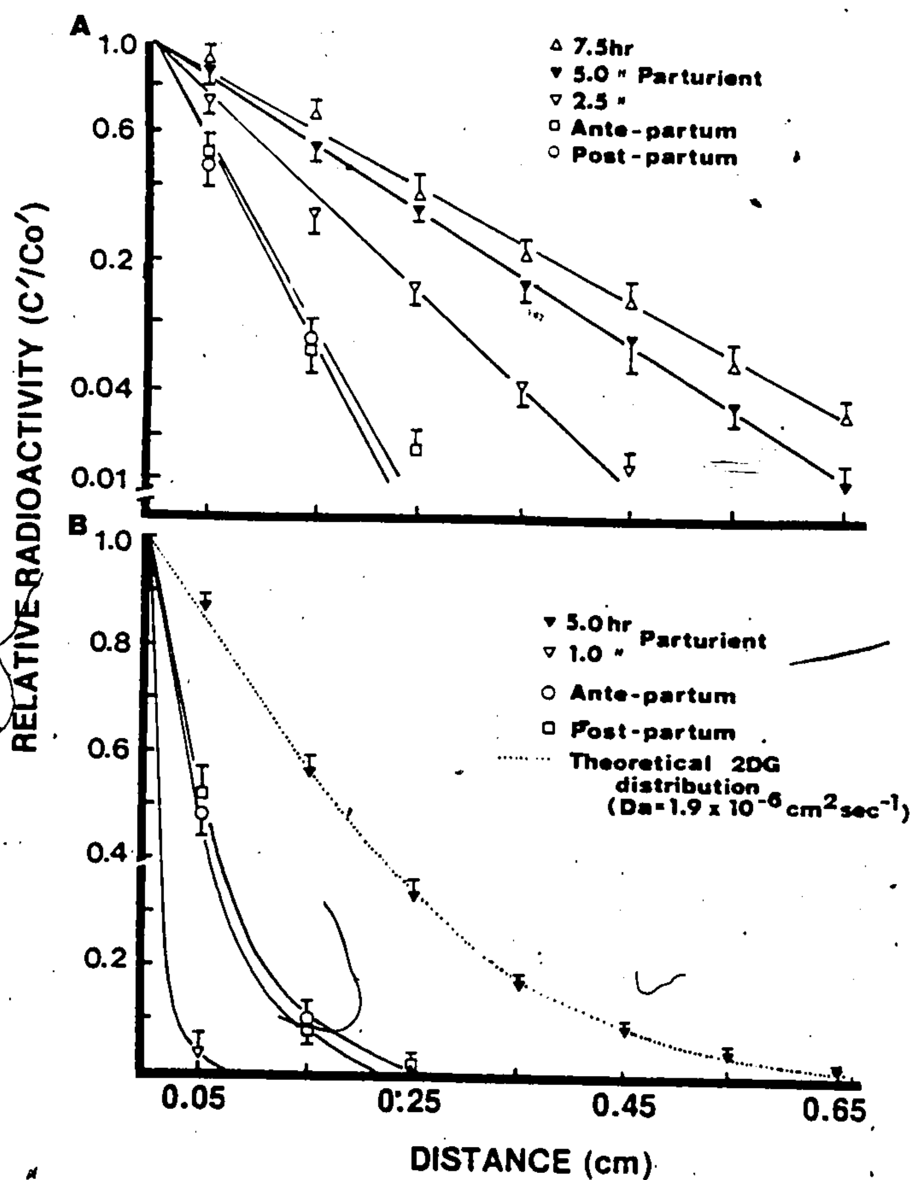

A
1.0
0.6
0.2
0.04
0.01
7.5hr
5.0 " Parturient
2.5 "
Ante-partum
Post-partum
B
1.0
0.8
0.6
0.4
0.2
5.0hr
1.0 "
Parturient
Ante-partum
Post-partum
Theoretical 2DG distribution
(Da=1.9 x 10⁻⁶ cm²sec⁻¹)
RELATIVE RADIOACTIVITY (C'/Co')
0.05
0.25
0.45
0.65
DISTANCE (cm)

Figure 3.1b shows that the data points obtained for a 5-hour diffusion experiment using parturient tissues fit a theoretically predicted distribution for 2-DG calculated using equation 2.1 (see Chapter 2, section 2.3) and a trial value of 1.9 x 10^{-6} cm^2 sec^{-1} for Da. This implies that equation 2.1 adequately described the experimental conditions and that the assumptions outlined in section 2.3 above were not violated. This point was further confirmed by the results of other control experiments. Firstly, it was shown that 2-DG movement in delivering tissues was Fickian in that Da remained constant during 2.5, 5.0, and 7.5 hour diffusion experiments (Table 3.1). Secondly, the limited diffusion of 2-DG into the nonexposed tissue at the end of the 1 hour uptake period as indicated in Figure 3.1b, suggests that a rectangular distribution of tracer in the exposed tissue at t = 0 was achieved. Finally, the fact that the longitudinal distribution of 2DG could be fitted to a straight line ($r \geq 0.90$) when plotted on probability graph paper (Fig. 3.1a) provides evidence that loss of tracer from the diffusible pool either as a result of chemical reaction, binding or sequestration (Schantz and Lauffer, 1962) by cytoplasmic components, or movement across the plasma membrane was not a significant factor in these experiments. Indeed, had a loss of tracer occurred due to any one of these mechanisms, then the content of radioactivity in each slice would have been less than that predicted by equation 2.1, and the resultant distribution of tracer would have assumed a concave profile on probability paper.

The qualitative data shown above for 2-DG distribution were

TABLE 3.1: THE APPARENT DIFFUSION COEFFICIENT (Da) FOR 2-DG IN MYOMETRIAL TISSUES FROM RATS ON VARIOUS DAYS OF PREGNANCY.

TISSUE	N*	Da ± SE. x 10^{-6} cm^2 sec^{-1}
1) Ante-partum		
Day 17	9(5)	0.20 ± 0.08[1]
Day 18	18(7)	0.18 ± 0.07[1]
Day 19	7(4)	0.22 ± 0.12[1]
Day 20	7(3)	0.24 ± 0.09[1]
2) Parturient		
5 hr+	46(26)	1.86 ± 0.16
2.5 hr	5(3)	2.05 ± 0.53[2]
7.5 hr	7(3)	1.98 ± 0.30[2]
3) Post-partum		
Day 2	10(4)	0.29 ± 0.04[1]
Day 3	4(3)	0.32 ± 0.09[1]

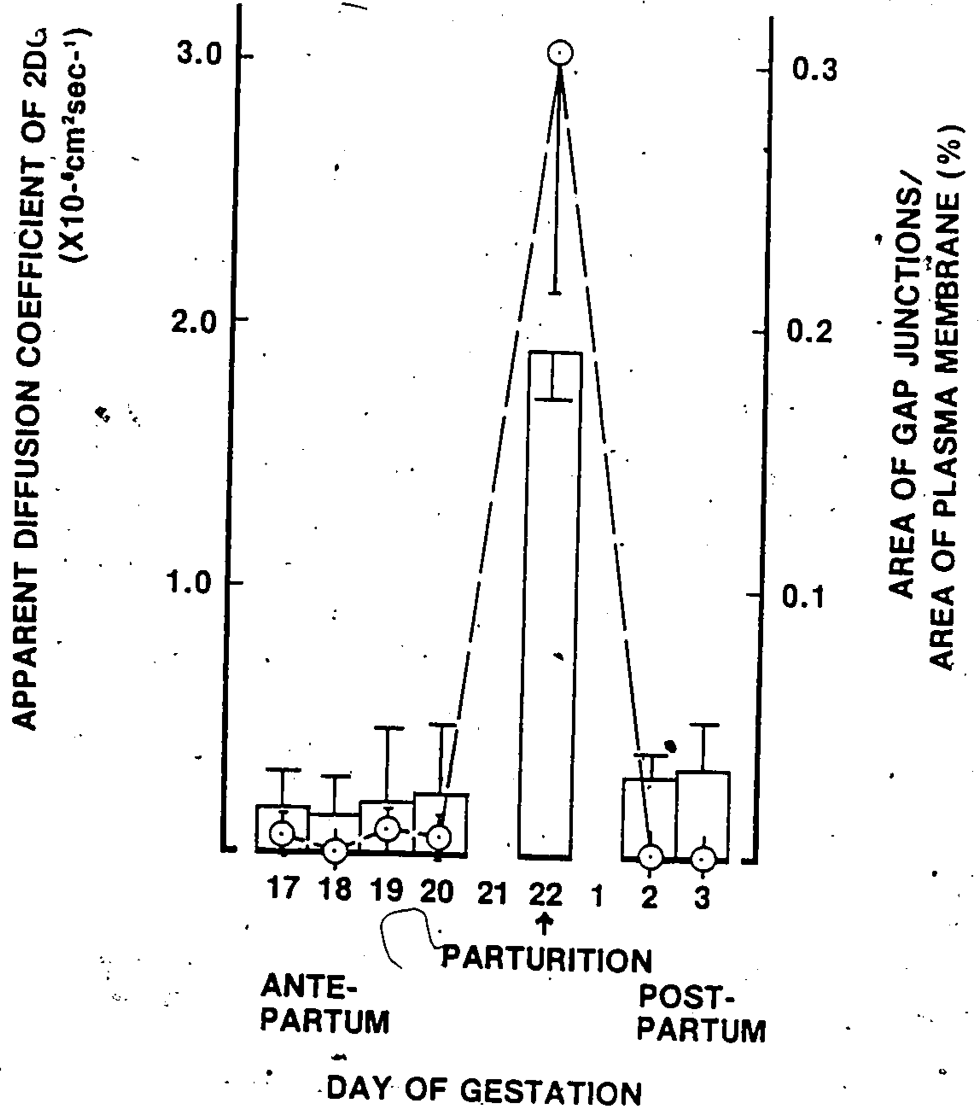
* n refers to the number of muscle strips employed whereas the numbers in the brackets indicates the number of animals.

\+ times refer to the length of the diffusion period for which the measurement of Da applies (all ante- and post-partum data are for a 5 hr period).

1 value of Da is significantly different ($P<0.05$) from that for parturient tissues and a 5 hr diffusion time.

2 value of Da is not significantly different ($P>0.05$) from that for parturient tissues and a 5 hr diffusion time.

FIGURE 3.2: The apparent diffusion coefficient (Da) of 2-DG (histogram) and the area of gap junction as a % of the plasma membrane (⊙) versus day of gestation. Da and the area of gap junctions are expressed as the means ± SE for several tissues.


APPARENT DIFFUSION COEFFICIENT OF 2DG
(X10-6cm2sec-1)
3.0
2.0
1.0
0.3
0.2
0.1
AREA OF GAP JUNCTIONS/
AREA OF PLASMA MEMBRANE (%)
17 18 19 20 21 22 1 2 3
PARTURITION
ANTE-
PARTUM
POST-
PARTUM
DAY OF GESTATION

reflected by different values for tracer diffusivity in the different tissues (Table 3.1). The mean value (± S.E.) for Da for 2-DG increased from 0.199 ± 0.03 x 10^{-6} cm^2sec^{-1} (n = 41 strips) in day 17-20 pregnant tissues to 1.86 ± 0.19 x 10^{-6} cm^2sec^{-1} (n = 46 strips) at parturition. Subsequent to delivery, the diffusivity of 2-DG decreased and Da was 0.296 ± 0.065 x 10^{-6} cm^2sec^{-1} (n = 14 strips) on days 2-3 post-partum. The values for tissues from rats on each day ante- and post-partum studied were not different (Fig. 3.2). The diffusion experiments indicate, therefore, that there is a significant ($P<0.05$), almost ten-fold increase and subsequent decrease in the diffusivity of 2-DG in the myometrium at term and following delivery of the fetuses, respectively (Fig. 3.2, Table 3.1).

3.3.1.2 Extracellular tracer diffusion in longitudinal myometrium. The problem of tracer diffusion through the extracellular space in the muscle strips was studied using ^{3}H-sucrose and ^{3}H-mannitol. The spatial distribution of sucrose and mannitol was shown to be the same in myometrial strips from days 17-20 pregnant, parturient, and days 2-3 post-partum rats after 5 hours for diffusion (Figs. 3.3 & 3.4). A theoretical plot of the longitudinal distribution of sucrose was made using equation 2.1 and is shown in Figure 3.3. This distribution was based on an interval of 5 hours for diffusion and a diffusion coefficient in the extracellular space of 3.0 x 10^{-6} cm^2 sec^{-1} or ½ of the value determined for sucrose in water (Kushmeric and Poldolsky, 1968; Nicholson & Phillips, 1981). The measured quantity of sucrose in each slice was consistently less than predicted by equation 2.1 indicating that there was a loss of sucrose

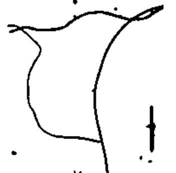
from the diffusible pool within the 'washed' tissues. That this loss of sucrose resulted from constant perfusion of inactive Krebs solution through the 'cold' compartment is evident from experiments in which no washing was conducted (Fig. 3.3). In this case, the distribution of sucrose did not decay substantially over the length of the tissue.

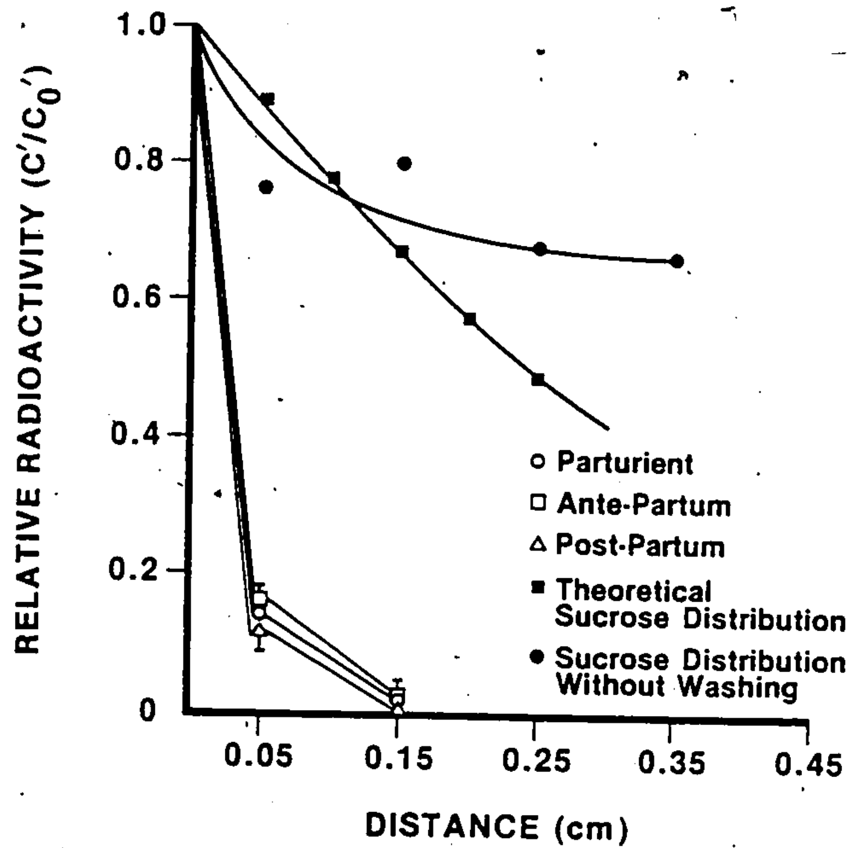
3.3.1.3 2-DG and sucrose diffusion in hepatic portal vein longitudinal smooth muscle. Longitudinal diffusion experiments were conducted using hepatic portal vein smooth muscle. These experiments failed to demonstrate any difference in the redistribution of 2-DG and sucrose in the 'washed' tissue after 5 hours for diffusion (Fig. 3.5). Furthermore, the distribution of sucrose was similar to that noted in myometrial strips (compare Fig. 3.5 and 3.3). The similar distribution of the intracellular and extracellular tracers suggests that the limited quantity of 2-DG detected in the 'washed' tissue resulted from diffusion through the extracellular space.

3.3.2 Structural Analysis

3.3.2.1 GJ area and muscle fiber size in longitudinal myometrium. The structural analysis of tissue samples fixed immediately after excision (zero time) revealed few, and sometimes no GJs in tissues from days 17-20 ante- and days 2-3 post-partum rats (Table 3.2). In contrast, GJs were abundant in tissues removed from delivering rats (Table 3.2, Fig. 3.6). The area of GJs as a percentage of the smooth muscle cell plasma membrane at zero time was less than 0.01% prior to term, approximately 0.2 - 0.4% during parturition, and less than 0.01% after 24 hours post-partum (Fig. 3.2). These results are consistent with those of Garfield et al. (1977, 1978, 1979a,b,

FIGURE 3.3: The longitudinal distribution of ^{3}H-sucrose in day 17-20 pregnant, parturient, and day 2-3 post-partum myometrial strips (n=6 for each group).





1.0
0.8
0.6
0.4
0.2
0
RELATIVE RADIOACTIVITY (C'/C0')
0.05
0.15
0.25
0.35
0.45
DISTANCE (cm)
Parturient
Ante-Partum
Post-Partum
Theoretical Sucrose Distribution
Sucrose Distribution Without Washing

FIGURE 3.4: The longitudinal distribution of ^{3}H-mannitol in day 17-20 pregnant, parturient, and day 2-3 post-partum myometrial strips (n=6 for each group).

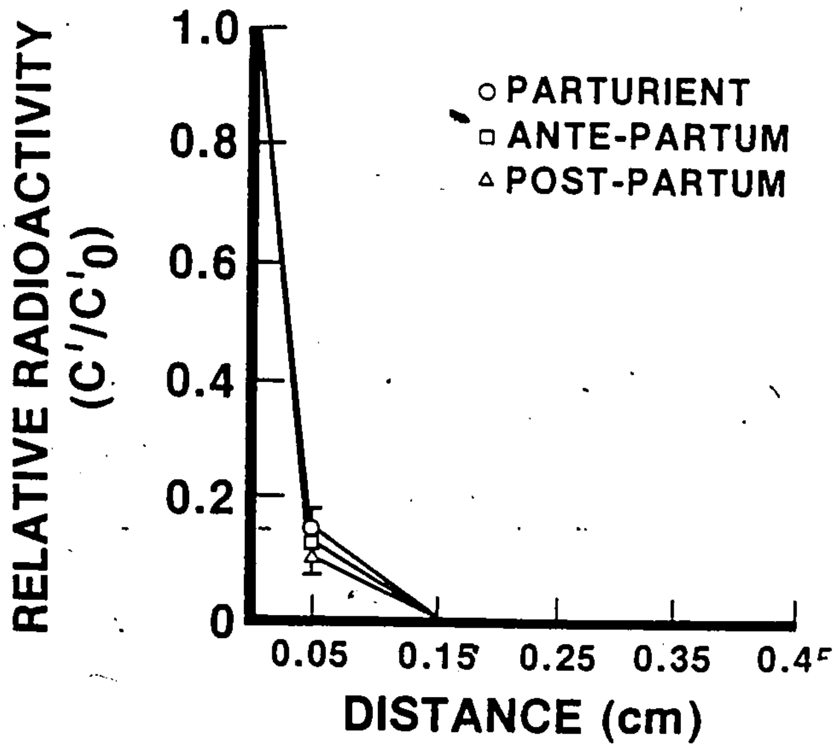
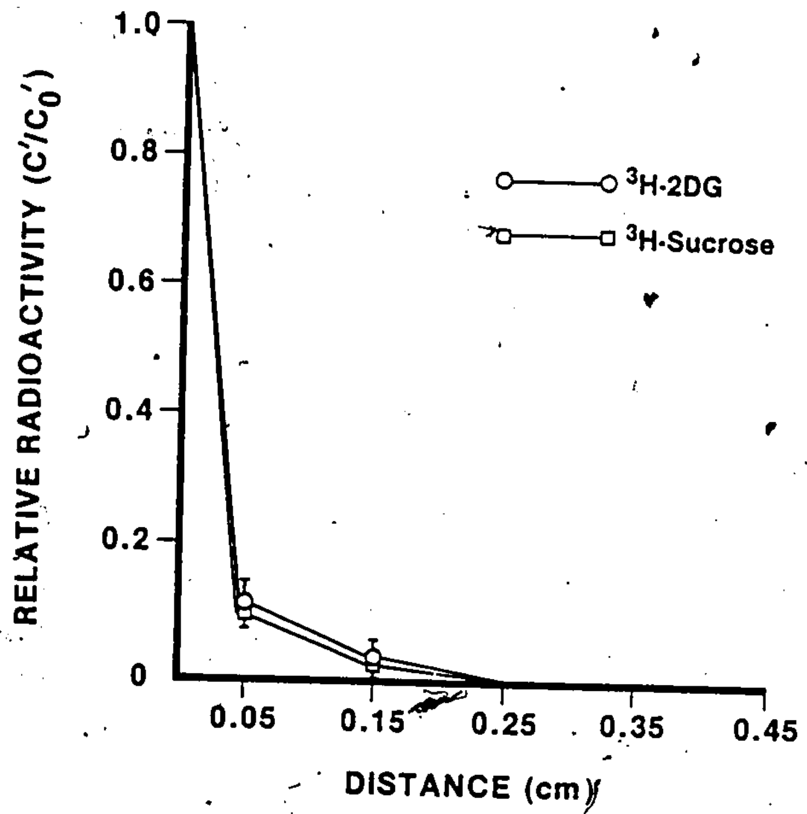

RELATIVE RADIOACTIVITY
(C'/C'0)
1.0
0.8
0.6
0.4
0.2
0
○ PARTURIENT
□ ANTE-PARTUM
△ POST-PARTUM
0.05
0.15
0.25
0.35
0.45
DISTANCE (cm)

FIGURE 3.5: The longitudinal distribution of 2-DG (n=10) and ^{3}H-sucrose (n=7) in strips of longitudinal hepatic portal vein muscle.



1.0
0.8
0.6
0.4
0.2
0
RELATIVE RADIOACTIVITY (C'/C'0)
3H-2DG
3H-Sucrose
0.05
0.15
0.25
0.35
0.45
DISTANCE (cm)

1980a,b) and show that GJs occupy a significantly ($P<0.05$) smaller portion of the plasma membrane in tissues from nonparturient compared to delivering animals.

The measurements of the sizes of the smooth muscle cells in the myometrium from ante-partum rats were 438 ± 16.5 μm in length and 4.93 ± 0.09 μm in diameter, whereas those in parturient tissues were 466 ± 28.1 μm and 5.06 ± 0.09 μm, respectively. Thus, the smooth muscle cells in parturient tissues were not significantly ($P>0.05$) larger than those in ante-partum animals.

Structural analysis for GJs in the tissues used in the diffusion experiments revealed that GJs had formed between the smooth muscle cells in the ante-partum tissues during the 6 hour incubation period in Krebs solution (Table 3.2). There were significantly ($P<0.05$) more GJs, and these occupied a significantly ($P<0.05$) greater area of the plasma membrane compared to samples from the same animals but fixed immediately after removal (Table 3.2). Conversely, there was no difference ($P>0.05$) in GJs between parturient tissues fixed at zero time and those used in the diffusion experiments (Table 3.2). It is important to note, however, that although GJs were present in ante-partum tissues after the experiments, they still occupied a considerably smaller area of the plasma membrane than was the case for the parturient tissues (either before or after the diffusion experiments) (Table 3.2).

3.3.2.2 GJ area in hepatic portal vein. GJs were not visible between smooth muscle cells in the longitudinal muscle tissues of rabbit portal vein fixed either prior to, or following, the diffusion

TABLE 3.2: GAP JUNCTIONS IN THE MYOMETRIUM AND HEPATIC PORTAL VEIN *IN VIVO* AND AFTER THE DIFFUSION EXPERIMENTS *IN VITRO*.

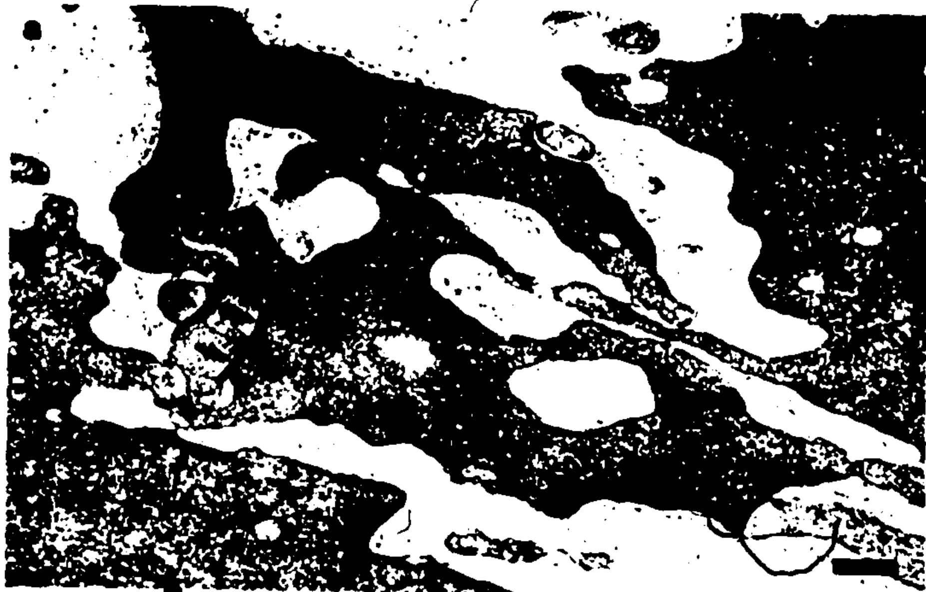
TISSUE	N	LENGTH OF MEMBRANE (in μms)	#GJs	#GJs/1000μm MEMBRANE	AVERAGE GJ SIZE (in μms)	FRACTIONAL AREA OF GJs AS A %
1. Ante-partum						
before	4(0)	6,055.4	0	0	0	0[1]
after	14(13)	20,251.8	51	2.56±0.36	0.11±0.02	0.104±0.02[2]
2. Parturient						
before	3(3)	4,147.8	30	7.23±1.08	0.29±0.03	0.299±0.05[3]
after	9(9)	13,364.6	83	6.22±1.07	0.24±0.02	0.257±0.04[3]
3. Portal Vein						
before	5(0)	7,462.9	0	0	0	0
after	5(0)	7,211.3	0	0	0	0

Where N is the number of tissues examined and the number of these containing GJs indicated in the brackets. Membrane length represents the total length of plasma membrane measured in 20-24 nonoverlapping micrographs of each tissue; the frequency, average size, and fractional percentage of the plasma membrane occupied by GJs are expressed as a mean ± SE.

[1,2,3] values with the same superscript are not significantly different at $P>0.05$ whereas values with different superscripts are significantly different at $P<0.05$.

FIGURE 3.6: Electron micrographs of gap junctions between uterine smooth cells. 3.6a: A low magnification of several gap junctions (arrows) between cytoplasmic processes of adjacent smooth muscle cells (s). X50,000. (the bar in this figure and in all subsequent micrographs represents 0.5 μm).

3.6b: A high magnification of a gap junction illustrating the typical septa-laminar appearance of these cell-to-cell contacts (between arrows). x200,000.



experiments (Table 3.2). It would seem that GJs did not form in the portal vein tissue during the in vitro incubation period.

3.3.3 2-DG, Mannitol and Sucrose Uptake and Efflux

The accumulation of 2-DG was studied using myometrial tissues from ante-partum, parturient, and post-partum rats. The pattern of 2-DG accumulation was the same in these different tissues (Fig. 3.7). 2-DG uptake was initially rapid but reached a stable plateau within 45-60 minutes (Fig. 3.7). The uptake of mannitol and sucrose by the different tissues was similar and did not exceed the radioactive content of the bathing solution. On the other hand, the tissue content of 2-DG was consistently greater than that of the bathing solution, indicating that it had access to a compartment from which sucrose and mannitol were excluded. These results are qualitatively similar to earlier observations on the uptake of sorbitol and another glucose substitute, 3-O-methylglucose (Arnquist, 1972), and justify the assumption (see Chapter 2, section 2.3 above) that tracer accumulation reached a stable level within 1 hour.

The patterns of 2-DG, mannitol and sucrose efflux from myometrial tissues are shown in Figure 3.8. In each variety of tissue, 2-DG displayed an initial rapid efflux which abated with time leaving a relatively stable residual content of radioactivity which was lost only very slowly during subsequent washing (Fig. 3.8). In contrast, sucrose and mannitol efflux from the tissue samples always continued until there was only a small residual quantity of tracer, if any, remaining in the tissue samples (Fig. 3.8). These results suggest that mannitol and sucrose remained in the extracellular space, whereas 2-DG

FIGURE 3.7: Uptake of (A) 2-DG (n=4 in each group), or (B) mannitol ● (n=3) and sucrose ○ (n=3), by myometrial tissues versus incubation time. Uptake is expressed as the percentage of radioactivity in a mg of tissue compared to that in a ml of bathing solution at the end of each incubation period. (Each point in this figure and in all subsequent efflux curves represents the mean ± SE.).

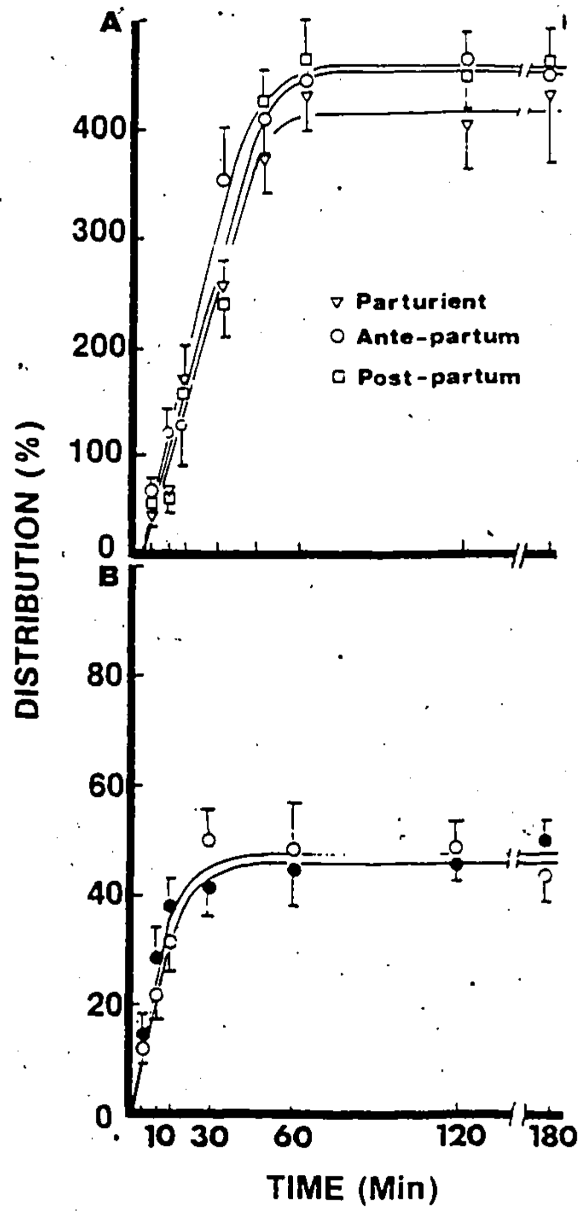
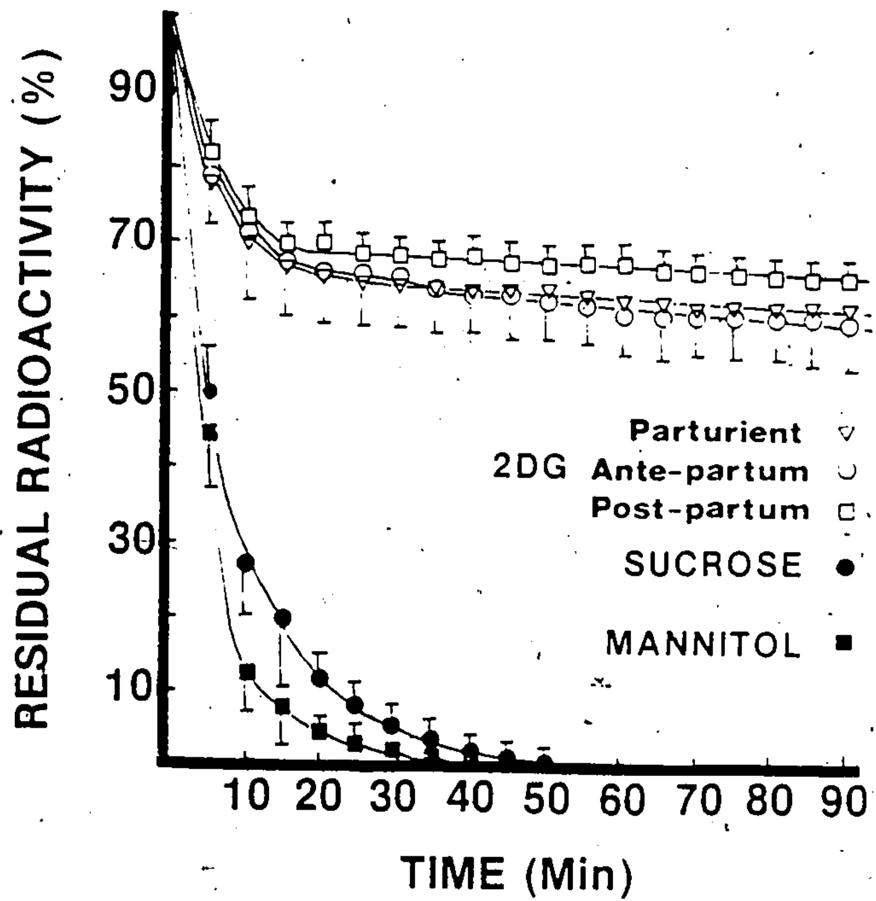

A
B
DISTRIBUTION (%)
400
300
200
100
0
80
60
40
20
0
▽ Parturient
○ Ante-partum
□ Post-partum
10
30
60
120
180
TIME (Min)

FIGURE 3.8: Efflux of 2-DG, mannitol and sucrose from myometrium as a function of wash time (n=3 in each group). Shown is the residual radioactivity, expressed as a percentage of the content at time zero, which remained in the tissues at the end of each interval.


RESIDUAL RADIOACTIVITY (%)
90
70
50
30
10
Parturient
2DG Ante-partum
Post-partum
SUCROSE
MANNITOL
10 20 30 40 50 60 70 80 90
TIME (Min)

entered a compartment which was isolated from the extracellular space, and from which this tracer could efflux only very slowly.

3.4 DISCUSSION

The experiments detailed in this chapter demonstrate the relationship between the area of GJs and the extent of direct intercellular communication in rat myometrial and rabbit hepatic portal vein smooth muscles. The acquisition of GJs between uterine smooth muscle cells at parturition is concomitant with an increase in the direct cell-to-cell transfer of 2-DG (see Figs. 3.1 & 3.2). Furthermore, the decrease in GJs following delivery of the fetuses is accompanied by a decrease in 2DG diffusivity in the myometrium (Figs. 3.1 & 3.2). GJs could not be identified in the longitudinal muscle of the hepatic portal vein nor was there any evidence of cell-to-cell exchange of 2-DG (3.5). These results are consistent with the hypothesis that (1) GJs are the morphological correlate of the site for direct intercellular communication in smooth muscle and (2) that the precipitous appearance of GJs in the myometrium at parturition may permit synchronized and coordinated muscular activity by facilitating the diffusion of small ions, metabolites, regulatory molecules and secondary messengers between the millions of uterine smooth muscle fibers.

3.4.1 GJs Between Uterine Smooth Muscle Cells

Few, and sometimes no GJs were observed in zero time ante- and post-partum myometrial tissues, however, they were abundant in tissues removed from parturient rats (Fig. 3.2 & Table 3.2). This is similar to previous studies on the rat and other mammals (Garfield et al.,

1977, 1978, 1979a,b, 1980a,b). In all instances, GJs have been found to be absent or present in low frequency in the myometrium throughout gestation and to develop in large numbers just prior to the onset of parturition. They are present in high frequency and large size during delivery of the fetuses, but disappear within 24 hours post-partum.

The formation of GJs between smooth muscle cells in ante-partum uterine tissues during in vitro incubation in Krebs solution as seen in this study is similar to that observed in studies using Minimum Essential Medium (Garfield et al., 1980a,b). It remains to be shown whether the GJs appeared prior to and/or during the diffusion experiments. However, the fact that significant numbers of GJs have been observed to develop in these ante-partum tissues has important implications for the present and other studies on the physiology of the pregnant myometrium. It seems possible that cell-to-cell communication in ante-partum tissues was improving with time during the diffusion experiments. Thus, the value of Da for 2-DG in the ante-partum myometrium very likely represents an overestimate of the extent of intercellular communication _in vivo_ where GJs are present in significantly fewer numbers and where the potential for channel permeability regulation by hormones is possible. The experiments using portal vein tissues demonstrated that in the absence of GJs and any _in vitro_ formation of junctions the distribution of 2-DG was similar to that of the extracellular tracers (Fig. 3.5). These results suggest that in the absence of any _in vitro_ GJ formation, the distribution of 2-DG in the nonparturient tissues would have mimicked that of mannitol and sucrose and would have been considerably less than that observed.

In any case, it is evident that the *in vitro* development of GJs obscures, rather than causes, any differences in the movement of small molecules between smooth muscle cells in parturient and nonparturient uterine tissues.

The development of GJs in tissues so recently removed from nonparturient animals implies that caution should be exercised when studying these tissues *in vitro*. Clearly, the formation of even a few cell-to-cell channels will provide a basis for metabolic and electrical coupling and perhaps alter the electrical and mechanical properties of the tissue. For example, *in vitro* formation of junctions may have contributed to previous observations of good electrical coupling in the ante-partum myometrium (Daniel & Lodge, 1973; Kuriyama & Suzuki, 1976; Zelcer & Daniel, 1979; Sims et al., 1982) and/or the failure to identify any change in coupling at term (Daniel & Lodge, 1973; Kuriyama & Suzuki, 1976; Zelcer & Daniel, 1979).

3.4.2 Route of 2-DG Diffusion

Small molecules can diffuse through tissues along intracellular, extracellular, or a combination of both pathways. The longitudinal redistribution of 2-DG in the myometrial strips can be attributed to diffusion in the intracellular compartment alone. Sucrose and mannitol remain in the extracellular space and their longitudinal distribution in the myometrium tissues was found to be consistently less than that of 2-DG. If an extracellular route had been solely employed by 2-DG, its distribution would have been similar to that of the extracellular tracers, as was observed in the case of the portal vein tissues. In addition, it was observed that the spatial distribution and

diffusivity of 2-DG were greater in parturient compared to ante- and post-partum myometrial tissues, but a similar change was not observed for sucrose and mannitol. These results indicate that 2-DG had access to a pathway from which sucrose and mannitol were excluded and that this pathway was not continuous with the extracellular space. It follows, therefore, that 2-DG diffused between smooth muscle cells along intercellular pathways for the direct transfer of small molecules from cell-to-cell.

An increase in the diffusivity of 2-DG in the intracellular compartment of myometrial tissues could have resulted from several possible changes in the properties of the tissue, however, this change is attributed to the increase in GJs per smooth muscle cell. A change in Da due to differences in tracer availability or loss across the plasma membrane was precluded by studies which showed that the influx and efflux of 2-DG was similar in tissues from ante-partum, delivering and post- partum rats (Figs. 3.7 & 3.8). According to the theoretical description of 2-DG in the myometrium outlined in section 3.4.5 below, the magnitude of Da is also influenced by several structural parameters including the length (Ω) and cross-sectional area (A_c) of the smooth muscle cells. Myometrial tissues from days 17-20 ante-partum and parturient rats were employed in order that there would be only minimal, if any, structural differences (eg., cell size and/or organization) between the strips due to growth of the myometrium or fetuses (Afting & Elce, 1978; Knox & Lister-Rosenoer, 1978). Indeed, the slight differences in length and cross-sectional area of the cells in ante-partum and parturient tissues described in this study (section

3.3.2.1 above) are certainly insufficient to account for the difference in 2-DG diffusivity. The model in section 3.4.5 below also shows that Da is influenced by the rate of diffusion of 2-DG in the cytoplasm (D_s). Thus, it is possible that the change in Da may have resulted from a marked decrease in the viscosity of the smooth muscle cell cytoplasm at term. Since D_s was not measured in this study, this possibility cannot be entirely dismissed. However, a change in the cytoplasmic resistivity at term was not observed by Sims et al. (1982) suggesting the absence of any change in cytoplasmic viscosity. Furthermore, if one assumes that there is no change in the rate of cell-to-cell movement, then in order to obtain the 10-fold increase in D_s required to account for the observed change in Da, the cytoplasmic viscosity must decrease by an order of magnitude. Such decrease in viscosity seems highly unlikely, however, given that there is a substantial increase in the content of various cytoplasmic constituents, including soluble proteins at term (Needham & Schoenberg, 1967). For this reason, the change in 2-DG diffusivity noted in this study is attributed to the marked increase in the effective area of cell-to-cell channels, A_j, due to the addition of permeable connexons to the junctional membranes between the smooth muscle cells.

The change in Da observed in post-partum tissues is very difficult to interpret because a significant decrease in the cell length (Ω) and an interruption of intracellular pathways due to cellular death are a consequence of the contraction of the myometrium following expulsion of the fetuses and the onset of myometrial involution, respectively.

For this reason the decrease in Da subsequent to parturition may very likely have been due to a combination of factors including a change in the area of GJs.

The ability of small molecules to diffuse between the cytoplasmic compartments of adjacent cells without entering the extracellular space and the necessity of GJs for this exchange are established for cells in culture (see Hooper & Subak-Sharpe, 1981). However, considerably less data showing metabolite transfer between cells, and the potential role of GJs in this process, are available for intact vertebrate tissues. This study provides indirect evidence that GJs are the morphological correlate of the cell-to-cell pathway in smooth muscle in that there is a concomitant increase in the cell-to-cell diffusion of 2-DG and GJs in the myometrium at parturition. Furthermore, the failure to detect any transfer of 2-DG between smooth muscle cells of the longitudinal layer of portal vein lacking GJs, implies that these structures are necessary for direct metabolite communication. These observations are novel because they provide the first quantitative evidence for a relationship between the extent of direct intercellular communication and the area of GJs between a single cell type in a whole tissue preparation. Previous attempts to provide similar evidence using a regenerating liver model were complicated by variations in the pattern of junctional contacts between hepatocytes (Meyer et al. 1980), a difficulty not encountered in the myometrium.

The movement of a variety of low molecular weight tracers has been demonstrated qualitatively in several tissues (e.g. Goodenough et al.,

1980; Iwatuski & Peterson, 1979) and quantitatively in small preparations of myocardium (Weidmann, 1966; Imanaga, 1974; Weingart, 1974; Tsien & Weingart, 1976). The value of Da obtained in the present experiments for 2-DG (MW. 164.2) (1.86×10^{-6} cm^2sec^{-1}) movement in the parturient myometrium is similar to that determined for tetraethyl ammonium ions (TEA^+; MW. 130, 2.0×10^{-6} cm^2sec^{-1}) in the heart (Weingart, 1974). These data imply that the movement of these molecules through the intracellular compartment is rather rapid and only 3-fold slower than in water.

The morphometric measurements imply that the increased diffusivity of 2DG in the myometrium at term resulted from the addition of GJs to the plasma membrane of uterine smooth muscle cells. However, junctional communication is also subject to regulation in many systems by a mechanism which is thought to involve a modulation of the permeability of the GJ channels (Loewenstein, 1981; Spray et al., 1984). Thus, a similar alteration in Da for 2-DG in the myometrium could also result from a change in the diameter of existing cell-to-cell channels, either as the result of an all-or-none opening of channels or, an iris-like dilation of channels. Indeed, it is possible that such mechanisms may contribute to the maintenance of asynchronous activity during the development of large numbers of junctions within the 24-36 hours prior parturition, and as well, in promoting the onset of coordinated activity during labor. Control of myometrial GJ permeability is dealt with in the subsequent chapters.

3.4.3 The Role of GJs and Direct Intercellular Communication in Uterine Smooth Muscle

The sudden development of GJs between smooth muscle cells at parturition has significant implications for myometrial function during labor. It has been recognized for some time that the electrical and mechanical activity in different regions of the uterus becomes coordinated and synchronized immediately prior to parturition and is maintained in that state throughout labor (Kao, 1977; Fuchs, 1978; Liggins, 1979; Csapo, 1981). That GJs are required for normal, effective labor is supported by numerous observations of these structures in the uterine muscle of term and pre-term animals in labor (Garfield et al., 1977, 1978, 1982). Moreover, if GJs are induced to form prematurely, or if their formation is prevented through experimental manipulations, then pre-term labor or the prolongation of pregnancy result, respectively (Garfield et al., 1978; L.W. MacKenzie & R.E. Garfield, unpublished observations). This requirement for GJs and the temporal association between the acquisition of electrical synchrony and GJ development led Garfield and co-workers (1977, 1978) to propose that the junctions serve to couple the millions of uterine smooth muscle cells into an electrical syncytium. However, the published electrophysiological evidence is conflicting. Indeed, of four reports dealing with coupling in the myometrium, only that of Sims et al. (1982) identified an increase in the length constant for the spatial decay of electrotonic potentials and reduced junctional impedance in longitudinal muscle at term. The present study uses a totally different technique, which shows improved direct intercellular communication at term and which does not involve the inherent difficulties that are associated with studying the role of GJs in

electrical coupling in smooth muscle *in vitro*. The reasons for the failure of some previous studies to identify any change in coupling at term remain to be determined. Several possible explanations can be advanced; incomplete separation of tissues into groups with and without GJs, an *in vitro* formation of GJs, and/or technical difficulties inherent in the use of the Abe-Tomita technique.

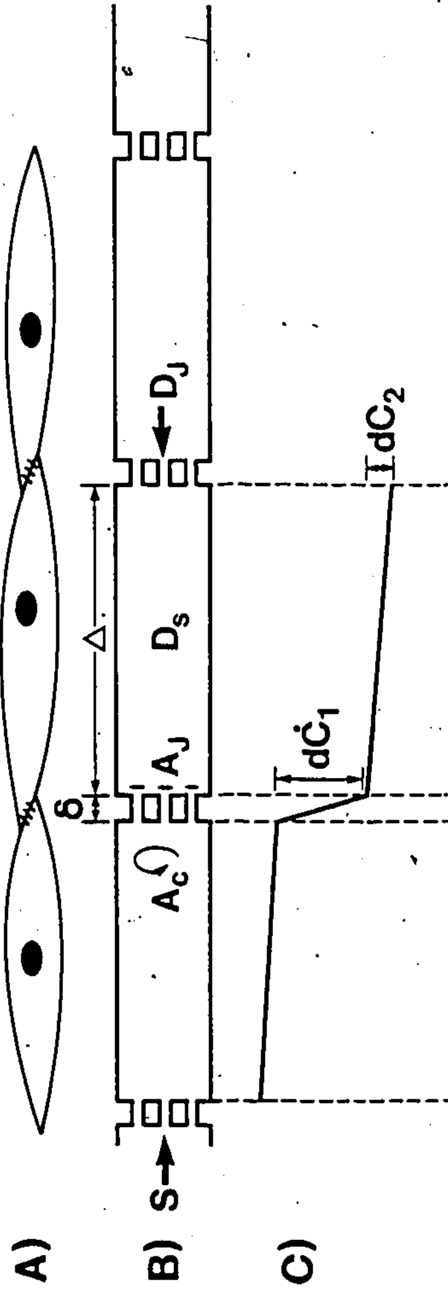
The physiological significance of metabolic coupling *per se* to uterine function during parturition remains to be established but would seem to warrant further study. It may be that metabolite and regulatory molecule transfer in the myometrium had a similar role to that proposed by Loewenstein (1981) for communication in other cell types; namely, that it facilitates an equalization of chemical potentials in the myometrium and prevents variations in metabolism and biosynthesis during the acquisition and maintenance of parturient muscular activity. Alternatively, a cell-to-cell exchange of secondary messengers or propagation of junction potentials through GJs could also serve as the basis for a cellular communication and amplification of responses to hormonal and neurogenic stimuli.

3.4.4 Calculation of a Theoretical Value of Da for 2-DG

The problem considered in this section is the derivation of a theoretical description of diffusion through myometrial tissue which may be used to calculate a theoretical or expected value for Da. The model provides an equation which calculates Da in terms of several parameters for which values were obtained in this study or from available published literature. The model assumes that (1) 2-DG diffusion through bundles in a strip of myometrium is equivalent to

solute movement along adjacent, parallel cables composed of a single chain of smooth muscle cells positioned end-to-end (i.e diffusion in a single chain of fibers is equivalent to that in all others), (2) the aqueous cell-to-cell pathway for the exchange of solute molecules is provided by a central hydrophilic channel of uniform dimensions within each connexon in the GJ, and (3) all channels in each GJ are patent and possess the same dimensions. In calculating the diffusivity of tracer in the cell-to-cell channel, correction factors were incorporated into the model to account for the steric and frictional interactions between the tracer molecule and channel because the molecular dimensions of 2-DG approach those of the channel (Pappenheimer et al., 1951). The regions of cell-to-cell contact are shown to be exclusively at the ends of the smooth muscle cells and the cells do not overlap in Figure 3.9. GJs do not demonstrate this distribution in the myometrium (R.E. Garfield, unpublished observations) or intestinal smooth muscle (Gabella & Blundell, 1981) nor do the muscle fibers show such a strict end-to-end arrangement in either tissue. If the junctions are on the lateral surfaces rather than the ends of the muscle cells, then there will be a greater number of junctional barriers per unit distance. Thus, the assumption of no overlap may have resulted in to a slight overestimate in the calculation of a the theoretical value for Da. However, placing the GJs at the ends of the cells is appropriate at this time since the structural data required to account for the degree of lateral cell-cell overlap and junctional contact between the myometrial fibers is lacking.

FIGURE 3.9: A chain of smooth muscle cells linked by gap junctions (A) is shown to be equivalent to a cylinder with a series of porous membranes (B). In (B) a solute (S) enters at one end and begins to diffuse longitudinally through the cylinder, where δ is the length of the channel across the porous membrane, Ω, the length of the compartments between the membranes (equivalent to cell length), D_s, the diffusion coefficient of the solute in the cytoplasm, D_j, the diffusion coefficient of the solute in the channel, A_j, the effective area of gap junction channel per cell, and A_c, the cross-sectional area of the muscle fibers. The effect of the porous membrane is to produce a step-like concentration curve of the solute (shown in C) along the cylinder where dC_1 and dC_2 are the differences in solute concentration across the junctional membrane and cytoplasm, respectively, of the cells.


A)
B)
C)
S
D_J
D_s
A_J
A_c
δ
Δ
dĊ_1
dC_2

Assuming that longitudinal diffusion of 2-DG in a strip of myometrium is equivalent to that in a single chain or cable of cells with a uniform core (Fig. 3.9a), then movement of 2-DG is subject to the serial barriers imposed by the cytoplasm and GJ channels, D_s and D_j, respectively (Fig. 3.9b), and for this reason the concentration curve of solute in the cells has a step-like appearance (Fig. 3.9c). From Fick's Law, the flux (J) across any one cell in the chain is;

$$-J = Da\ ((dC_1+dC_2)/(\Omega+\delta)) \qquad (3.1)$$

where Da is as described above, dC_1 and dC_2 represent the difference in 2-DG concentration across the GJ channel and cytoplasm, respectively (Fig. 3.9c) whereas Ω and δ are the lengths of the smooth muscle cell and channel, respectively (Fig. 9b). However, if the diffusion of solute along the entire chain is considered, then according to the continuity of the flux rule, the flux at any one point on the chain is;

$$-J = Da((dC_1+dC_2)/(\Omega+\delta)) = D_s(dC_2) = D_j(dC_1) \qquad (3.2)$$

giving three expressions for the flux of solute. If one solves for dC_1 and dC_2, and subsequently inverts the equation, an expression for Da is obtained;

$$1/Da = (1/(\Omega+\delta))\ ((\Omega/D_s) + (\delta/D_j)) \qquad (3.3)$$

Since in smooth muscle cells, as in all cells, δ (nm) is very much less than Ω (um), it is possible to eliminate δ from $(\Omega+\delta)$ and obtain a new expression;

$$1/Da = (1/D_s) + (\delta/\Omega D_j) \quad (3.4)$$

if it is assumed that the junctional channels are filled with cytoplasm, then;

$$D_j = (D_s)(A_j)/(A_c) \quad (3.5)$$

where A_j and A_c are the effective area of G_j channel per cell and the cross-sectional area of the muscle cells respectively (Fig. 3.9b). Thus, Da is determined by the variables D_s, Ω, δ, A_c, and A_j;

$$D_a = (D_s)/(1+(\delta A_c/\Omega A_j)) \quad (3.6)$$

The following values obtained in this study were used for Ω (450 µm), δ (15 nm), and A_c (78.5 µm^2). Since D_s for 2-DG was not measured in this study it was assumed to be 3.3×10^{-6} cm^{2}sec^{-1}, or about ½ that of glucose in water. The cytoplasmic viscosity of most cell types is such that it reduces the diffusivity of solutes by a factor of 2-3 from that in free aqueous solution (Kushmerick & Podolsky, 1969; Mastro et al., 1984). The value for the effective area of cell-to-cell channel per cell, A_j, was determined by calculating the fractional area of the junctional membrane actually occupied by cell-to-cell

channels and by accounting for steric and fractional hindrance to the movement of permeant molecules through these channels. The area of junctional membrane per cell is about 13.2 μm^2 or 0.2% of the 6600 μm^2 of plasma membrane making up the surface area of the muscle fibers. The packing density of connexons, hence cell-to-cell channels, in myometrial GJs was determined by counting the number of connexons, or particles, within measured areas of gap junctional membrane in of freeze-fracture replicas (kindly provided by Dr. M.S. Kannan). The density was found to be approximately 7000 per μm^2, which is comparable to that determined for intestinal muscle (Gabella & Blundell, 1981). Thus, in 13.2 μm^2 of GJ membrane there are 92,400 particles and 92,400 cell-to-cell channels. The exact dimensions of the cell-to-cell channel are unknown but the effective radius is thought to lie between 0.75 and 1.0 nm (Loewenstein, 1981). These values were used to calculate upper and lower limits for the area of channel per cell, 0.163 μm^2 or 0.29 μm^2. Correction coefficients for steric and frictional interactions introduced into the calculation of the effective area of channel for 2-DG diffusion, A_j, include variables for the molecular radius of the permeant molecule (σ) and that of the channel (r). The magnitude of the steric hindrance (Φ) at the channel opening is;

$$\Phi = (1 - \sigma/r)^2 \qquad (3.7)$$

and the frictional drag between the molecule and channel wall ($\emptyset$) is;

$$\emptyset = (1-2.019(\sigma/r)+2.09(\sigma/r)^3-0.95(\sigma/r)^5 \qquad (3.8)$$

A value of 0.4 nm was used for the molecular radius of 2-DG and the two limiting values of 0.75 and 1.0 nm for the channel radius. If the channel is 0.75 nm, then Φ and $\emptyset$ are 0.218 and 0.199, respectively. For a 1.0 nm channel they are 0.36 and 0.316, respectively. The effective area of the GJ channels per cell for 2-DG diffusion is the product of the area of the channel per cell, Φ, and $\emptyset$. For a tissue with 0.75 nm channels, A_j is 0.007 μm^2 and for one with 1.0 nm channels, A_j is 0.033 μm^2.

In theory, therefore, Da for 2-DG in the delivering myometrium should lie between 2.4×10^{-6} and 3.1×10^{-6} $cm^2\ sec^{-1}$ for the limiting channels sizes of 0.75 and 1.0 nm, respectively. The empirical value of $1.86 \pm 0.13 \times 10^{-6}$ cm^2sec^{-1} obtained in the diffusion experiments is slightly lower. However, given that the model is an oversimplification, this slight difference is quite reasonable. Several factors probably contributed to the difference in Da values, the most likely being that (1) a proportion of the GJ channels present in the membrane are closed under the conditions in the bathing chamber, (2) the assumption that the cells are end-to-end probably underestimates the number of junctional barriers per unit length and gives rise to a slight overestimate of Da, and/or (3) the value of Ds (3.3×10^{-6} cm^2sec^{-1}) used above is slightly greater than the actual diffusivity of 2-DG in the cytoplasm. The magnitude of Ds would be reduced if the cytoplasmic viscosity is greater than ½ that of water and/or if the presence of intracellular components (eg. myofilaments

and organelles) in the muscle cells increases the tortuosity of the pathway for 2-DG diffusion through the cytoplasm.

3.5 SUMMARY

It has been recognized for some time that parturition follows a series of precise changes in the levels of certain circulating and local hormones (Fuchs, 1978; Liggins, 1979; Thorburn & Challis, 1979). Furthermore, it was suggested that the appearance of GJs at term is probably evoked by these hormonal alterations (Garfield et al, 1978; Puri & Garfield, 1982). The data presented in this chapter provide evidence which is consistent with the hypotheses that (1) GJs serve as sites for the transfer of small molecules between smooth muscle cells and (2) there is markedly improved cell-to-cell coupling in the myometrium during parturition. Improved coupling offers one mechanism whereby the rate of pressure development by the uterine wall may increase at term. The presence of many large GJs may facilitate more effective contractile activity by promoting more rapid and extensive propagation of stimulating electrical activity from cell-to-cell throughout the myometrium. Thus, hormonal regulation of the extent of structural coupling in the myometrium would appear to represent one mechanism by which intercellular interactions and the coordination of electrical and mechanical activity in the uterine wall may be controlled during pregnancy and parturition.

CHAPTER 4:

REGULATION OF MYOMETRIAL GAP JUNCTION PERMEABILITY BY Ca++ AND CALMODULIN

4.1 INTRODUCTION

The development of GJs in the myometrium is influenced by circulating and local hormones (Garfield et al., 1978, 1980a,b; Puri & Garfield, 1982; MacKenzie et al., 1983; MacKenzie & Garfield, 1985). This endogenous control of structural coupling represents one possible mechanism for producing alterations in intercellular communication. It remains to be shown whether the extent of communication between uterine smooth muscle cells is also modulated by mechanisms which influence the functional properties of the GJs after they have formed.

Elevated levels of intracellular free calcium ion ($[Ca^{++}]_i$) have been demonstrated to inhibit electrical and metabolite communication, referred to as uncoupling, in all cell types studied (Loewenstein, 1981). However, the effect of this ion on coupling in smooth muscle has never been adequately studied. Furthermore, despite the abundance of data which demonstrate Ca^{++}-induced uncoupling in other tissues, the mechanism by which Ca^{++} influences the junctions is poorly understood.

This chapter describes the results of experiments in which the two-compartment bathing chamber technique was used to determine whether elevated $[Ca^{++}]_i$ and the calcium-dependent regulatory protein, calmodulin (Cheung, 1980, 1982; Means et al., 1982), may participate in the regulation of cell-to-cell diffusion of 2-DG in longitudinal myometrial tissues removed from rats during delivery. In order to determine whether elevated $[Ca^{++}]_i$ reduces coupling in uterine smooth muscle, myometrial tissues from delivering rats were exposed to the

calcium ionophore, A23187. The role of calmodulin was investigated using two structurally different calmodulin antagonists, chlorpromazine (Weiss et al., 1980; Means et al., 1982) and calmidazolium (Van Belle, 1981). The data indicate the presence of a Ca^{++}-calmodulin mechanism for inhibiting coupling in the myometrium in the absence of a change in the area of GJs per smooth muscle cell.

4.2 METHODS AND MATERIALS

The methods used in this chapter to document cell-to-cell diffusion of 2-DG were identical to those described in detail previously (see Chapter 2.0). Only myometrial tissues from delivering rats were used in this study. All drug treatments (described below) were initiated during the equilibration period prior to the addition of tracer, by adding drugs to, or altering the Ca^{++} composition of, the physiological salt solution perfused through the 'cold' compartment.

To determine the effect of $[Ca^{++}]_i$ and calmodulin on cell-to-cell diffusion of 2-DG in strips of parturient myometrium, the 'cold' compartment of the bathing apparatus was perfused with Krebs solutions containing calcium ionophore, A23187 (1 μM, Calbiochem) and/or calmodulin antagonist drugs. In all instances, tissues were initially equilibrated in Krebs solution prior to treatment with normal Krebs or Krebs containing either (1) ionophore (1.0 μM), (2) ionophore (1.0 uM) and no added Ca^{++}, (3) ionophore (1 uM), no added Ca^{++}, and EGTA (1.0 mM), (4) no added Ca^{++} and EGTA (1.0 mM), (5) ionophore (1 μm) and varied concentrations of either chlorpromazine (Sigma) (0.01, 1.0, 100.0 μM) or calmidazolium (Calbiochem) (0.001, 0.01, 0.1, 10 μM), (5)

varied concentrations of chlorpromazine (0.001, 0.1, 1.0, 100.0 μM), calmidazolium (0.001, 0.1, 10.0μM), or the local anesthetics, lidocaine (1.0 mM) or procaine (1.0 mM). All drugs were dissolved in 100% EtOH or DMSO, which were then diluted to a maximum of 0.05% in the Krebs solutions. Neither EtOH nor DMSO influenced coupling when added alone (see Table 4.1).

Several tissue samples from strips treated with A23187, calmodulin antagonist, or Ca^{++}-free in the diffusion experiments were processed, photographed and the area of GJs as a percentage of the smooth muscle cell plasma membrane quantitated according to procedures described in detail above (section 2.5).

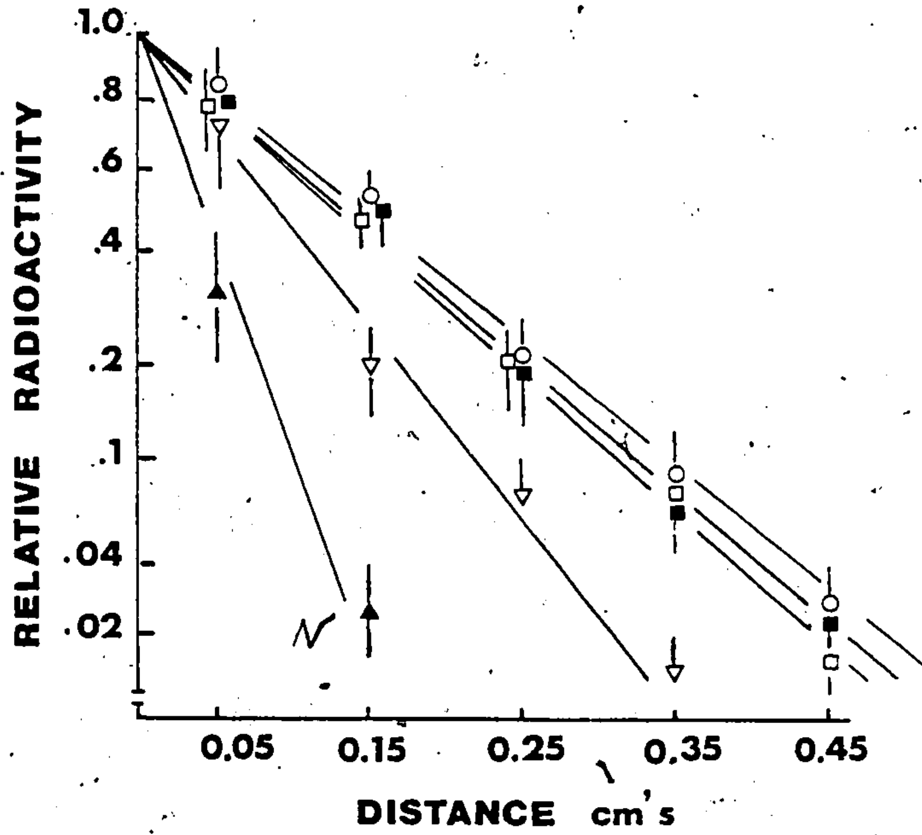
Efflux studies were conducted under similar experimental conditions to those in the diffusion experiments and according to methods previously described (section 2.4). Following a brief wash in nonradioactive Krebs, the samples were sequentially transferred at five minute intervals through a series of vials containing either (1) normal Krebs solution or saline with (2) A23187 (1 μM), (3) A23187, 1.0 mM EGTA and no added Ca^{++}, (4) 1.0 mM EGTA and no added Ca^{++}, or (5) chlorpromazine (100 μM).

4.3 RESULTS

4.3.1 Diffusion Experiments

4.3.1.1 Calcium ionophore reduces 2-DG diffusivity. To investigate whether $[Ca^{++}]_i$ influences cell-to-cell diffusion of 2-DG, myometrial strips from parturient rats were exposed to the calcium ionophore, A23187 (1 μM), a drug known to raise intracellular Ca^{++} in many cell types and elicit contraction in smooth muscle (eg. Watson,

FIGURE 4.1: Longitudinal distribution of 2-DG in parturient myometrial tissues exposed to A23187 in 2.5 mM Ca^{++} (▲) (n=8) or low Ca^{++} (ie. no added Ca^{++} or EGTA) (▽) (n=8) Krebs solution, Ca^{++}-free (1 mM EGTA) and A23187 (■) (n=9), Ca^{++}-free (1 mM EGTA) (□) (n=8), or normal (○) (n=8) Krebs solutions.



1.0
.8
.6
.4
.2
.1
.04
.02
RELATIVE RADIOACTIVITY
0.05
0.15
0.25
0.35
0.45
DISTANCE cm's

1978). Figure 4.1 and Table 4.1 depict the longitudinal distribution and apparent diffusion coefficient (Da) of 2-DG in strips treated with ionophore. The distribution of 2-DG was considerably reduced from that observed for control strips and was only slightly greater than that previously shown for sucrose and mannitol in Chapter 3.0 (compare Figs. 3.3 & 3.4 with 4.1). Da for 2-DG was significantly ($P<0.05$) and almost 10-fold less in strips treated with ionophore compared to that obtained for control samples (Table 4.1). That A23187 facilitated an inward flux of Ca^{++} ion from the extracellular pool is evident from the dependency of the ionophore effect on extracellular Ca^{++}; the distribution and Da for 2-DG were significantly ($P<0.05$) greater in strips treated with ionophore in Ca^{++}-free (ie. no added Ca^{++} and 1.0 mM EGTA) or low Ca^{++} (ie. no added Ca^{++} but no EGTA) solutions compared to values obtained using solutions with Ca^{++} (ie. 2.5 mM) (Fig. 4.1, Table 4.1). The distribution of 2-DG in tissues exposed to Ca^{++}-free solution alone was slightly, but not significantly, reduced compared to that of controls (Fig. 4.1). A similar observation of slightly reduced coupling in Ca^{++}-free solutions was made previously (Flagg-Newton & Loewenstein, 1979; see also Spray & Bennett, 1985). A23187 did not affect sucrose diffusion in the extracellular space (not shown).

4.3.1.2 Calmodulin antagonists block the effects of ionophore. Calmidazolium and chlorpromazine were found to block the effects of A23187 (1.0 μM) on 2-DG diffusivity (Figs. 4.2a & 4.2b, respectively and see Table 4.1). The distribution of 2-DG was greater in tissues treated with a combination of ionophore and calmodulin antagonist

TABLE 4.1: THE APPARENT DIFFUSION COEFFICIENT (Da) FOR 2-DG IN PARTURIENT MYOMETRIAL TISSUES WITH ELEVATED $[Ca^{++}]_i$

TREATMENT	[ ]	N*	Da ± SE. x 10^{-6} cm^2 sec^{-1}
1a. Control	-	14(8)	1.72 ± 0.15
b. A23187	1 μM	8(5)	0.18 ± 0.03[2]
c. A23187 & Ca^{++} free	1 μM	9(5)	1.65 ± 0.12[1]
d. Ca^{++} free		8(4)	1.56 ± 0.18[1]
e. DMSO	0.1%	7(3)	1.69 ± 0.22[1]
f. EtOH	0.1%	4(2)	1.81 ± 0.15[1]
2a. Control	-	19(12)	1.97 ± 0.21
b. A23187	1 μM	4(2)	0.16 ± 0.02[2]
c. A23187 & low Ca^{++}		8(3)	0.53 ± 0.15[2]
d. A23187 1 μM & Calmidazolium			
	0.001 μM	4(3)	0.47 ± 0.12[2]
	0.01 μM	3(2)	0.90 ± 0.17[2]
	0.1 μM	3(3)	1.10 ± 0.17[2]
	10 μM	4(3)	1.89 ± 0.09[1]
e. A23187 1 μM & Chlorpromazine			
	0.001 μM	4(2)	0.23 ± 0.14[2]
	0.1 μM	4(3)	0.71 ± 0.20[2]
	1 μM	8(4)	1.14 ± 0.13[2]
	100 μM	8(3)	1.41 ± 0.11[2]
f. Calmidazolium	100 μM	6(3)	0.23 ± 0.06[2]
g. Chlorpromazine	100 μM	6(4)	0.29 ± 0.04[2]
h. Lidocaine	100 μM	4(2)	1.61 ± 0.11[1]
i. Procaine	100 μM	4(2)	1.71 ± 0.12[1]

* n refers to the number of muscle strips employed whereas the numbers in the brackets indicates the number of animals.

1\. values of Da for treated tissues that were not significantly different (P>0.05) from that for control, untreated tissues.

2 values of Da for treated tissues which were significantly different (P<0.05) from that for control untreated tissues.

compared to those exposed to ionophore alone. This effect was found to be dose-dependent and at the highest concentration of calmidazolium, the value of Da for 2-DG obtained was not significantly different ($P>0.05$) from that for control tissues (Fig. 4.3, Table 4.1). EC_{50} values were 0.05 μM and 1.0 μM for calmidazolium and chlorpromazine, respectively. Rather surprisingly, however, the distribution and Da for 2-DG were reduced in tissues treated with both of these calmodulin antagonists alone (Table 4.1) and this effect of chlorpromazine and calmidazolium was found to be dose-dependent (Figs. 4.4 and 4.5). In contrast, the distribution of 2-DG was not significantly ($P>0.05$) affected by treatment with two other hydrophobic drugs, lidocaine (100 μM) and procaine (100 μM) (Table 4.1).

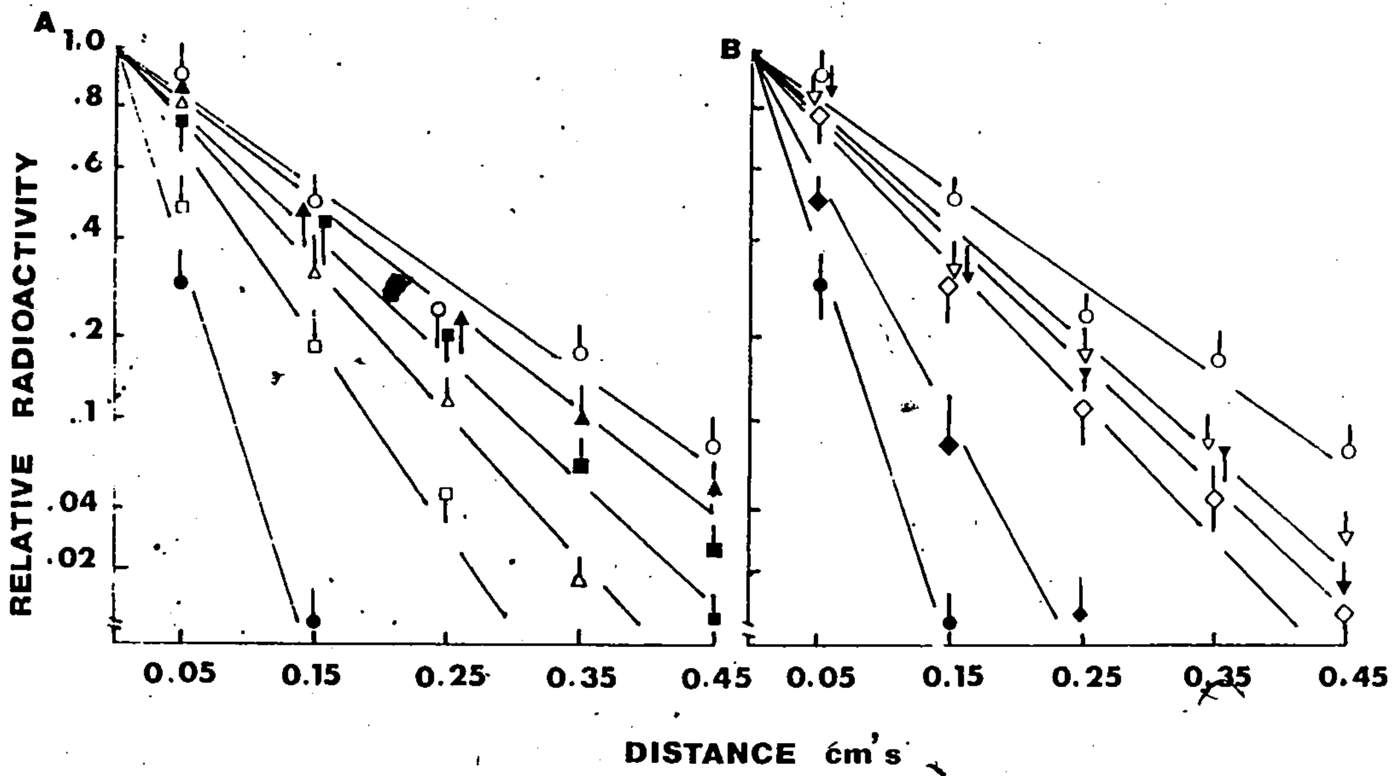
4.3.2 Effect of Ionophore and Calmodulin Antagonist on Structural Coupling

GJs in the tissues used in the diffusion experiments are summarized in Table 4.2. No significant difference ($P>0.05$) in the area of junctional membrane as a percentage of the plasma membrane were noted amongst the tissues exposed to A23187 (1.0 μM), chlorpromazine (100.0 μM), or Ca^{++}-free-EGTA (1.0 mM) compared to untreated muscle strips (Table 4.2). The reduced diffusivity of 2-DG was not, therefore, the result of an alteration in the extent of structural coupling in the myometrium.

4.3.3 Effect of Ionophore and Calmodulin Antagonist on 2-DG Efflux

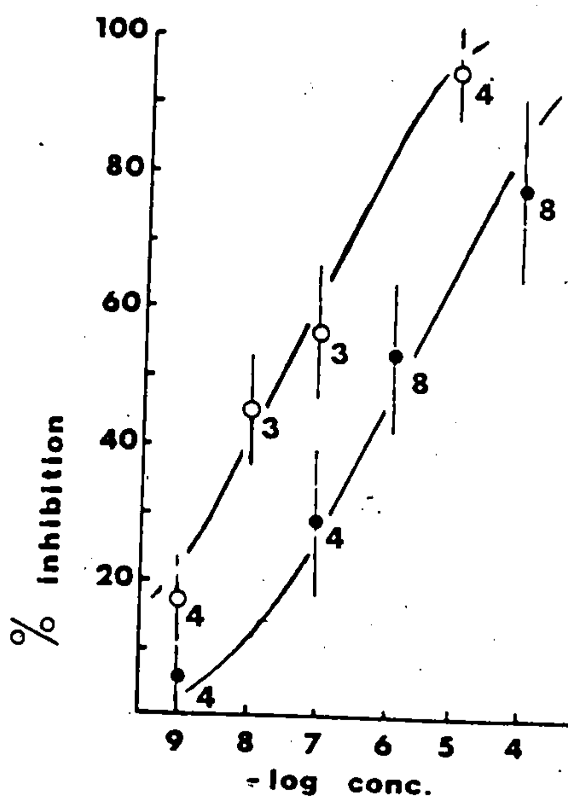
The pattern of 2-DG efflux from tissue samples exposed to calcium ionophore (1.0 μM), calmodulin antagonist (chlorpromazine, 100.0 μM), or Ca^{++}-free-EGTA (1.0 mM) solutions was similar to that of tissues

FIGURE 4.2: Longitudinal distribution of 2-DG in parturient myometrial tissues treated with A23187 (●) (n=8) or A23187 and varied concentrations of either (A) calmidazolium or (B) chlorpromazine .
Calmidazolium in μMs; 0.001 (□)(n=4), 0.01 (△)(n=3), 0.1 (■)(n=3), and 10.0 (▲)(n=4).
Chlorpromazine in μMs; 0.001 (◆)(n=3), 0.1 (◇)(n=4), 1.0 (▼)(n=8), and 100.0 (▽)(n=8).
Control untreated (○) (n=19)



A
B
RELATIVE RADIOACTIVITY
1.0
.8
.6
.4
.2
.1
.04
.02
0.05
0.15
0.25
0.35
0.45
DISTANCE cm's

FIGURE 4.3: Dose-response curves for the inhibition of A23187-induced uncoupling by calmidazolium (○) and chlorpromazine (●). (n for each point is as indicated)



100
80
60
40
20
% Inhibition
4
3
3
4
8
8
4
4
9
8
7
6
5
4
-log conc.

FIGURE 4.4: Longitudinal distribution of 2-DG in parturient myometrial tissues exposed to varied concentrations of chlorpromazine (in μM; 0.01 (▽)(n=5), 0.1 (▼)(n=4), 1.0 (○)(n=7), 10.0 (■)(n=7), and 100.0 (□)(n=6)) or normal Krebs solution (●)(n=6).

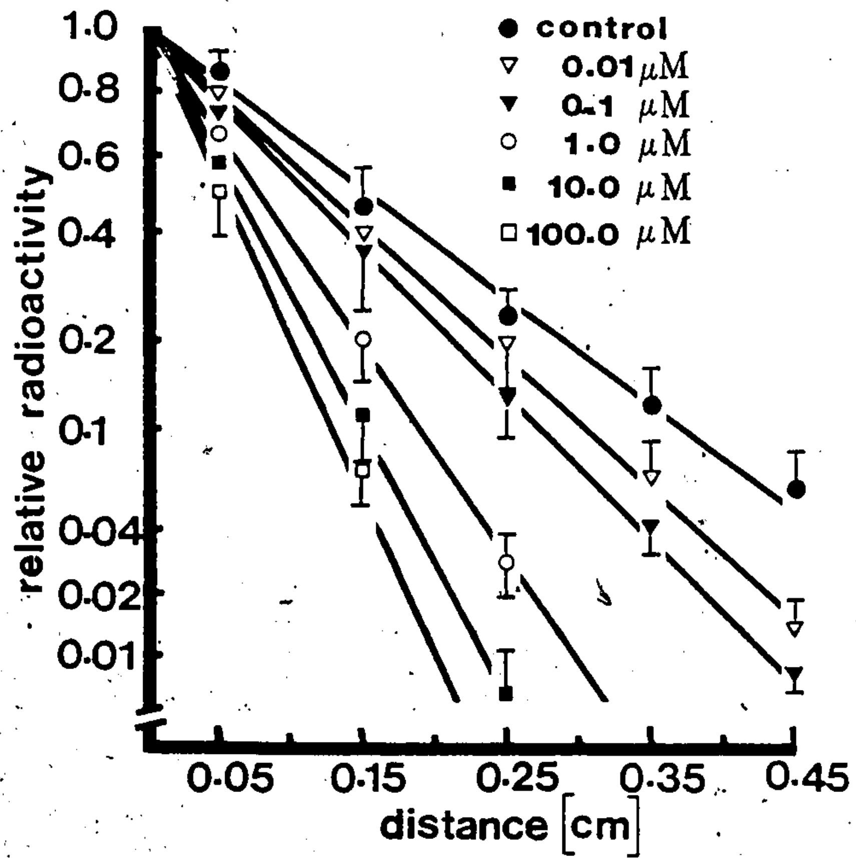
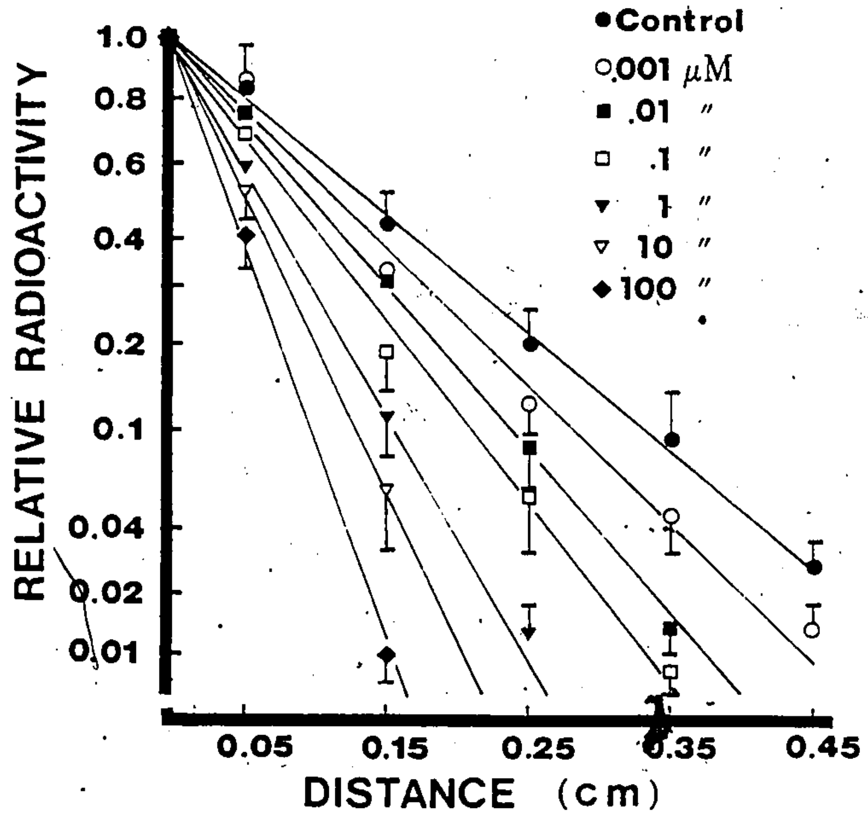

1.0
0.8
0.6
0.4
0.2
0.1
0.04
0.02
0.01
relative radioactivity
control
0.01 μM
0.1 μM
1.0 μM
10.0 μM
100.0 μM
0.05
0.15
0.25
0.35
0.45
distance [cm]

FIGURE 4.5: Longitudinal distribution of 2-DG in parturient myometrial tissues exposed to varied concentrations of calmidazolium (in μM; 0.001 (○)(n=7), 0.01 (■)(n=6), 0.1 (□)(n=5), 1.0 (▼)(n=7), 10.0 (▽)(n=7), 100.0 (◆)(n=7) or normal Krebs solution (●)(n=4).



●Control
○.001 µM
■ .01 "
□ .1 "
▼ 1 "
▽ 10 "
◆ 100 "
RELATIVE RADIOACTIVITY
1.0
0.8
0.6
0.4
0.2
0.1
0.04
0.02
0.01
0.05
0.15
0.25
0.35
0.45
DISTANCE (cm)

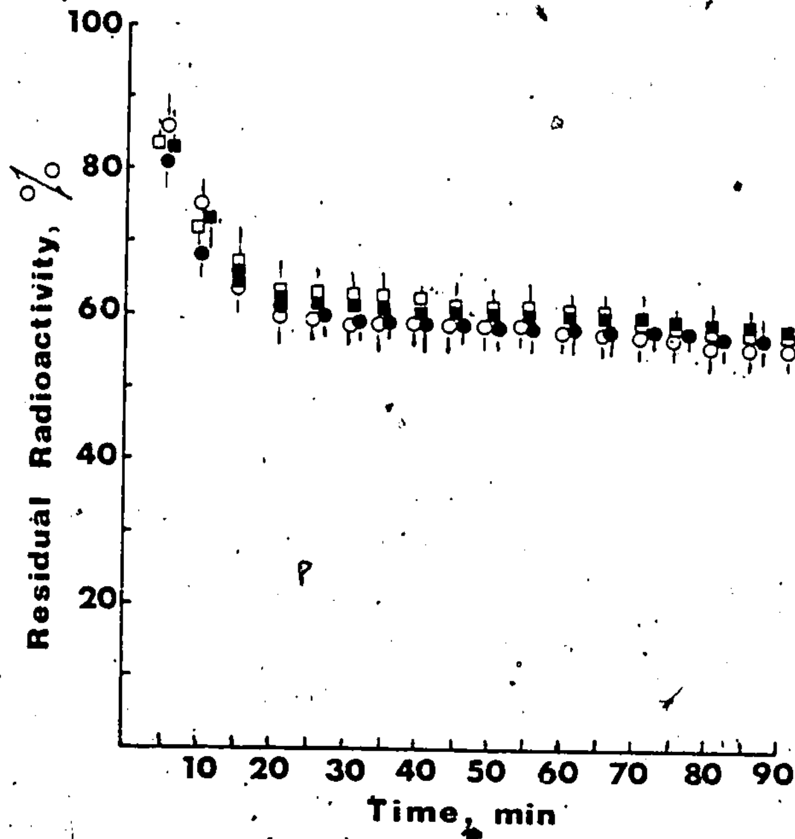
TABLE 4.2: GAP JUNCTIONS IN PARTURIENT MYOMETRIUM AFTER TREATMENT WITH IONOPHORE (Iono.), CHLORPROMAZINE (Chlor.), OR Ca^{++} FREE SOLUTIONS, DURING THE DIFFUSION EXPERIMENTS

TISSUE	N	LENGTH OF MEMBRANE (in μms)	#GJs	#GJs/1000μM MEMBRANE	AVERAGE GJ SIZE (in μms)	FRACTIONAL AREA OF GJs AS A %
1. Contr.	6(6)	8,712	44	5.27 ± 0.9	0.20 ± 0.02	0.24 ± 0.09
2. Iono. (1 μM)	3(3)	4,838	47	9.72 ± 1.1	0.14 ± 0.01	0.29 ± 0.06[1]
3. Chlor. (100 μM)	9(9)	11,594	69	5.43 ± 0.8	0.22 ± 0.02	0.25 ± 0.08[1]
4. Ca-Free (1 mM EGTA)	4(3)	5,251	37	6.14 ± 2.4	0.16 ± 0.01	0.28 ± 0.11[1]

where N is the number of tissues examined and the number of these containing GJs indicated in the brackets. Membrane length represents the total length of plasma membrane measured in 20-24 nonoverlapping micrographs of each tissue; the frequency, average size, and fractional percentage of the plasma membrane occupied by GJs are expressed as a mean ± SE.

[1] values were not significantly different ($P>0.05$) from control tissues.

FIGURE 4.6: Efflux of 2-DG from parturient myometrial tissues exposed to A23187 (1 μM, ●), Ca^{++}-free (1 mM EGTA, □), Ca^{++}-free (1 mM EGTA) and A23187 (■), or normal Krebs solution (○) (n=3 for each group).


100
80
60
40
20
Residual Radioactivity, %
10
20
30
40
50
60
70
80
90
Time, min

exposed to normal Krebs (Fig. 4.6). In all instances, 2-DG displayed an initial rapid efflux, which abated with time, leaving a residual content of radioactivity that was trapped in a compartment from which the tracer was lost only slowly. This result suggests that the drug treatments which were found to alter the longitudinal distribution of 2-DG in the diffusion experiments did not affect the manner in which 2-DG was lost from the intracellular compartment.

4.4 DISCUSSION

The experiments reported in this chapter utilized a calcium ionophore, A23187 and two structurally unrelated calmodulin antagonists; the phenothiazine, chlorpromazine (Weiss et al., 1980; Means et al., 1982), and calmidazolium (Van Belle, 1981), to determine whether direct intercellular communication between parturient rat uterine smooth muscle cells is influenced by the level of cytosolic Ca^{++} and the calcium-dependent regulatory protein, calmodulin. The data indicate that elevated $[Ca^{++}]_i$ uncouples smooth muscle cells in the absence of a change in the extent of GJs between the smooth muscle cells. Furthermore, calmodulin may play a direct role in this process by conferring Ca^{++} sensitivity to the GJs, as well as, exerting a second, more indirect influence on coupling. That the alterations in the extent of cell-to-cell communication occurred in the absence of a change in the area of GJs suggests an uncoupling mechanism dependent on decreased junctional channel permeability.

4.4.1 Mechanism for Altered 2-DG Diffusivity

In a previous chapter (section 3.4) evidence was provided that GJs represent the morphological correlate of the site for 2-DG diffusion

between smooth muscle cells in the myometrium and that the area of functional junctions per cell is an important determinant of the rate of tracer diffusion through myometrial muscle strips. However, it was demonstrated that the diffusivity of 2-DG, indicated by the magnitude of Da, is also dependent upon several additional factors which are indicated in the following equation (see section 3.4.5, eqn. 3.6):

$$Da = Ds/(1 + \delta A_c/\Omega A_j) \qquad (3.6)$$

where Da is the apparent diffusion coefficient of the solute, Ds, the diffusion coefficient of the solute in the cytoplasm, δ and Ω, the lengths of the cell-to-cell GJ channel and the smooth muscle cells, respectively, and A_c and A_j, the cross-sectional area of the smooth muscle cells and the effective area of cell-to-cell channel per cell, respectively. Thus, a decrease in Da such as was observed following treatment with A23187 and the calmodulin antagonists could result from several changes in the myometrium other than an alteration in the extent of structural or functional coupling. However, the reduced diffusivity of Da is attributed to a change in the GJs rather than a nonjunctional component of the muscle cells.

Several points argue in favor of this interpretation. Reduced 2-DG diffusivity could result from a decrease in the size of the smooth muscle cells (i.e. either Ω or Ac) because of the presence of more junctional barriers to longitudinal diffusion per unit length of the muscle strip. However, all tissues were pinned at their *in vivo* length in the baths so that significant changes in these parameters were

unlikely. Secondly, a 10-fold increase in cytoplasmic viscosity and subsequent decrease in Ds would be expected to reduce the rate of 2-DG movement by the extent which was observed in ionophore treated tissues. Since Ds was not measured in this study it is impossible to entirely dismiss this possibility. However, previous studies demonstrated the ability of ionophore to inhibit cell-to-cell diffusion of fluorescent tracers between smooth muscle cells (M.G. Blennerhassett, M.S. Kannan, R.E. Garfield, unpublished observations), and insect epithelial cells (Lees-Miller & Caveney, 1982; Safranyos & Caveney, 1985), but these treatments did not affect the movement of fluorescent tracers through the cytoplasmic compartment. For this reason it seems unlikely that the drugs could induce a 10-fold, and in the case of the calmodulin antagonists, a dose-dependent, change in cytoplasmic viscosity sufficient to account for the alterations in 2-DG diffusivity. Finally, a more rapid loss of tracer from the muscle cells as a result of the drug treatments does not seem to have contributed to the decreased longitudinal distribution of 2-DG. Efflux studies conducted under conditions identical to those in the diffusion experiments failed to produce any difference in the pattern of tracer loss from treated and control tissues.

For these reasons, the reduced diffusivity of 2-DG would appear to have resulted from a change in the effective area of functional cell-to-cell channel per muscle cell, represented by the variable, A_j, in eqn. 3.6. A_j would be expected to decrease if the area of GJs per cell is reduced by the withdrawal of connexons from the plasma membrane or, alternatively, if there is a decrease in the permeability of the

channels. Since the area of GJs in tissues used in the diffusion experiments was not affected by the various drugs used in this study (Table 4.2), the treatments probably evoked a drop in the permeability of the cell-to-cell channels. This change in permeability may be provided by a switch to an all-or-none closed state (Spray et al., 1984), or by a partial, iris-like closure of the channel sufficient to exclude the tracer (Ito et al., 1974; Deleze & Loewenstein, 1976; Loewenstein et al., 1978). A change in the arrangement of proteins comprising the channels (Unwin & Zamphigi, 1980; Unwin & Ennis, 1983), or a gating mechanism at the cytoplasmic surface of the channels (Spray et al., 1984; Makowski, 1985), are two possible mechanisms by which the permeability of the junctional channels could be modulated.

4.4.2 Uncoupling Induced by Elevated $[Ca^{++}]_i$

The data presented in this chapter demonstrates the ability of elevated $[Ca^{++}]_i$, produced by ionophore treatment, to uncouple uterine smooth muscle cells. The increase in $[Ca^{++}]_i$ produced by A23187 was not measured in this study, however, the failure of ionophore to markedly influence coupling in tissues bathed in a Ca^{++}-free (1.0 mM EGTA) medium in this study is consistent with the idea that the drug produced a Ca^{++}-mediated effect on coupling. The ability of elevated $[Ca^{++}]_i$ to influence the permeability of GJs and the extent of cell-to-cell coupling in other tissues is well recognized (Loewenstein, 1981), however, this study represents the first well-documented demonstration of an influence of Ca^{++} on coupling in smooth muscle tissues.

Although this study demonstrates an effect of $[Ca^{++}]_i$ on coupling

in uterine smooth muscle it does not indicate the critical concentration required for uncoupling. However, there is some evidence that $[Ca^{++}]_i$ must be substantially elevated before uncoupling will occur. The physiological range for Ca^{++} in smooth muscle cells is thought to be 0.1 to 1.0-5.0 μM (see Brading, 1981). Complete uncoupling, as indicated by a similar distribution of 2-DG and ^3H-sucrose, was observed in this study in tissues treated with A23187 in 2.5 mM Ca^{++} Krebs solutions. It is probable that the level of $[Ca^{++}]_i$ exceeded the physiological range for this ion in the general cytoplasm of the smooth muscle cells in these tissues. However, partial uncoupling was observed in myometrial tissues treated with solutions containing ionophore and low Ca^{++} (no added Ca^{++} and no EGTA; Table 4.1). In this case, it is probable that $[Ca^{++}]_i$ was at or very close to the level of this ion in the extracellular space, or about 1.0-10.0 μM (ie. the concentration of Ca^{++} due to contamination in the absence of EGTA buffering). Tissues treated with a physiologically relevant concentration of oxytocin (0.1 nM) possess phasic mechanical activity but this has no effect on coupling, as measured by the present methods (see Chapter 6.0), or with microelectrode and fluorescent dye transfer techniques (M.G. Blennerhassett, M.S. Kannan and R.E. Garfield, unpublished observations). However, very high doses of oxytocin (1 uM), which produce tetanic contractions, were found to markedly reduce coupling (Da for 2-DG was $0.441 \pm 0.033 \times 10^{-6}$ $cm^2 sec^{-1}$ in 6 strips). This suggests that uncoupling does not occur within the range of $[Ca^{++}]_i$ produced during phasic contractile activity but can occur when $[Ca^{++}]_i$ is elevated sufficiently to produce tetanic contractions.

Thus, uncoupling of smooth muscle cells appears to be initiated at rather high $[Ca^{++}]_i$. Similarly, high levels of $[Ca^{++}]_i$ are apparently required for uncoupling in cardiac muscle (≈50 μM; Dahl and Isenberg, 1980) and in Fundulas embryos (≈100 μM; Spray et al., 1982, 1984). For this reason Spray et al. (1982, 1984) have questioned the physiological relevance of Ca^{++}-induced uncoupling mechanisms. However, until precise, parallel measurements of coupling and $[Ca^{++}]_i$ within the microenvironment at the junctions are made it would seem unwarranted to make any firm conclusions in this regard.

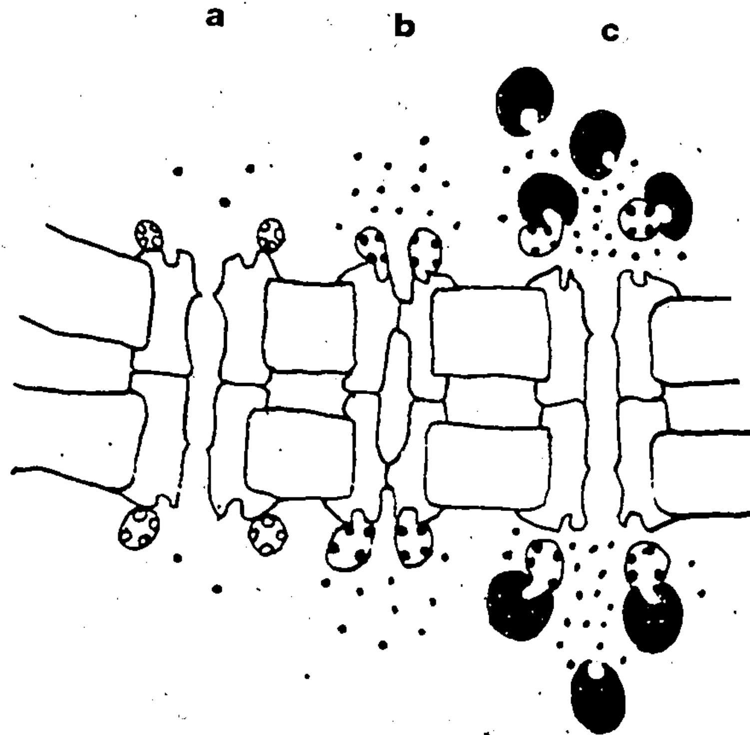
The present observation of A23187-induced uncoupling in myometrial tissues is similar to that reported for invertebrate neurons (Baux et al., 1978), insect epithelial cells (see Loewenstein, 1981), and several varieties of mammalian cells in culture (Flagg-Newton & Loewenstein, 1979). In addition, several other experimental procedures have been employed to alter $[Ca^{++}]_i$, for example, plasma membrane rupture, intracellular injection of calcium, inhibition of oxidative phosphorylation or glycolysis, and inhibition of the sodium pump in cardiac muscle with ouabain (see Loewenstein, 1981; De Mello, 1982b). In all cases, a rise in cytosolic free $[Ca^{++}]$ is accompanied by a measurable drop in either ionic or metabolic coupling. In some instances, the uncoupling produced by elevated $[Ca^{++}]_i$ was dissociated from that brought about by reduced cystolic pH (Rose & Rick, 1978; Spray et al., 1982, 1984). Furthermore, treatment of pancreatic acinar cells with secretagogues was demonstrated to elicit a Ca^{++}-mediated drop in cell communication (Iwatuski & Petersen, 1978a,b; Findlay & Petersen, 1982). This observation and the experiments using oxytocin

mentioned above, suggest that there may also be specific agonist-receptor mediated mechanisms that raise $[Ca^{++}]_i$ sufficient to alter coupling between cells. However, despite this abundance of data, rather little is understood about the mechanism whereby Ca^{++} produces uncoupling.

4.4.3 Roles of Calmodulin in the Control of GJ Permeability

The present observations that both chlorpromazine and calmidazolium block the effects of A23187 on 2-DG movement in the myometrium raises the possibility that the Ca^{++}-induced uncoupling mechanism may involve an intermediate regulatory protein. Specifically, the present data suggest that a Ca^{++}-calmodulin interaction may be involved in the ionophore stimulated alteration of myometrial GJ permeability (see Fig. 4.7). It seems most likely that chlorpromazine and calmidazolium blocked the actions of A23187 on 2-DG diffusion between myometrial cells by preventing an interaction between activated calmodulin and the junctional uncoupling mechanism (Fig. 4.7). Whether this uncoupling mechanism is embodied in the junctions or is a separate, perhaps enzymatic, process remains to be determined. However, it has been shown that calmodulin interacts directly with isolated liver GJ proteins in a Ca^{++}-dependent fashion (Hertzberg & Gilula, 1981), and as well, that it can interact with, and elicit a Ca^{++}-dependent conformational change in, isolated lens junctions (Girsch & Peracchia, 1985a,b). This is consistent with a direct interaction between activated calmodulin and GJs. The interaction between Ca^{++}-calmodulin- and the junctions is probably similar to that described for calmodulin-dependent enzyme systems

FIGURE 4.7: Diagrammatic representation of the possible interaction between Ca^{++} (●), calmodulin ( ), calmodulin antagonists ( ), and a gap junction uncoupling mechanism. A) The postulated gating mechanism is open at resting Ca^{++} (●) levels. Calmodulin is in its inactive state ( ) and does not interact with the junctions to produce channel closure. B) At higher Ca^{++} levels, calmodulin is activated ( ), and can interact with the junctions to induce channel closure. C) Calmodulin antagonist drugs ( ) may interact with the activated calmodulin preventing the regulatory protein from affecting the gap junctions, hence, normal coupling is observed despite high Ca^{++}.


a
b
c

(Cheung, 1980, 1982; Means et al., 1982). That is, elevated $[Ca^{++}]_i$ alters the functional properties of the junctions by inducing a conformational change in, and activating, calmodulin, which is then able to interact with the uncoupling mechanism (Fig. 4.7). Activation of calmodulin is thought to occur when this regulatory protein has bound 3 or 4 calcium ions (calmodulin-$(Ca^{++})_{3-4}$) (Cheung 1980, 1982). It is possible that the activated complex of Ca^{++}-calmodulin interacts with, and induces a conformational change in, either the proteins forming the cell-to-cell channels or a gating mechanism at the cytoplasmic opening of the channel (Fig. 4.7). This sequence of events may be blocked by chlorpromazine and calmidazolium because these drugs bind to, and compete with the uncoupling mechanism for, the activated Ca^{++}-calmodulin complex (Fig. 4.7). Indeed, the EC_{50} values for the inhibition of the ionophore induced uncoupling by chlorpromazine (1.0 μM) and calmidazolium (0.05 μM) are consistent with values reported for these drugs in the antagonism of other Ca^{++}-calmodulin dependent processes (Weiss et al., 1980; Van Belle, 1981).

That an intermediate regulatory molecule such as calmodulin may participate in the mechanism which regulates GJ permeability is also suggested by other studies. Calmodulin antagonists have been demonstrated to block the uncoupling effects of elevated $[Ca^{++}]_i$ in cardiac fibers (Wojtczak, 1984) and of reduced cytosolic pH in amphibian embryos (Peracchia et al., 1983; Peracchia, 1984). These drugs were also found to reduce coupling between cells in an insect epithelium (Lees-Miller & Caveney, 1982; Safranyos & Caveney, 1985). This latter observation was interpreted to indicate that in some

instances an interaction between calmodulin and the junctions may be necessary to maintain coupling (see below). Johnston and Ramon (1981) failed to observe any junctional sensitivity to Ca++ or H+ in crayfish axons in which the cytoplasmic constituents were removed by internal perfusion. These authors concluded that a soluble intermediate regulatory molecule had been removed from the junctions preventing the uncoupling action of perfusates with high [Ca^{++}] or [H^{+}] content. However, Spray et al. (1982) did not identify any such dependency on a soluble intermediate in similar studies using Fundulas blastomeres. The reasons for this discrepancy remain to be determined.

Recently, Girsch and Peracchia (1985a,b) incorporated the postulated channel forming protein of lens reticular cells, MP 26, into lipid vesicles. It was demonstrated that the influx of sucrose into the vesicles (presumably through channels provided by MP 26) was reduced by solutions of high Ca^{++} content, but only in the presence of calmodulin (Girsch & Peracchia, 1985a). Moreover, chelation of Ca^{++} by EGTA was shown to reinitiate sucrose influx, suggestive of a release from Ca^{++}-calmodulin permeability control. As noted by Hertzberg (1985), the fact that intermediate molecules have not been identified in samples of isolated junction proteins does not preclude their involvement in permeability regulation nor an association with junctional proteins. The relatively harsh conditions utilized to isolate the junctions may remove any associated regulatory molecules. Indeed, Manjunath et al. (1984) provide evidence for the presence of a 15-17K protein associated with the cytoplasmic surface of cardiac GJs and the removal of this component during most isolation procedures. It

is interesting in this context that calmodulin is reported to have a molecular weight of about 16.7K daltons (Cheung, 1980, 1982).

That the calmodulin antagonists should lower the diffusivity of 2-DG in the myometrium when added in the absence of ionophore is confusing in that no effect was the expected result of these experiments. The effect of the antagonists on coupling in tissues without elevated Ca^{++} levels suggests that, in addition to a direct involvement in the Ca^{++}-induced uncoupling mechanism, calmodulin may also be involved in a second, less direct pathway for the control of junctional communication in the myometrium. The effect of these drugs on 2-DG diffusion is apparently not due to a nonspecific action of the drugs on the smooth muscle cells. Firstly, both chlorpromazine and calmidazolium had a dose-dependent effect on coupling (Fig. 4.7). Secondly, an attempt was made to separate the calmodulin-related effects of the phenothiazine drugs from their ability to disrupt the integrity of plasma membranes (Seeman, 1972) and to interact with specific membrane receptors (see Van Belle, 1981). Diffusion experiments were conducted in the presence of lidocaine and procaine, two local anesthetics which are thought to possess similar membrane perturbing properties to the phenothiazines (Seeman, 1972). However, an alteration in coupling in tissues treated with either of these drugs was not observed. Chlorpromazine and other phenothiazines interact with α-adrenoceptors, as well as, histamine H_1, dopamine and serotonin receptors (see Van Belle, 1981), and it was possible, therefore, that the loss of communication could have been produced by a receptor-mediated mechanism. For this reason the effect on coupling

of the nonphenothiazine derivative, calmidazolium, which does not appear to interact with these receptors (Van Belle, 1981) was also tested. As noted above, both types of calmodulin antagonist were found to produce uncoupling. For these reasons it seems that the influence of chlorpromazine and calmidazolium on coupling in the myometrium was due to an inhibition of calmodulin activity.

An inhibition of coupling by calmodulin antagonists would be expected if a direct interaction between calmodulin and GJs was required for coupling in the myometrium. For example, if calmodulin binding to the junctions were necessary for the channels to exist in an open, functional state. However, this explanation cannot account for all of the present observations. A similar inhibition of cell-to-cell communication by calmodulin antagonists to that identified in this study was reported for epithelial cells in an insect epidermis (Less-Miller & Caveney, 1982; Safranyos & Caveney, 1985). It was postulated that the antagonists blocked a direct interaction between calmodulin and GJs that was necessary for open channels. The interaction was proposed to be analogous to that observed for calmodulin and some adenylate cyclase activities. That is, a biphasic interaction involving a stimulation of coupling by calmodulin in conditions of low $[Ca^{++}]_i$ (ie. <1.0 μM) but inhibition to successively greater degrees as Ca^{++} is increased (see Lees-Miller & Caveney, 1982). The uncoupling produced by calmodulin antagonist was postulated to have resulted because the drug prevented an interaction between calmodulin and the GJs at low Ca^{++} (Lees-Miller & Caveney, 1982). However, this explanation does not explain the results of the present

study. If a direct interaction between calmodulin and myometrial GJs was required for coupling to occur, then an inhibition of communication by the calmodulin antagonists would be expected regardless of the level of $[Ca^{++}]_i$. Since normal coupling was observed in myometrial tissues treated with a combination of ionophore and calmodulin antagonist, a direct interaction was probably not involved. This experiment combining A23187 with the calmodulin antagonists was apparently not attempted by Lees-Miller and Caveney (1982).

It is possible that the calmodulin antagonists influence coupling in the myometrium by altering the activity of a second, more indirect and, as yet unidentified, regulatory pathway. For example, these drugs could inhibit the activity of phosphodiesterase (an enzyme well-known to be dependent on calmodulin for activity (Cheung, 1980, 1982; Means et al., 1982)), leading to an increase in the level of adenosine 3'-5' monophosphate (cAMP) and a cAMP-mediated decrease in cell-to-cell coupling. That coupling between myometrial cells is inhibited by a variety of treatments which elevate intracellular cAMP (ie. dibutyryl cAMP, forskolin, isoproterenol), including an inhibition of phosphodiesterase with theophylline (see Chapter 5, section 5.3.1), is consistent with this mechanism. Interestingly, cAMP is also reported to uncouple the cells in the insect epidermis (Caveney, 1980). It is tempting to suggest, therefore, that an inhibition of phosphodiesterase may account for the observation of uncoupling by calmodulin antagonists in this tissue as well.

4.5 SUMMARY

The data reported in this chapter provide evidence which is

consistent with a modulation of intercellular communication between uterine smooth muscle cells by alterations in the permeability of the cell-to-cell channels comprising GJs. Furthermore, this study shows that coupling is decreased by elevated $[Ca^{++}]_i$, and that the Ca^{++}-dependent regulatory protein, calmodulin, may be directly involved in this process, possibly conferring Ca^{++}-sensitivity to the junctional uncoupling mechanism. The data also suggest that calmodulin participates in a second, more indirect, pathway for GJ permeability regulation. This pathway may involve control of cAMP degradation in the smooth muscle cells.

CHAPTER 5:

REGULATION OF MYOMETRIAL GJ PERMEABILITY BY cAMP

5.1 INTRODUCTION

β-Adrenoceptor agonists (ie. primarily β_2 adrenoceptor agonists) inhibit spontaneous activity in the myometrium and premature labor in animals and humans (Korenman & Krall, 1977; Lipshitz, 1981). There is limited evidence that relaxin can also act as a tocolytic agent (Schwabe et al., 1978) and it was proposed that endogenous relaxin serves to inhibit coordinated contractility in the rat myometrium during the final 24-36 hours of pregnancy (Downing & Sherwood, 1985a,b). These agonists are thought to elevate the intracellular concentration of cyclic adenosine 3'5' monophosphate ($[cAMP]_i$) in uterine muscle fibers (Vesin & Harbon, 1974; Korenman & Krall, 1977; Sanborn et al., 1980; Harbon et al., 1984) but the mechanism by which they inhibit spontaneous activity in the myometrium is poorly understood and remains controversial. It is conceivable that this reduction in spontaneous activity resulted because the spread of electrical activity from pacemaker regions was suppressed by a cAMP-mediated inhibition of functional coupling between myometrial cells. For this reason the effect of elevated $[cAMP]_i$ on 2-DG diffusion in the delivering myometrium was studied using the two-compartment bathing chamber technique.

The data show that elevated $[cAMP]_i$, produced by dibutyryl cAMP, 8-bromo cAMP, forskolin, or theophylline, reduces the diffusivity of 2-DG between uterine smooth muscle cells. The effects of agonists which are thought to elevate $[cAMP]_i$ in the myometrium are reported in the subsequent chapter in relation to the possible presence of a

endogenous mechanism for regulating GJ permeability (see Chapter 6, section 6.3).

5.2 METHODS AND MATERIALS

The diffusion experiments described in this chapter were conducted using longitudinal tissues from delivering rats according to the methods described above (Chapter 2). All drug treatments were conducted by adding the various agents to the perfusate in the 'cold' compartment. Treatment was initiated during the last 10 minutes of the equilibration period and continued during the diffusion period. The tissues were exposed to dibutyryl cAMP (1.0, 0.5, 0.01 mM) (drugs purchased from Sigma Chemicals unless otherwise indicated), 8-bromo cAMP (0.1 mM), 5'AMP (1.0 mM), cAMP (1.0 mM), butyrate (1.0 mM), theophylline (1.0 mM), and forskolin (1.0 μM) (Calbiochem).

Small samples of the tissues used in the diffusion experiments were processed for electron microscopy and the quantitation of GJs according to the methods described above (section 2.5). 2-DG efflux studies were also conducted according to the methods used above (section 2.4) to determine whether cAMP had an effect on the transmembrane movement of phosphorylated 2-DG.

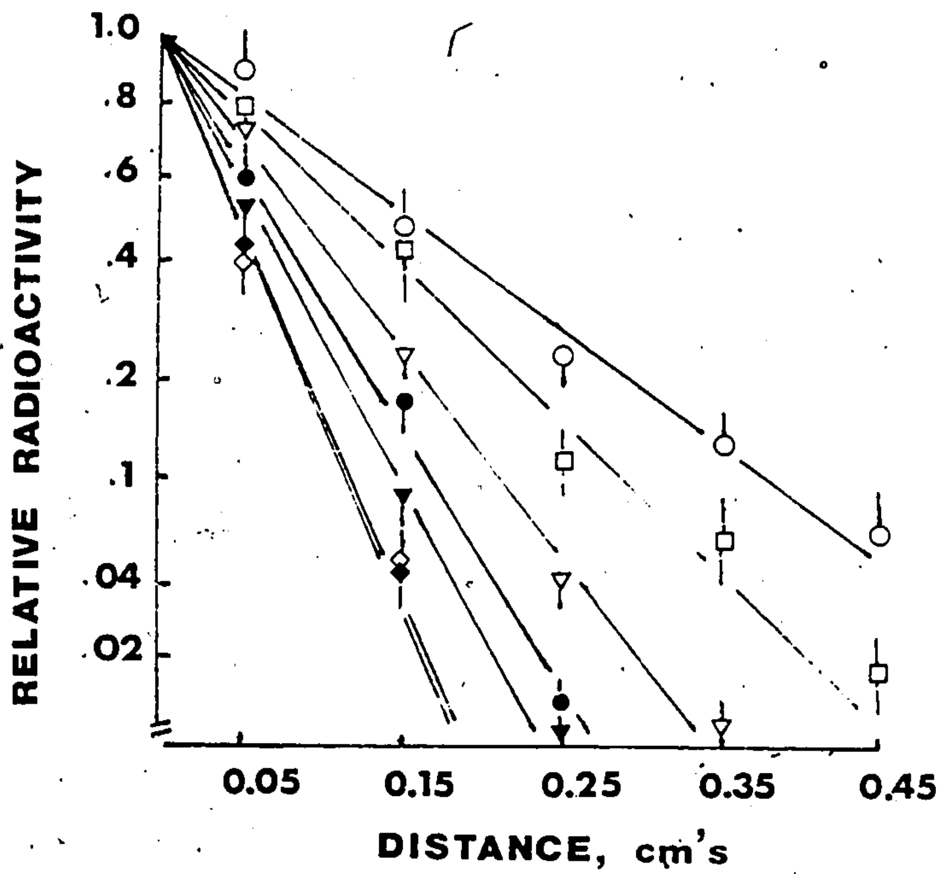
5.3 RESULTS

5.3.1 Diffusion Experiments

5.3.1.1 Effect of an exogenous source of cAMP on 2-DG diffusivity.

In order to determine whether elevated $[cAMP]_i$ had an influence on junctional communication in the myometrium, tissues from parturient rats were exposed to several agents, which elevate intracellular levels of this cyclic nucleotide. Figure 5.1 demonstrates the effect

FIGURE 5.1: Longitudinal distribution of 2-DG in parturient myometrial tissues treated with dibutyryl cAMP (0.01 mM, □, n=3; 0.5 mM, ▽, n=4; 1.0 mM, ▼, n=10), 8-bromo cAMP (0.1 mM, ●, n=10) or untreated (○). The distribution of ^3H-sucrose in tissues exposed to dibutyryl cAMP (1.0 mM, ◇, n=3) versus untreated strips (◆, n=4) is also indicated.



1.0
.8
.6
.4
.2
.1
.04
.02
RELATIVE RADIOACTIVITY
0.05
0.15
0.25
0.35
0.45
DISTANCE, cm's

TABLE 5.1: THE APPARENT DIFFUSION COEFFICIENT (Da) FOR 2-DG IN PARTURIENT MYOMETRIAL TISSUES WITH ELEVATED $[cAMP]_i$

TREATMENT	[ ]	N*	Da ± SE. $\times 10^{-6}$ cm^2 sec^{-1}
1a. Control		7(4)	1.58 ± 0.18
b. Db-cAMP	0.01 mM	3(2)	1.06 ± 0.06[2]
	0.5 mM	4(2)	0.59 ± 0.09[2]
	1.0 mM	10(5)	0.23 ± 0.09[2]
c. 8-Br cAMP	0.1 mM	10(6)	0.30 ± 0.03[2]
d. Forskolin	1.0 µM	10(6)	0.49 ± 0.07[2]
e. Theophylline	1.0 mM	5(3)	0.86 ± 0.09[2]
f. Forskolin & theophylline	as above	6(3)	0.34 ± 0.08[2]
2a. Control		4(3)	1.46 ± 0.12
b. Butyrate	1.0 mM	4(2)	1.33 ± 0.14[1]
c. 5' cAMP	1.0 mM	3(2)	1.26 ± 0.16[1]
d. cAMP	1.0 mM	4(3)	1.50 ± 0.21[1]

* n refers to the number of muscle strips employed whereas the numbers in the brackets indicates the number of animals.

1 values of Da for treated tissues that were not significantly different ($P>0.05$) from that for control, untreated tissues.

2 values of Da for treated tissues which were significantly different ($P<0.05$) from that for control untreated tissues.

of providing an exogenous source of cAMP on the distribution of 2-DG. Dibutyryl cAMP (db-cAMP) (0.01, 0.5 & 1.0 mM) or 8-bromo cAMP (8br-cAMP) (0.1 mM), which cross the plasma membrane and yield free cAMP when hydrolyzed by endogenous esterases, reduced the distribution of 2-DG compared to that in control, untreated tissues (Fig.5.1). The diffusivity of 2-DG was similarly, and significantly ($P<0.05$) reduced by db-cAMP and 8br-cAMP (Table 5.1). That db-cAMP at the three concentrations used in this study produced a dose-dependent reduction in the distribution (Fig. 5.1) and diffusivity (Table 5.1) of 2-DG implies that the degree of uncoupling is dependent on the intracellular concentration of this second messenger.

In contrast to the effects of db-cAMP and 8br-cAMP structurally related compounds, such as cAMP (1.0 mM), 5'AMP (1.0 mM), and butyrate (1.0 mM) did not influence 2-DG diffusion in the myometrium and the value of Da for each group was not significantly different ($P>0.05$) from that for untreated tissues (Fig. 5.2 & Table 5.1). The inability of these compounds to influence 2-DG diffusion is consistent with a modulation of coupling by cAMP. Furthermore, since db-cAMP and 8br-cAMP cross the plasma membrane relatively quickly, but cAMP enters only slowly, if at all, it appears that elevated $[cAMP]_i$ is necessary for the alteration in 2-DG diffusivity.

5.3.1.2 **Effect of altered cAMP synthesis or degradation.** Forskolin is thought to interact with the catalytic subunit of adenylate cyclase and stimulate the synthesis of cAMP by this enzyme (de Souza et al., 1983). The distribution (Fig. 5.3) and value of Da (Table 5.1) for 2-DG in tissues treated with forskolin (1.0 μM) were

FIGURE 5.2: Longitudinal distribution of 2-DG in parturient myometrial tissues treated with cAMP (1.0 mM, ▲, n=4), 5'AMP (1.0 mM, ▽, n=3), butyrate (1.0 mM, □, n=4) or untreated (○, n=4).

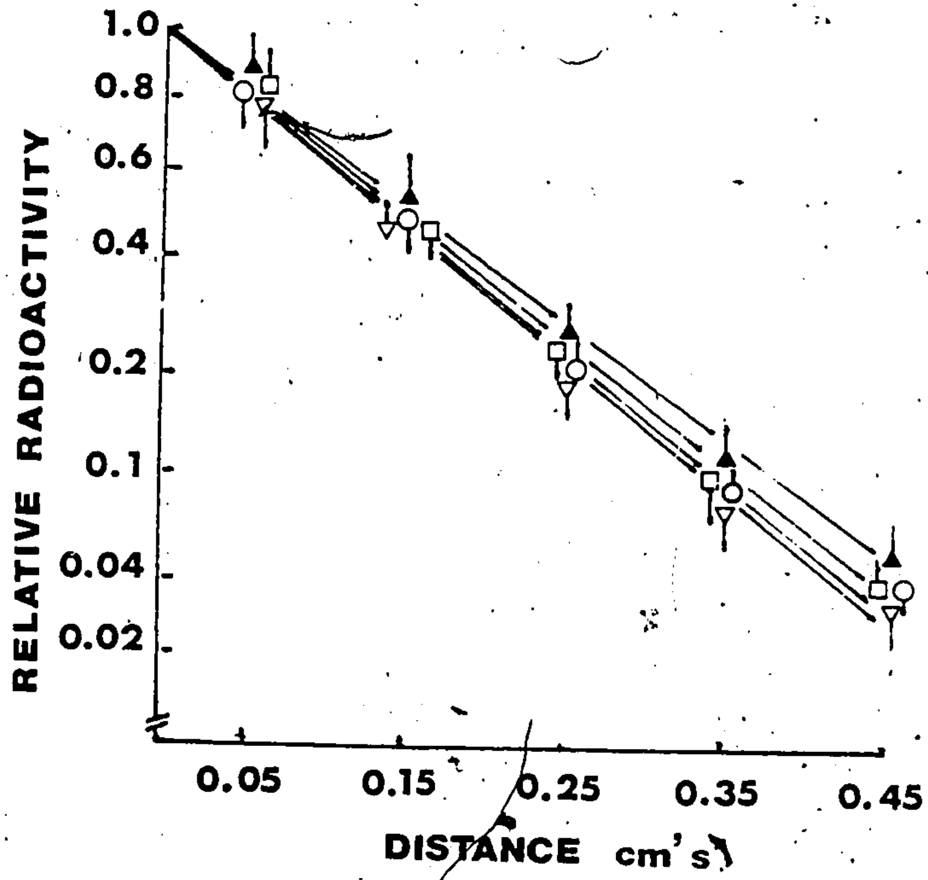
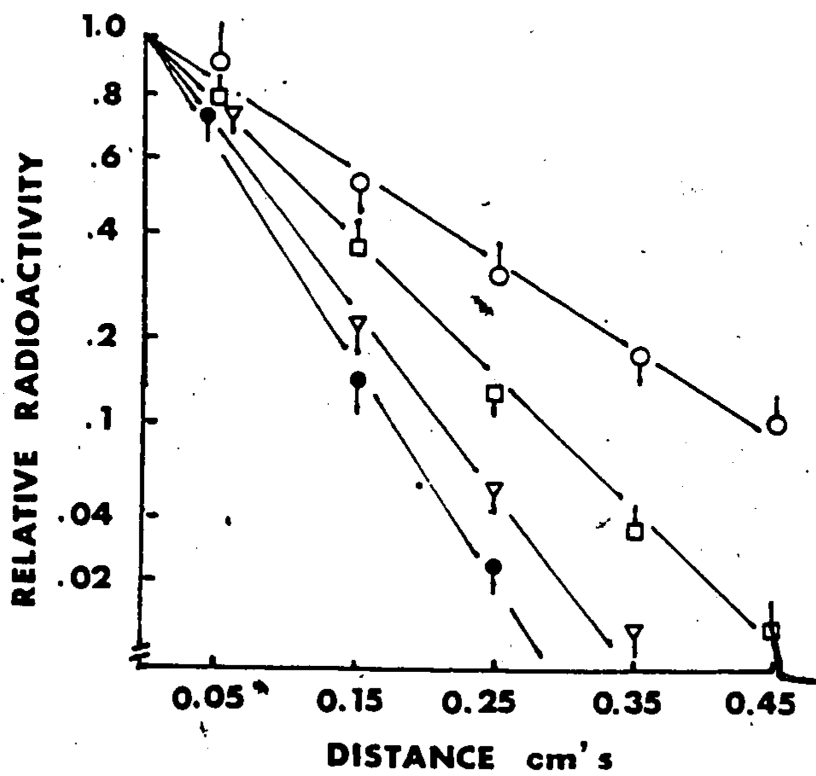

RELATIVE RADIOACTIVITY
1.0
0.8
0.6
0.4
0.2
0.1
0.04
0.02
0.05
0.15
0.25
0.35
0.45
DISTANCE cm's

FIGURE 5.3: Longitudinal distribution of 2-DG in parturient myometrial tissues exposed to theophylline (1.0 mM, □, n=5), forskolin (1.0 μM, ▽, n=10), forskolin and theophylline (1.0 μM and 1.0 mM, ●, n=6) or untreated (○, n=7).


1.0
.8
.6
.4
.2
.1
.04
.02
RELATIVE RADIOACTIVITY
0.05
0.15
0.25
0.35
0.45
DISTANCE cm's

significantly ($P<0.05$) reduced compared to that for untreated strips. Similarly, inhibition of the cAMP-degrading enzyme, phosphodiesterase, with theophylline (1.0 mM) also significantly ($P<0.05$) reduced the diffusivity of 2-DG in the delivering myometrium (Fig. 5.3 & Table 5.1). The concept that the degree of inhibition of 2-DG diffusion is dependent on $[cAMP]_i$ is further supported by the observation that an additive, and significantly greater ($P<0.05$) reduction of the distribution (Fig. 5.3) and diffusivity (Table 5.1) is obtained when tissues are simultaneously exposed to forskolin (1.0 μM) and theophylline (1.0 mM).

5.3.2 Effect of Elevated [cAMP]i on Structural Coupling

5.3.2.1 Lack of an effect of [cAMP]i on GJ area. Small portions of several myometrial strips treated with db-cAMP (1.0 mM) and forskolin (1.0 μM) in the diffusion experiments were examined in the electron microscope and the extent of structural coupling compared to that in several control tissues. GJs were observed in all tissues examined. The fractional percentage of the plasma membrane occupied by GJs between the smooth muscle cells was not found to be different in the three treatment groups (Table 5.2). The average size of the junctions was slightly but not significantly greater, and their frequency slightly but not significantly lower, in tissues treated with db-cAMP and forskolin compared to controls (Table 5.2). These results indicate that the alterations in 2-DG diffusivity cannot be explained on the basis of an alteration in the extent of structural coupling.

5.3.2.2 Forskolin stimulates altered GJ morphology. Whereas no

TABLE 5.2: GAP JUNCTIONS IN PARTURIENT MYOMETRIUM AFTER TREATMENT WITH DIBUTYRYL cAMP (DBcAMP) OR FORSKOLIN (Forsk.) DURING THE DIFFUSION EXPERIMENTS

TISSUE	N	LENGTH OF MEMBRANE (in μms)	#GJs	#GJs/1000μM MEMBRANE	AVERAGE GJ SIZE (in μms)	FRACTIONAL AREA OF GJs AS A %
1. Contr.	3(0)	4,065	47	12.5 ± 2.3	0.17 ± 0.13	0.36 ± 0.22
2. DBcAMP (1 mM)	7(1)	7,858	63	7.92 ± 2.0	0.29 ± 0.02	0.49 ± 0.25[1]
3. Forsk. (1 μM)	5(5)	4,641	36	8.75 ± 1.6	0.26 ± 0.02	0.44 ± 0.19[1]

Where N is the number of tissues examined and the number of these containing GJs with dense granules indicated in the brackets. Membrane length represents the total length of plasma membrane measured in 20-24 nonoverlapping micrographs of each tissue; the frequency, average size, and fractional percentage of the plasma membrane occupied by GJs are expressed as a mean ± SE.

[1] values were <u>not</u> significantly different (P>0.05) from control tissues.

change in the area of GJs was found after the above treatments, a significant difference in the morphology of the junctions was noted in tissues treated with forskolin (1.0 μM) and with other agonists (relaxin, isoproterenol, and carbacyclin, see Chapter 6.0) which stimulate adenylate cyclase activity in the myometrium. Electron dense deposits of varied size and shape were found to be associated with the cytoplasmic surface of several junctions in four of the five tissues treated with forskolin which were examined (Table 5.2). Figure 5.4 shows several examples of the granules in tissues treated with forskolin and the agonists employed in Chapter 6.0. Occasionally, the deposits appeared to be precisely paired in position and size on opposite sides of the region of cell-to-cell contact within the two smooth muscle cells (Fig 5.4d). In addition, small deposits were also observed on the membrane beside the junctions and at localized positions on the nonjunctional membrane (Fig. 5.4c). Similar structures were not observed in the control tissues and only two small deposits were present in one tissue treated with db-cAMP (Table 5.2). Similar deposits were previously observed between uterine smooth muscle cells (Garfield et al., 1980b), as well as, between Leydig cells, granulosa cells, and cultured Lesch-Nyhan cells (Larsen, 1975, 1977a). In all instances, the presence of the deposits was not dependent on lead citrate or uranyl acetate staining (Larsen, 1975, 1977a; Garfield et al., 1980b).

5.3.3 Lack of an Effect of Elevated [cAMP]i on 2-DG Efflux

Small samples of myometrium from delivering rats were loaded with 2-DG and efflux studies were conducted using saline solutions

FIGURE 5.4: Electron micrographs of opaque deposits on gap junctions in parturient myometrial tissues used in the diffusion experiments. A) Electron opaque deposits (arrows) on two gap junctions in a tissue exposed to isoproterenol (1.0 μM; see chapter 6.0). x55,000.
B) A high magnification of a gap junction with a large opaque deposit in a tissue treated with forskolin (1.0 μM). x140,000.
C) Two small deposits (arrows) on the membrane beside a gap junction in a tissue exposed to relaxin (0.1 μg/ml). x66,000.
D) Several opaque deposits of which 3 are paired across the gap junction (arrows) in a tissue treated with forskolin (1.0 μM). x80,000.

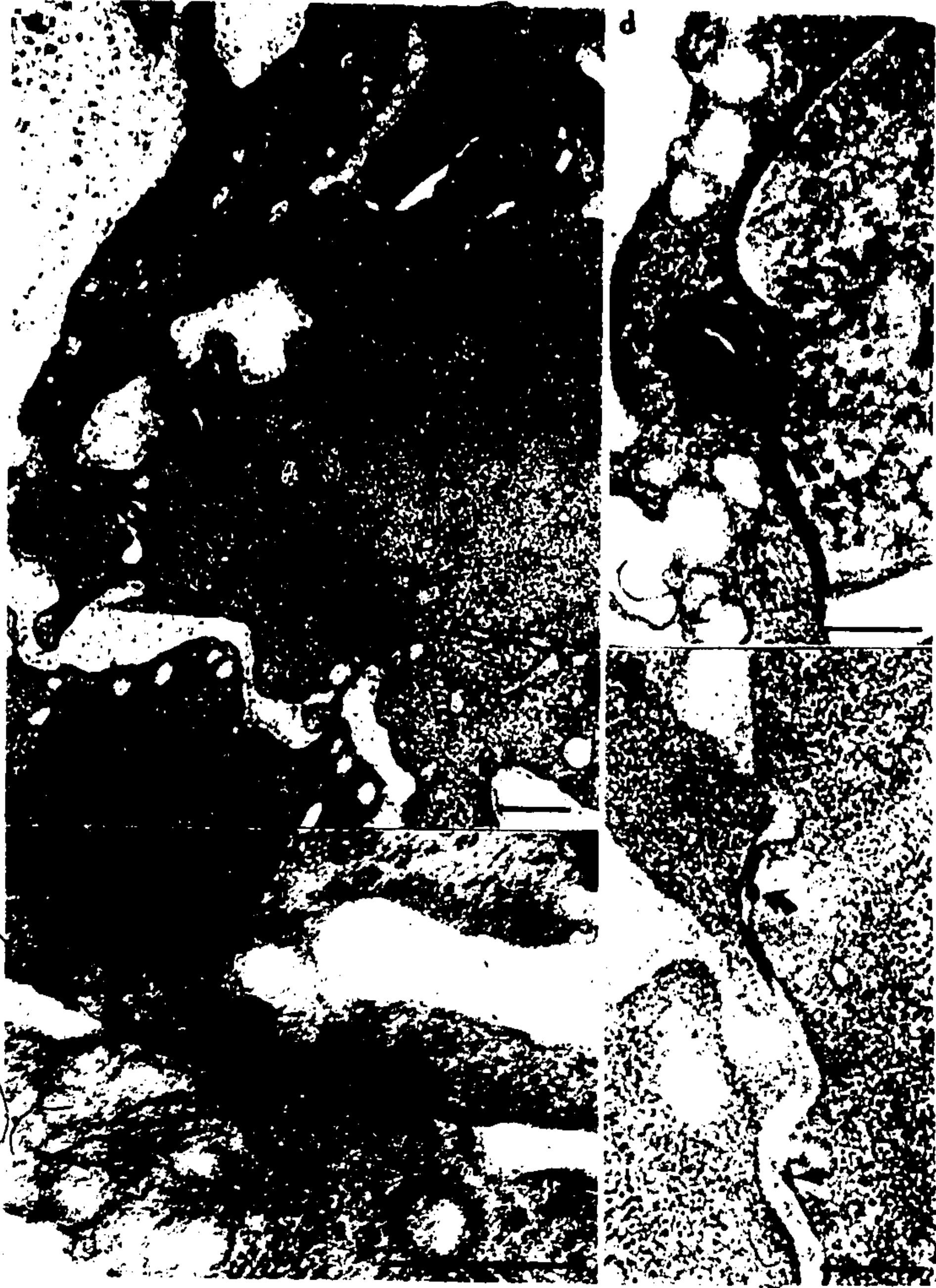
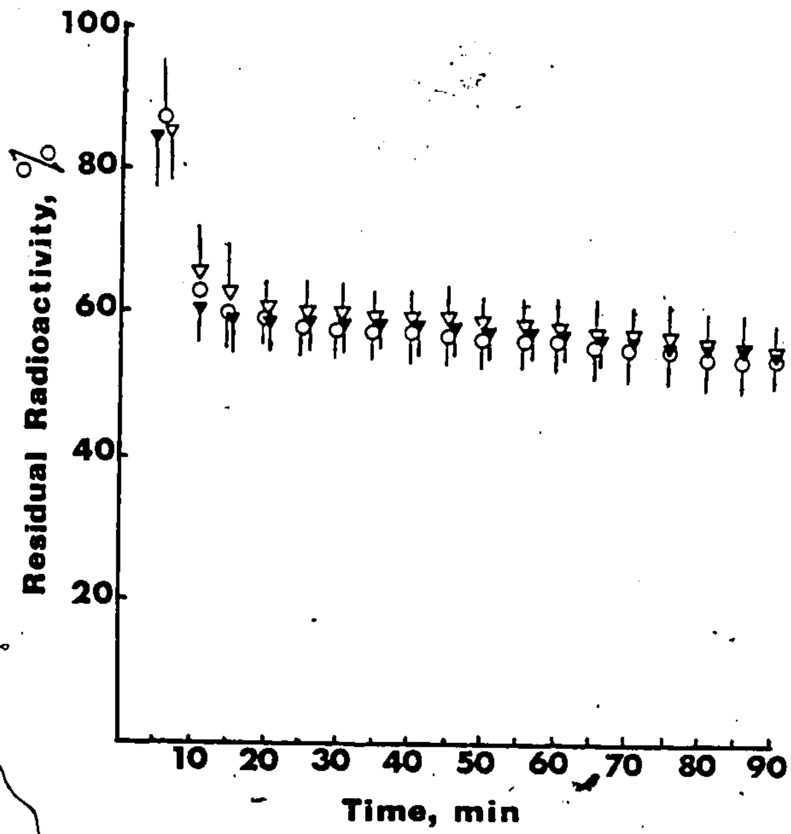


FIGURE 5.5: Efflux of 2-DG from parturient myometrial tissues exposed to dibutyryl cAMP (1.0 mM, ▼), forskolin (1.0 μM, ▽) or untreated (○) (n=4 for each group).


100
80
60
40
20
Residual Radioactivity, %
10 20 30 40 50 60 70 80 90
Time, min

containing db-cAMP (1.0 mM) or forskolin (1.0 μM). The profile of 2-DG efflux in these treated tissues was similar to that for untreated samples (Fig. 5.5), indicating that there was no change in the rate of transmembrane movement of the tracer in tissues with elevated $[cAMP]_i$ or stimulated cyclase activity.

5.4 Discussion

The data presented in this chapter indicate that the diffusivity of 2-DG in myometrial strips from delivering rats is reduced by treatments which elevate $[cAMP]_i$ and provide evidence for a modulation of GJ permeability by cAMP. This control of coupling by cAMP may contribute to an inhibition of spontaneous activity by agonists which elevate the intercellular levels of this second messenger. In addition, the presence of electron dense deposits in tissues in which adenylate cyclase was presumably stimulated suggests a possible association between this enzyme and myometrial GJs.

5.4.1 Modulation of Functional Coupling by [cAMP]i

The alterations in 2-DG diffusivity described in this chapter resulted from a reduction in GJ channel permeability. As noted in previous chapters (sections 3.4 & 4.4), the diffusivity of 2-DG in the myometrium is affected by several factors in addition to the number, size, and function of the GJs. However, it is unlikely that a change in these nonjunctional variables contributed to the depressed 2-DG diffusion in tissues with elevated $[cAMP]_i$. As noted previously, the tissues were maintained at a fixed length in the diffusion chambers so that a change in the size of the muscle cells was precluded. Increased loss of tracer from cells with elevated $[cAMP]_i$ cannot account for the

altered 2-DG diffusivity because the efflux experiments failed to reveal increased 2-DG washout from the treated tissues. Furthermore, the observation of a structural alteration in the GJs within tissues treated with forskolin also lends credence to the belief that a change at the level of the junctions produced the alteration in tracer diffusivity.

The experiments described in this chapter imply that regardless of whether $[cAMP]_i$ is elevated by, stimulating its synthesis by adenylate cyclase, inhibiting its degradation with phosphodiesterase, or providing an exogenous, additional source of cAMP, the effect is to decrease the longitudinal diffusion of 2-DG in the delivering myometrium. This is further supported by data presented in the subsequent chapter (Chapter 6) which demonstrate that the diffusivity of 2-DG (Fig. 6.1 & Table 6.1) is also reduced by physiologically relevant agents, including isoproterenol (a nonselective β-agonist), relaxin, and the stable analog of prostacyclin (PG I_2), carbacyclin, which are thought to elevate $[cAMP]_i$ in myometrial tissues by interacting with specific membrane receptors coupled to adenylate cyclase (Korenman & Krall, 1977; Sanborn et al., 1980; Vesin & Harbon, 1974; Vesin et al., 1979; Harbon et al., 1984). However, the mechanism by which cAMP reduces the permeability of myometrial GJs remains to be identified. Indeed, understanding the relationship between elevated $[cAMP]_i$ and alterations in functional coupling is difficult in light of the fact that (1) there are only limited experimental data showing an effect of cAMP on coupling and, (2) this second messenger reduces GJ permeability in some tissues (eg. the myometrium (Cole & Garfield,

1985) and retinal horizontal cells, Laufer & Salas, 1981; Piccolino et al., 1982; Teranishi et al., 1982) but improves coupling in others (eg. cardiac muscle, Estape & De Mello, 1983, De Mello, 1982a, 1984; hepatocytes, Saez et al., 1985).

It is possible that the modulation of GJ permeability in the myometrium by cAMP involves the phosphorylation of the cell-to-cell channels by a cAMP-dependent protein kinase. The elevated levels of cAMP may enhance the activity of a cAMP protein kinase leading to decreased channel permeability. That cAMP-dependent kinase activity will label isolated liver junctions _in vitro_ when provided with P^{32}-radiolabelled ATP (Saez et al., 1985) is consistent with the idea that the GJ proteins may be phosphorylated by this kinase. Phosphorylation of the junctions might alter the permeability of the channels directly, or alternatively, it could cause a change in the sensitivity of the uncoupling mechanism to intracellular Ca^{++} or pH. This latter mechanism is equivalent to the sensitivity modulation of enzyme activity described by Rasmussen and Waisman (1983). Phosphorylation of myometrial GJs might increase the sensitivity of the channel gating mechanism to Ca^{++}-activated calmodulin, shift the pCa curve for junctional uncoupling to the left, and produce an inhibition of communication at or near resting $[Ca^{++}]_i$. This postulated mechanism is analogous to that which regulates smooth muscle myosin light chain kinase activity (see Rasmussen & Waisman, 1983). Phosphorylation of light chain kinase results in increased affinity for Ca^{++}-activated calmodulin and enhanced enzyme activity at resting $[Ca^{++}]_i$.

Modulation of junctional sensitivity to $[Ca^{++}]_i$ and $[H^+]_i$ by means

of GJ channel phosphorylation also provides a simple, attractive explanation for the divergent effects of cAMP on coupling in different tissues. Phosphorylation of the channel gating mechanism could increase sensitivity to Ca^{++} or pH in some cell types, such as smooth muscle and retinal horizontal cells, but decrease the sensitivity in others, such as cardiac fibers. Opposing changes in channel permeability following phosphorylation are well established for other varieties of membrane channel (Levithan et al., 1883; Nestler & Greengard, 1983). Furthermore, this mechanism can explain the ability of elevated $[cAMP]_i$ to improve coupling in cardiac fibers despite producing a simultaneous elevation $[Ca^{++}]_i$. De Mello (1983) suggested that elevated $[Ca^{++}]_i$ produced by cAMP does not reduce coupling in cardiac cells because this ion does not have access to the cytoplasmic compartment subjacent to the junctions. This explanation was advanced despite the fact that this same author previously demonstrated that iontophoretic injection of Ca^{++} into the cell interior with microelectrodes has a potent uncoupling effect (see De Mello, 1982b). Conversely, if it is assumed that the phosphorylation of cardiac junctions reduces the sensitivity of the junctions to Ca^{++} and shifts the pCa^{++} curve for uncoupling to the right, then the junctions would become insensitive to the elevated $[Ca^{++}]_i$ produced by cAMP, and there would be no need to invoke the rather tenuous compartment hypothesis. Although clearly speculative, this postulated sensitivity modulation of coupling by junctional phosphorylation represents a useful working hypothesis for further experimentation.

5.4.2 **Possible Association between GJs and Adenylate Cyclase.** This

chapter documents the presence of electron opaque deposits associated with many GJs in muscle strips treated with forskolin. Similar deposits were also observed in subsequent experiments in which myometrial tissues were exposed to relaxin, isoproterenol and carbacyclin during 2-DG diffusion experiments (see chapter 6, section 6.3 below): GJ-associated occlusions similar to those described in this study were previously observed in the myometrium (Garfield et al., 1980b), between smooth muscle cells in the inflamed intestinal wall of a parasite-infected rat (I. Berezin, J.E.T. Fox, E.E. Daniel, unpublished observations), and between Leydig cells, granulosa cells, and Lesch-Neyhan cells in culture (Larsen, 1975, 1977a). However, their relationship, if any, to the functional properties of the junctions was never defined. The present data imply that the deposits reflect (1) the stimulation of an endogenous mechanism for reducing GJ permeability which is intimately associated with the junctions and, (2) an association between adenylate cyclase and myometrial GJs and the participation of this enzyme in an endogenous uncoupling mechanism.

It seems likely that the electron opaque deposits represent precipitates of Ca^{++} and PO_4^-. Electron microprobe analysis indicates the presence of both calcium and phosphate in the granules (Larsen, 1977a; R.E. Garfield, unpublished observations). The presence of Ca^{++} is also implied by the previous observation that the formation of the deposits is precluded if calcium is left out of the fixative (Larsen, 1975, 1977a; Garfield et al., 1980b). Larsen (1975, 1977a) postulated that glutaraldehyde fixation in calcium-containing solutions (such as

were used in this study) may permit continued enzymatic activity and liberation of phosphates at the site of the junctions which subsequently interact with Ca^{++} to form the electron dense precipitates.

Adenylate cyclase represents an obvious candidate for the phosphate-liberating enzyme activity associated with the junctions in the myometrium. Adenylate cyclase liberates PO_4^- during the synthesis of cAMP by hydrolysis of ATP and stimulation of cyclase activity is apparently related to the presence of deposits on myometrial GJs. Only two small deposits were observed on a single GJ in only one tissue treated with cAMP and no deposits were not found in control tissues. In contrast, the deposits were relatively abundant in tissues in which adenylate cyclase was presumably stimulated; either directly, with forskolin, or indirectly, by relaxin, isoproterenol and carbacyclin (see Chapter 6, section 6.3.2) and their specific cyclase-coupled receptors. Furthermore, opaque precipitates were previously reported to be abundant in myometrial tissues exposed to isoproterenol or isoxuprine (a β_2-selective adrenoceptor agonist), but absent in control and db-cAMP treated samples (Garfield et al., 1980b). Thus, the deposits appear to result from the stimulation of adenylate cyclase activity, but not from the subsequent elevation in $[cAMP]_i$. It should be noted that these deposits are also occasionally observed in tissues fixed immediately after removal from the animal (R.E. Garfield, unpublished observations). Evidently, their formation can be stimulated by mechanisms present *in vivo*.

Although the present data indicate a relationship between

adenylate cyclase and the junctions, the precise site of the cyclase remains to be determined. Perhaps, the enzyme is dispersed within the inner layer between the junctional connexons and, as well as, within the nonjunctional membrane adjacent to the GJs. Although the electron opaque deposits were most frequently observed over the junctional membranes, figure 5.4c clearly shows their presence immediately adjacent to the junctions and similar albeit much smaller precipitates were frequently observed on the nonjunctional membrane at some distance from the GJs. This indicates that adenylate cyclase is not necessarily an integral component of the GJs proteins. Receptor-coupled forms of adenylate cyclase are thought to be associated with the inner layer of the plasma membrane (Gilman, 1984; Harden, 1984; Schramm & Selinger, 1984). Thus, it seems that the enzyme is distributed in the inner leaflet, both around the connexons, within the junctions, and within the nonjunctional membrane immediately adjacent to the GJs.

5.5 SUMMARY

This chapter demonstrates that elevated $[cAMP]_i$ reduces cell-to-cell diffusion of 2-DG in the delivering rat myometrium. Moreover, the extent of the uncoupling produced by cAMP appears to be directly related to the cytoplasmic concentration of this second messenger. Electron opaque deposits, possibly containing Ca^{++} and PO_4^-, were observed to be associated with the junctional membrane in tissues treated with forskolin and other agonists (see Chapter 6) which stimulate adenylate cyclase. It seems possible that cAMP levels at the junctions may be locally elevated by an adenylate cyclase which is

associated with the GJs. The significance of reduced intercellular communication produced by cAMP to myometrial contractility is described in the subsequent chapter.

CHAPTER 6:

EVIDENCE FOR PHYSIOLOGICAL REGULATION OF MYOMETRIAL GJ PERMEABILITY

6.1 INTRODUCTION

The results presented in previous chapters demonstrate a role for the GJs in providing sites for cell-to-cell coupling between uterine smooth muscle cells. The regulation of GJ development by circulating and local hormones during gestation represents one mechanism whereby the extent of functional coupling in the myometrium may be regulated physiologically. The data reported in chapters 4 & 5 imply that the functional properties of the junctions can be modulated by experimental conditions which elevate $[Ca^{++}]_i$ or $[cAMP]_i$. However, whether either second messenger participates in an endogenous regulation of GJ permeability remains to be shown. Indeed, although there is an abundance of data showing alterations in functional coupling in a wide variety of cell types following various experimental manipulations (see Loewenstein, 1981; De Mello, 1982b), only a few studies provide evidence which is consistent with a physiologically relevant modulation of GJ permeability (eg. Teranishi et al., 1982). The experiments reported in this chapter sought to determine whether an endogenous mechanism, dependent on specific circulating or local hormones, transmitters, or autacoids, is present for regulating intercellular communication in the myometrium by producing alterations in GJ permeability.

The two compartment bathing chamber was employed to study the effects of several agents, including relaxin (porcine), isoproterenol (nonselective β-adrenoceptor agonist), carbacyclin (stable prostacyclin analog), prostaglandins PG E_2 and PG $F_{2\alpha}$, oxytocin,

indomethacin (cyclooxygenase (prostaglandin synthetase) inhibitor), 5,8,11,14 eicosatetraynoic acid (ETYA; lipoxygenase inhibitor), on cell-to-cell diffusion of 2-DG in myometrial tissues from delivering rats.

The data presented in this chapter are consistent with the presence of an endogenous mechanism for altering GJ permeability. This mechanism may be dependent upon specific agonists which alter cAMP production at the junctions to reduce or enhance myometrial GJ permeability.

6.2 METHODS AND MATERIALS

The diffusion experiments described in this chapter were conducted using longitudinal tissues from delivering rats according to the methods described above (see Chapter 2). All agonist treatments were conducted by adding the various agents to the perfusate in the 'cold' compartment. Treatment was initiated during the last 10 minutes of the equilibration period and continued during the diffusion period. The tissues were exposed to relaxin (0.1 ng/ml) (this porcine relaxin was kindly provided by Dr. O.D. Sherwood, Dept. of Physiology and Biophysics, University of Illinois), carbacyclin (1.0 uM), (stable analog of prostacyclin, PG I_2; Upjohn Chemicals), prostaglandin E_2 (1.0 μM) (PG E_2; Upjohn Chemicals), prostaglandin $F_{2\alpha}$ (1.0 μM) (PG $F_{2\alpha}$; Upjohn Chemicals), indomethacin (5 μM) (Sigma Chemicals), 5,8,11,14 eicosatetraynoic acid (ETYA) (30 μM) (Hoffman LaRoche), oxytocin (0.01, 1000 nM) (Vega Pharmaceuticals), and isoproterenol (nonselective β-adrenoceptor agonist; 1.0 μM; Sigma Chemicals).

Small samples of the tissues used in some of these diffusion

experiments were processed for electron microscopy and the quantitation of GJs according to the methods described above (section 2.5).

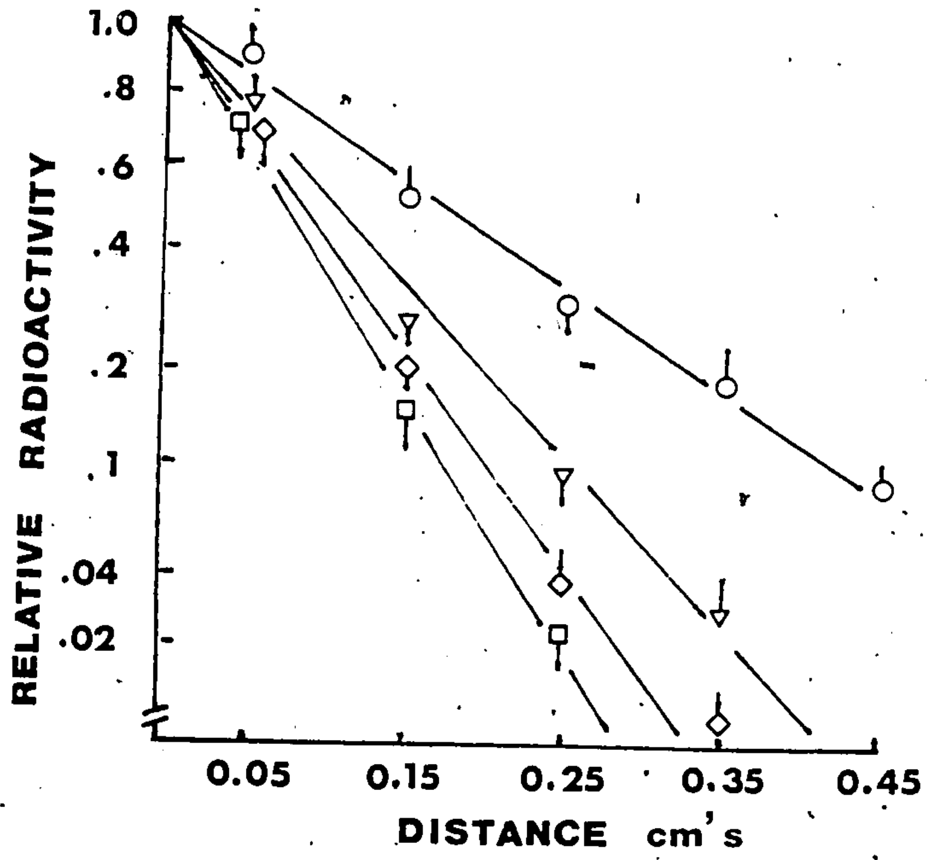
6.3 RESULTS

6.3.1 Diffusion Experiments

6.3.1.1 Agonists which elevate [cAMP]i reduce 2-DG diffusivity. Several agonists which may participate in the regulation of uterine contractility during pregnancy and parturition are reported to stimulate adenylate cyclase activity and elevate $[cAMP]_i$ in myometrial smooth muscle cells. Included in this group are relaxin, β-adrenoceptor agonists, such as isoproterenol, and prostaglandins, such as prostacyclin (PG I_2) and PG E_2 (Vesin & Harbon, 1974; Korenman & Krall, 1977; Sanborn et al., 1980; Harbon et al., 1984). When longitudinal myometrial muscle strips were exposed to either relaxin (0.1 µg/ml or ≈ 0.02 µM assuming a molecular weight of about 6000 g/mole (Schwabe et al., 1978), the stable PG I_2 analog, carbacyclin (1.0 µM), PG E_2 (1.0 µM) or isoproterenol (a nonselective β-adrenoceptor agonist), the distribution (Figs. 6.1 & 6.2) and Da (Table 6.1) for 2-DG were significantly reduced in comparison to untreated tissues. These data are consistent with the idea that myometrial GJ permeability can be altered by agonist-receptor mediated stimulation of adenylate cyclase activity and elevation in $[cAMP]_i$.

6.3.1.1 Effect of oxytocin and PG F2α on 2-DG diffusivity. In an attempt to determine whether any agents important to the control of labor enhance coupling in the myometrium during delivery, diffusion experiments were conducted using delivering tissues exposed to

FIGURE 6.1: Longitudinal distribution of 2-DG in parturient myometrial tissues exposed to carbacyclin (1.0 µM, ▽, n=8), relaxin (0.1 µg/ml, ◇, n=8), isoproterenol (1.0 µM, □, n=8) or untreated (○, n=8).



RELATIVE RADIOACTIVITY
1.0
.8
.6
.4
.2
.1
.04
.02
0.05
0.15
0.25
0.35
0.45
DISTANCE cm's

TABLE 6.1: THE APPARENT DIFFUSION COEFFICIENT (Da) FOR 2-DG IN PARTURIENT MYOMETRIAL TISSUES TREATED WITH PROSTAGLANDINS, OXYTOCIN, ISOPROTERENOL, RELAXIN, INDOMETHACIN AND 5,8,11,14 EICOSATETRAYNOIC ACID (ETYA).

TREATMENT	[ ]	N*	Da ± SE. $\times 10^{-6}$ cm^2 sec^{-1}
1a. Control		8(5)	1.62 ± 0.19
b. Relaxin	0.1 μg/ml	8(5)	0.39 ± 0.10[2]
c. Isoproterenol	1 μM	8(4)	0.30 ± 0.16[2]
d. Carbacyclin	1 μM	8(5)	0.73 ± 0.09[2]
2a. Control		3(3)	1.68 ± 0.19
b. PG E_2	1 μM	7(5)	0.73 ± 0.05[2]
c. PG $F_{2\alpha}$	1 μM	6(3)	1.65 ± 0.12[1]
d. Oxytocin	0.1 nM	3(2)	1.82 ± 0.07[1]
	1 μM	8(5)	0.49 ± 0.05[2]
3a. Control		3(3)	1.56 ± 0.14
b. Indomethacin	5 μM	7(4)	1.51 ± 0.17[1]
c. 5,8,11,14 ETYA	30 μM	8(3)	0.43 ± 0.06[2]

* n refers to the number of muscle strips employed whereas the numbers in the brackets indicates the number of animals.

1 values of Da for treated tissues that were not significantly different (P>0.05) from that for control, untreated tissues.

2 values of Da for treated tissues which were significantly different (P<0.05) from that for control untreated tissues.

oxytocin and PG $F_{2\alpha}$. Both of these agonists stimulate uterine electrical and mechanical activity and are thought to play a role in the facilitation of labor (Fuchs, 1978; Thorburn & Challis, 1979). At a physiologically relevant concentration of oxytocin (0.1 nM) no significant (P>0.05) alteration in the distribution (Fig. 6.2) or Da (Table 6.1) for 2-DG was noted. However, at a considerably higher dose (1.0 μM), oxytocin significantly (P<0.05) reduced the distribution (Fig. 6.2) and Da (Table 6.1) for 2-DG. PG $F_{2\alpha}$ did not influence (P>0.05) the distribution or the diffusivity of 2-DG (Fig. 6.2 & Table 6.1) at the relatively high concentration of 1.0 μM.

6.3.1.3 Effect of indomethacin and ETYA on 2-DG diffusivity. The possibility that metabolites of arachidonic acid (AA), other than the prostaglandins employed above, may enhance myometrial GJ permeability in the delivering myometrium was explored using indomethacin and eicosatetraynoic acid (ETYA) during 2-DG diffusion experiments. At a concentration of 5.0 μM, indomethacin effectively prevents the synthesis of prostaglandins from AA in myometrial tissues by inhibiting the enzyme cyclooxygenase (eg. Vesin et al., 1984). However, this drug did not influence (P.0.05) the diffusivity of 2-DG in the delivering myometrium (Fig. 6.3 & Table 6.1). In contrast, ETYA (20.0 μM), which inhibits lipoxygenase (as well as cyclooxygenase) metabolism of AA (eg. Vesin et al., 1984), was found to reduce (P<0.05) the distribution (Fig. 6.4) and diffusivity (Table 6.1) of 2-DG in the delivering myometrium. This implies that an eicosanoid product of the lipoxygenase cascade, perhaps a leukotriene, but not the cyclooxygenase pathway of AA metabolism, may be involved in promoting cell-to-cell coupling in the delivering myometrium.

FIGURE 6.2: Longitudinal distribution of 2-DG in parturient myometrial tissues exposed to prostaglandin $F_{2\alpha}$ (1.0 μM, □, n=6), prostaglandin E_2 (1.0 μM, ■, n=9) oxytocin (0.1 nM, ▽, n=3; 1.0 μM, ▼, n=8) or untreated (○, n=3).

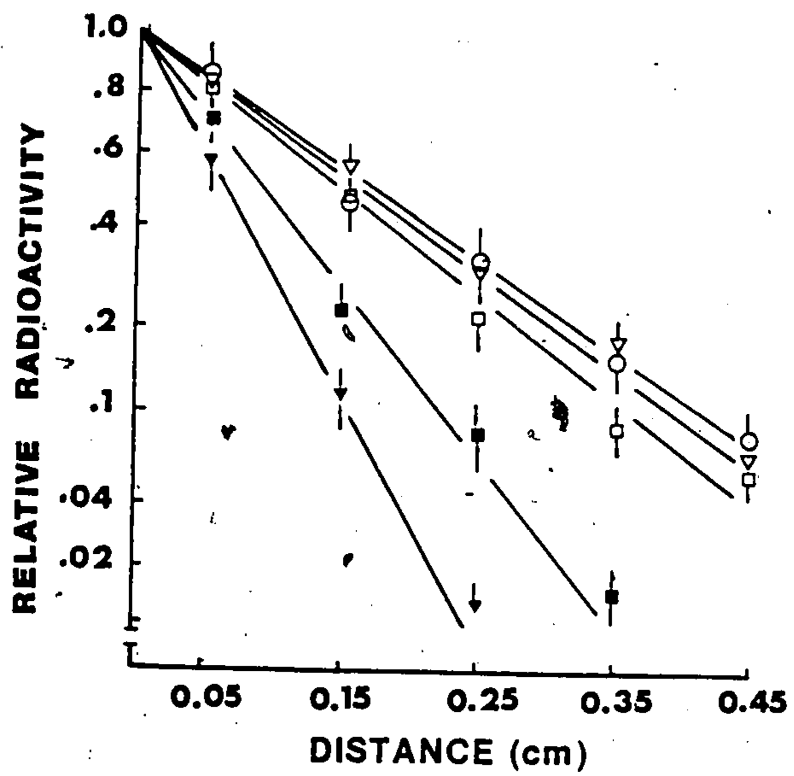
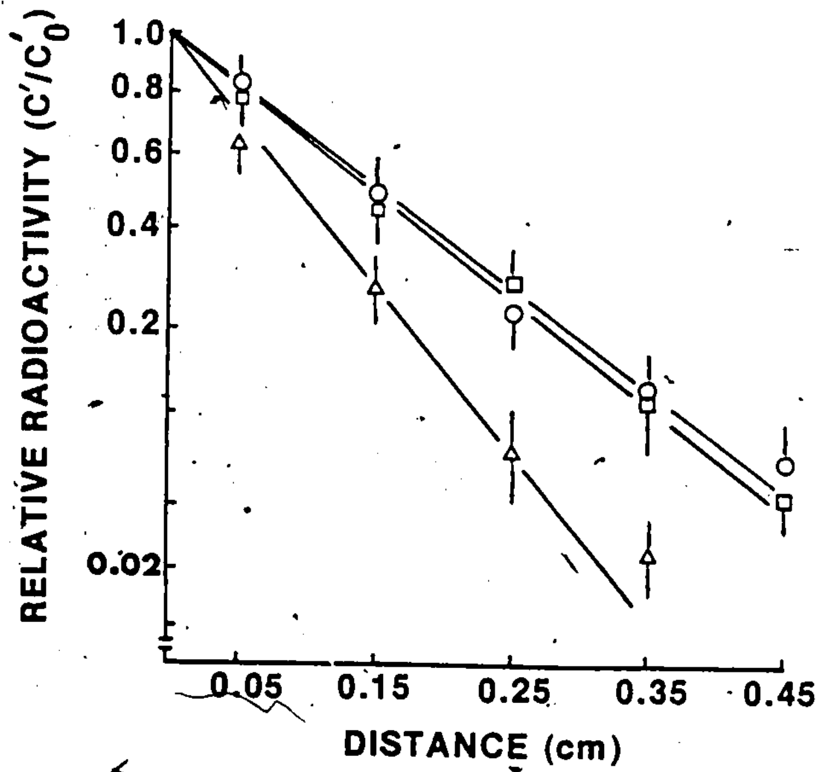

1.0
.8
.6
.4
.2
.1
.04
.02
RELATIVE RADIOACTIVITY
0.05
0.15
0.25
0.35
0.45
DISTANCE (cm)

FIGURE 6.3: Longitudinal distribution of 2-DG in parturient myometrial tissues exposed to indomethacin (5 μM, □, n=7), 5,8,11,14 eicosatetraynoic acid (30 μM, △, n=8), or untreated (○, n=3).



RELATIVE RADIOACTIVITY (C'/C'0)
1.0
0.8
0.6
0.4
0.2
0.02
0.05
0.15
0.25
0.35
0.45
DISTANCE (cm)

TABLE 6.2: GAP JUNCTIONS IN PARTURIENT MYOMETRIUM AFTER TREATMENT WITH ISOPROTERENOL (Isopr.), RELAXIN (Relax.), CARBACYCLIN (Carba.), OR 5,8,11,14 EICOSATETRAYNOIC ACID (ETYA)

TISSUE	N	LENGTH OF MEMBRANE (in µms)	#GJs	#GJs/1000µM MEMBRANE	AVERAGE GJ SIZE (in µms)	FRACTIONAL AREA OF GJs AS A %
1. Contr.	3(0)	4,065	47	12.5 ± 2.4	0.17 ± 0.13	0.36 ± 0.22
2. Isopr. (1 µM)	4(4)	5,055	50	9.97 ± 1.6	0.18 ± 0.01	0.36 ± 0.17[1]
3. Relax. (1 µg/ml)	4(3)	2,961	45	15.4 ± 5.0	0.27 ± 0.06	0.38 ± 0.19[1]
4. Carba. (1 µM)	3(2)	3,069	36	11.9 ± 1.6	0.12 ± 0.01	0.30 ± 0.16[1]
5. ETYA (30 µM)	3(3)	3,326	41	12.5 ± 2.0	0.19 ± 0.02	0.38 ± 0.02[1]

Where N is the number of tissues examined and the number of these containing GJs with dense granules indicated in the brackets. Membrane length represents the total length of plasma membrane measured in 20-24 nonoverlapping micrographs of each tissue; the frequency, average size, and fractional percentage of the plasma membrane occupied by GJs are expressed as a mean ± SE.

[1] values were not significantly different (P>0.05) from control tissues.

6.3.2 Structural Analysis

6.3.2.1 Lack of any effect on GJ area. Small portions of some of the tissues used in the diffusion experiments were examined for GJs. Table 6.2 shows that the area of GJs in untreated, control tissues was not different from that of strips exposed to relaxin (1.0 µg/ml), isoproterenol (1.0 µM), carbacyclin (1.0 µM) or ETYA (1.0 µM). Thus, a change in structural coupling cannot account for the alterations in 2-DG diffusivity produced by these agents.

6.3.2.2 Alterations in GJ morphology. Similar electron dense deposits to those described in chapter 5 (section 5.3.2) were found to be associated with GJs in tissues treated with isoproterenol, relaxin and carbacyclin, and ETYA. Two of the micrographs in Figure 5.4 are of tissues treated with isoproterenol and relaxin.

6.4 DISCUSSION

The results reported in this chapter provide evidence that cAMP and agonists which elevate the intracellular content of this second messenger may participate in an endogenous mechanism for decreasing GJ permeability in myometrial tissues. Furthermore, the data suggest that an eicosanoid(s) may serve to depress the synthesis of cAMP at the junctions and maintain functional coupling. The presence of an endogenous mechanism for altering the exchange of small ions and metabolites between uterine smooth muscle cells has important implications for the physiological control, and pharmacological manipulation, of coordinated uterine contractility.

6.4.1 Control of Functional Coupling by Physiologically Relevant Agonists

The decrease in coupling observed with relaxin, isoproterenol, carbacyclin, and PG E_2 on 2-DG diffusion in the myometrium described in this chapter were probably mediated by a receptor-induced activation of adenylate cyclase and production of cAMP. Each of these agonists was previously demonstrated to elicit increased adenylate cyclase activity and cAMP production in myometrial plasma membrane preparations and/or cells (Vesin & Harbon, 1974; Korenman & Krall, 1977; Vesin et al., 1979; Sanborn et al., 1980; Harbon et al., 1984). Furthermore, tissues exposed to relaxin, isoproterenol and carbacyclin demonstrated electron opaque deposits associated with myometrial GJs (Fig. 5.4). These precipitates were identical to those previously described to demonstrate an apparent relationship with a forskolin stimulated adenylate cyclase activity (Chapter 5, section 5.3.2). Thus, depressed functional coupling and the formation of deposits at the GJs in delivering myometrium would seem to result from the stimulation of a receptor-coupled adenylate cyclase activity which is apparently associated with the GJs. Moreover, these data provide evidence which is consistent with the hypothesis that agonists relevant to the physiological control of pregnancy and parturition can influence the function of myometrial GJs.

Alterations in junctional communication produced by a physiological (or pharmacological) control of GJ permeability can be expected to have an influence on coordinated uterine contractility during the period of junction formation at term, and subsequently, during labor. Synchronous contractions observed in the uterus are triggered by action potentials which propagate throughout the

myometrium from sites of spontaneous generation within pacemaker regions (see section 1.4.4). Given that the junctions participate in cell-to-cell coupling, then reduced GJ permeability would be expected to impair the propagation of these activating currents. This would tend to isolate electrical events within pacemaker regions and inhibit spontaneous activity, thus rendering the myometrium quiescent, asynchronous, and incapable of generating sufficient intrauterine pressure for effective expulsion of the fetus(es). On the other hand, enhanced GJ permeability and functional coupling between uterine muscle fibers would be expected to promote propagation and lead to a more synchronous and rapid generation of tension by myometrial muscle fibers.

The mechanism whereby isoproterenol, relaxin, and other agonists which elevate cAMP levels, influence spontaneous uterine contractility is poorly understood and controversial. The present data suggest a modulation of intercellular coupling by cAMP may be involved. It was previously suggested that an activation of adenylate cyclase, and elevation of $[cAMP]_i$, may elicit relaxation of the uterus by either; (1) potentiating Ca^{++} extrusion across the plasma membrane or uptake into intracellular compartments, (2) altering the configuration of propagating action potentials to reduce Ca^{++} influx (eg. inhibition of the plateau potential in circular muscle fibers by relaxin, Chamley & Parkington, 1984), or (3) by inhibiting the interaction between contractile filaments (Korenman & Krall, 1977; Hardman, 1981). On the basis of the present observations it seems likely that the effects of relaxin, β-adrenoceptor agonists and other similar agonists are not

limited to Ca^{++}-handling or myofilament interaction. An isolation of spontaneous electrical activity within pacemaker regions as a result of depressed GJ permeability may also contribute to a state of uterine quiescence produced by these agents.

In contrast to the present observation that relaxin inhibits intercellular communication in the myometrium, it was previously reported that this hormone improves the coordination of myometrial activity. This was based on the observation that relaxin elicits an increase in the rate of rise of pressure cycles in ovariectomized, nonpregnant rats in vivo (Downing et al., 1980; Bradshaw et al., 1981). However, the improved coordination was actually observed after either one (Bradshaw et al., 1981) or twenty-four (Downing et al., 1980) hours following exposure to relaxin, and not when the hormone was present.

That GJ permeability and the extent of functional coupling in the myometrium can be modulated by stimulating endogenous receptor-coupled mechanisms with appropriate concentrations of naturally occurring agonists is significant in that it provides evidence for the presence of a physiologically relevant mechanism. Indeed, control of GJ permeability by endogenous relaxin may account in part for the maintenance of pregnancy during the final 24-36 hours of gestation in the rat. The uterus of the rat displays a very marked quiescence during this period (Fuchs 1969, 1978; Porter, 1984; Downing & Sherwood, 1985) despite the fact that GJs begin to develop in large numbers and are present in the myometrium at this time (Puri and Garfield, 1982). On the basis of the results presented in Chapter 3,

these structures should, if functional, provide the basis for cell-to-cell communication, coordinated contractility and the onset of labor. However, the levels of circulating relaxin peak at this time (Sherwood et al., 1980; Downing & Sherwood, 1985a) and are apparently responsible for producing the quiescence (Porter & Downing, 1978; Porter, 1984; Downing & Sherwood, 1985b). The data presented in this chapter imply that this inhibition of spontaneous activity may be due, at least in part, to the regulation of GJ permeability by relaxin. A similar decrease in GJ permeability may also contribute to the ability of relaxin and β-adrenoceptor agonists to inhibit term and preterm labor in animals and humans. It is also significant in this regard that the adrenergic innervation of the uterus of most species displays a marked degeneration during pregnancy (Marshall, 1981).

6.4.2 Possible Mechanisms for Enhancing GJ Permeability

Although the majority of the experiments in this and previous chapters document conditions in which cell-to-cell coupling is depressed, the observation that ETYA impairs coupling is consistent with the presence of a mechanism(s) for enhancing GJ permeability.

The inhibition of 2-DG diffusion by ETYA (Fig. 6.4 & Table 6.1) indicates that a product(s) of the lipoxygenase pathway may promote the presence of open, patent GJ channels, enhancing and maintaining coupling during labor. However, in the absence of any data showing either a potentiation of coupling, or a reversal of the effects of ETYA, by a specific eicosanoid, some skepticism of this mechanism seems warranted. Indeed, the presence of electron opaque deposits on junctions in the ETYA-treated tissues implies that the reduction in

coupling produced by this drug was due to the same mechanism responsible for the uncoupling in muscle strips exposed to agonists which activate adenylate cyclase. Although there is no published evidence showing a direct stimulation of adenylate cyclase activity by ETYA, a forskolin-like effect of this drug on coupling cannot be ruled out.

On the other hand, the deposits may reflect the mechanism by which GJ permeability is increased by lipoxygenase products. It is possible that these agents do not interact with the GJs directly to open or dilate the channels and enhance functional coupling. Rather, they may release a tissue uncoupled by cAMP by inhibiting the activity of adenylate cyclase produced for example, by relaxin or carbacyclin. As noted above, the presence of electron dense deposits implies that there may be elevated adenylate cyclase activity at the junctions in tissues treated with ETYA. It follows, therefore, that in the absence of this drug, an eicosanoid(s) may activate an inhibitory mechanism which prevents this adenylate cyclase activity. The lipoxygenase products could influence adenylate cyclase activity either by a direct inhibition produced by heterologous desensitization (Harden, 1984; Krall et al., 1984;) or activation of inhibitory G-proteins (Jakobs, 1979; Gilman, 1984), or alternatively, by an indirect inhibition mediated by the cGMP system (Vesin et al., 1982; Harbon et al., 1983). It should also be noted that although oxytocin or PG $F_{2\alpha}$ were not found to promote coupling in this study, the possibility that they participate in a stimulatory mechanism cannot be entirely dismissed. If their influence on junctional communication is relatively short in

duration then a significant alteration in the diffusivity of 2-DG would not be observed over the rather long incubation period required for the diffusion experiments. Similarly, if PG $F_{2\alpha}$ only blocks the effect of a second agent, such as a β-agonist, PG I_2, or relaxin, then a change in coupling may not be observed when they are added on their own. Myometrial adenylate cyclase activity is known to be subject to the combined effects of stimulatory and inhibitory agonists, for example, PG $F_{2\alpha}$ and oxytocin reduce the output of cAMP following β-adrenoceptor stimulation by isoproterenol (eg. Bhalla et al., 1972). Similarly, although PG E_2 was shown to reduce coupling in this study its actions _in vivo_ may be to desensitize adenylate cyclase to stimulation by β-adrenoceptor or relaxin. This is similar to one aspect of the postulated role for this prostaglandin in the control of myometrial contractility (Krall et al., 1984).

Control of functional coupling in the myometrium may be similar, therefore, to the regulation of myometrial contractility during pregnancy and parturition. That is, the extent of coupling may be determined by the combined effects of inhibitory and stimulatory factors. The influence of inhibitory factors, such as relaxin and prostacyclin, may predominate during pregnancy, but be offset by stimulatory agonists, such as eicosaniods, during labor.

6.4.3 Two Different Mechanisms Control the Extent of Functional Coupling in the Myometrium

Previous studies postulated that the precipitous development of GJs at the end of term is a focal event in the conversion of the rat myometrium into an active, reactive and synchronously contracting

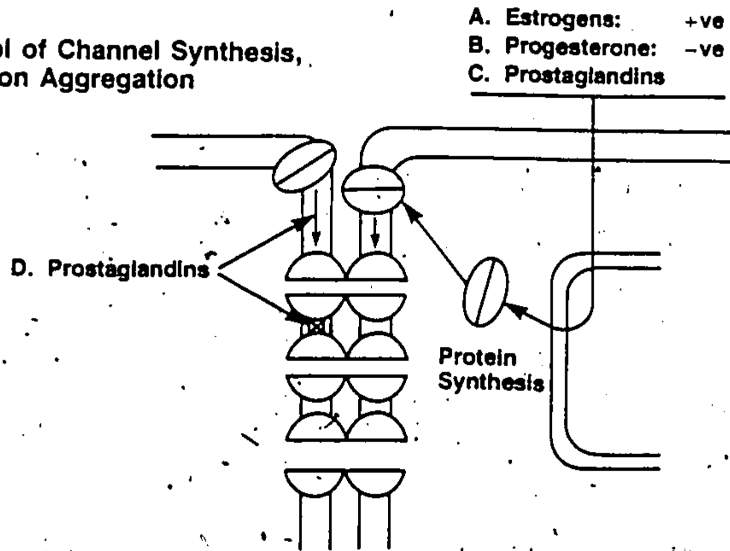
organ at the end of term (Garfield et al., 1977, 1978). The data provided in Chapter 3 indicate that the elaboration of GJs improves functional coupling of myometrial fibers during labor. This alteration in GJs and the extent of cell-to-cell communication in the myometrium appears to be controlled by the changing hormonal environment of the uterus at term. There is evidence that circulating and local levels of prostaglandins, thromboxanes and the steroid hormones, estrogen and progesterone, may be important in the control of GJ development (Garfield et al., 1980a,b). Thus, there is an endogenous control mechanism, dependent on circulating and local hormones which modulates the extent of functional coupling in the myometrium by producing alterations in the extent of structural coupling between uterine smooth muscle cells (see Fig. 6.4).

This study provides evidence for the presence of an additional, second level for the physiological control of functional coupling in the myometrium. The data are consistent with a mechanism, dependent on circulating and local hormones, which produces alterations in the permeability of the cell-to-cell channels, hence, the functional properties of the GJs after they have formed between the smooth muscle cells (see Fig. 6.4). It is evident that some agonists may modulate both the development and function of the GJs (eg. prostacyclin) and others only their function (eg. relaxin and β-adrenoceptor agonists). This second mechanism may be primarily responsible for the inhibition of synchronous activity in the rat myometrium during the final 24-36 hours of gestation and for stimulating and maintaining coordinated activity in the uterine wall during labor.

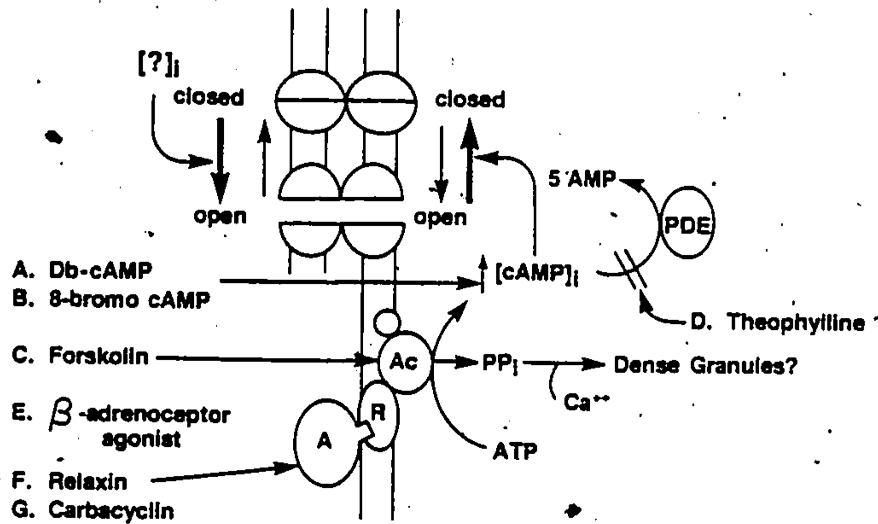
FIGURE 6.4: A schematic representation of the possible mechanisms regulating alterations in structural and functional coupling in the myometrium. Possible roles are indicated in 1) for estrogen, progesterone and prostaglandins in controlling the synthesis, insertion and/or aggregation of connexons into gap junctions based on Garfield et al., 1980a,b and MacKenzie & Garfield, 1985). The postulated mechanisms for control of GJ permeability are shown in 2). Dibutyryl (Db-cAMP) and 8-bromo cAMP are shown to elevate intracellular levels of cAMP ($\uparrow[cAMP]_i$) directly, whereas theophylline inhibits cAMP degradation by phosphodiesterase (PDE) and forskolin stimulates its synthesis by directly interacting with adenylate cyclase (Ac). Agonists (A), such as isoproterenol, relaxin and carbacyclin are shown to interact with receptors (R) that are functionally coupled to adenylate cyclase. Stimulation of cyclase activity by these agonists is postulated to elevate intracellular cAMP and result in the liberation of pyrophosphate at the junction. Pyrophosphate may precipitate with calcium ions (Ca^{++}) to form the electron opaque deposits along the membrane at the Junctions. A possible role for eicosanoids (?) in promoting enhanced gap junction permeability is also indicated. (From Colé & Garfield, 1985c).

Model for Regulation of Structural and Functional Coupling in the Myometrium

1. Control of Channel Synthesis, Insertion Aggregation



2. Control of Channel Permeability



Physiological control of myometrial GJ permeability is postulated to participate in the regulation of coordinated contractile activity in the uterus of the rat, however, it seems probable that it is also relevant to control of coupling in the uterus of other species, and in other smooth muscle tissues. That GJs in the rat myometrium are sparse until just a short time (24-36 hrs) prior to parturition when they increase rapidly is significant. This suggests that control of functional coupling is primarily limited to this brief period associated with parturition and is probably secondary to the regulation of GJ development. However, in other animals such as the guinea pig, sheep, and, human, GJs are reported to be present in greater numbers throughout pregnancy and, in at least the former two species, the development of the junctions appears to occur over a much more prolonged time period (Garfield et al., 1979a,b; Garfield & Hayashi, 1981; R.E. Garfield, unpublished observations). In the case of these species, it would seem that physiological control of GJ permeability may be of greater importance and may represent the primary mechanism for inhibiting intercellular communication during gestation and promoting synchronized and coordinated uterine contractility at term.

The myometrium is unique in comparison to other smooth muscles in that it appears to be the only muscle to undergo drastic physiological alterations in structural coupling. For this reason, it is tempting to suggest that control of GJ permeability may be the primary mechanism for regulating the presence of coordinated activity in other sparsely innervated smooth muscle tissues. Furthermore, in light of the present

observations, the presence of electron dense deposits associated with GJs between smooth muscle cells in the parasite-infected rat gut (I. Berezin, J.E.T. Fox, and E.E. Daniel, unpublished observations), implies the elaboration of a regulatory mechanism, and perhaps concomitant alterations in functional coupling, which may not be present in the healthy animal.

6.5 SUMMARY

The data presented in this chapter are consistent with the presence of an endogenous mechanism which regulates myometrial GJ permeability. This mechanism may involve relaxin, β-adrenoceptor agonists, prostaglandins, and lipoxygenase products. Inhibition of intercellular communication during GJ formation in late gestation may be produced by agents which stimulate adenylate cyclase and elevate $[cAMP]_i$. It is postulated that during labor the effects of these agents may be offset by stimulatory agonists. Furthermore, it is postulated that modulations in GJ permeability may be relevant to the control of coordinated activity in myometrial tissues of other species and in other smooth muscle tissues.

CHAPTER 7: CONCLUSION

7.1 GENERAL CONCLUSIONS AND FUTURE QUESTIONS

It is apparent that the evolution of intense, regular, and synchronous contractions in the myometrium at the end of gestation is determined by many factors (Fuchs, 1969, 1978; Liggins, 1979; Thorburn & Challis, 1979). It is thought that a sequence of hormonal changes prior to and during the onset of labor is required to increase excitability, reactivity and contractility of the myometrium (Thorburn & Challis, 1979; Csapo, 1981). More recently, evidence was provided that this hormonal cascade also stimulates the formation of many large gap junctions (GJs) between the smooth muscle cells of the myometrium at term (Garfield et al., 1978, 1980a,b; Puri & Garfield, 1982; Mackenzie & Garfield, 1985). This thesis contributes to our understanding of the role of the GJs in the myometrium and how the function of these structures may be regulated during pregnancy and parturition.

In this thesis a technique was developed to study the role of GJs in providing sites for cell-to-cell diffusion of a radiolabelled glucose analog in the myometrium. The results support the hypothesis that the proliferation of GJs in the myometrium at term provides for markedly improved cell-to-cell communication between uterine smooth muscle cells during delivery. This increased capacity to synchronize cellular activities, by means of a direct exchange of small ions and molecules, probably contributes to the coordination of electrical, metabolic, and mechanical activity in the millions of muscle fibers in the uterine wall during labor and, hence, to the effective delivery of the fetus(es).

This study also supplies the first evidence that the functional properties of GJs in smooth muscle can be altered. It was demonstrated that the extent of functional coupling in tissues with GJs can be manipulated pharmacologically by altering intracellular levels of free calcium ion and cyclic AMP. However, it is more significant that this study also provides support to the contention that an endogenous mechanism is present for controlling the permeability of the GJ cell-to-cell channels in the myometrium. Alterations in functional coupling were shown to occur in myometrial tissues exposed to specific local and circulating hormones that are thought to play a role the regulation of pregnancy and parturition. Thus, in addition to providing for an increase in GJs in the myometrial tissues, the sequence of hormonal changes at term may also provide for an alteration in the functional properties of the GJs. This integrated regulation of the presence and function of GJs by endogenous hormonal mechanisms probably provides for a precise control of synchronous activity in the myometrium during pregnancy and parturition. On the basis of the present and previous observations it would appear that the extent of intercellular communication in the myometrium before the onset of labor is suppressed by the relative paucity of GJs and depressed channel permeability. Conversely, synchronization of cellular activities during parturition may be promoted by both an increase in the number and the permeability of the GJs.

It is tempting to speculate that some conditions of abnormal uterine contractility, such as occur in dysfunctional labor or premature labor, may result from aberrations in the control mechanisms

at either level of myometrial GJ regulation. Knowledge of the mechanisms for control of GJ permeability and the extent to which alterations in these mechanisms contribute to conditions of abnormal contractility may provide the basis for a rational approach to the development of specific therapies for their treatment.

Although this study has provided evidence for the participation of GJs in cell-to-cell communication in smooth muscle and for the presence of a physiological mechanism for myometrial GJ permeability control by intracellular cyclic AMP, it only represents an initial step in understanding these phenomena. Clearly, there are several pertinent questions which remain to be answered.

- Firstly, are similar mechanisms for the control of GJs to those identified in this study present in other animals and humans and what is the extent of their contribution to the control of synchronous activity?

- Can a causal relationship between alterations in GJ permeability and a modulation of the pattern of uterine contractility be obtained?

- What is the contribution of the cascade of hormonal alterations at term to the control of GJ permeability?

- What specific lipoxygenase product(s) is involved in the regulation of GJ permeability and how is its influence manifest?

- Are agonists which manipulate cyclic AMP levels the only physiologically relevant agents for control of myometrial GJ permeability?

- What is the contribution of alterations in GJ permeability to the control of coordinated activity in other smooth muscle tissues?

- And finally, are alterations in functional coupling important in pathological conditions of abnormal smooth muscle contractility and, if so, then what is the contribution of the permeability control mechanism to the altered coupling?

7.2 SUMMARY

1. The diffusivity of a low molecular weight radiolabelled molecule, [^{3}H-] 2-deoxy-D-glucose (2-DG) in small preparations of longitudinal myometrium was measured and found to be considerably greater in muscle strips removed from rats during delivery (many GJs) compared to tissues from animals not in delivery (few GJs).

2. The movement of 2-DG could not be accounted for by extracellular diffusion nor could the change in diffusivity at term be attributed to an alteration in cell size, intracellular diffusion or tracer efflux across the plasma membrane.

3. Longitudinal diffusion of 2-DG in the myometrium occurred within the intracellular compartment of the smooth muscle cells and between the muscle fibers along a pathway isolated from the extracellular space. That an increase in cell-to-cell diffusion of 2-DG was observed concomitant to the proliferation of GJs supports the idea that the junctions provide the cell-to-cell pathway and that there is markedly improved coupling in the myometrium during labor.

4. GJs could not be identified between smooth muscle cells in the longitudinal muscle of rabbit hepatic portal vein nor was there evidence of cell-to-cell diffusion of 2-DG. Evidently, GJs are necessary for metabolite communication between smooth muscle cells.

5. 2-DG diffusivity in parturient tissues was reduced when

intracellular levels of free calcium ion were elevated by treatment with calcium ionophore A23187. This decrease in cell-to-cell exchange of tracer occurred in the absence of a change in the area of GJs, cell size or tracer efflux across the plasma membrane and was attributed to a reduction in the permeability of the cell-to-cell channels comprising the GJs.

6. Calmodulin antagonist drugs blocked the effects of ionophore on 2-DG diffusion suggesting that the calcium-sensitivity of the uncoupling mechanism is dependent on calmodulin. Calmodulin may also play a role in the regulation of other intracellular processes, such as control of cAMP levels, and thus have an additional, more indirect, influence on the extent of functional coupling.

7. Elevated intracellular levels of cAMP reduced diffusivity of 2-DG in the absence of any change in the area of GJs in myometrial tissues from parturient rats. Regardless of whether cAMP was elevated by supplying an exogenous source, stimulating its synthesis with forskolin, or inhibiting its breakdown with theophylline, depressed cell-to-cell exchange of 2-DG was the result.

8. The formation of electron dense deposits at the GJs in tissues in which adenylate cyclase activity was presumably stimulated (eg. treatment with forskolin) indicates that this enzyme may have a rather intimate association with the junctions.

9. 2-DG diffusivity was also reduced by local (eg. prostacyclin) and circulating (eg. relaxin) hormones which elevate cAMP levels in uterine smooth muscle cells. This is consistent with the presence of an endogenous mechanism for the control of myometrial GJ permeability during pregnancy and parturition.

10. It is postulated that the extent of intercellular communication and, presumably, the ability to coordinate electrical and contractile activity, between uterine smooth muscle cells is regulated by physiological mechanisms at two levels; (1) the previously described control of the area of GJs between the cells and, (2) the novel mechanism documented in this thesis, which involves control of the permeability of the cell-to-cell channels comprising the GJs.

REFERENCES

1. Abe, Y. 1970. The hormonal control and the effects of drugs on the electrical and mechanical activity of the uterus. In: Smooth Muscle, eds. E. Bulbring, A.F. Brading, A.W. Jones, and T. Tomita, Edward Arnold Ltd., London, pp.396-417.

2. Abe, Y. and T. Tomita. 1968. Cable properties of smooth muscle. J. Physiol. (Lond.) 196:87-100.

3. Afting, E.G. and J.S. Elce. 1978. DNA in the rat uterus myometrium during pregnancy and post-partum involution. Analyt. Biochem. 86:90-99.

4. Anderson, G., T. Kawarabayshi, and J.M. Marshall. 1981. Effect of indomethacin and aspirin on uterine activity in pregnant rats; comparison of circular and longitudinal muscle. Biol. Reprod. 24:359-372.

5. Arnqvist, H.J. 1972. Characteristics of monosaccharide permeability in arterial tissue and smooth muscle; effect of insulin. Acta Physiol. (Scand.) 85:217-227.

6. Auerbach, A.A.. and M.V.L. Bennett. 1969. A rectifying synapse in the central nervous system of a vertebrate.

J. Gen. Physiol. 53:211-237.

7. Bhalla, R.C., B.M. Sanborn, S.G. Korenman. 1972. Hormonal interactions in the uterus: Inhibition of isoproterenol-induced accumulation of adenosine 3'5' cyclic monophosphate by oxytocin and prostaglandins. Proc. Natl. Acad. Sci. (USA) 69:3761-3764.

8. Barr, L., W. Berger, M.M. Dewey. 1968a. Electrical transmission at the nexus between smooth muscle cells. J. Gen. Physiol. 51:347-368.

9. Barr, L., M.M. Dewey, W. Berger. 1968b. Propagation of action potentials and the structure of the nexus in cardiac muscle. J. Gen. Physiol. 48:797-823.

10. Baux, G., M. Simonneau, L. Tauc, and J.P. Segundo. 1978. Uncoupling of electronic synapse by calcium. Proc. Natl. Acad. Sci. (USA) 75:4577-4581.

11. Bennett, M.V.L. 1977. Electrical transmission: a functional analysis and comparison to chemical transmission. In: Cellular Biology of Neurons, vol. 1, Sect. 1, Handbook of Physiology, The Nervous System, ed. E. Kandel, Williams and Wilkins Inc., Baltimore, pp. 357-416.

12. Berezin, I., E.E. Daniel, R.E. Garfield. 1982. Ovarian hormones are not necessary for post partum regression of gap junctions. Can. J. Physiol. Pharmacol. 60:1567-1572.

13. Bozler, E. 1938. Electrical stimulation and conduction of excitation in smooth muscle. Amer. J. Physiol. 122:614-623.

14. Brink, P.R. 1983. Effect of deuterium oxide on nexal membrane channel permeability. J. Membr. Biol. 71:79-87.

15. Brink, P.R. and M.M. Dewey. 1978. Nexal membrane permeability to anions. J. Gen. Physiol. 72:69-78.

16. Brink, P.R. and M.M. Dewey. 1980. Evidence for a fixed charge in the nexus. Nature (Lond.) 285:101-102.

17. Brink, P.R., V. Verselis, and L. Barr. 1984. Solvent-solute interactions within the nexal membrane. Biophys. J. 45:121-124.

18. Brink, P.R., S.W. Jaslove, L. Barr. 1985. Evidence for ion-dye competition for occupancy within intercellular channels. Biophys. J. 47:448a.

19. Brading, A.F. 1981. Ionic distribution and mechanisms of transmembrane ion movements in smooth muscle. In: Smooth Muscle: An Assessment of Current Knowledge, eds. E. Bulbring, A.F. Brading, A.W. Jones, and T. Tomita, Edward Arnold Ltd., London, pp.171-198.

20. Bradshaw, J.M.C, S.J. Downing, A. Moffatt, J.C. Hinton, and D.G. Porter. 1981. Demonstration of some of the physiological properties of rat relaxin. J. Reprod. Fert. 63:145-153.

21. Burnstock, G. 1970. The structure of smooth muscle and its innervation. In: Smooth Muscle, eds. E. Bulbring, A.F. Brading, A.W. Jones, and T. Tomita, William Clowes and Sons, London, pp. 1-69.

22. Caspar, D.L.D., D.A. Goodenough, L. Makowski, and W.C.

Phillips. 1977. Gap junction structures. I: Correlated electron microscopy and x-ray diffraction. J. Cell Biol. 74:605-628.

23. Caveney, S. 1985. The role of gap junctions in development. Ann. Rev. Physiol. 47:319-335.

24. Chamley, W.A. and H.C. Parkington. 1984. Relaxin inhibits the plateau component of the action potential in the circular myometrium of the rat. J. Physiol. (Lond.) 353:51-65.

25. Cheung, W.Y. 1980. Calmodulin plays a pivotal role in cellular regulation. Science 207:19-27.

26. Cheung, W.Y. 1982. Calmodulin: an overview. Fed. Proc. 41:2253-2257.

27. Cole, W.C. and R.E. Garfield. 1984. A23187 and calmodulin antagonists inhibit metabolic coupling between parturient rat myometrial smooth muscle cells. Biophys. J. 45:23a

28. Cole W.C., R.E. Garfield. 1985a. Elevated intracellular cAMP reduces intercellular communication between rat uterine smooth muscle cells. Biophys. J. 47:448a.

29. Cole, W.C. and R.E. Garfield. 1985b. Alterations in coupling in uterine smooth muscle. Gap Junctions, ed. D.C. Spray and M.V.L. Bennett, Cold Spring Harbor Press, in press.

30. Cole, W.C. and R.E. Garfield. 1985c. Evidence for physiological regulation of myometrial gap junction permeability. Science, submitted.

31. Cole, W.C., R.E. Garfield, J.S. Kirkaldy. 1985. Gap junctions and direct intercellular communication between rat uterine smooth muscle cells. Amer. J. Physiol. 249:C20-31.

32. Crank, J. 1956. The Mathematics of Diffusion, 1st edn., Oxford, Clarendon Press.

33. Csapo, A.I. 1981. Force of Labor. In: Principles and Practice of Obstetrics and Perinatology, eds. L. Iffy and H.A. Kaminetzky. John Wiley and Sons, New York, pp. 761-802.

34. Dahl, G. and G. Isenberg. 1980. Decoupling of heart muscle cells: correlation with increased cytoplasmic calcium

activity and with nexus ultrastructure. J. Membr. Biol. 53:63-75.

35. Dahl, G., R. Azarnia, R. Werner (1980). De novo construction of cell-to-cell channels. In Vitro 16:1068-1075.

36. Daniel, E.E. and S. Lodge. 1973. Electrophysiology of myometrium. In: Uterine Contraction, ed. J.B. Josimovich, Wiley-Interscience. New York, pp.19-35.

37. Daniel, E.E., V.P. Daniel, G. Duchon, R.E. Garfield, M. Nichols, S.K. Malhotra, and M.K. Oki. 1976. Is the nexus necessary for cell-to-cell coupling of smooth muscle? J, Membr. Biol. 28:207-239.

38. Deleze, J. and W.R. Loewenstein. 1976. Permeability of a cell junction during intracellular injection of divalent cations. J. Membr. Biol. 28:71-86.

39. De Mello, W.C. 1982a. Changes in cell-to-cell coupling in the cardiac cycle. Physiologist 25:197-203.

40. De Mello, W.C. 1982b. Cell-to-cell communication in the heart and other tissues. Prog. Biophys. molec. Biol. 39:147-182.

41. De Mello, W.C. 1983. The role of cAMP and Ca on the modulation of junctional conductance: an integrated hypothesis. Cell Biol. Int. Rep. 7:1033-1040.

42. De Mello, W.C. 1984. Effect of intracellular injection of cAMP on the electrical coupling of mammalian cardiac cells. Biochem. Biophys. Res. Commun. 119:1001-1007.

43. Demianczuk, N., M.E. Towel, R.E. Garfield. 1984. Myometrial electrophysiologic activity and gap junctions in the pregnant rabbit. Amer. J. Obstet. Gynecol. 149:485-491.

44. Downing, S.J. and O.D. Sherwood. 1985a. The physiological role of relaxin in the pregnant rat. I: The influence of relaxin on parturition. Endocrinol. 116:1200-1205.

45. Downing, S.J. and O.D. Sherwood. 1985b. The physiological role of relaxin in the pregnant rat. II: The influence of relaxin on uterine contractile activity.

Endocrinol. 116:1206-1214.

46. Downing, S.J. and O.D. Sherwood. 1985c. The physiological role of relaxin in the pregnant rat. III: The influence of relaxin on cervical extensibility. Endocrinol. 116:1215-1220.

47. Downing, S.J., J.M.C. Bradshaw, D.G. Porter. 1980. Relaxin improves the coordination of rat myometrial activity _in vivo_. Biol. Reprod. 23:899-903.

48. Elbrink, J. and I. Bihler. 1975. Membrane transport: its relation to cellular metabolic rates. Science 188:1177-1184.

49. Estapé, E. and W.C. De Mello. 1983. Cyclic nucleotides and calcium: their role in the control of cell communication in the heart. Cell Biol. Int. Rep. 7:91-97.

50. Findlay, I. and O.H. Peterson. 1982. Acetylcholine-evoked uncoupling restricts the passage of Lucifer yellow between pancreatic acinar cells. Cell Tiss. Res. 225:633-638.

51. Flagg-Newton, J.L. and W.R. Loewenstein. 1979. Experimental depression of junctional membrane permeability in mammalian cell culture. A study with tracer molecules in the 300 to 800 dalton range. J. Membr. Biol. 50:65-100.

52. Flagg-Newton, J.L., I. Simpson, and W.R. Loewenstein. 1979. Permeability of the cell-to-cell channels in the mammalian cell junction. Science 205:404-406.

53. Flagg-Newton, J.L., G. Dahl, W.R. Loewenstein. 1981. Cell junctions and cyclic AMP: I. Upregulation of junctional membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. J. Membr. Biol. 63:105-121.

54. Fuchs, A.R. 1969. Uterine activity in late pregnancy and during parturition in the rat. Biol. Reprod. 1:344-353.

55. Fuchs, A.R. 1978. Hormonal control of myometrial function during pregnancy and parturition. Acta Endocrinol. (Scand.) 221 (suppl):1-71.

56. Gabella, G. 1976. Quantitative morphological study of smooth muscle cells of the guinea-pig taenia coli. Cell Tiss. Res. 170:161-186.

57. Gabella, G. and D. Blundell. 1981. Gap junctions of the muscles of the small and large intestine. Cell Tiss. Res. 219:469-488.

58. Garfield, R.E. and E.E. Daniel. 1974. The structural basis of electrical coupling (cell-to-cell contacts) in rat myometrium. Gynecol. Invest. 5:284-300.

58. Garfield, R.E. and R.H. Hayashi. 1981. Appearance of gap junctions in the myometrium of women in labor. Amer. J. Obstet. Gynec. 140:254-260.

60. Garfield, R.E., S. Sims, E.E. Daniel. 1977. Gap junctions: Their presence and necessity in myometrium during parturition. Science 198:958-960.

61. Garfield, R.E., S. Sims, M.S. Kannan, E.E. Daniel (1978). Possible role of gap junctions in activation of myometrium during parturition. Amer. J. Physiol. 235:C168-179.

62. Garfield, R.E., S. Rabideau, J.R.G. Challis, E.E. Daniel. 1979a. Ultrastructural basis for maintenance and termination of pregnancy. Amer. J. Obstet. Gynec. 133:308-315.

63. Garfield, R.E., S. Rabideau, J.R.G. Challis, E.E. Daniel. 1979b. Hormonal control of gap junction formation in sheep myometrium during parturition. Biol. of Reprod. 21:999-1007.

64. Garfield, R.E., M.S. Kannan, E.E. Daniel. 1980a. Gap junction formation in myometrium: control by estrogens, progesterone and prostaglandins. Amer. J. Physiol. 238:C81-89.

65. Garfield, R.E., D. Merrett, A.K. Grover. 1980b. Gap junction formation and regulation in myometrium. Amer. J. Physiol. 239:C217-228.

66. Garfield, R.E., C.P. Puri, and A.I. Csapo. 1982. Endocrine, structural, and functional changes in the uterus during premature labor. Amer. J. Obstet. Gynecol. 142:21-27.

67. Gilman, A.F. 1984. G proteins and dual control of adenylate cyclase. Cell 36:577-579.

68. Gilula, N.B., O.R. Reeves, and A. Steinbach. 1972. Metabolic coupling and cell contacts. Nature (Lond.) 235:262-265.

67. Girsch, S.J. and C. Peracchia. 1985. Lens cell-to-cell channel protein. I: Self-assembly into liposomes and permeability regulation by calmodulin. J. Membr. Biol. 83:217-225.

68. Girsch, S.J. and C. Peracchia. 1985. Lens cell-to-cell channel protein. II: Conformational change in the presence of calmodulin. J. Membr. Biol. 83:227-233.

69. Goodenough, D.A, J.S.B. Dick, and J.E. Lyons. 1980. Lens metabolic cooperation: a study of mouse lens transport and permeability visualized with freeze-substitution autoradiography and electron microscopy. J. Cell Biol. 86: 576-589, 1980.

70. Gorski, J. and F. Gannon. 1976. Current models of steroid hormone action: a critique. Ann. Rev. Physiol. 38:425-450.

71. Harbon, S., Z. Tanfin-Tougui, L. Dokhac. 1984. Control of cyclic AMP content of the rat myometrium. β-Adrenergic-, PG E_2-, and prostacyclin-induced stimulation and desensitization. In: Uterine Contractility, eds. S. Bottari, J.P. Thomas, A. Vokaer, and R. Vokaer, Masson Publ. Inc., New York, pp. 53-68.

72. Harden, T.K. 1983. Agonist-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase. Pharmacol. Rev. 35:5-32.

73. Hardman, J.G. 1981. Cyclic nucleotides and smooth muscle contraction: some conceptual and experimental considerations. In: Smooth Muscle: an Assessment of Current Knowledge, eds. E. Bulbring, A.F. Brading, A.W. Jones, and T. Tomita, Edward Arnold Ltd., London, pp.171-198.

74. Hermsmeyer, K. 1982. Angiotensin II increases electrical coupling mammalian ventricular myocardium. Circ. Res. 47:524-529.

75. Hertzberg, E.L. 1985. Antibody probes in the study of gap junctional communication. Ann. Rev. Physiol. 47:305-319.

76. Hertzberg, E.L. and N.B. Gilula. 1981. Liver gap junctions and lens fiber junctions: comparative analysis and calmodulin interaction. Cold Spring Harbor Symp. Quant. Biol. 46:639-645.

77. Hertzberg, E., D.C. Spray, M.V.L. Bennett. 1985. Reduction of gap junctional conductance by microinjection of antibodies against the 27-kDa liver gap junction polypeptide. Proc. Natl. Acad. Sci. (USA), 82:2412-2416.

78. Holman, M.E. and T.O. Neild. 1979. Membrane properties. Brit. Med. Bull. 35:235-241.

79. Hooper, M.L. and J.H. Subak-Sharpe. 1981. Metabolic cooperation between cells. Int. Rev. Cytol. 69:45-104.

80. Imanaga, I. 1974. Cell-to-cell diffusion of procion yellow in sheep and calf Purkinje fibers. J. Memb. Biol. 16:381-388.

81. Ito, S., E. Sato, and W.R. Loewenstein. 1974. Studies on the formation of a permeable junction. II: Evolving junctional conductance and junctional insulation. J. Membr. Biol. 19:339-355.

82. Iwatsuki, N. and O.H. Petersen. 1978a. Pancreatic acinar cells: acetylcholine-evoked electrical uncoupling and its ionic dependency. J. Physiol. (Lond.) 274:81-96.

83. Iwatsuki, N. and O.H. Petersen. 1978b. Electrical coupling and uncoupling of exocrine acinar cells. J. Cell Biol. 79:533-545.

84. Iwatsuki, N. and O.H. Petersen. 1979. Direct visual observation of cell-to-cell coupling: transfer of fluorescent probes in living mammalian pancreatic acini. Pflugers Arch. 380:277-288.

85. Jakobs, K.H. 1979. Inhibition of adenylate cyclase by hormones and neurotransmitters. Mol. Cell. Endocrinol. 16:147-156.

86. Johnston, M.F. and F. Ramon. 1981. Electrotonic coupling in internally perfused crayfish segmented axons. J. Physiol. 317:509-518.

87. Johnson, R., J. Hammer, J.D. Sheridan, and J.P. Revel. 1974. Gap junction formation between reaggregated Novikoff hepatoma cells. Proc. Nat. Acad. Sci. (USA) 71:4535-4543.

88. Kao, C.Y. 1977. Electrophysiological properties of uterine smooth muscle. In: Biology of the Uterus, ed. R.M. Wynn, Plenum Press, New York, pp. 423-496.

89. Knox, W.E. and L.M. Lister-Rosenoer. 1978. Timing of gestation in rats by fetal and maternal weights. Growth 42:43-53.

90. Korenman, S.G. and J.F. Krall. 1977. The role of cyclic AMP in the regulation of smooth muscle contraction in the uterus. Biol. Reprod. 16:1-17.

91. Krall, J., J.D. Barrett, N. Jamgotchian, and S.G. Korenman. 1984. Interaction of prostaglandin E_2 and β-adrenergic catecholamines in the regulation of uterine smooth muscle motility and adenylate cyclase in the rat. J. Endocrinol. 102:329-336.

92. Kuriyama, H. 1981. Excitation-contraction coupling in various visceral smooth muscles. In: Smooth Muscle: and Assessment of Current Knowledge, eds. E. Bulbring, A.F. Brading, A.W. Jones, and T. Tomita, Edward Arnold Ltd., London, pp.171-198.

93. Kuriyama, H. and H. Suzuki. 1976. Changes in electrical properties of rat myometrium during gestation and following hormonal treatments. J. Physiol. 260:315-333.

94. Kushmeric, M.J. and R.J. Podolsky. 1969. Tonic mobility in muscle cells. Science 166:1297-1298.

95. Larsen, W.J. 1975. Opaque deposits on gap junctions in membranes after glutaraldehyde fixation. J. Cell Biol. 67:801-813.

96. Larsen, W.J. 1977a. Gap junctions and hormone action. In: Transport of Ions and Water in Epithelia, eds.

B.J. Wall, J.L. Oschman, B. Moreton, and B. Gupta. Academic Press, London, pp. 333-361.

97. Larsen, W.J. 1977b. Structural diversity of gap junctions: a review. Tiss. Cell 9:373-395.

98. Larsen, W.J. 1983. Biological implications of gap junction structure, distribution and composition. Tiss. Cell 15:645-671.

99. Laufer, M. & R. Salas (1981). Intercellular coupling and retinal horizontal cell receptive field. NeuroSci. Lett. 7:5339.

100. Lauffer, M.A. 1961. Theory of diffusion in gels. Biophys. J. 1:205-213.

101. Lees-Miller, J.P. and S. Caveney. 1982. Drugs that block calmodulin activity inhibit cell-to-cell coupling in the epidermis of Tenebrio molitor. J. Membr. Biol. 69:233-245.

102. Levithan, I.B., J.R. Lemos, and I. Novak-Hofer. 1983. Protein phosphorylation and the regulation of ion channels. Trends Neurosci. Dec. 1983, pp. 496-499.

103. Liggins, G.C. 1979. Initiation of parturition. Br. Med. Bull. 35:145-150.

104. Lipshitz, J. 1981. Beta-adrenergic agonists. Semin. Perinatol. 5:252-265.

105. Lodge, S. and J. Sproat. 1981. Resting membrane potentials of pacemaker and nonpacemaker areas in rat uterus. Life Sci. 28:2251-2256.

106. Loewenstein, W.R. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. Physiol. Rev. 61:829-913.

107. Loewenstein, W.R., Y. Kanno, S.J. Socolar. 1978. Quantal jumps of conductance during formation of membrane channels at cell-cell junction. Nature (Lond.) 274:133-136.

108. MacKenzie, L.W. and R.E. Garfield. 1985. Hormonal control of gap junctions in the myometrium. Amer. J. Physiol. 248:C296-308.

109. MacKenzie, L.W., C.P. Puri, R.E. Garfield. 1983. Effects

of estradiol-17B and prostaglandins on rat myometrial gap junctions. Prostaglandins 26:925-944.

110. Makowski, L., D.L.D. Caspar, W.C. Phillips, T.S. Baker, and D.A. Goodenough. 1984. Gap junction structures. IV: Variation and conservation in connexon conformation and packing. Biophys. J. 45:208-218.

111. Manjunath, C.K., G.E. Goings and E. Page. 1984. Cytoplasmic surface and intramembrane components of rat heart gap junctional proteins. Amer. J. Physiol. 246:H865-875.

121. Marshall, J.M. 1962. Regulation of activity in uterine smooth muscle. Physiol. Rev. 42(suppl. 5):213-227.

122. Marshall, J.M. 1981. Effects of ovarian steriods and pregnancy on adrenergic nerves of the uterus and oviduct. Amer. J. Physiol. 240:C165-174.

123. Mastro, A.M., M.A. Babich, W.D. Taylor, and A.D. Keith. 1984. Diffusion of a small molecule in the cytoplasm of mammalian cells. Proc. Natl. Acad. Sci. (USA) 81:3414-3418.

124. Means, A.R., J.S. Tash, and J.G. Chafouleas. 1982. Physiological implications of the presence, distribution, and regulation of calmodulin in eukaryotic cells. Physiol. Rev. 62:1-39.

125. Merk, F.B., C.R. Botticelli, J.T. Albright. 1972. An intercellular response to estrogen by granulosa in the rat ovary: an electron microscopic study. Endocrinol. 90:992-1007.

126. Merk, F.B., P.W.L. Kwan, I. Leav. 1980. Gap junctions in the myometrium of hypothysectomized estrogen-treated rats. Cell Biol. Int. Rep. 4:287-294.

127. Meyer, D.J., S.B. Yancey, J.P. Revel. 1981. Intercellular communication in normal and regenerating rat liver: a quantitative analysis. J. Cell Biol. 91:505-523.

128. Mossman, H.W. 1977. Comparative anatomy. In: Biology of the Uterus, ed. R.M. Wynn, Plenum Press, New York, pp. 19-34.

129. Needham, D.M. and C.F. Schoenberg. 1967. Biochemistry of the myometrium. In: Cellular Biology of the Uterus, ed. R.M. Wynn, Meredith Publ. Co., New York, 1967.

130. Nestler, E.J. and P. Greengard. 1983. Protein phosphorylation in the brain. Nature (Lond.) 305:583-588.

131. Nicholson, C. and J.M. Phillips. 1981. Ion diffusion modified by tortuosity and volume fraction in the extracellular microenvironment of the rat cerebellum. J. Physiol. (Lond.) 321:225-257.

132. Pappenheimer, J.R., E.M. Renkin, and L.M. Borrero. 1951. Filtration, diffusion and molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability. Amer. J. Physiol. 167:13-46.

133. Peracchia, C. 1980. Structural correlates of gap junction permeation. Inter. Rev. Cytol. 66:81-146.

134. Peracchia, C. 1984. Communicating junctions and calmodulin: inhibition of electrical uncoupling in Xenopus embryo by calmidazolium. J. Membr. Biol. 81:49-58.

135. Peracchia, C., G. Bernardini, and L.L. Peracchia. 1983. Is calmodulin involved in the regulation of gap junction permeability. Phlugers Arch. 399:152-154.

136. Piccolino, M., M.J. Neyton, P. Witkovsky, H.M. Gerschenfeld. 1982. Gama-aminobutyric acid antagonists decreases junctional communication between L-horizontal cells of the retina. Proc. Natl. Acad. Sci. 79:3671-3675.

137. Pollack, G.H. 1976. Intercellular coupling in the atrioventricular node and other tissues of the rabbit heart. J. Physiol. 255:275-298.

138. Porter, D.G. and S.J. Downing. 1978. Evidence that a humoral factor possessing relaxin-like activity is responsible for uterine quiescence in the late pregnant rat. J. Reprod. Fert. 52:95-102.

139. Porter, D.G. 1984. Relaxin and myometrial activity. In: Uterine Contractility, eds. S. Bottari, J.P. Thomas, A. Vokaer, and R. Vokaer, Masson Publ. Inc., New York, pp. 265-273.

140. Puri, C.P. And R.E. Garfield. 1982. Changes in hormone levels and gap junctions in the rat uterus during pregnancy and parturition. Biol. Reprod. 27:967-975.

141. Rasmussen, H. and D.M. Waisman. 1983. Modulation of cell function in the calcium messenger system. Rev. Physiol. Biochem. Pharmacol. 95:111-150.

142. Reynolds, S.R.M. 1949. Physiology of the Uterus, 2nd. edition, P.B. Hoeber Inc., New York.

143. Rose, B. and W.C. Loewenstein. 1976. Permeability of a cell junction and the localized free calcium concentration. A study with aequorin. J. Membr. Biol. 28:87-119.

144. Rose, B. and R. Rick, 1978. Intracellular pH, Intracellular free Ca, and junctional cell-to-cell coupling. J. Membr. Biol. 44:377-415.

145. Saez, J.C., D.C. Spray, M.V.L. Bennett, and E.L Hertzberg. 1985. Phosphorylation of gap junction protein from liver. Biophys. J. 47:504a.

145. Sandborn, B.M., H.S. Kuo, N.W. Weisbrodt, O.D. Sherwood. 1980. The interaction of relaxin with the rat uterus. I. Effects on cyclic nucleotide levels and spontaneous contractile activity. Endocrinol. 106:1210-1215.

146. Sanfillipo, J.S., J. Teichman, T.R. Melvin, C.O. Osyamkpe, J.L. Wittliff. 1983. Influence of certain prostaglandin synthetase inhibitors on cytoplasmic estrogen receptors in the uterus. Amer. J. Obstet. Gynecol. 145:100-104.

147. Schantz, E.F. and M.A. Lauffer. 1962. Diffusion measurements in agar gel. Biochemistry 1:658-663.

148. Schramm, M. and Z. Selinger. 1984. Message transmission: Receptor controlled adenylate cyclase system. Science 225:1350-1356.

149. Schwabe, C., B.G. Steinetz, G. Weiss, G. Segaloff, J.K. McDonald., E. O'Byrne, J. Hochman, B. Carrier, and L. Goldsmith. 1978. Relaxin. Rec. Prog. Horm. Res. 34:123-211.

150. Seeman, P. 1972. The membrane actions of anaesthetics and tranquilizers. Pharmacol. Rev. 24:583-655.

151. Sheridan, J.D. and M.M. Atkinson. 1985. Physiological roles of permeable junctions: some possibilities. Ann. Rev. Physiol. 47:337-353.

152. Sheridan, J.D., M. Hammer-Wilson, D. Preus and R.G. Johnson. 1978. Quantitative analysis of low resistance junctions between cultured cells and correlation with gap junctional areas. J. Cell Biol. 76:532-544.

152. Sherwood, O.D., V.E. Crnekovic, W.L. Gordon, and J.E. Rutherford. 1980. Radioimmunoassay of relaxin throughout pregnancy and during parturition in the rat. Endocrinol. 104:691-698.

153. Simpson, I., B. Rose, and W.R. Loewenstein. 1977. Size limit of molecules permeating the junctional membrane channels. Science 195:294-296.

154. Sims, S., E.E. Daniel and R.E. Garfield. 1984. Improved electrical coupling in uterine smooth muscle is associated with increased numbers of gap junctions at parturition. J. Gen. Physiol. 80:353-375.

155. Smith, D.E. and J. Gorski. 1968. Estrogen control of uterine glucose metabolism. J. Biol. Chem. 243:4169-4174.

156. Smith, D.E. and M.S. Smith. 1971. Properties of estrogen-sensitive uterine sugar metabolism: specificity of inhibitory sugars. Endocrinol. 88:218-223.

157. Socolar, S.J. and W.R. Loewenstein. 1982. Methods for studying transmission through permeable cell-to-cell junctions. In: Methods in Membrane Biology, vol. 10, ed. K. Korn, Plenum Press, New York, pp. 121-127.

160. Sokoloff, L. 1981. Localization of functional activity in the central nervous system by measurement of glucose utilization with radioactive deoxyglucose. J. Cerebral Blood Flow Metab. 1:7-36.

161. de Souza, N.J., A.N. Dohadwalla, and J. Reden. 1983. Forskolin: A labdane diterpenoid with antihypertensive, positive ionotropic, platlet aggregation inhibitory, and adenylate cyclase activating properties. Med. Research. Rev. 3:201-219.

162. Sperelakis, N. 1969. Lack of electrical coupling between contiguous myocardial cells in vertebrate hearts. In: Comparative Physiology of the Heart: Current Trends, ed. F.V. McCann, Birkhauser-Verlag, Basel, pp.135-165.

163. Sperelakis, N. 1979. Propagation mechanisms in heart. Ann. Rev. Physiol. 41:441-457.

164. Spray, D.C. and M.V.L. Bennett. 1985. Physiology and pharmacology of gap junctions. Ann. Rev. Physiol. 47:281-303.

165. Spray, D.C., J.H. Stern, A.L. Harris, and M.V.L. Bennett. 1982. Gap junctional conductance: comparison of sensitivities to H^+ and Ca^{++} ions. Proc. Natl. Acad. Sci. (USA) 79:441-445.

166. Spray, D.C., R.L. White, A. Campos de Carvalho, A.L. Harris, and M.V.L. Bennett. 1984. Gating of gap junction channels. Biophys. J. 45:219-230.

167. Staehelin, L.A. 1974. Structure and function of intercellular junctions. Int. Rev. Cytol. 39:191-283.

168. Teranishi, T., K. Negishi, S. Kato. 1982. Dopamine

modulates S-potential amplitude and dye coupling between external horizontal cells in carp retina. Nature (Lond.) 301:243-246.

169. Thorburn, G.D. and J.R.G. Challis. 1979. Endocrine control of parturition. Physiol. Rev. 59:863-918.

170. Tomita, T. 1970. Electrical properties of smooth muscle. In: Smooth Muscle, eds. E. Bulbring, A.F. Brading, .W. Jones, and T. Tomita, Edward Arnold Ltd., London, pp.396-417.

171. Tsien, R.W. and R. Weingart. 1976. Inotropic effect of cyclic AMP in calf ventricular muscle studied by a cut-end method. J. Physiol. 260:117-141.

172. Unwin, P.N.T. and G. Zampighi. 1980. Structure of the junction between communicating cells. Nature (Lond.) 283:545-549.

173. Unwin, P.N.T. and P.D. Ennis. 1983. Calcium-mediated changes in gap junction structure: evidence from low angle x-ray pattern. J. Cell Biol. 97:1459-1466.

174. Unwin, P.N.T. and P.D. Ennis. 1984. Two configurations

of a channel-forming membrane protein. Nature (Lond.) 307:609-613.

175. Vassort, G. 1981. Ionic currents in longitudinal muscle of the uterus. In: Smooth Muscle: and Assessment of Current Knowledge, eds. E. Bulbring, A.F. Brading, A.W. Jones, and T. Tomita, Edward Arnold Ltd., London, pp.171-198.

176. Van Belle, H. 1981. R 24571: a potent inhibitor of calmodulin activated enzymes. Cell Calcium 2:483-494.

177. Vesin, M.F. and S. Harbon. 1974. The effect of epinephrine, prostaglandins, and their antagonists on adenosine 3'5' monophosphate concentrations and motility of the rat uterus. Mol. Pharmacol. 16:457-462.

178. Vesin, M.F., L. Dokhac, S. Harbon. 1979. Prostacyclin as an endogenous modulator of adenosine cyclic 3'-5' monophosphate in the myometrium and endometrium. Mol. Pharmacol. 16:823-840.

179. Vesin, M.F., S. Bourgoin, D. Leiber, S. Harbon. 1984. Lipoxygenase and cyclooxygenase products of arachidonic acid in uterus. Selective interaction with the cGMP and cAMP systems. Prostaglan. Leuko. Med. 13:75-78.

180. Wathes, D.C. and D.G. Porter. 1982. Effect of uterine distention and estrogen treatment on gap junction formation in the myometrium of the rat. J. Reprod. Fert. 65:497-505.

181. Watson, E. 1978. Effects of ionophores A23187 and X537A on vascular smooth muscle activity. Eur. J. Pharmacol. 52:171-178.

182. Weibel, E.R. 1973. Stereological techniques for electron microscope morphometry. In: Principles and Techniques of Electron Microscopy: Biological Applications, vol. 3, ed. M.A. Hayat, Van Nostrand Reinhold, New York, pp. 237-297.

183. Weibel, E.R., W. Staubli, H.R. Gnagi, and F.A. Hess. 1969. Correlated morphometric and biochemical studies on the liver cell. I: Morphometric model, stereologic methods and normal morphometric data for rat liver. J. Cell Biol. 42:68-95.

184. Weidmann, S. 1966. Diffusion of radiopotassium across intercalated disks of mammalian cardiac muscle. J. Physiol. 187:323-342.

185. Weingart, R. 1974. The permeability to tetraethylammonium ions of the surface membrane and the intercalated disks of sheep and calf myocardium. J. Physiol. 240:741-762.

186. Weiss, B., W. Prozialeck, M. Cimino, M. Sellinger-Barnette, and L. Wallace. 1980. Pharmacological regulation of calmodulin. Ann. N.Y. Acad. Sci. 356:319-345.

187. Williams, E.H. and R.L. De Haan. 1981. Electrical coupling among heart cells in the absence of ultrastructurally defined gap junctions. J. Cell Biol. 60:237-248.

188. Wojtczak, J. 1984. Effects of general and local anesthetics on intercellular communication in the heart muscle. Biophys. J. 45:22a.

189. Zampighi, G. and P.N.T. Unwin. 1979. Two forms of isolated gap junctions. J. Mol. Biol. 135:451-464.

190. Zelcer, E. and E.E. Daniel. 1979. Electrical coupling in rat myometrium during pregnancy. Can. J. Physiol. Pharmacol. 57:490-495.