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A Comparison of insulin/IGF 1 and progesterone-induced meiotic maturation of Xenopus laevis Oocytes

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A COMPARISON OF INSULIN/IGF-1 AND PROGESTERONE-INDUCED MEIOTIC MATURATION OF *Xenopus laevis* OOCYTES

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Full-grown *Xenopus laevis* oocytes are arrested in prophase of meiosis. Both the steroid progesterone, and the peptide hormone insulin, can induce the resumption of meiosis in *Xenopus* oocytes. Previous studies indicated that insulin and progesterone may induce meiosis by separate pathways. It appeared that insulin took 2 to 4 hours longer than progesterone to induce the meiotic reductive division. This time differential indicated that the insulin pathway may involve one or more steps that are not required in the progesterone pathway leading to meiosis. However, when oocytes were stored for 12 - 14 hours in Modified Barth's Solution (MBS) they underwent meiosis at the same rate when stimulated with either insulin or progesterone. Furthermore, all of the physiological changes that I measured, such as an increase in intracellular pH, protein synthesis and protein phosphorylation were identical in timing and in magnitude in oocytes stimulated with either insulin or progesterone. Therefore, it appears that the insulin and progesterone pathways converge early after the signal transduction process across the oocyte plasma membrane and use a common pathway leading to GVBD.

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CHAPTER I

INTRODUCTION

A. Purpose

Oocytes in all vertebrates complete an extended growth phase while in the ovary. During this growth phase, the oocytes are immature and are not ready for fertilization by sperm. At the end of the growth period, the oocytes undergo a brief maturation process in response to hormonal stimulation, which prepares them for fertilization and normal embryonic development. This maturation process is called oocyte meiotic maturation.

The pituitary gland releases the hormone LH (luteinizing hormone) which triggers oocyte meiotic maturation in all vertebrates. A number of studies have been done using oocytes of many species to determine how LH triggers oocyte maturation. Several biochemical changes in reponse to LH stimulation have been measured in oocytes from amphibians, mice, rats, and monkeys. Many of these biochemical changes appear to be similar in all oocytes.

In amphibians, LH induces the follicle cells, which surround the oocyte, to synthesize and release progesterone. Progesterone then induces meiotic maturation in the oocytes. Meiotic maturation is a series of biochemical and physiological changes within the oocyte that allow the oocyte to re-enter the meiotic cell cycle from prophase arrest. A culminating morphological indicator of meiotic maturation is germinal vesicle breakdown (GVBD). GVBD is the migration of the germinal vesicle (nucleus) to the apex of the animal pole and

dissolution of the nuclear membrane. Some of the biochemical changes that occur due to

progesterone stimulation, from the time of signal transduction to GVBD are: a transient increase in the level of intracellular ionized calcium, a drop in the intracellular level of cAMP, an increase in the intracellular pH, an increase in the rate of protein synthesis, and an increase in total protein phosphorylation.

More recently, the peptide hormones insulin and insulin-like growth factor-1 (IGF-1) have also been shown to induce oocyte maturation. Insulin, IGF-1 and progesterone however, vary in the amount of time it takes from the time of hormone addition until germinal vesicle breakdown. Insulin/IGF-1 appear to take 2-4 hours longer than does progesterone to induce GVBD. While some physiological and biochemical events have been documented in regards to insulin and progesterone, it is still uncertain why this time differential exists. Where in the meiotic maturation process, from signal transduction to GVBD, does insulin differ from progesterone to account for the greater length of time it takes for insulin to induce maturation?

Our working hypotheses are that insulin and progesterone are either using completely separate pathways or that insulin induces one or more events before converging with the progesterone pathway, and this accounts for the differential response time. The goal of my thesis, therefore, is to compare the rate of physiological changes induced by progesterone and insulin in an attempt to explain why a time differential exists.

B. Literature Review

Meiotic cell division is of fundamental biological importance. It is the process leading to the maturation of the egg and the generation of the haploid chromosome content necessary for the continuation of the species after fertilization. The reinitiation of meiotic maturation (removal of the prophase arrest) is often triggered by hormonal stimulation

(Eckberg, 1988). This process of oocyte meiotic maturation has been studied in a variety of vertebrate and invertebrate organisms, but the process has been investigated most intensively in amphibians (Smith, 1989).

Amphibian oocytes are very large single cells, growing to a diameter of 1.3 mm in *Xenopus laevis* . Oogenesis in *Xenopus* is an asynchronous process. Oocytes of different sizes exist in the ovary at any given time, and can be classified into various developmental stages (I-VI) according to morphological and metabolic criteria (Dumont, 1972). When oocytes reach a diameter of 1.2-1.3 mm (stage VI), they become competent to undergo the process of oocyte maturation in response to progesterone. LH from the pituitary stimulates the follicle cells that surround the full-grown oocyte, causing them to synthesize and release progesterone. Progesterone, in turn, triggers the meiotic maturation process within the oocyte. This involves several biochemical changes and the migration of the germinal vesicle (nucleus) to the apex of the animal pole, breakdown of the germinal vesicle (GVBD), chromosome condensation, completion of Meiosis I with elimination of a polar body and arrest at second meiotic metaphase as an unfertilized egg. This meiotic maturation process can also be induced in full-grown stage VI oocytes *in vitro* by progesterone (Wasserman and Smith, 1978; Masui and Clark, 1979; Maller, 1983).

The site of action of progesterone in the full-grown oocyte appears to be at the plasma membrane of the oocyte. This is different from most steroid target cell interactions wherein the steroid is translocated into the nucleus and alters gene transcription (Beato, 1989). Microinjection of progesterone into the oocyte does not induce the meiotic maturation process (Masui and Markert, 1971; Smith and Ecker, 1971), but surface exposure does. Furthermore, when progesterone is covalently bound to polystyrene beads, and cannot enter the full-grown oocyte, meiotic maturation still occurs (Ishikawa *et al.*, 1977; Godeau

et al., 1978). Also, researchers using photoaffinity labelling have identified a progesterone receptor in plasma membrane preparations (Sadler and Maller, 1982; Blondeau and Baulieu, 1984). Therefore, the progesterone receptor appears to be located at the plasma membrane and the signal is transduced across the membrane to trigger the meiotic maturation process within the oocyte cytoplasm.

The cytoplasmic events following progesterone signal transduction can be defined on a temporal basis, that is, between the time of steroid addition until the time of germinal vesicle breakdown.The first event after progesterone addition in a full-grown stage VI oocyte is a transient increase in the level of intracellular free calcium (Wasserman *et al.*, 1980). This event has been reported to occur 40 seconds after the addition of progesterone, and the calcium appears to be released from an intracellular pool (Wasserman *et al.*, 1980). The second event is a drop in the intracellular level of cAMP (Speaker and Butcher, 1977; Maller *et al.*, 1979; Cicerelli and Smith, 1985). This decrease in intracellular cAMP is due to a down-regulation of the catalytic subunit of adenylate cyclase resulting in the decreased synthesis of cAMP from ATP (Mulner *et al.*, 1979; Sadler and Maller, 1981; Finidori-Lipicard *et al.*, 1982). This event takes place 15 minutes after the addition of progesterone.

An increase in intracellular pH (pHi) starts by 2 hours after progesterone addition. In the literature, there appears to be a discrepancy about the magnitude of the increase in pHi . This difference may be attributed to priming animals with pregnant mare serum gonadotropin (PMSG) prior to measurement. Oocytes from animals that are unprimed have a basal pHi of 7.2 to 7.3. Upon application of the steroid the intracellular pH increases to 7.6 or 7.7 (Houle and Wasserman, 1983; Wasserman and Houle, 1984; Wasserman *et al.*,

1984). Oocytes from animals that have been primed appear to have a basal pHi of 7.4 to 7.5 and increase to 7.6 or 7.7 (Lee and Steinhardt, 1981; Cicerelli *et al.*, 1983; Stith and Maller, 1984). Therefore, in oocytes from unprimed animals an increase of 0.4-0.5 pH units occurs in response to progesterone.

The increase in intracellular pH is due to stimulation of the Na^+/H^+ antiporter in the plasma membrane of full-grown stage VI oocytes (Wasserman and Houle, 1984; Stith and Maller, 1985). An increased efflux of H^+ ions from the oocyte cytoplasm is a result of stimulation of the Na^+/H^+ antiporter (Cicerelli *et al.*, 1983). If the Na^+/H^+ antiporter is inhibited by replacing extracellular sodium with choline chloride or using the inhibitor amiloride, then the progesterone-induced pHi increase does not occur (Wasserman and Houle, 1984; Stith and Maller, 1985). Although the importance of the magnitude of the increase in pHi is uncertain, the pHi is critical. If the pHi is maintained at or below 7.0 with either carbon dioxide or weak acids GVBD is completely blocked (Belle *et al.*, 1982; Houle and Wasserman, 1983).

Another event in progesterone-stimulated full-grown stage VI oocytes is a two to threefold increase in the rate of protein synthesis by 4 hours after addition of hormone (Wasserman *et al.*, 1982). The protein synthetic rate increase is due to recruitment of additional maternal mRNA onto polysomes and is not due to an increase in translational efficiency (Richter *et al.*, 1982). Progesterone-induced GVBD requires protein synthesis. Inhibiting protein synthesis with cycloheximide (>10 μg/ml) blocks GVBD in full-grown stage VI oocyte (Wasserman and Masui, 1975b; Ziegler and Masui, 1976; Wasserman *et al.*,1986). The progesterone-induced increase in the rate of protein synthesis is mainly due to the synthesis of the same proteins made in prophase-arrested oocytes (Ballantine *et al.*, 1979; Wasserman *et al.*, 1982), however four or five new peptides may be synthesized

(Younglai *et al.*, 1981, 1982). An increase in total protein phosphorylation takes place by 5 hours after progesterone stimulation. There appears to be a threefold increase in total protein phosphorylation (Maller *et al.*, 1977), and a six to eightfold increase in ribosomal protein phosphorylation (Wasserman *et al.*, 1986).

Progesterone was thought to be the only hormone that acts directly on the oocyte to induce meiotic maturation. However, Baulieu and collaborators reported that insulin at high concentrations could mimic progesterone by inducing meiotic maturation in *Xenopus laevis* oocytes (El-Etr *et al.*, 1979). It was also reported that GVBD proceeded faster with progesterone than with insulin, and therefore may suggest that the two hormones trigger meiotic division by two different pathways (El-Etr *et al.*, 1979). Initially it was thought that the response to insulin was mediated through the insulin receptor (El-Etr *et al.*, 1979). However, the location of the receptor was uncertain i.e. it could be on the follicle cells that surround the oocyte or on the oocyte plasma membrane. The question remained where does insulin interact with its receptor to induce oocyte maturation.

Microinjection of insulin had no effect on GVBD, therefore it seemed likely that insulin acted via a surface receptor interaction (Maller and Koontz, 1981). In addition, removal of follicle cells by pronase did not abolish the ability of insulin to induce oocyte maturation (Maller and Koontz, 1981). Therefore, it appears that insulin works via a surface receptor at or near the surface of the oocyte plasma membrane and not on the follicle cells. However, Maller and Koontz found that insulin bound with low affinity to the plasma membrane. It was believed that the binding site may be a specific insulin receptor with a low affinity, or the site may represent binding to another hormone receptor having some structural similarity to the insulin receptor (Maller and Koontz, 1981). Also, it was reported that the EC_{50} for insulin (effective concentration to produce 50% GVBD in

the responding oocytes) was 10 times greater than the dissociation constant (K_D) for a high-affinity insulin receptor. This suggested that insulin induction might be mediated through a non-insulin receptor, particularly since insulin generally has a maximal biological effect at low receptor occupancy levels (Kono and Barham, 1971). In addition, the time course of insulin maturation was essentially unaffected by the presence of anti-insulin receptor antibodies (Maller and Koontz, 1981). Previous reports indicate that, in a variety of other cell systems, the mitogenic effects of insulin apparent at high insulin concentrations are mediated by insulin binding to insulin-like growth factor receptors, termed IGF receptors (Zapf *et al.*, 1975; Zapf *et al.*, 1978a, b; Rinderknecht and Humbel, 1976; Megyesi *et al.*, 1974). Insulin binding to IGF-1 receptors is unaffected by anti-insulin receptor antibodies (Maller and Koontz, 1981). Evidence that IGF-1 receptors are involved is supported by the ability of IGF-1 to stimulate meiotic maturation in amphibian oocytes at physiological concentrations [10^{-10} M] (Maller and Koontz, 1981).

Competitive hormone binding studies with partially purified receptors from *Xenopus* oocytes suggest that the oocyte possesses high affinity binding sites for IGF-1, but not for insulin (Janicot *et el.*, 1991). In hexose uptake studies by Janicot and collaborators it was found that IGF-1 activates hexose uptake by oocytes with an EC_{50} of 3 nM (the concentration of IGF-1 required to induce 50% of maximal hexose uptake) which is identical with the K_D (3 nM) of the IGF-1 receptor. However, insulin activated hexose uptake with an EC_{50} of 200-250 nM, suggesting activation through IGF-1 receptors (Janicot *et al.*, 1991). Therefore, it appears that most if not all of the effects of both insulin and IGF-1 in *Xenopus* oocytes may be exerted through the IGF-1 receptor, since IGF-1 is 50-100 times more potent than insulin in stimulating these processes (Janicot and Lane,

1989).

To further characterize the oocyte's response to insulin and to determine if insulin may work via a different receptor than progesterone, the role of p21 *ras* was analyzed (Korn *et al.*, 1987; Deshpande and Kung, 1987; Davis and Sadler, 1992). The protein product of the *ras* cellular proto-oncogene is a membrane-bound, guanine nucleotide-binding protein that is thought to function in growth control of cells (Korn *et al.*, 1987; Deshpande and Kung, 1987; Davis and Sadler, 1992). Previous studies have suggested that p21 mediates the cell's response to insulin (Korn *et al.*, 1987). Monoclonal antibodies (6B7 and Y13-259) against p21 injected into oocytes inhibited their response to insulin, but there was no inhibition of their response to progesterone (Korn *et al.*, 1987; Deshpande and Kung, 1987). The finding that a monoclonal antibody to p21 specifically inhibits insulin, but not progesterone-induced maturation, seems to show that insulin and progesterone induce oocyte maturation by acting through separate signal transduction pathways, and indicates that p21 *ras* is part of the pathway used by insulin (Korn *et al.*,1987; Deshpande and Kung, 1987).

A transient increase in intracellular free calcium takes place early after progesterone stimulation in *Xenopus* oocytes (Wasserman *et al.*, 1980). Thus far no studies have been reported on the role of calcium in insulin/IGF-1-stimulated oocytes. As in the case for progesterone where a decrease in the intracellular level of cAMP is required for GVBD, it has also been reported that the effects of insulin and IGF-1 require a drop in intracellular cAMP (Sadler and Maller, 1987). When the phosphodiesterase inhibitors, theophylline, papaverine, or isobutylmethylxanthine (IBMX) were used with insulin and IGF-1 stimulated oocytes there was inhibition of GVBD (Sadler and Maller, 1987). Also, cholera toxin which stimulates adenylate cyclase, increasing cAMP levels, is able to block GVBD

induced by insulin suggesting that a decrease in cAMP is also necessary for insulin-induced GVBD (Stith and Maller, 1984).

Preliminary measurements of intracellular pH (pHi) in insulin-stimulated full-grown stage VI *Xenopus* oocytes have been performed (Stith and Maller, 1984). The concentration of insulin required for half-maximal GVBD also produced an increase in the intracellular pH. The basal pHi was 7.43 and a final pHi, after insulin stimulation, was 7.62 (Stith and Maller, 1984). However, the study done by Stith and Maller was done on oocytes from only one animal. Further analysis is necessary to verify this study, and to determine if an increase in the intracellular pH does occur in response to insulin.

As mentioned earlier progesterone-induced oocyte maturation appears to involve a two to threefold increase in the rate of protein synthesis (Wasserman *et al.*, 1982), and the synthesis of four or five new proteins (Younglai *et al.*, 1981, 1982). The same type of extensive analysis has not been done during insulin-induced oocyte maturation. Thus, the rate of total protein synthesis and whether newly synthesized proteins are required for insulin-induced GVBD has yet to be determined.

While total protein phosphorylation has been examined during progesterone-induced oocyte maturation, it appears that only ribosomal S6 phosphorylation has been studied in insulin-induced oocytes (Stith and Maller, 1984). Increasing concentrations of insulin caused an increase in S6 phosphorylation, with half-maximal increase in phosphorylation at an insulin concentration equivalent to the EC_{50} for GVBD (Stith and Maller, 1984). Therefore, it appears that S6 phosphorylation is associated with the maturation response promoted by both insulin and progesterone.

While the progesterone response has been analyzed and characterized quite thoroughly, the insulin response has not. With the exception of the IGF-1 receptor, cAMP

and S6 studies in *Xenopus* oocytes, the characterization of the insulin pathway leading to oocyte maturation has been deficient. Therefore, a number of physiological changes that were known to take place in progesterone-treated oocytes were analyzed in insulin-treated oocytes. The goal of this work was to compare these changes in progesterone- and insulin-treated oocytes to determine a cause for the time differential in the oocytes' response to insulin or progesterone.

CHAPTER II

MATERIALS and METHODS

A. Oocyte Isolation and Media

Ovarian lobes were surgically removed from *Xenopus laevis* (NASCO, Ft. Atkinson, Wisconsin) that were anesthesized by hypothermia (30 min. in ice water). The lobes were transferred to Modified Barth's Solution (MBS): 73 mM NaCl, ± 10 mM $NaHCO_3$, 1mM $MgCl_2$, 0.5 mM $CaCl_2$, 1mM KCl, 25 mM Hepes, pH 7.8 (Sadler and Maller, 1988). Stage VI oocytes (Dumont, 1972) were manually defolliculated with #5 watchmakers forceps and cultured in MBS. Enzyme digestion of follicle cells by Collagenase or Pronase was not used so that no damage to receptors would be sustained. Animals were not primed with human chorionic gonadotropin (hCG).

B. Hormones

Progesterone (Sigma, St. Louis, MO.) was used at $1x\ 10^{-6}$ M for all treatments. Porcine insulin, sodium salt, and human recombinant IGF-1 (Calbiochem, La Jolla, CA.) were used at concentrations ranging from $1x10^{-11}$ M to $1x10^{-4}$ M (see below).

C. Dose Response Curves for Insulin and IGF-1

Dose response curves for insulin and IGF-1 induced oocyte maturation were done to compare the EC_{50} with previous reports using oocytes from primed animals (El-Etr *et al.*,

1979; Maller and Koontz, 1981) and to gain knowledge of the optimal hormone concentration in which to carry out subsequent experiments.

1. Insulin

Oocytes were incubated for 14-18 hours at 20°C in concentrations of porcine insulin ranging from $1x10^{-4}$ M to $1x10^{-11}$ M and scored according to the percent that achieved germinal vesicle breakdown (% GVBD). The oocytes response to progesterone served as the standard to which all events and parameters of insulin induction were compared. Progesterone was also used as a control to ensure the quality of the oocytes. Progesterone at $1x10^{-6}$ M should always induce 90-100% GVBD. If not, then it is highly probable that failure to induce GVBD was due to the poor quality of the oocytes analyzed and not the hormone being examined.

2. Insulin-Like Growth Factor-1(IGF-1)

Oocytes were incubated for 14-18 hours at 20°C in concentrations of IGF-1 ranging from $1x10^{-7}$ M to $1x10^{-11}$ M and scored for the %GVBD. Progesterone was also used as a control to ensure the quality of the oocytes.

D. Kinetics of Insulin/IGF-1 vs. Progesterone-Induced GVBD

These experiments were done to confirm previous reports that a time differential existed between insulin-induced GVBD, and progesterone-induced GVBD. In addition, several experiments have been performed with oocytes taken from primed animals (Maller and Koontz, 1981; Stith and Maller,1984; Sadler and Maller, 1989). It was necessary to confirm that a time differential also occurs in oocytes taken from unprimed animals.

Based on the dose response curves for Insulin and IGF-1, the lowest dose that induced 90-100% GVBD was used for the timing of GVBD for each hormone. Oocytes were incubated at 20°C for each treatment and scored every hour for percent GVBD. Fifty percent of the progesterone-stimulated oocytes achieved GVBD ($GVBD_{50}$) between 4-6 hours, and therefore served as a reference marker to compare with the $GVBD_{50}$ times for insulin and IGF-1.

E. Experimental Design

The questions asked were in what way are the pathways used by progesterone and insulin to induce oocyte maturation similar and where does the time differential between the two occur? There are two alternative hypotheses:

Hypothesis I: The pathways used by progesterone and insulin from signal transduction to GVBD are completely separate pathways that take different times to complete.

Hypothesis II: The pathway used by insulin following signal transduction involves one or more steps before converging with the progesterone pathway leading to GVBD.

The timing and characterization of a series of physiological and biochemical changes during progesterone-induced meiotic maturation has been documented for Stage VI oocytes. A chronology of events following the addition of progesterone are: a transient increase in the level of intracellular free calcium takes place within 40-60 seconds (Wasserman *et al.*, 1980), a drop in the intracellular level of cAMP at 15 minutes (Speaker

and Butcher, 1977; Maller *et al.*, 1979), an increase in the intracellular pH starts by 2 hours (Houle and Wasserman, 1983), an increase in the rate of protein synthesis by 4 hours (Wasserman et al., 1982), an increase in total protein phosphorylation by 5 hours (Maller *et al.*, 1977), and germinal vesicle breakdown (GVBD) by 6 hours. Our strategy was to compare these physiological changes and their timing in insulin- and progesterone-stimulated oocytes. Due to the lack of equipment, and time limitations, we were unable to analyze any changes in intracellular calcium or cAMP levels. Furthermore, these events take place between 40 sec. and 15 min. after progesterone-stimulation. These very rapid physiological changes may be unique to the progesterone signal transduction pathway and cannot account for the 2-4 hours extra time for insulin-induced GVBD. However, several other physiological events were analyzed in insulin- and progesterone- stimulated oocytes: pHi, protein synthesis, protein phosphorylation and GVBD kinetics. There are at least two possible outcomes to our studies:

a) If all the physiological events induced by progesterone and insulin, from signal transduction to GVBD are different, then hypothesis I would be supported, and hypothesis II would be rejected.

b) If some physiological events following signal transduction for progesterone and insulin are different in the beginning, but all changes are the same after some common event, then hypothesis II would be supported, and hypothesis I would be rejected.

If successful, this analysis should tell us how similar or different the two hormones are in inducing oocyte maturation. However, upon completing this analysis an event not yet determined may be responsible for the time differential between the progesterone and

insulin pathways. Further characterization of the insulin pathway would be necessary to resolve this problem (see Discussion).

1. RNA Synthesis Requirement for Insulin-Induced GVBD

These experiments were carried out using the RNA synthesis inhibitor Actinomycin D. It has previously been shown that treatment of oocytes with either Actinomycin D or α-Amanitin inhibits hCG - but not progesterone-induced GVBD. Actinomycin D at 100 μg/ml inhibits 95% of total oocyte RNA synthesis (Wasserman *et al.*, 1974), but has no effect on progesterone-induced GVBD. Whether or not insulin requires RNA synthesis within the oocyte had to be determined.

Oocytes were pre-treated with Actinomycin D (100ug/ml) for 2 hours at 20°C before application of hormones and continuously thereafter. Oocytes were incubated for 14-18 hours at 20°C in the presence of: porcine insulin ($1x10^{-5}$M) ± Actinomycin D, progesterone ($1x10^{-6}$ M) ± Actinomycin D, or hCG (50 IU/ml) ± Actinomycin D. HCG-treated oocytes were not defollicullated because this hormone works via the follicle cells. Since hCG-treated oocytes require RNA synthesis they served as a positive control, and no GVBD should occur in the presence of Actinomycin D plus hCG.

2. Oocyte pHi Determination and Kinetics of the pHi Increase

Intracellular pH (pHi) was determined by the uptake of tracer amounts of the weak acid ^{14}C - dimethyloxazolodine 2,4 dione, DMO (NEN), a method originally described by Waddell and Butler (1959). Control experiments validating the ^{14}C-DMO technique in oocytes have been previously reported (Houle and Wasserman, 1983). Uptake experiments indicated that at least 2 hours are needed for ^{14}C-DMO to reach an equilibrium

between the intracellular and extracellular environments across the plasma membrane (Houle and Wasserman, 1983)(see Figure5). ^{14}C- DMO (2.5 µl/tube) was dried under nitrogen to remove the ethyl acetate solvent (stock 50 µCi/500 µl). ^{14}C- DMO was resuspended in 1.0 ml MBS, vortexed, and poured into a 2 ml multiwell plate (final concentration of 0.25 µCi/ml). For each treatment 20 oocytes were placed in each well and labelled. After incubation in ^{14}C- DMO for 12-14 hours at 20°C, hormones were added. Oocytes plus hormone were then incubated for an additional 2 to 8 hours at 20°C.

After incubation the media and oocyte ^{14}C-DMO DPM were determined. In triplicate, 10 µl of each medium were placed in a scintillation vial with 10 ml Biodegradable Counting Scintillant (BCS, Amersham) and mixed. In triplicate, groups of 5 oocytes at a time were washed 3x10 seconds in MBS, placed in a scintillation vial, excess fluid was withdrawn with a pasteur pipette, 0.5 ml of NCS (tissue solubilizer, Amersham) was added, and incubated at 60°C for 30 minutes or until all oocytes were digested. After digestion the vials were incubated for 5 minutes at -20°C, then 10 ml of cold Toluene-PPO (scintillation cocktail, Amersham) was added to the vials and mixed. The media and oocytes were counted in a Beckman LS-7000 liquid scintillation counter. CPM data from the counter were converted to DPM and this was used to calculate the pHi by using the following formula (Waddell and Butler, 1959):

$$pHi = pKa + \text{Log}\left(\frac{[DMO]\ \text{inside}}{[DMO]\ \text{outside}} \left(10^{pHo-pka} + 1\right) - 1 \right)$$

pHi	= intracellular pH
pHo	= outside pH (Modified MBS)=7.80
pKa of DMO	= 6.32
[DMO]inside	= concentration of DMO inside the oocyte
	= DPM/µl (water volume of a stage VI oocyte is 0.45µl)
[DMO]outside	= concentration of DMO outside the oocyte (medium)
	= DPM/µl

The intracellular pH for progesterone and insulin-treated oocytes was compared every two hours after the addition of hormone until GVBD.

3. Protein Synthesis Requirement for Insulin-Induced GVBD

Progesterone-stimulated oocytes require protein synthesis in order to undergo GVBD (Wasserman and Masui, 1975b). Cycloheximide at 10 µg/ml inhibits protein synthesis 96% and blocks GVBD in progesterone-stimulated *Xenopus* oocytes (Wasserman *et al.*, 1986). Whether protein synthesis is necessary for insulin-induced GVBD had to be investigated. Therefore, oocytes were pre-treated in cycloheximide at 0.01-10 µg/ml (Sigma) for 2 hours at 20°C. Progesterone or insulin was then added and the oocytes were scored for % GVBD after 14 hours incubation.

4. Rate of Protein Synthesis in Insulin-Treated Oocytes

A two to three fold increase in the rate of protein synthesis has been reported for progesterone-treated oocytes (Wasserman et al, 1980). A quantitative analysis of protein synthesis in progesterone- and insulin-stimulated oocytes was performed. Control, progesterone, and insulin-treated oocytes were microinjected with 20 nl of ^{3}H-leucine (4 Ci/mmole, Amersham) delivering 4.4 x 10^{5} dpm and 50 pmoles of leucine per oocyte. At various times after hormone addition oocytes were injected, incubated for 10 min. and then fixed in TCA. Two groups of oocytes (5 oocytes/group) for each treatment and time point were washed three times in MBS and placed in a tube containing 3 ml of cold 10% TCA (w/v) and 1 mM leucine. Oocytes were then homogenized in the cold TCA + leucine. A 100 µl aliquot was taken from the homogenate and placed in a scintillation vial along with 10 ml biodegradable counting scintillant (BCS, Amersham). This was used to

calculate the total amount of isotope retained within the group of oocytes. The remaining homogenate was vortexed, and filtered through GF/A filters. Filters were then washed 2 times with 5 ml TCA and then 5 ml 95% ethanol. Filters were dried, placed in a scintillation vial with 10 ml toluene/PPO and counted in a Beckman LS 7000 scintillation counter. Both the 100 µl aliquot and filter were counted for each sample treatment and time point.

The % of total ^{3}H-leucine that was incorporated into newly synthesized protein was calculated using the following formula:

$$\% \text{ Incorporation} = \frac{\text{Filter CPM} \times 1.034}{100\mu\text{l CPM} \times 30} \times 100$$

5. Two Dimensional Gel Electrophoretic Analysis of Newly Synthesized Proteins

Qualitative examination of the newly synthesized proteins in progesterone and insulin-stimulated oocytes was performed. Control oocytes (no hormone) were microinjected with ^{35}S-methionine (1.8-2.0 x 10^{6} cpm NEN) and incubated in MBS for 1 hour. Progesterone and insulin-treated oocytes were divided into two groups per time point, 4 oocytes per group, and injected with [^{35}S]-methionine at one-hour intervals from time zero (addition of hormone) to GVBD. After microinjection, oocytes were incubated for 1 hour at 20°C in MBS. The oocytes were then homogenized in one ml of protein extraction buffer: 100 mM NaCl, 10mM ß-mercaptoethanol, 0.5% Triton-X-100, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 20 mM Tris, pH 7.5. After centifugation at 12,500 x g, 10 min the supernatant was drawn off and one ml of 100% ethanol was added to precipitate proteins for 18 hours at -20°C.

Protein precipitates were centrifuged 12,500 x g, 20 min and the pellets were dessicated for one hour. Protein precipitates were then dissolved in sample buffer containing Bio-Lytes (BIO RAD) for gel isoelectric focusing (IEF) according to O'Farrell (1975). The first-dimension IEF gel was then applied to a second-dimension 10% SDS Laemmli gel (1970). After electrophoresis, fluorography was performed. Fluorography involved the placement of the gel in glacial acetic acid for 15 min., then 20% PPO-acetic acid for 60 min., then ddH_2O for 5 min. The gels were then dried for 2 hours at 80°C. After drying, the gels were put up with XAR-5 film (Kodak) and stored at -85°C. After film development the 2-D arrays of newly synthesized proteins were compared.

6. Quantitation of Protein Phosphorylation in Insulin-Treated Oocytes

A threefold increase in total protein phosphorylation had been measured in progesterone-induced oocytes (Maller, 1977). To examine whether this change also occurred during insulin-induced maturation, oocytes were labelled in neutralized ^{32}P-orthophosphate at 50 µCi/ml from time zero to GVBD with or without hormones. For each time point and each treatment four oocytes were homogenized in 3 ml of 7.5% TCA + 1.0% pyrophosphate. Total uptake of ^{32}P was determined by taking a 100 µl aliquot from the homogenate, and placing it in a scintillation vial with 10 ml BCS. The remaining homogenate was heated at 90°C for 15 minutes to destroy RNA and DNA that had ^{32}P incorporated into the nucleotides. The homogenate was then cooled and vacuum filtered through GF/A glass fiber filters. The filters were washed with TCA + pyrophosphate, and washed two times with 5 ml of chloroform:methanol (3 vols.:1 vol.) to remove ^{32}P-containing phospholipids. Filters were then washed with ethanol and dried under a heat lamp. Both the 100 µl aliquot and the filter were counted for each time point and

treatment. Calculation of the % of total ^{32}P-orthophosphate that was incorporated into newly phosphorylated proteins was calculated using the following formula:

$$\% \text{ Incorporation} = \frac{\text{Filter CPM} \times 1.034}{100\mu\text{l CPM} \times 30} \times 100$$

7. Electrophoretic Analysis of Phosphorylated Proteins in Insulin-Treated Oocytes

To examine protein phosphorylation qualitatively, oocytes were labelled in ^{32}P-orthophosphate at 100 μCi/ml from time zero until GVBD (9hours) with or without hormones. For each time point, and each treatment, four oocytes were homogenized in one ml of protein extraction buffer, and then centrifuged (12,500 x g, 10 min.). After centrifugation the supernatant was drawn off and one ml of 100% ethanol was added to precipitate proteins for 18 hours at -20°C.

Protein precipitates were centrifuged at 12,500 x g, 20 min., and the pellets were dessicated for 1 hour. Samples were resuspended in SDS sample buffer, and then loaded onto a 10% Laemmli gel (1970). After electrophoresis gels were dried for 2 hours at 80°C and put up with XAR-5 film (Kodak) and stored at -85°C. After film development the phosphorylated protein bands were compared.

F. Statistical Analysis

Experiments were replicated using oocytes from a minimum of three different animals on 3 different days (n=3). All values are the mean ± S.E. of the mean. The data were analyzed by using Model II ANOVA, Randomized Block, and comparison of the slopes of two lines (Zar, 1984). Different treatments were compared using Tukey's multiple comparisons test. The analysis was done on MYSTAT.

CHAPTER III

RESULTS

Part I

A. Dose Response Curves for Insulin and IGF-1

Dose response curves for insulin and IGF-1 induced oocyte maturation were performed to determine the EC_{50} values (effective concentration of hormone to induce GVBD in 50% of the oocytes) for oocytes taken from unprimed (not injected with pregnant mare serum gonadotropin, PMSG) animals. Previous studies used oocytes from PMSG-primed animals reported an EC_{50} of 1-7 x 10^{-8} M for insulin and 2-8 x 10^{-10}M for IGF-1 (El-Etr *et al.*, 1979; Maller and Koontz, 1981; Hainaut *et al.*, 1991). Oocytes were incubated in MBS for 14-18 hours in concentrations of insulin or IGF-1 ranging from 1x10^{-4}M to 1x10^{-11}M and scored according to the percent that achieved germinal vesicle breakdown (%GVBD). As shown in Figure 1, the EC_{50} for insulin was 5x 10^{-8}M and the EC_{50} values ranged from 2-8 x 10^{-8}M. As shown in Figure 2, a similar analysis was done with IGF-1, and produced an EC_{50} of 3 x 10^{-10}M and ranged from 1-7 x 10^{-10}M. From these results, insulin at a concentration of 1 x 10^{-5}M was used in all subsequent experiments to achieve 90-100% GVBD. Previous reports indicated that, in a variety of other cell systems, the mitogenic effects of insulin at high concentrations are mediated by insulin binding to IGF receptors (Zapf *et al.*, 1975; Zapf *et al.*, 1978a, b; Rinderknecht and Humbel, 1976; Megyesi *et al.*, 1974). It appeared that most if not all of the effects of both

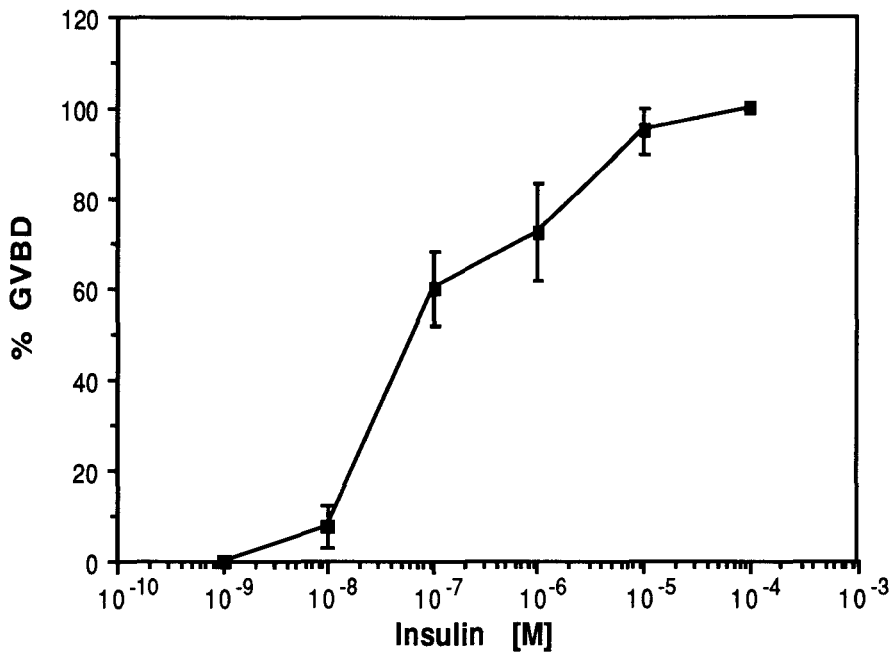


Figure 1. Dose response curve for insulin-induced oocyte maturation. Oocytes were incubated for 14-18 hours in MBS medium at 20°C with concentrations of insulin ranging from $1x10^{-4}$M to $1x10^{-11}$M (10 oocytes/concentration/animal) and scored according to the percent that achieved germinal vesicle breakdown (% GVBD). The insulin EC_{50} (effective concentration to achieve 50% GVBD) was $5x10^{-8}$M. The data are expressed as the mean ± S.E. of the mean, where n=4 animals. Data points without error bars represent data that were identical for all experiments.

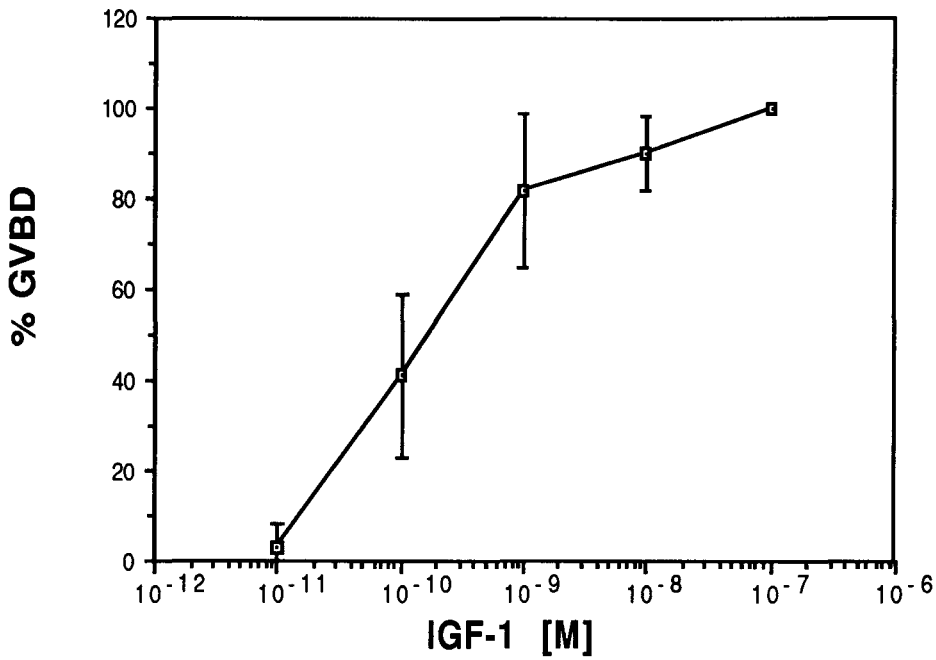


Figure 2. Dose response curve for IGF-1 (insulin like growth factor-1) induced oocyte maturation. Oocytes were incubated for 14-18 hours in MBS at 20°C in concentrations of IGF-1 ranging from $1x10^{-7}M$ to $1x10^{-11}M$ (10 oocytes/concentration/animal) and scored according to the percent that achieve germinal vesicle breakdown (% GVBD). The EC_{50} for IGF-1 (effective concentration to achieve 50% GVBD) was $3x10^{-10}M$. The data are expressed as the mean ± S.E. of the mean, where n=4 animals. Data points without error bars represent data that were identical for all experiments.

insulin and IGF-1 in *Xenopus* oocytes were exerted through the IGF-1 receptor (Janicot and Lane, 1989). Therefore all subsequent experiments were done with insulin instead of IGF-1 because insulin was 6000 times less costly than IGF-1.

B. Kinetics of GVBD in Insulin vs. Progesterone-Treated Oocytes

It has been reported that the timing of oocyte maturation was faster in oocytes that were stimulated with progesterone than in oocytes that were stimulated with insulin (El-Etr *et al.*, 1979; Stith and Maller, 1984). However, their studies were done on primed (injected with PMSG) animals prior to insulin treatment. Therefore it was necessary to determine if these same results could have been confirmed using oocytes from unprimed animals. Oocytes were treated with either insulin or progesterone within 1 to 2 hours after they were isolated from the animal. Figure 3 shows that progesterone-treated oocytes achieved germinal vesicle breakdown (GVBD) 2 hours faster than did the insulin-treated oocytes. This experiment was repeated 8 times (see Part II), and in each case the kinetics of GVBD in progesterone-treated oocytes was always faster than in the insulin-treated oocytes. These data confirmed previous reports and therefore seemed to be reliable. In some cases, insulin-stimulated oocytes took 2-4 hours longer to achieve GVBD than did progesterone-stimulated oocytes.

C. RNA Synthesis Requirement for Insulin-Induced GVBD

One potential reason why insulin took longer than progesterone to induce GVBD may have been due to the requirement for extra biochemical steps in the insulin pathway. Previous reports indicated that treatment of oocytes with Actinomycin D inhibits hCG-induced GVBD, but did not inhibit progesterone-induced GVBD. Actinomycin D at

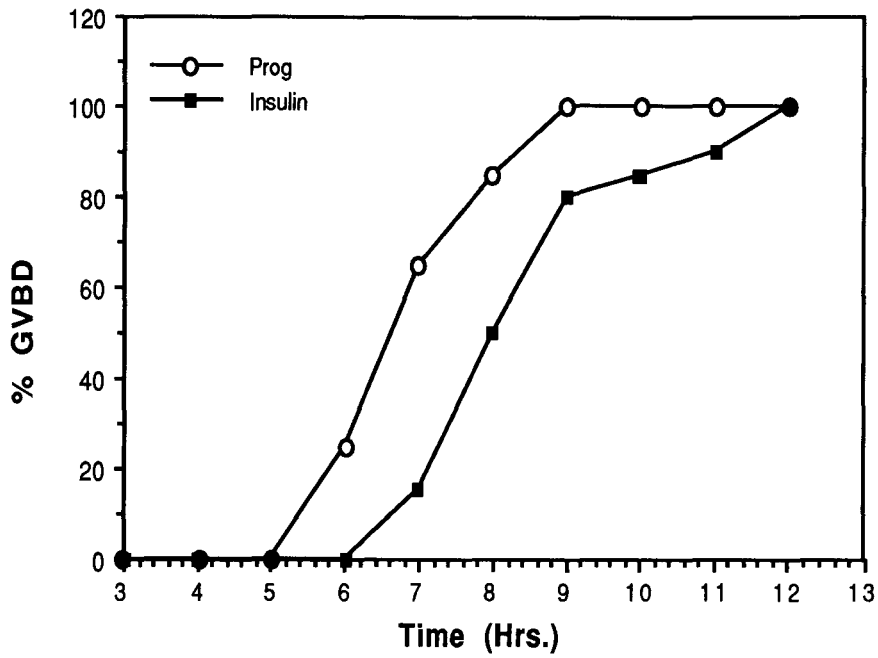


Figure 3. Kinetics of GVBD in progesterone- or insulin-treated oocytes. Oocytes freshly isolated from the animal were incubated for 12 hours at 20°C in MBS medium containing either insulin ($1x10^{-5}M$) or progesterone ($1x10^{-6}M$). Oocytes were scored every hour according to the percent that achieved germinal vesicle breakdown (% GVBD). The data are for 20 oocytes/treatment from one animal.

100 μg/ml inhibits 95% of total oocyte RNA synthesis, but had no effect on progesterone-induced GVBD (Wasserman *et al.*, 1974). Does insulin action require RNA synthesis to achieve GVBD, as is the case with hCG, or is insulin action RNA synthesis-independent, as is the case with progesterone? To answer this question oocytes were pre-treated with Actinomycin D (100 μg/ml) for 2 hours before application of hormones and continuously thereafter. Oocytes were incubated for 14-18 hours in the presence of : insulin ($1x10^{-5}$M) ± Actinomycin D, progesterone ($1x10^{-6}$M) ± Actinomycin D, or human chorionic gonadotropin (hCG) (50 IU/ml) ± Actinomycin D. Figure 4 shows that neither insulin nor progesterone required RNA synthesis to induce GVBD, but hCG did require RNA synthesis to induce GVBD. Therefore, both insulin and progesterone were working at a post-transcription level to induce GVBD.

D. Oocyte pHi Determination and the Kinetics of the pHi Increase

Progesterone-treated oocytes from unprimed animals (not injected with PMSG) underwent an increase in their pHi of 0.4-0.5 pH units. Their pHi increased from a basal intracellular pH of 7.2 - 7.3 to 7.6 - 7.7 (Houle and Wasserman, 1983; Wasserman and Houle, 1984: Wasserman *et al.*, 1984). To determine if insulin also caused an increase in intracellular pH in oocytes from unprimed animals, oocytes were incubated in MBS containing ^{14}C-DMO for 12-14 hours and then stimulated with insulin ($1x10^{-5}$M) or progesterone ($1x10^{-6}$M) for 8 hours. Oocytes were stored in MBS for 12-14 hours prior to treatment because of the inability to carry out the entire experiment in a day. Intracellular pH was determined by the uptake of tracer amounts of the weak acid ^{14}C-dimethyloxazolodine 2,4 dione, (DMO, Figure 5). Figure 6 shows that there was an increase in pHi in both insulin and progesterone-treated oocytes. Both oocyte groups

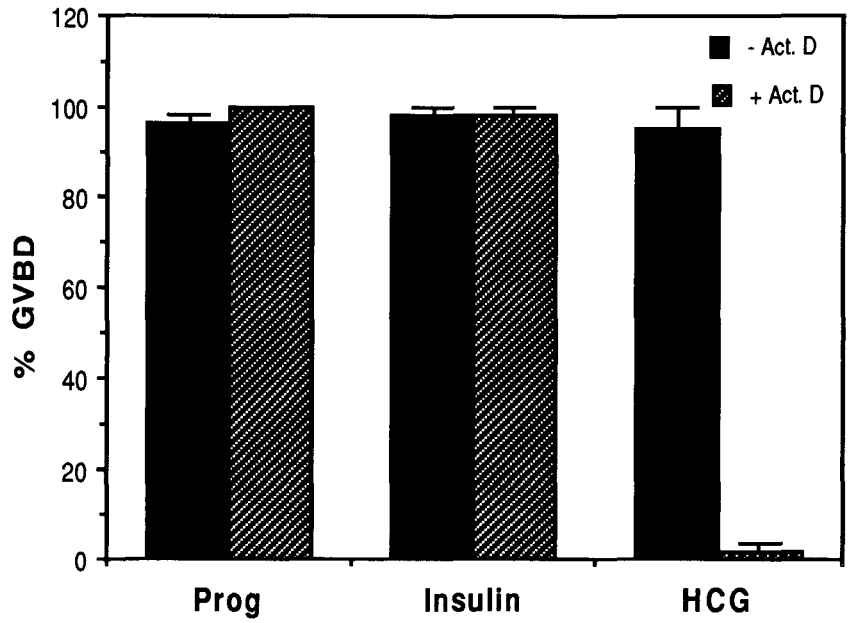


Figure 4. Actinomycin D, an RNA synthesis inhibitor, blocks hCG - but not insulin- or progesterone-induced oocyte maturation. Oocytes were pre-treated with Actinomycin D (100μg/ml) for 2 hours at 20°C before application of hormones and continuously thereafter. Oocytes were incubated in MBS for 14-18 hours at 20°C in the presence of: insulin ($1x10^{-5}$M) ± Actinomycin D, progesterone ($1x10^{-6}$M) ± Actinomycin D, or human chorionic gonadotropin (hCG) (50 IU/ml) ± Actinomycin D. Oocytes (10 oocytes/ treatment/animal) were scored according to the percent that achieved germinal vesicle breakdown (% GVBD). The data are expressed as the mean ± S.E. of the mean, where n=3 animals. Data points without error bars represent data that were identical for all experiments.

exhibited a significant increase from a basal pHi of 7.3-7.4 to 7.7-7.8 at the time of GVBD ($p<0.001$).

Since it appeared that progesterone and insulin induced similar increases in oocyte pHi, the kinetics of the pHi increase were determined for both treatments. Oocytes were incubated in MBS with ^{14}C-DMO for 12-14 hours and then stimulated with progesterone (10^{-6}M) or insulin (10^{-5}M) for 2-6 hours. pHi measurements were determined every 2 hours until the oocytes achieved 100% GVBD. Figure 7 shows that both insulin- and progesterone-treated oocytes had similar trends for the increase in pHi, but insulin may have induced a more rapid increase in pHi.

E. Protein Synthesis Requirement for Insulin-Induced GVBD

There appeared to be a two to threefold increase in the rate of protein synthesis in progesterone-treated oocytes (Wasserman *et al.*, 1982). Furthermore, progesterone-induced GVBD required protein synthesis. Inhibiting protein synthesis with cycloheximide (>10 μg/ml) blocked progesterone-induced GVBD in full-grown stage VI oocytes (Wasserman and Masui, 1975b; Wasserman *et al.*, 1986). In order to determine if insulin-induced GVBD required protein synthesis, oocytes were pre-treated in varying concentrations of cycloheximide (0.001-10 μg/ml) for 2 hours, and then stimulated with insulin (10^{-5}M) or progesterone (10^{-6}M). Oocytes were scored for %GVBD after a 14-hour incubation period. Figure 8 shows that both insulin and progesterone required protein synthesis to induce GVBD. Cycloheximide inhibited GVBD in insulin-treated oocytes with an IC_{50} (concentration that inhibits 50% of the oocytes) of 0.09 μg/ml and progesterone-treated oocytes with an IC_{50} of 0.2 μg/ml.

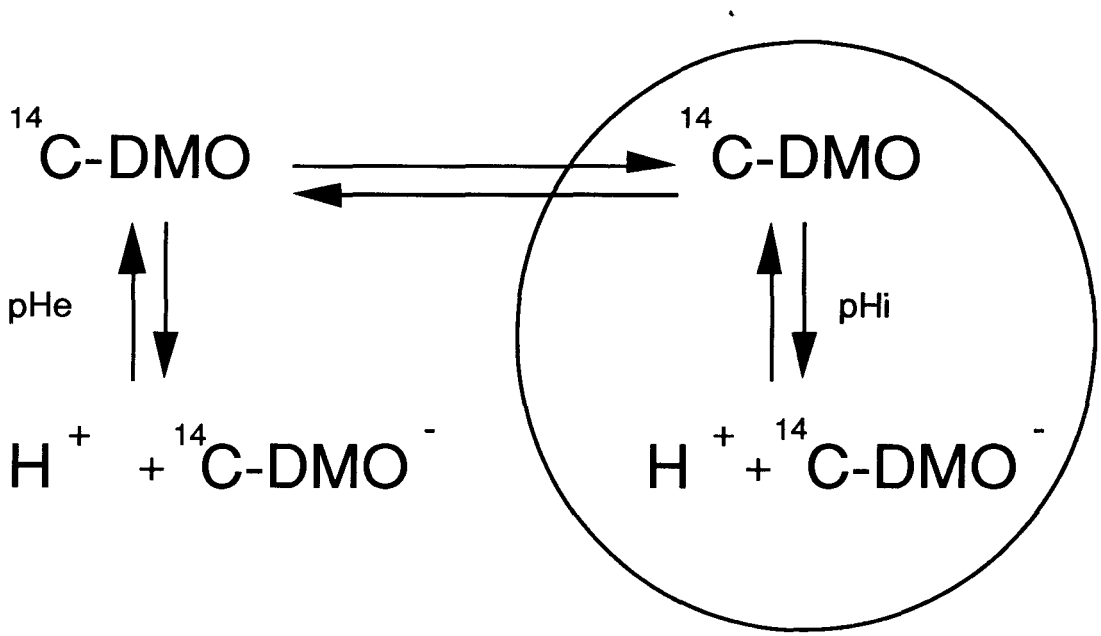


Figure 5. DMO equilibration across the oocyte plasma membrane. Oocytes were incubated in MBS medium containing ^{14}C-DMO (0.25μCi/ml) at 20°C to allow for equilibration. The equilibrium of DMO across the cell membrane will be established according to the extracellular pH (pHe) and intracellular pH (pHi) environments.

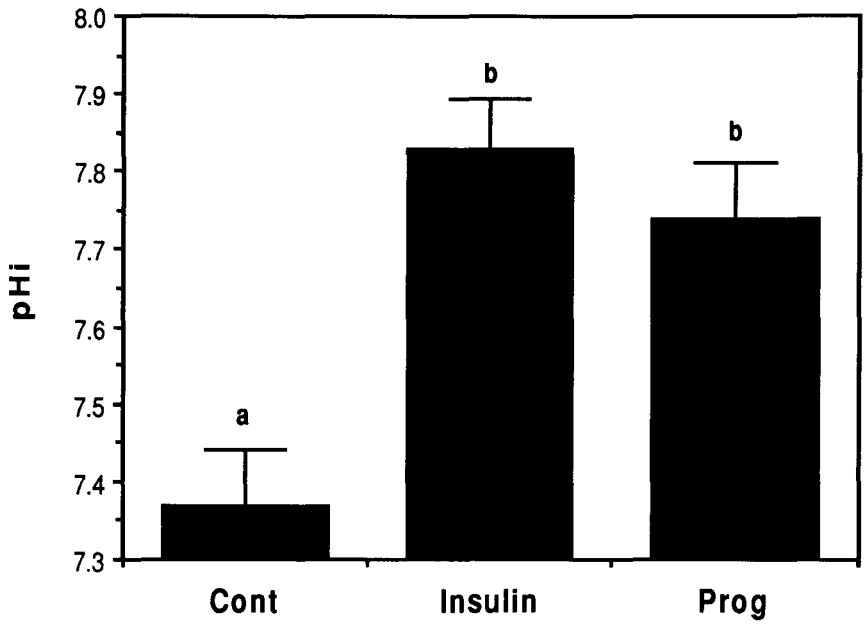


Figure 6. Insulin and progesterone induce a similar increase in oocyte intracellular pH. Oocytes were incubated in MBS containing ^{14}C-DMO and MBS for 12-14 hours at 20°C. Oocytes (15 oocytes/treatment/animal) were either left untreated (control), or treated with insulin ($1x10^{-5}$M) or progesterone ($1x10^{-6}$M) for 8 hours. The concentration of ^{14}C-DMO inside the oocyte cytoplasm and in the external medium was used to calculate the intracellular pH. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are expressed as the mean ± S.E. of the mean, where n=3 animals.

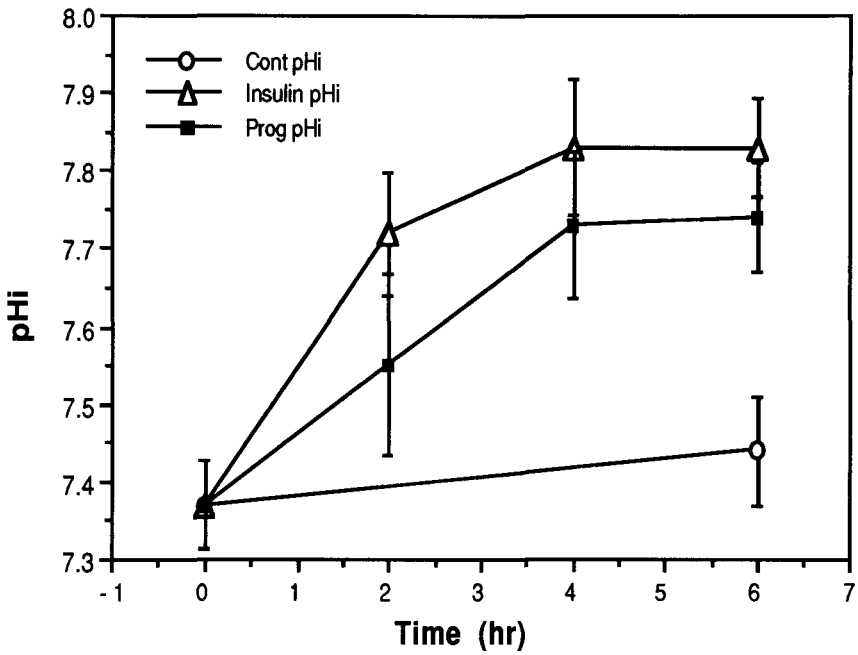


Figure 7. Insulin and progesterone induce an increase in oocyte intracellular pH with similar kinetics. Oocytes (15 oocytes/treatment/animal) were incubated in MBS medium containing ^{14}C-DMO for 12-14 hours at 20°C. Oocytes were then either left untreated (control), or treated with insulin ($1x10^{-5}$M) or progesterone ($1x10^{-6}$M) and incubated for an additional 0, 2, 4, or 6 hours. The concentration of ^{14}C-DMO inside the oocyte cytoplasm and in the external medium was used to calculate the intracellular pH. The data are expressed as the mean ± S.E. of the mean, where n=3 animals.

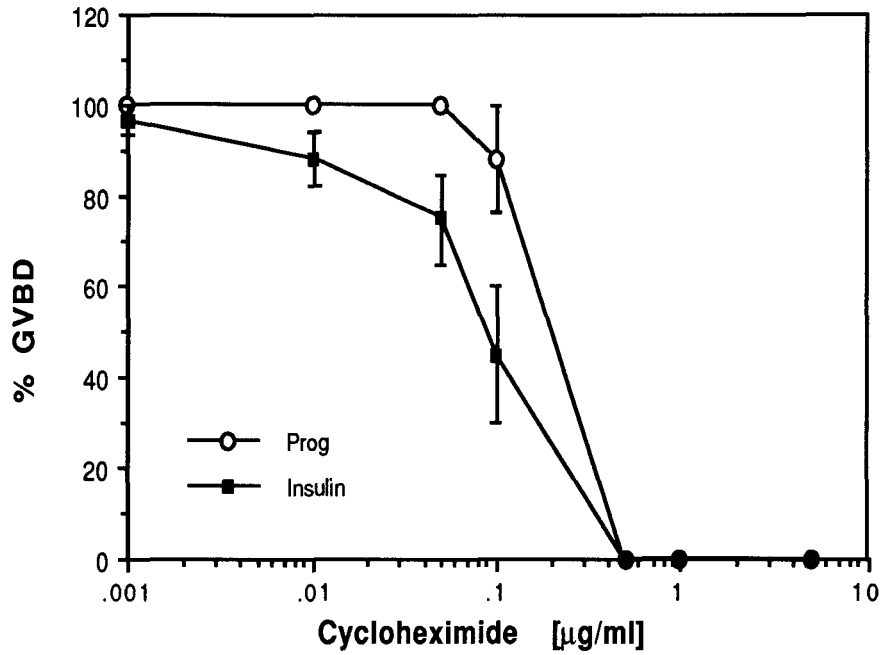


Figure 8. Cycloheximide, a protein synthesis inhibitor, blocks GVBD in both insulin- and progesterone-treated oocytes. Oocytes (20 oocytes/treatment/animal) were pre-treated in MBS medium containing cycloheximide (0.001-10 µg/ml) for 2 hours at 20°C. Progesterone ($1x10^{-6}$M) or insulin ($1x10^{-5}$M) was then added and incubated for 12-14 hours. Oocytes were then scored according to the percent that achieved germinal vesicle breakdown (% GVBD). Cycloheximide had a significant inhibitory effect on insulin- and progesterone-induced oocyte maturation ($p<0.001$). The data are expressed as the mean ± S.E. of the mean, where n=3 animals. Data points without error bars represent data that were identical for all experiments.

F. Rate of Protein Synthesis in Insulin-Treated Oocytes

A two to threefold increase in the rate of protein synthesis had been reported for progesterone-treated oocytes (Wasserman *et al.*, 1980). Did insulin treatment also cause a similar increase in oocyte protein synthesis? To answer this question oocytes were incubated in MBS for 12-14 hours and then treated with no hormone (control), progesterone (10^{-6}M), or insulin (10^{-5}M). At 2, 4, and 6 hours after hormone addition oocytes were microinjected with 20 nl of ^{3}H-leucine ($4.4x10^{5}$ dpm/oocyte), and pulse labelled for 10 minutes. Total protein synthesis was measured by calculating the % of total ^{3}H-leucine within the oocyte that was incorporated into newly synthesized protein. Figure 9 shows that total protein synthesis in insulin-stimulated oocytes was significantly different from the control ($p<0.001$), but was similar to the increase in total protein synthesis in progesterone-treated oocytes ($p>0.05$). Furthermore, the increase in protein synthesis appeared to occur with similar kinetics under both hormonal treatments (Figure 9).

G. Two Dimensional Gel Analysis of Proteins Synthesized in Insulin-Treated Oocytes

It appeared that insulin and progesterone were similar in their requirements for protein synthesis and rates of total protein synthesis. The progesterone-induced increase in protein synthesis has been shown to be mostly due to the increased synthesis of the same proteins made in prophase-arrested oocytes (Ballantine et al., 1982; Wasserman *et al.*, 1982), but four or five new peptides may have been synthesized (Younglai *et al.*, 1981, 1982). Does insulin induce a similar increase in the same types of proteins as progesterone? To answer this question, oocytes were treated with no hormone (control), progesterone (10^{-6}M), or insulin (10^{-5}M) and then microinjected with ^{35}S-methionine at one-hour intervals from the time of hormone addition (time zero) to 100% GVBD. Figure

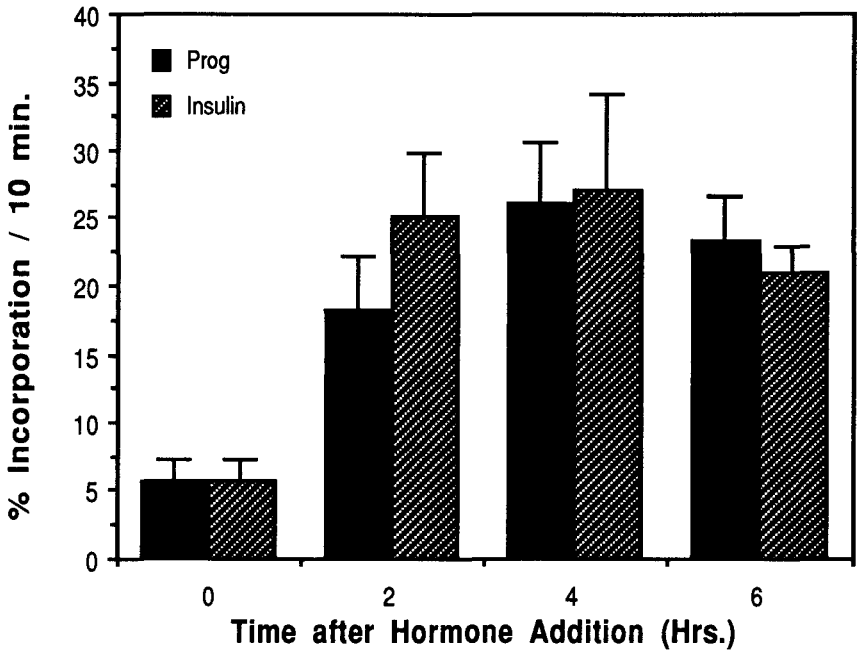


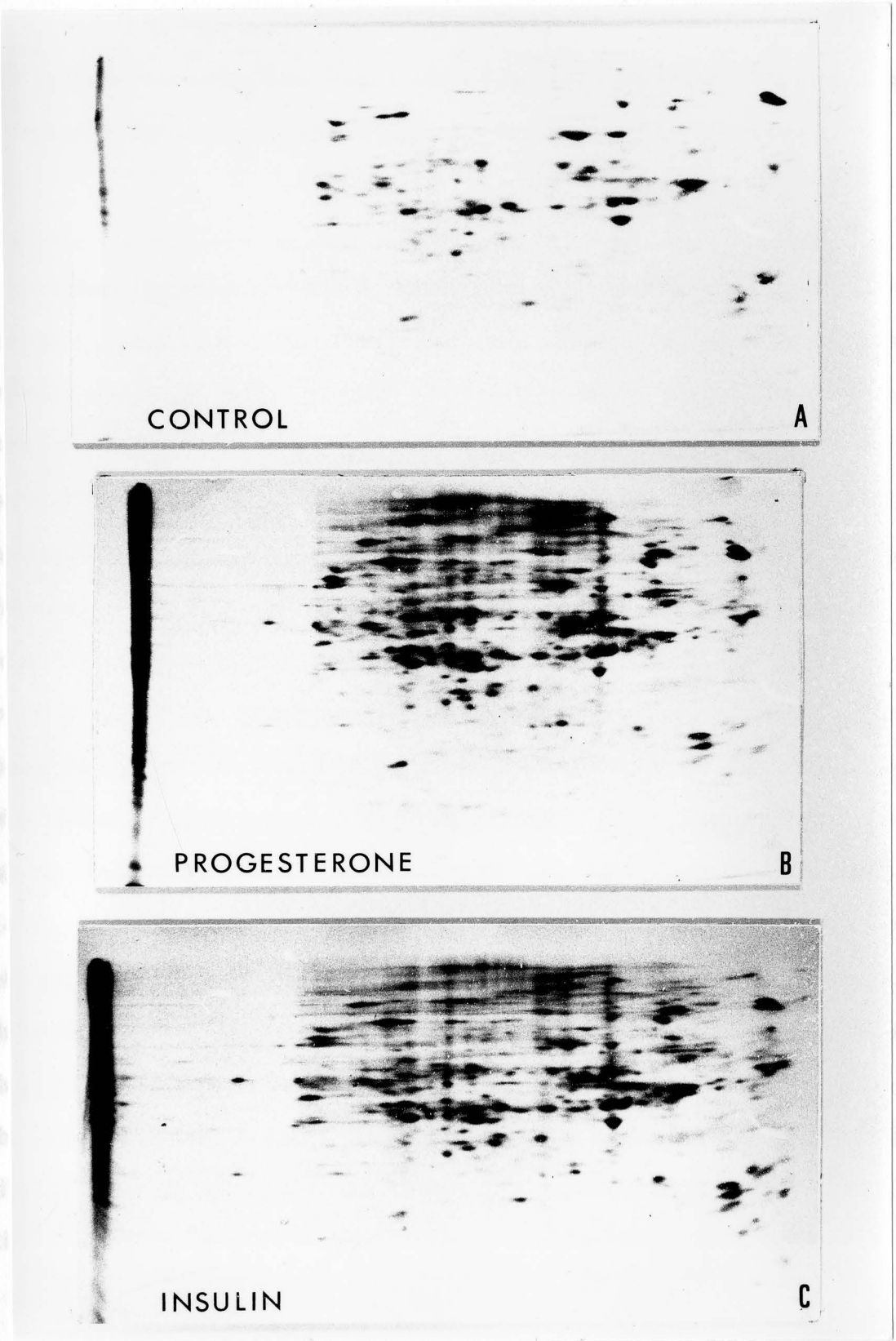
Figure 9. Rates of protein synthesis (% incorporation/10 min. pulse) are similar in insulin- and progesterone-treated oocytes. Oocytes were stored in MBS medium for 12-14 hours and then treated with insulin ($1x10^{-5}M$) or progesterone ($1x10^{-6}M$) for 0 to 6 hours. At 0, 2, 4, or 6 hours oocytes (6 oocytes/treatment/animal) were microinjected with ^{3}H-leucine and their rates of protein synthesis (expressed as % incorporation per 10 min. pulse) were measured. Total protein synthesis in insulin-stimulated oocytes is significantly different from the control ($p<0.001$), but is similar to the increase in total protein synthesis in progesterone treated oocytes ($p>0.05$). Both insulin and progesterone stimulated protein synthesis with similar kinetics. The data are expressed as the mean ± S.E. of the mean, where n=3 animals.

10 shows the 2-D gel profiles of newly synthesized proteins for control oocytes (Figure 10A), insulin-treated oocytes (Figure 10B), and progesterone-treated oocytes (Figure 10C) at the time of GVBD. The 2-D gel protein profiles were compared via visual inspection. Insulin and progesterone appeared to induce a similar array of newly synthesized proteins. There did not appear to be any additional proteins synthesized in insulin-treated oocytes that did not also appear in progesterone-stimulated oocytes. Two Dimensional gel protein patterns were examined for every hour of maturation up to GVBD , and again there appeared to be no differences between the two hormone treatments (data not shown).

H. Quantitation of Protein Phosphorylation in Insulin-Treated Oocytes

Once it had been determined that protein synthesis appeared to be similar for insulin- and progesterone-treated oocytes, the next step was to analyze the effects of the hormones on stimulating protein phosphorylation. A two to threefold increase in total protein phosphorylation had been measured in progesterone-treated oocytes (Maller, 1977). To examine whether insulin-induced maturation is also associated with this same increase in protein phosphorylation, oocytes were labelled in MBS medium containing ^{32}P-orthophosphate at 50 μCi/ml from addition of hormone (time zero) to GVBD. Oocytes were removed from labelling every hour after the addition of hormones. The % of total ^{32}P-orthophosphate taken up by the oocytes that was incorporated into newly phosphorylated proteins was calculated for the control (no hormone), insulin- and progesterone-treated oocytes. As Figure 11 shows there was a significant difference in the level of protein phosphorylation between the control and the insulin-treated oocytes ($p<0.001$), and between the control and progesterone-treated oocytes ($p<0.001$). However, there is no significant difference in the level of protein phosphorylation between

Figure 10. 2D gel electrophoretic analysis of newly synthesized proteins in control, insulin and progesterone-treated oocytes. Oocytes (8 oocytes/treatment/hour/animal) were stored in MBS for 12-14 hours and then treated with either A) no hormone (control), B) progesterone ($1x10^{-6}M$), or C) insulin ($1x10^{-5}M$). At 0, 1, 2, 3, 4, 5, 6 (data not shown), or 7 hours after hormone addition the oocytes were microinjected with ^{35}S-methionine and incubated for one hour. Proteins were extracted and analyzed by gel isoelectric focusing (IEF). The first-dimension gel was then applied to a second-dimension 10% SDS PAGE. After electrophoresis fluorography was performed. There was no qualitative difference in the proteins synthesized by progesterone and insulin-treated oocytes.


CONTROL
A
PROGESTERONE
B
INSULIN
C

the progesterone- and insulin-treated oocytes ($p>0.05$). Therefore, both insulin- and progesterone-induced a two to threefold increase in total protein phosphorylation.

I. Electrophoretic Analysis of Phosphorylated Proteins in Insulin-Treated Oocytes

Insulin-treated oocytes exhibit the same two to threefold increase in total protein phosphorylation as progesterone-treated oocytes. However, it had to be determined whether insulin also caused the same proteins to be phosphorylated as progesterone. To examine the protein phosphorylation profiles in insulin- and progesterone-treated oocytes, oocytes were labelled in MBS medium with ^{32}P-orthophosphate at 100 μCi/ml and treated with no hormone (control), insulin (10^{-5}M), or progesterone (10^{-6}M) from time zero (addition of hormone) until 100% GVBD (9 hours). Oocytes were removed from the respective treatment and proteins were isolated at hours 1,3,5,7,9. Figure 12 shows a side by side comparison of the phosphorylated protein profiles. Columns 1-5 show phosphorylated proteins isolated from insulin-stimulated oocytes, and columns 6-10 show phosphorylated proteins from progesterone-stimulated oocytes. Size comparisons of phosphorylated proteins were made relative to ^{14}C-methylated protein markers: lysozyme, 14,300; carbonic anhydrase, 30,000; ovalbumin, 46,000; bovine serum albumin, 69,000; phosphorylase b 92,500; myosin, 200,000. Examination via visual inspection indicated that both insulin- and progesterone-stimulated similar types of protein phosphorylation and there did not appear to be any significant difference between the two treatments. A few of the proteins that were phosphorylated in both treatments are: H-1 histone, 22,000-25,000; ribosomal protein S6, 32,000; mos kinase, 39,000; MAP kinase, 42,000-44,000; S6 kinase, 92,000 (based on previous studies done on progesterone-treated oocytes).

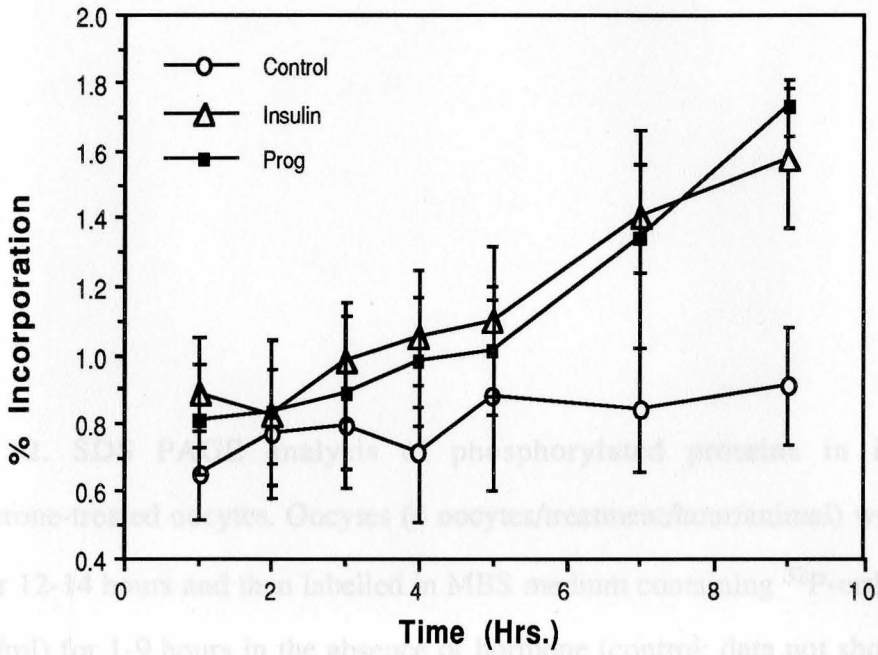
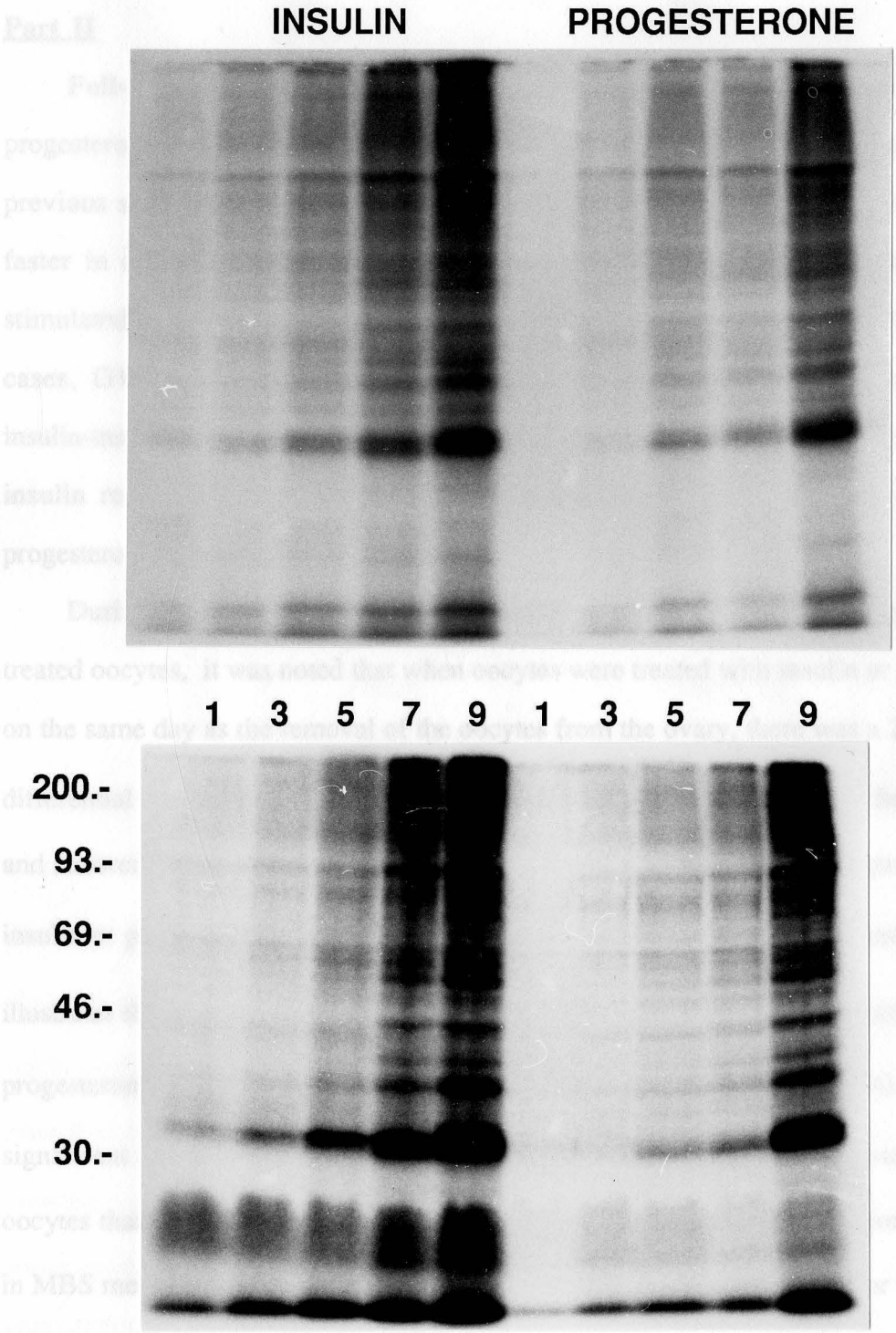


Figure 11. Both insulin and progesterone stimulate protein phosphorylation in *Xenopus* oocytes. Oocytes were stored in MBS medium for 12-14 hours at 20°C. They were then labelled in MBS medium containing ^{32}P-orthophosphate (50 μCi/ml) for 1 to 9 hours in the absence of hormone (control), or treated with insulin ($1x10^{-5}$M) or progesterone ($1x10^{-6}$M). The % of total ^{32}P-orthophosphate taken up by the oocytes (8 oocytes/treatment/hour/animal) that was incorporated into newly phosphorylated proteins was calculated at hours 1, 2, 3, 4, 5, 7, and 9. Insulin- and progesterone-stimulated oocytes exhibited a similar increase in the level of total protein phosphorylation with similar kinetics. There is no significant difference in the levels of protein phosphorylation within insulin- and progesterone-treated oocytes ($p>0.05$). The data are expressed as the mean ± S.E. of the mean, where n=3 animals.

Figure 12. SDS PAGE analysis of phosphorylated proteins in insulin and progesterone-treated oocytes. Oocytes (4 oocytes/treatment/hour/animal) were stored in MBS for 12-14 hours and then labelled in MBS medium containing ^{32}P-orthophosphate (50 μCi/ml) for 1-9 hours in the absence of hormone (control; data not shown), insulin ($1x10^{-5}$M), or progesterone ($1x10^{-6}$M). At 1, 3, 5, 7, and 9 hours after hormone addition proteins were extracted and analyzed by one-dimesional SDS PAGE. There was no qualitative difference in the proteins phosphorylated in insulin and progesterone-treated oocytes.


INSULIN
PROGESTERONE
1 3 5 7 9 1 3 5 7 9
200.-
93.-
69.-
46.-
30.-

Part II

Full-grown *Xenopus laevis* oocytes undergo meiotic maturation in response to progesterone stimulation, and in response to insulin and IGF-1 stimulation. Some of these previous studies reported that the timing of germinal vesicle breakdown (GVBD) was faster in oocytes that were stimulated with progesterone than in oocytes that were stimulated with insulin or IGF-1 (El-Etr *et al.*, 1979; Stith and Maller, 1984). In some cases, GVBD took place 4 hours faster in progesterone-treated oocytes than in insulin-treated oocytes (Figure 3). This time differential seemed to indicate that perhaps the insulin response pathway involved one or more steps that are not required by the progesterone pathway leading to GVBD.

During our investigation of the physiological changes in progesterone and insulin-treated oocytes, it was noted that when oocytes were treated with insulin or progesterone on the same day as the removal of the oocytes from the ovary, there was a 2-4 hour time differential for $GVBD_{50}$ (Figure 3). However if the oocytes were removed from the ovary and allowed to incubate for 12-14 hours in MBS medium and then stimulated with either insulin or progesterone, the time differential to achieve $GVBD_{50}$ disappeared. Figure 13 illustrates the time of $GVBD_{50}$ for oocytes that were treated with either insulin (10^{-5}M), or progesterone (10^{-6}M) shortly after they were isolated from the animal (Fresh). There was a significant time difference between the $GVBD_{50}$ for insulin- and progesterone-treated oocytes that were freshly isolated ($p<0.001$). Oocytes that were incubated for 12-14 hours in MBS medium (Overnight) and then treated with either insulin (10^{-5}M) or progesterone (10^{-6}M), and had no significant time difference between the $GVBD_{50}$ for insulin and progesterone-treated oocytes ($p>0.50$).

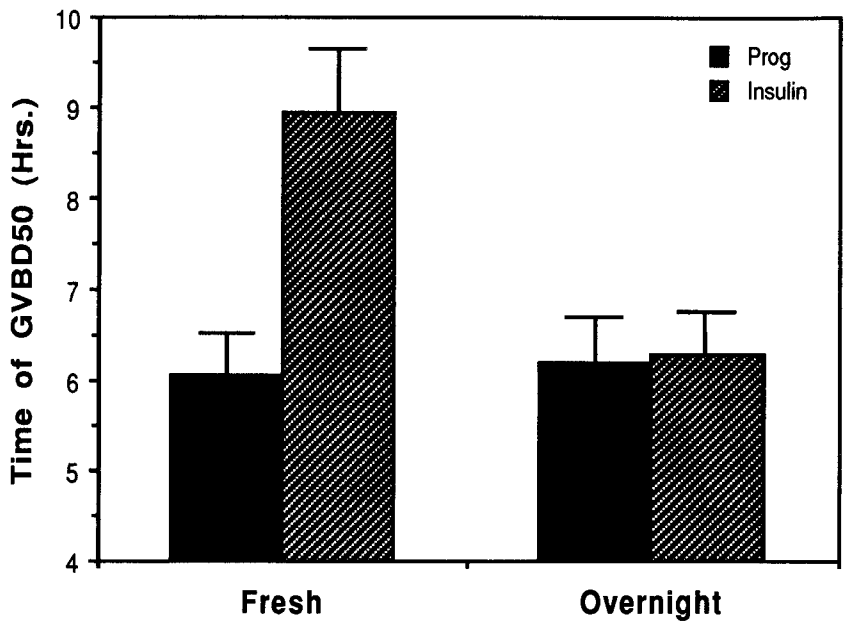
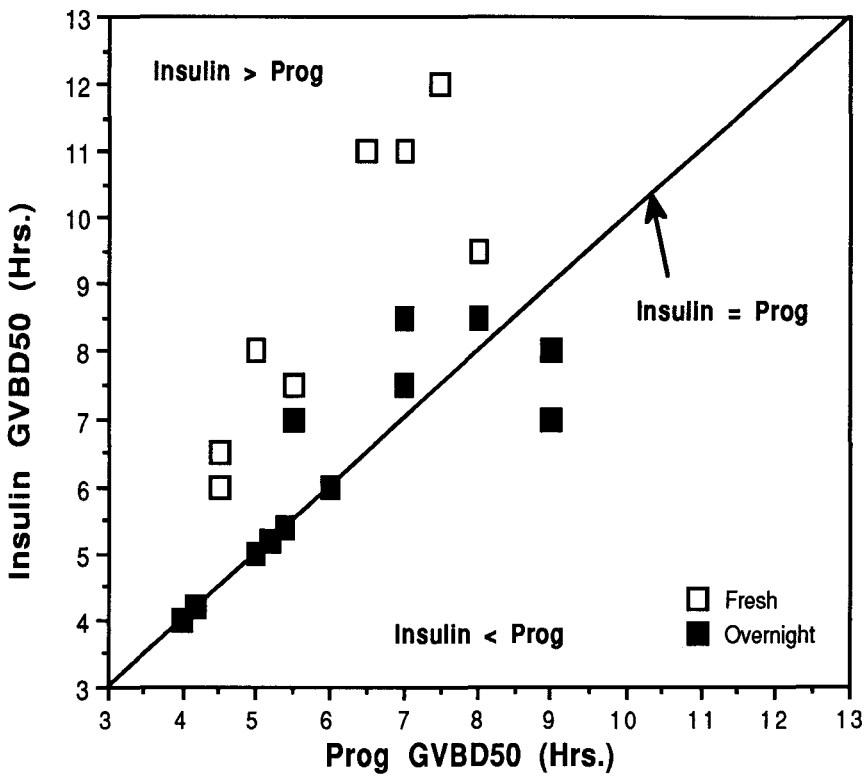


Figure 13. Storage time in MBS medium affects the timing of $GVBD_{50}$ in insulin-stimulated oocytes. Oocytes that were recently isolated from animals (Fresh) were incubated in MBS medium at 20°C with insulin ($1x10^{-5}M$) or progesterone ($1x10^{-6}M$) and scored every hour for the percent GVBD, and the time for $GVBD_{50}$ was determined. Additional oocytes were stored in MBS medium for 12-14 hours (Overnight), and then treated with insulin ($1x10^{-5}M$) or progesterone ($1x10^{-6}M$). Oocytes (20 oocytes/treatment/hour/animal) were scored every hour for the percent GVBD and $GVBD_{50}$ timing determined. Freshly isolated oocytes took 2 to 4 hours longer to respond to insulin than to progesterone. Oocytes that were stored in MBS medium for 12-14 hours prior to hormone addition no longer exhibited this time differential. The data are expressed as the mean ± S.E. of the mean, where n=8 animals (Fresh) and n=12 animals (Overnight).

Figure 14 shows the timing of $GVBD_{50}$ for insulin- and progesterone-treated oocytes for all experiments in which oocytes were treated from either recently isolated oocytes (Fresh) or oocytes stored for 12-14 hours (Overnight). Figure 14 shows the timing of $GVBD_{50}$ for 8 experiments in which freshly isolated oocytes were treated with insulin or progesterone, and the timing of $GVBD_{50}$ for 12 experiments in which the oocytes were treated with insulin or progesterone after the oocytes were first stored for 12-14 hours in MBS medium.

Figure 14. Storage time in MBS medium affects the timing of GVBD in insulin stimulated oocytes. Recently isolated oocytes (Fresh) or oocytes that were stored overnight in MBS (Overnight) were incubated in MBS medium at 20°C with insulin ($1x10^{-5}M$) or progesterone ($1x10^{-6}M$) (20 oocytes/treatment/hour/animal) and scored every hour for the percent GVBD and the timing for $GVBD_{50}$ was determined. Data points that fall directly on the diagonal line indicate those experiments where insulin and progesterone took the same time to induce GVBD. Data points above the diagonal line indicate those experiments where insulin took longer than progesterone to induce GVBD. Data points below the diagonal indicate cases where progesterone took longer than insulin. Freshly isolated (Fresh) oocytes took 2 to 4 hours longer to respond to insulin than to progesterone (❑). Oocytes that were stored in MBS medium for 12-14 hours (Overnight) prior to hormone addition no longer exhibited this time differential (■). The data are expressed as the mean ± S.E. of the mean, where n=8 animals (Fresh) and n=12 animals (Overnight).


Insulin > Prog
Insulin = Prog
Insulin < Prog
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Prog GVBD50 (Hrs.)
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CHAPTER IV

DISCUSSION

PART I

All vertebrate oocytes must undergo meiotic maturation before they can be fertilized by sperm. Oocyte maturation not only involves the meiotic reductive divisions, but it also includes a number of physiological changes that prepare the oocyte for fertilization and subsequent embryonic development. Many of these physiological changes within the oocyte are similar in all vertebrate species that have been examined.

Full-grown *Xenopus lævis* oocytes undergo meiotic maturation in reponse to progesterone stimulation (Maller, 1983; Wasserman *et al.*, 1986). Progesterone-treated oocytes undergo a series of physiological changes which include: a transient increase in the level of intracellular ionized calcium, a drop in the intracellular level of cAMP, an increase in the intracellular pH, an increase in the absolute rate of protein synthesis, and an increase in total protein phosphorylation.

Several labs have shown that insulin-like growth factor-1 (IGF-1) and insulin can mimic progesterone by inducing meiotic maturation in *Xenopus* oocytes (Baulieu, 1984; El-Etr *et al.*, 1979; Maller and Koontz, 1981; Davis and Sadler, 1992). It was noted however, that GVBD in progesterone-treated oocytes occured faster than in insulin-treated oocytes (El-Etr *et al.*, 1979; Stith and Maller, 1984). In some cases, GVBD took place 4 hours earlier in progesterone-stimulated oocytes than in insulin-stimulated oocytes. Therefore, it was believed that perhaps oocytes treated with insulin require an additional

step or steps to achieve GVBD that are not required in progesterone-stimulated oocytes. One lab hypothesized that the insulin and progesterone oocyte response pathways differ at some point between the increase in intracellular pH and GVBD, and this accounts for the timing differential (Stith and Maller, 1984). However, a systematic comparison of the physiological changes in insulin- and progesterone-treated oocytes leading to GVBD had not been investigated. In addition, other labs investigated insulin-induced oocyte maturation using animals that had been primed with PMSG prior to insulin treatment of the oocytes *in vitro* (Maller and Koontz, 1981; Stith and Maller, 1984; El-Etr *et al.*, 1979; Davis and Sadler, 1992). Priming may alter certain physiological responses within the oocytes and also affect the timing of the response pathways. Therefore, it was necessary to perform our investigation on oocytes taken from unprimed animals since the timing of events in the meiotic maturation pathway were critical to our investigation.

A number of physiological changes that were known to take place in progesterone-treated oocytes were analyzed in insulin-treated oocytes. The goal was to compare these changes in progesterone and insulin-treated oocytes to determine how similar or different the responses were and to determine the reason for the time differential in the oocytes' response to the two hormone treatments.

A. Dose Response Curves for Insulin and IGF-1:

Dose response curves were performed for insulin- and IGF-1-induced oocyte maturation to determine the dose that would be used in all subsequent experiments. Previous studies had used oocytes from primed animals, which may alter the effective concentration of insulin and/or IGF-1 needed to induce maturation. In fact, there was a

slight difference in the EC_{50} for insulin-treated oocytes taken from primed or unprimed animals. Several labs using primed animals reported EC_{50} values that ranged from $1x10^{-8}M$ to $6x10^{-8}M$ for insulin (El-Etr *et al.*, 1979; Maller and Koontz, 1981; Hainaut *et al.*, 1991). Our data showed a mean insulin EC_{50} of $5x10^{-8}M$, which falls in the upper range of the previous reports, indicating that a slightly higher concentration of insulin may be required to stimulate oocytes taken from unprimed animals. Priming animals with PMSG is known to potentiate the oocytes' response to progesterone. Priming may do the same for insulin-treated oocytes.

B. Kinetics of GVBD in Insulin- vs. Progesterone-Treated Oocytes

In determining the EC_{50} for insulin-induced oocyte maturation it was noted that a time differential existed between the amount of time it took insulin to induce GVBD and progesterone to induce GVBD. Thus, an analysis of the GVBD kinetics for the two hormones was performed. In these experiments oocytes were isolated from the animals and immediately treated with insulin or progesterone. This protocol was repeated 8 times and in each case it was noted that the insulin-treated oocytes took 2 to 4 hours longer to achieve GVBD than did the progesterone-treated oocytes. Our data using freshly isolated oocytes from unprimed animals confirmed previous reports that the timing of GVBD was faster in oocytes that were stimulated with progesterone than in oocytes that were stimulated with insulin (El-Etr *et al.*, 1979; Stith and Maller, 1984). Priming animals with PMSG does not appear to be the reason for this time differential. Therefore, some other physiological event or events must be responsible for this timing difference.

C. RNA Synthesis Requirement for Insulin-Induced GVBD

One potential reason why insulin took longer than progesterone to induce GVBD may be due to the possibility that insulin required new transcription within the oocyte to induce GVBD. Previous studies had already shown that progesterone-treated oocytes do not require RNA synthesis to achieve GVBD, but that hCG-treated oocytes do require RNA synthesis in the follicle cells that surround the full grown oocyte to induce GVBD (Wasserman and Masui, 1974). Actinomycin D, an RNA synthesis inhibitor, did not block GVBD in oocytes stimulated with either insulin or progesterone, but did block oocytes stimulated with hCG. Therefore, there was no apparent difference in the RNA synthesis requirement for progesterone and insulin-induced oocyte maturation. In previous experiments it was demonstrated that even when the nucleus of a progesterone-treated oocyte was removed, cytoplasmic events associated with maturation still occured (Masui and Markert, 1971; Wasserman *et al.*, 1980). Therefore, progesterone is working at a post-transcriptional level to induce oocyte maturation. Thus, it appears that insulin is also working at a post-transcriptional level, and any proteins that need to be synthesized are translated from maternal RNAs that are stored in the oocyte cytoplasm during oogenesis. Therefore, RNA synthesis cannot account for the timing differential in the oocytes' response to the two hormones.

D. Oocyte pHi Determination and the Kinetics of the pHi Increase

An increase in intracellular pH from a basal pHi of 7.2-7.3 to 7.6-7.7 has been reported for progesterone-stimulated oocytes (Houle and Wasserman, 1983; Stith and Maller, 1984). Our data support these previous reports. The intracellular pH in

progesterone-treated oocytes is critical for the timing of GVBD. If the increase in pHi reaches 7.7, then GVBD takes place in approximately 6 hours. If the pHi is kept at 7.2, however, then GVBD takes place in 12 hours (Wasserman *et al.*, 1986). It had also been reported that insulin-stimulated oocytes taken from a single primed animal underwent an increase in the intracellular pH (Stith and Maller, 1984). If the increase in pHi was slower in insulin-treated oocytes than in progesterone-treated oocytes, then this could account for the slower oocyte response to insulin. Our data using oocytes from unprimed animals also show that insulin can induce an increase in oocyte pHi. However, Stith and Maller (1984) reported that insulin was slower than progesterone in inducing GVBD, but both hormones were able to increase the oocyte intracellular pH at essentially the same rate. They speculated that the increased time required for insulin-induced maturation was due to a maturational event following the increase in pHi. We found that the increase in pHi in insulin or progesterone-treated oocytes occured with similar kinetics. In fact, in some instances, insulin induced an increase in oocyte pHi more rapidly than progesterone did.

Unexpectedly, during the course of our pHi investigations we noted that the time differential for GVBD between the two hormone treatments disappeared. The reason for the timing disappearance seems to be due to the amount of time the oocytes were first stored in MBS medium prior to their being stimulated with insulin or progesterone. During the analysis of pHi, oocytes were removed from the animal and stored in MBS medium containing ^{14}C-DMO for 12-14 hours. When insulin or progesterone was subsequently added (after the 12-14 hour storage time) and the oocyte pHi measured, both insulin and progesterone-treated oocytes underwent GVBD with similar kinetics. (for further discussion see Part II)

Even though the time difference disappeared when the oocytes were stored in MBS

medium, we decided to continue our studies. Our second goal was to compare the physiological changes in insulin- and progesterone-treated oocytes to see how similar or different they were.

E. Protein Synthesis Requirement for Insulin-Induced GVBD

Protein synthesis is required for progesterone-induced GVBD (Wasserman *et al.*, 1982). Inhibiting protein synthesis with cycloheximide completely blocked oocyte maturation (Wasserman and Masui, 1975b; Wasserman *et al.*, 1986). Our cycloheximide results indicate that protein synthesis is also required for insulin-induced GVBD. Furthermore, it appeared that insulin-stimulated oocytes were more sensitive to cycloheximide inhibition than progesterone-stimulated oocytes. The specific reason for this is unknown. Previous studies have shown, however, that oocytes undergo a decrease in their permeability to exogenous substances during oocyte maturation (Wasserman and Masui, 1975). Perhaps this permeability change takes place faster in progesterone-treated oocytes than in insulin-treated oocytes. Therefore a higher external dose of cycloheximide may be required to produce a concentration of cycloheximide within progesterone-treated oocytes equivalent to that found in insulin-treated oocytes.

Mos protein kinase is synthesized from stored maternal mRNA in response to progesterone stimulation (Sagata *et al.*, 1989). Furthermore, the translation of Mos is necessary for both progesterone- and insulin-induced GVBD. The injection of Mos antisense oligonucleotides blocks GVBD in response to both hormones (Sagata *et al.*, 1988, 1989). In fact, Mos is the only protein that must be synthesized to induce GVBD, since the injection of Mos protein into cycloheximide-treated oocytes induces GVBD (Yew *et al.*, 1992). Both insulin and progesterone require the synthesis of p39 Mos kinase to

induce GVBD, while other proteins required for GVBD are already present in the oocyte cytoplasm. Therefore, the inhibition of oocyte maturation by cycloheximide is most likely due to the inhibition of c-Mos synthesis.

F. Rate of Protein Synthesis in Insulin-Treated Oocytes

Both progesterone- and insulin-induced oocyte maturation require protein synthesis. Knowing this, we then wanted to determine if insulin caused an increase in the rate of oocyte protein synthesis as did progesterone. Previous reports had shown that a two to threefold increase in the rate of protein synthesis takes place in progesterone-treated oocytes (Wasserman *et al.*, 1980; Wasserman *et al.*, 1986). Our data indicate that insulin induces a similar two to threefold increase in protein synthesis. In addition, this increase in protein synthesis occurred with similar kinetics in oocytes treated with either hormone. The increase in protein synthesis in progesterone-treated oocytes involves the recruitment of stored mRNA onto polysomes (Richter *et al.*, 1982). It would appear that both insulin and progesterone treatments lead to the "unmasking" (dissociation of RNA binding proteins) and utilization of stored maternal mRNA. A similar recruitment of maternal mRNA has been seen in sea urchin eggs. The utilization of mRNA in sea urchin eggs may be regulated by the intracellular pH. Studies showed that artificially increasing the pHi from 6.9 to 7.4 caused an increase in protein synthesis, and that a change in pHi activates the translational apparatus of the egg. It has been suggested that the alkanization of the sea urchin egg cytoplasm serves to both "unmask" the maternal mRNA and to activate translational initiation factors (Winkler and Steinhardt, 1981; Hille *et al.*, 1985; Danilchik,1986). Perhaps, the increase in pHi measured in progesterone- and insulin-treated *Xenopus* oocytes also leads to the "unmasking" of mRNA and activation of tranlational components.

G. 2D Gel Analysis of Proteins Synthesized in Insulin-Treated Oocytes

It had been reported that the two to threefold increase in total protein synthesis in progesterone-stimulated oocytes was mainly due to the increased synthesis of the same proteins made in prophase-arrested oocytes (Ballantine *et al.*, 1982; Wasserman *et al.*, 1982). However, four or five new peptides may be different (Younglai *et al.*, 1981, 1982). Through careful visual inspection of 2-D gel protein profiles it did not appear that insulin-stimulated oocytes synthesized any proteins that were not also being synthesized in progesterone-stimulated oocytes. Therefore, the same set of maternal mRNAs appeared to be "unmasked" and translated in both progesterone- or insulin-treated oocytes. We could not identify the c-Mos protein on the gels because we do not know its isoelectric point (pI).

H. Quantitation of Protein Phosphorylation in Insulin-Treated Oocytes

Our investigation into oocyte protein synthesis did not indicate any difference between insulin and progesterone treatments. We then examined protein phosphorylation to determine if there was any difference in oocytes treated with either hormone. It had been reported that there is a threefold increase in total protein phosphorylation during progesterone-induced maturation (Maller, 1977). Our data indicated that a similar increase in total protein phosphorylation takes place in insulin-treated oocytes. Furthermore, it appeared that the phosphorylation increase occured with similar kinetics in response to insulin or progesterone. Therefore, a similar increase in protein kinase activity and/or a decrease in protein phosphatase activity takes place in both progesterone- and insulin-treated oocytes. While the fold increase in total protein phosphorylation was similar for progesterone and insulin treatments, it did not indicate whether the same proteins were

being phosphorylated. To answer this question we did an electrophoretic analysis of the proteins that were phosphorylated under both hormone treatments.

I. Electrophoretic Analysis of Phosphorylated Proteins in Insulin-Treated Oocytes

A number of proteins are known to be phosphorylated in progesterone-stimulated oocytes. The main regulator of oocyte maturation is Maturation Promoting Factor (MPF), which consists of p34 kinase and cyclin B. MPF is maintained in prophase-arrested oocytes as inactive pre-MPF by inhibitory phosphorylations (Segata *et al.*, 1993). Following progesterone stimulation, a cascade of protein phosphorylation takes place that leads to the formation of active MPF from pre-MPF. p39 c-mos kinase is synthesized and phosphorylated which then phosphorylates and activates p69 Raf-1 kinase. Raf-1 phosphorylates and activates p45 MAP kinase kinase which in turn phosphorylates and activates p42 MAP kinase. MAP kinase phosphorylates and activates S6 kinase, S6 kinase phosphorylates 40S ribosomes (Posada and Cooper, 1992; Posada *et al.*, 1993; Nebrada and Hunt, 1993). Raf-1 kinase also activates cdc25 which in turn activates p34 kinase which induces GVBD. This protein phosphorylation cascade has been observed in progesterone-stimulated oocytes, but has not been reported yet for insulin-stimulated oocytes. However, careful visual examination of the protein phosphorylation gel profiles indicated that insulin induces the same protein phosphorylation patterns as progesterone. Based solely on a molecular weight comparison of the phosphorylated proteins, both insulin and progesterone appear to turn on a similar kinase phosphorylation pathway. Additional experiments will have to be carried out to verify the kinase cascade in insulin-treated oocytes.

Part II

Other labs have reported that the timing of oocyte maturation was faster in oocytes that were stimulated with progesterone than in oocytes that were stimulated with insulin (El-Etr *et al.*, 1979; Stith and Maller, 1984). In some cases, insulin-stimulated oocytes took 2-4 hours longer to achieve GVBD than did progesterone-stimulated oocytes. Our initial data confirmed these results. When oocytes were treated with either insulin or progesterone within 1 to 2 hours after they were isolated from the animal, progesterone-treated oocytes achieved GVBD 2-4 hours faster than did the insulin-treated oocytes. This experiment was repeated 8 times, and in each case the kinetics of GVBD in progesterone-treated oocytes was always faster than in the insulin-treated oocytes.

We found, however, that if oocytes were first stored in MBS medium for 12 to 14 hours and then treated with either insulin or progesterone, the time differential for $GVBD_{50}$ almost completely disappeared. This experiment was repeated 12 times and overall there was no significant difference in the timing of GVBD for insulin- or progesterone-treated oocytes. While insulin-treated oocytes exhibited a significant decrease in the timing of $GVBD_{50}$, the storage time in MBS medium appeared to have no significant effect on the timing of $GVBD_{50}$ in progesterone-treated oocytes.

It appears that the extra storage time in MBS medium prior to hormone treatment would explain the kinetic results for the following experiments: oocyte intracellular pH, protein synthesis, and protein phosphorylation. In each of these experiments, oocytes were first stored in MBS medium for 12 to 14 hours prior to hormonal treatment. In each case, the kinetics of the increase in pHi, or protein synthesis or protein phosphorylation as well as GVBD were very similar in insulin- and progesterone-treated oocytes.

It is uncertain why the 12 to 14 hour storage time in MBS medium accelerates the response of oocytes to insulin, but not their response to progesterone. There are, however, at least three possible explanations for this observed phenomenon. First, full-grown oocytes have IGF-1 receptors on their plasma membrane and insulin binds to these receptors to induce oocyte maturation *in vitro* (Janicot and Lane, 1989; Janicot *et al.*, 1991; Hainaut *et al.*, 1991). The extra storage time in MBS medium may allow for the increased synthesis and expression of additional IGF-1 receptors on the oocyte plasma membrane. This seems unlikely given the lengthy time of oogenesis (6-8 months) for receptor synthesis to take place. An extra 12 to 14 hours in MBS medium would seem to make little difference. Alternatively, recent studies have shown that insulin receptor substrate-1 (IRS-1) protein is a substrate of the IGF-1 receptor kinase (Chuang, *et al.*, 1993). *Xenopus* oocytes reponded more efficiently to insulin if they were microinjected with IRS-1 protein (Chuang *et al.*, 1993). Perhaps the incubation of defolliculated oocytes in MBS medium for 12 to 14 hours may stimulate the synthesis of IRS-1 protein within the oocyte. Finally, the IGF-1 receptors on the oocyte may not be available while the oocyte is in the ovary. These receptors may only play a role later in development after the egg is fertilized. One way to insure that these IGF-1 receptors are not utilized in the ovary would be to block them with an intra-ovarian inhibitor. When the oocytes are defolliculated *in vitro* or ovulated *in vivo* this inhibitor may start to dissociate from the IGF-1 receptor. The apparent accelerated response of stored oocytes to insulin may reflect the increased accessibility of the IGF-1 receptors on the oocyte plasma membrane. To the best of our knowledge, evidence for IGF-1 receptor inhibition is unknown in other cell systems. However, there is evidence for this type of receptor inhibition mechanism in *Xenopus* embryos. Activin receptors in *Xenopus* neurula embryos are "masked" by the protein

Follistatin. Follistatin prevents the ligand activin B from interacting with the activin receptor (Melton, personal communication, Cell Biology Meetings, 1993). Additional experiments will have to be carried out to test these three alternative hypotheses to see which one if any can possibly explain the accelerated response of "stored" oocytes to insulin stimulation.

If there is no intra-ovarian inhibitor of the IGF-1 receptor, does insulin and/or IGF-1 play a role in regulating oocyte maturation *in vivo*? Several observations indicate that insulin/IGF-1 may play this role *in vivo.*

1) Schuldiner *et al.* (1991) using reverse transcription PCR have identified two different non-allelic insulin mRNAs in *Xenopus* ovary. Therefore mRNAs for insulin I and II are present in non-pancreatic tissue. Whether these mRNAs get loaded onto polysomes and translated in the ovary is still unknown. In addition, it is not known whether these insulin mRNAs are in the oocyte itself or in the surrounding somatic cells within the ovary.

2) Chesnel *et al.* (1992) have found that the somatic cells surrounding the full-grown *Xenopus* oocyte synthesize and secrete a 5-7 kD protein. This protein is about the size of insulin or IGF-1. Furthermore, this secreted protein could augment the maturation-inducing effect of progesterone. This observation may be the first indication that *Xenopus* oocyte maturation is under the dual control of both progesterone and a protein that are synthesized by the somatic cells in the ovary.

3) Numerous studies have demonstrated that in the mammalian ovary, IGF-1 and IGF-2 are synthesized by the granulosa cells which surround the oocyte (Hammond *et al.*, 1985). IGF-1 and IGF-2 accumulate in the follicular fluid in response to gonadotropin stimulation of the granulosa cells (Adashi *et al.*, 1991).

4) A study has shown that the injection of IGF binding protein (BP3) into the follicular fluid of rat follicles significantly inhibited oocyte maturation and ovulation (Adashi *et al.*, 1990).

5) A recent study done on mice, that were made diabetic by injection of streptozotocin or alloxan, reported a significant delay in oocyte GVBD when compared to oocytes taken from non-diabetic mice (Diamond *et al.*, 1989).

6) Finally, in a recent IVF study on human oocytes it was found that the addition of IGF-1 to the culture medium significantly accelerated the kinetics of GVBD (Gomez *et al.*, 1993).

All of these studies provide circumstantial evidence for a role for insulin and/or IGF-1 in regulating oocyte maturation *in vivo*. Additional experiments will have to be done to directly establish this.

CHAPTER V

CONCLUSIONS

In summary, my study suggests that the insulin and progesterone meiotic maturation pathways converge early after the signal transduction process and use a common pathway leading to GVBD. To summarize our data Figure 15 shows that 1) Insulin interacts with IGF-1 receptors, while progesterone interacts with a progesterone receptors on the oocyte plasma membrane. 2) Both insulin and progesterone cause an increase in oocyte pHi. The timing and magnitude of the increase in pHi is similar in insulin and progesterone oocytes. 3) Both insulin and progesterone require protein synthesis to induce maturation. 4) Both insulin- and progesterone-treated oocytes synthesize the same proteins, and do so with similar kinetics. 5) Both insulin- and progesterone-treated oocytes phosphorylate the same proteins, and do so with similar kinetics. 6) Both insulin- and progesterone-treated oocytes undergo GVBD with similar kinetics when oocytes are stored in MBS medium for 12 to 14 hours prior to hormone treatment. Therefore, our conclusion is that insulin and progesterone pathways converge early to a common pathway leading to GVBD. Any differences would be early events during the signal transduction process. Further analysis will be required to determine if IRS-1 protein synthesis is required for insulin-induced maturation, and/or if the IGF-1 receptor is being "masked" by an intra-ovarian inhibitor.

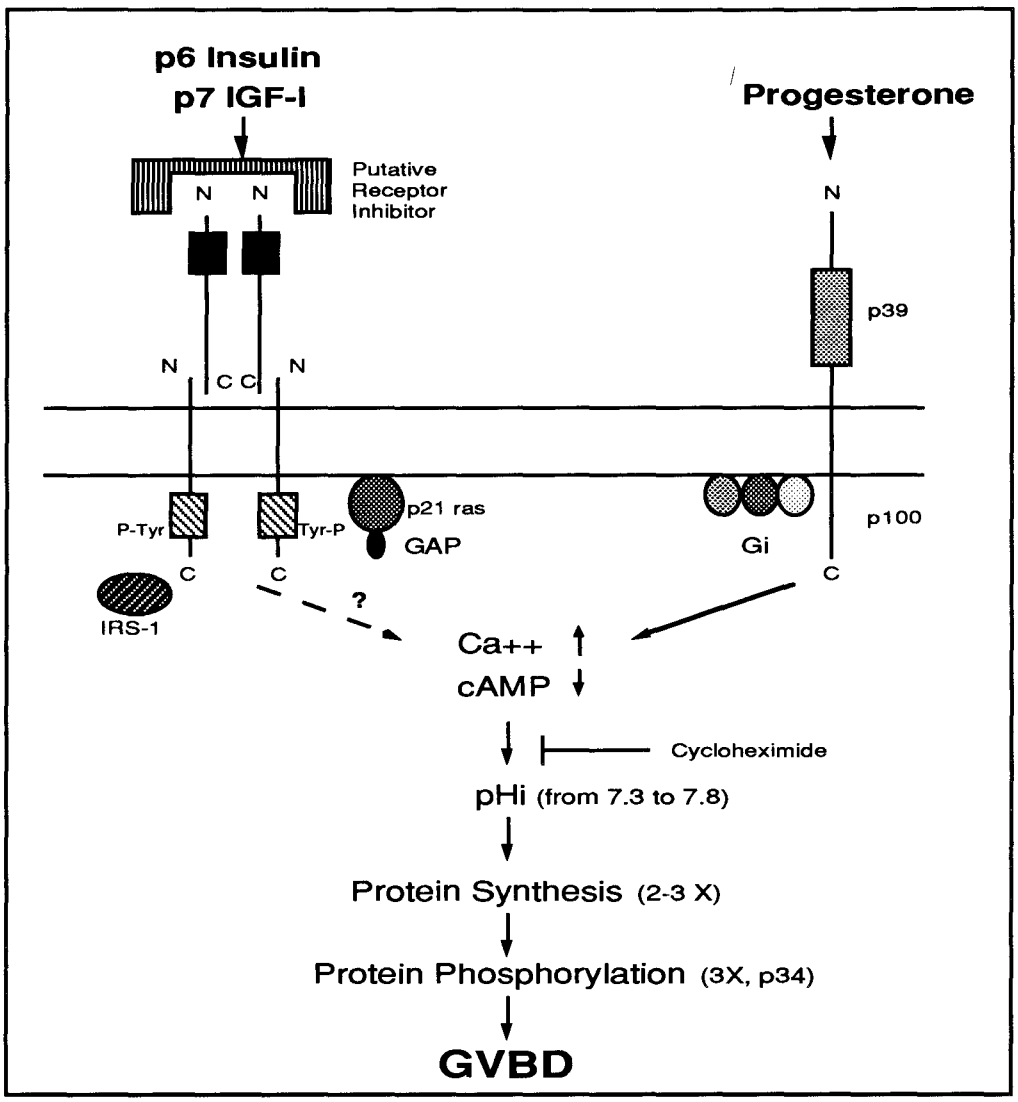

Figure 15. Proposed model for the insulin and progesterone meiotic maturation pathways. 1) Insulin interacts with IGF-1 receptors, while progesterone interacts with progesterone receptors. 2) Insulin- and progesterone-treated oocytes undergo a similar increase in intracellular pH. 3) Insulin- and progesterone-treated oocytes require protein synthesis, and synthesize the same proteins. 4) Insulin- and progesterone-treated oocytes phosphorylate the same proteins. 5) Insulin- and progesterone-treated oocytes undergo GVBD with similar kinetics. 6) Synthesis of IRS-1 protein may be required for insulin-induced maturation, and/or the IGF-1 receptor must be "unmasked".

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